Ex vivo PD-L1/PD-1 pathway blockade reverses dysfunction of circulating CEA specific T cells in pancreatic cancer patients.

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We are pleased to submit our manuscript describing the isolation of functional CEA691--specific CD8+ T cells from the peripheral blood and/or draining lymph nodes of 18 consecutive patients with carcinoma of the pancreas. We demonstrate for the first time that the antigen--specific function of these self--restricted tumor antigen--specific T cells can be enhanced by PD1/PDL1 pathway blockade. These findings support the clinical development of T cell mediated therapies in combination with checkpoint inhibitors for patients with an extremely poor prognosis malignancy.

ABSTRACT

Carcinoembryonic antigen (CEA) is a candidate target for cellular immunotherapy of pancreatic cancer (PC). In this study, we have characterised the antigen-specific function of autologous cytotoxic T lymphocytes (CTL) specific for the HLA-A2 restricted peptide, pCEA691-699, isolated from the peripheral T cell repertoire of PC patients and sought to determine if *ex vivo* PD-L1 & TIM3 blockade could enhance CTL function. $CD8^+$ T cell lines were generated from peripheral blood mononuclear cells (PBMCs) of 18 HLA-$A2^+$ patients with PC and from 15 healthy controls. *In vitro* peptide specific responses were evaluated by flow cytometry after staining for intracellular cytokine production and CSFE cytotoxicity assays using pancreatic cancer cell lines as targets. Cytokine secreting functional CEA691-specific CTL lines were successfully generated from 10 of 18 PC patients, with two CTL lines able to recognise and kill both CEA691 peptide-loaded T2 cells and CEA^+ HLA-$A2^+$ pancreatic cancer cell lines. In the presence of *ex vivo* PD-L1 blockade, functional CEA691-specific $CD8^+$ T cell responses, including IFN-γ secretion and proliferation, were enhanced and this effect was more pronounced on Ag-specific T cells isolated from tumor draining lymph nodes. These data demonstrate that CEA691-specific CTL can be readily expanded from the self-restricted T cell repertoire of PC patients and that their function can be enhanced by PD-L1 blockade.

INTRODUCTION

Pancreatic cancer (PC) remains a highly aggressive and difficult to treat malignancy. As such, it is one of the leading causes of cancer deaths worldwide (1). At the time of diagnosis approximately 30% of patients have locally advanced disease and a further 50% already have evidence of metastatic disease. A minority of patients (approximately 15%) are eligible for potentially curative surgery (such as pancreatico-duodenectomy, or the Whipple procedure), but the majority will die from recurrent disease (2, 3). Current 5-year overall survival rates are in the order of 5% with only marginal improvements in prognosis having been achieved in the last few decades (4-6).

For patients with un-resectable PC, gemcitabine and folfirinox-based chemotherapeutic regimens are the treatment of choice; however, efficacy is limited, and drug resistance and disease relapse are very common (7-9). There is therefore an urgent need to develop alternative therapeutic approaches for the treatment of PC.

Targeted immunotherapeutic approaches aim to improve the therapeutic index by maximising tumor cell death whilst minimising side effects. Promising results have recently been demonstrated for various advanced cancers, including melanoma, metastatic non-small-cell lung cancer, renal cell cancer and ovarian cancer (10) using a number of strategies including monoclonal antibodies, checkpoint inhibitors, vaccination and genetically modified immune cells (11-13). Cytotoxic T lymphocytes (CTL) play a pivotal role in cellular immunity and, by recognizing tumor-associated antigen (TAA)-derived short peptide epitopes presented by MHC-class I molecules, they can mediate target cell killing and induce protective anti-tumor immune responses (14).

Whilst a number of TAAs have been identified in the context of PC, including mesothelin (MSLN) (15) and the carcinoembryonic antigen (CEA) (16), both of which are widely expressed in PC cells, pancreatic tumors are generally considered to be non-immunogenic and resistant to immunotherapies (10, 17, 18). Additionally, pancreatic cancer cells actively contribute to local immune suppression in the tumor microenvironment through the production of anti-inflammatory cytokines such as TGF-β, IL-10 and IL-6 and/or the expression of negative regulatory molecules (18). Specifically, pancreatic cancer cells express high levels of programmed death ligand-1 (PD-L1) (19), an immunosuppressive molecule that, upon engagement with its receptor programmed death-1 (PD-1) on the surface of CTLs delivers inhibitory signals impairing T cell effector function (20). In murine models, it has been shown that anti-PDL1 and anti-CTLA-4 blocking antibodies, commonly referred to as checkpoint inhibitors, improved T cell

mediated anti-tumor immunity and prolong survival (21-23). Although supporting evidence from human clinical trials is currently lacking (24), it is expected that T cell-based immunotherapies in combination with checkpoint inhibitors may circumvent the immunosuppressive properties of the pancreatic tumor microenvironment, and such combinatorial approaches are likely to be required.

In this study we examined the *ex vivo* functional and phenotypic properties of CEA-specific T cells isolated from 18 consecutive HLA-$A2^+$ pancreatic cancer patients.

MATERIALS AND METHODS

Patients and samples

This study was approved by the Central London Research Ethics Committee (Study no 06/Q0512/106) and conducted in accordance with the Declaration of Helsinki. Written, informed consent was obtained from all patients.

Peripheral blood samples were collected from PC patients at three central hospitals: University College London Hospitals NHS Foundation Trust (UCLH); Royal Free London Hospital NHS Foundation Trust (RFH); and Charing Cross Hospital - Imperial College Healthcare NHS Foundation Trust. Detailed patient demographics and tumor characteristics are summarised in Table 1. In all cases the diagnosis of pancreatic carcinoma was confirmed by standard cytopathology or histopathology after biopsy, and the clinical stage was assigned using staging criteria described in the WHO histological classification of tumors of the exocrine pancreas (25). Anonymised peripheral blood mononuclear cells (PBMCs) were obtained from the National Blood Service from healthy controls.

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using standard methodology (Ficoll, Lymphoprep-Apogent Discoveries, Wilmslow, UK). Where possible, lymph node samples were collected from patients undergoing surgery and then mechanically disrupted to generate a single cell suspension prior to PBMC isolation.

Patient HLA-A2 status was determined by flow cytometric analysis after staining with a PE-conjugated, anti-HLA-A2 antibody (clone BB7.2; BD BioSciences, UK).

Peptides and tetramers

The HLA-A2 restricted peptides CEA691 (IMIGVLVGV), CMV pp65 (NLVPMVATV) and Telomerase540 (ILAKFLHWL) were synthesized by Mimotopes Pty Limited (Clayton Victoria, Australia) and dissolved in PBS at a stock concentration of 2 μmol/L prior to use. APC-labelled pCEA691/HLA-A*0201 tetramers (TCMetrix, Zurich, Switzerland) were used to detect CEA-specific T cells. Other HLA-A2 restricted peptides used in this study are detailed in supplementary Table 1 and were also synthesized by Mimotopes Pty Ltd.

Short-term T-cell expansion cultures

Short-term primary T-cell lines were generated as previously described (26). Briefly, isolated PBMC were cultured at 1.5×10^6 cells/mL in normal growth medium (NGM) consisting of RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 2 mM glutamine, 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated FCS (BioWest, Ringmer, United Kingdom). Peptide stimulation was performed at a final concentration of 2μM, in the presence of rhIL-2 (20 U/mL), and the cells were harvested after 9-10 days of culture.

Establishment of primary T-cell lines

PBMC were expanded over four rounds of peptide-specific stimulation, with cells being analysed by flow cytometry at the end of rounds one and four. Briefly, $3x10^6$ PBMC were initially resuspended in NGM at 1.5×10^6 cells/mL in a 24-well plate, in the presence of rhIL-2 (20 U/mL, Roche, Basel, Switzerland), rhIL-7 (2 ng/ml, R&D Systems, Abingdon, Oxfordshire, UK), rhIL-15 (5 ng/ml, R&D Systems) and rmIL-21 (0.5 ng/ml, R&D Systems). Peptide-specific stimulation was performed by adding pCEA691 or CMV pp65 directly into specific wells, at a final concentration of 10μM. After a first round of 7-9 days duration, cells were moved to re-stimulation. Three rounds of re-stimulation were performed as described below, each of which were 7-9 days duration.

Re-stimulation was conducted by re-suspending 5×10^5 cells in 2 ml of cytokine-supplemented NGM in a new 24-well plate, and stimulating them with irradiated (70 Gy) T2 cells (2×10^5) pulsed with the appropriate peptide, at a final concentration of 10μM. Irradiated (35 Gy) autologous PBMCs or PBMC from healthy HLA-$A2^+$ donors obtained from the National Blood Service (Colindale, UK) were used as feeder cells (2×10^6).

Cell lines

Six pancreatic cancer cell lines (MiaPaca-2, PK-45, Panc-1, KLM-1, Bx-Pc-3, and PK-1) were obtained from PIKEN BioResource centre (PIKEN BRC, Tsukuba, Japan). Among them, Panc-1, MiaPaca2, PK-1, and Bx-Pc-3 were used as target cells in cytotoxicity assays. Apart from MiaPaca2, all cell lines were maintained in normal growth medium

(NGM) containing RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 2 mmol/L glutamine, 1% penicillin plus streptomycin (Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated FCS (BioWest, Ringmer, United Kingdom). MiaPaca2 cells were cultured in DMEM (Invitrogen, Paisley, United Kingdom) with 1% penicillin plus streptomycin and 10% heat-inactivated FCS. The HLA-A2 positive T2 cell line was loaded with specific peptides and used as target cells were indicated. TAP deficient T2 cells have impaired presentation of HLA molecules with endogenous peptide, but can be efficiently loaded with exogenously peptides (27). The T2 cell line was maintained in NGM.

Cytotoxicity Assays

A carboxy fluorescein succinimydyl ester (CFSE) cytotoxicity assay was used to determine the antigen-specific cytotoxicity of expanded T cell lines. pCEA691 peptide-loaded T2 cells or HLA-A2 positive pancreatic cancer cell lines (known to express CEA, CEA^+) were used as target cells. T2 cells pulsed with irrelevant peptides and HLA-A2 negative pancreatic cancer cell lines were used as control target cells. 1 x 10^6 target cells were suspended in PBS/1%FCS at a concentration of 10^6/mL and then stained with CFSE. For sensitive targets, 0.5μl of CFSE stock solution (5M) was added to 1 ml of cell suspension, while for control targets, 0.5μl of diluted CFSE at 500μM was used. After 4 minutes' incubation at room temperature, 9mL of PBS/1%FCS was added to stop the reaction. The cells were washed with PBS then re-suspended at 5×10^4 cells/mL prior to setting up co-cultures with the effector cells. T cells (effectors) were added to round-bottomed 96-well plates to obtain a total volume of 200μL/well (28). Various E:T (effector : target) ratios were tested, including 100:1, 50:1, 20:1, 10:1, 5:1, 2.5:1, 1.25:1 and 0.625:1 respectively. Assay plates were incubated for 4 hours at 37 ° C, 5% CO2. Cells were washed in PBS prior to FACS analysis (FACSCalibur). For peptide titration assays, CFSE-stained T2 cells were loaded with variable concentrations of peptides, at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10-9, 10^{-10}, 10^{-11}, 10^{-12} M, respectively (29).

***In vitro* PD-L1 and TIM-3 blockade**

PBMC from 11 PC patients (where sufficient cells were available) and lymph node derived lymphocytes from one PC patient were cultured in the presence of CEA691 peptides and rh-IL-2 as described above, at a concentration of 2×10^6/mL in 200 μL of NGM. On day one, anti-PD-L1 and anti-TIM-3 antibodies (Mouse IgG, eBioscience, Hatfield, United Kingdom) were added to the wells, either separately, or in combination, at a final concentration of 10 μg/mL.

After 7 days of incubation at 37°C, the cells were harvested for functional analysis using intracellular cytokine staining.

Flow cytometry

The following antibody-fluorochrome combinations were used: CD3-PE-Cy7, CD8-Horizon v450, CD4-Horizon v500, IFN-γ-FITC, PD-1-PE, CD45RO-BV650 (all from BD Biosciences, Oxford, UK), CD62L-APC-Cy7 (eBioscience, Hatfield, UK); LAG-3-FITC (R&D Systems, Abingdon, UK), and TIM-3-AF700 (eBioscience, Hatfield, UK). *Ex vivo* surface staining was performed on 1×10^6 freshly isolated PBMC. Briefly, one microliter of a 1:50 dilution of each antibody was added to the cells and incubated for 30 minutes, at 4ºC, in the dark. Cells were washed twice with PBS/1% FCS and then resuspended in 200 μL PBS/1% FCS for data acquisition. Flow cytometry data acquisition was performed using a FACSCalibur. Propidium iodide (10 μg/mL) was added immediately prior to acquisition to discriminate dead cells from viable cells. Data analysis was performed using FlowJo (Treestar Inc., San Carlos, CA, version v10).

Intracellular cytokine staining assay

Intracellular cytokine staining was performed on cultured cells, either after short-term stimulation, or after four rounds of antigen-specific stimulation during the primary T-cell line establishment protocol (described above). Upon harvest, cultured cells were re-stimulated with 10 μmol/L of relevant peptide for a further 5 hours in the presence of 10 μg/mL Brefeldin A. Cells stimulated with an irrelevant peptide were used as negative controls, and cells stimulated with PMA (50ng/ml) + Ionomycin (500ng/ml) were used as positive controls. Cells were surface stained with anti-CD3, anti-CD4 and anti-CD8 antibodies, as described above, then permeabilized and fixed using FACS fix/perm solution (Invitrogen, Paisley, United Kingdom) prior to staining for intracellular cytokines with FITC-conjugated anti-IFN-γ, for 20 minutes, at 4ºC in the dark. Cells were washed with PBS/1% FCS and then resuspended in 200 μL PBS/1% FCS.

An immunological 'response/responder' was defined as a two-fold increase in the frequency of cytokine-producing cells in relation to that observed with the irrelevant peptide (Telomerase540). For example, if the frequency of IFN-γ producing $CD8^+$ T cells induced by CEA691 doubled that stimulated by control peptide at the end of 4 rounds pCEA691 specific stimulation, the response was defined as positive (i.e. a 'responder').

Statistical analysis

Statistical analysis was conducted using the SPSS software (SPSS for windows, version 21). Data sets were first tested for parametric distribution using the Skewness–Kurtosis and the homogeneity of variance tests. For parametric data, the T test was used to determine statistical significance; for non-parametric data distributions, the Mann–Whitney U test was applied. When comparing data sets between more than two groups, the one-way ANOVA test (for parametric data) or Kruskal–Wallis test (for nonparametric data) were used. Whenever an overall P value was statistically significant, post-hoc pairwise comparisons were performed with the Tukey Honest Significant Difference (HSD) method. P values < 0.05 were considered statistically significant.

RESULTS

CEA691-specific CTL responses can be generated from PBMCs of PC patients

We previously investigated antigen-specific CTL responses generated by short-term culture of PBMCs isolated from 13 PC patients and 10 healthy controls (Supplemental Table 1). These were stimulated with 3 CEA HLA-A2-restricted peptides (CEA605-613 YLSGANLNL, CEA691-699 IMIGVLVGV, and CEA694-702 GVLVGVALI) and one irrelevant peptide Telomerase540-548 ILAKFLHWL over 9-10 days. Antigen-specific IFN-γ production was most frequently observed in CEA691-responsive $CD8^+$ T cells, with a higher percentage of both patients and healthy controls generating functional responses than to other tested epitopes (Supplemental Figure 1A). In responders, the frequency of IFN-γ producing cells within the $CD3^+CD8^+$ T cell subset was not significantly higher in PC patients than in healthy controls (supplemental Figure 1B and 1C). This data was in agreement with previous findings indicating that immune tolerance to particular self-peptides may be incomplete (30).

Here, we show in Figure 1A the gating strategy for analysis of PBMCs from a single pancreatic cancer patient (CA11) after 4 rounds of expansion *in vitro.* An increase in the percentage of antigen-specific IFN-γ producing $CD8^+$ T cells was observed. After the 4th round of peptide stimulation, IFN-γ producing $CD8^+$ T cells were detectable in 5 out of 15 healthy controls and 10 out of 18 PC patients, with 4 examples of each shown in Figure 1B (healthy controls H01, H04, H07, H13) and Figure 1C (cancer patients CA07, CA11, CA12, CA18), respectively.

PC disease progression is associated with impaired CEA691-specific CTL responses

Six patients recruited for this study were classified as having stage IV PC (metastatic disease at presentation), whilst the rest were classified stages II (N=9) and III (N=3) PC (Figure 2A). CEA691-specific CTL responses were detected in 9 out of 12 patients with stage II-III disease, whilst only 1 out of 6 patients with stage IV disease had demonstrable functional CEA691-specific T cells responses as determined by IFN-γ secretion upon 4-round antigen specific stimulation (Figure 2B). Relative frequencies of CEA691-specific $CD8^+$ T cells were also significantly lower than that in patients with earlier stage disease II-III (Figure 2B). PC patients who had been treated with chemotherapy (Figure 2C) and patients with inoperable tumors (Figure 2D) also had lower frequencies of CEA691-specific CTLs compared to the non-chemo and surgical patient groups respectively.

CEA691-Specific CTL from pancreatic cancer patients can recognize and kill pancreatic cancer cell lines *in vitro*

T cell lines were generated by stimulating the PBMCs of PC patients using pCEA691 loaded T2 cells for 4 rounds. As described above, over 30% of $CD8^+$ T cells from 3 patients (CA07, CA11 and CA18) produced cytokines in response to pCEA691 re-stimulation after 4 rounds of expansion (Figures 1C). To examine their ability to recognize and induce tumor cell death, we utilized CFSE killing assays from T cell lines generated from CA07, CA 11 and CA18.

T2 cells loaded with 1mM (10^{-6}M) pCEA691 peptide (CFSEhi) or irrelevant peptide loaded T2 cells (CFSElo) were co-culture with CTL at different E:T ratios ranging from 100:1 to 0.6:1. Unstimulated HLA-$A2^+$ PBMCs were used as control effector cells (Supplemental Figure 2A). The results demonstrate that CEA691-specific CTLs generated from all these three patients (CA07, 11, 18) were able to recognize and kill CEA691 loaded T2 cells (Supplemental Figure 2B). Moreover, T cells from CA11 were able to kill T2 cells loaded with 10^{-9}M CEA691 (1nM), at a E:T ratio = 20:1, in peptide titration assay, in which T cell lines were stimulated with T2 cells loaded with 10^{-5} to 10^{-12}M (10mM to 1pM) CEA691 peptide for 4 h (E:T=20:1) (Supplemental Figures 2C and 2D). The T cell line generated from CA11 was shown to have moderate to high avidity with recognition of nano-molar (10^{-9}M) concentrations of CEA, which are comparable to the concentration of TAA expression on the surface of tumor cells (31).

Subsequently, we investigated the cytotoxic effector function of T cell lines following stimulation with pancreatic cancer cell lines *in vitro*. The expression of CEA and HLA-A2 in six pancreatic cancer cell lines was determined after staining with specific monoclonal antibodies and analysed by flow cytometry. MiaPaca-2 was negative for both CEA and HLA-A2; the Panc-1 cell line was HLA-A2 positive, but expressed low levels of CEA; Bx-Pc-3 and PK-1 were HLA-A2

positive cell lines with high expression of CEA (Figure 3A). Therefore, MiaPaca-2 was used as the negative control for both HLA-A2 and CEA, and PK-1, Bx-Bc-3 (HLA-A2+ and CEA+), together with Panc-1 (HLA-A2+ and CEA low) were used as specific targets. Figure 3B shows the relative reduction in the frequencies of PK-1 after co-culture with the CTL line (from patient CA11) and MiaPaca-2 cells, at different E:T ratios. T cell lines generated from CA07 and CA11 patients lysed PK-1 and Bx-Pc-3 cancer cell lines, but not Panc-1, demonstrating their CEA691-specific cytotoxicity. (Figure 3C). No cytotoxicity against pancreatic cancer cell lines was observed using unstimulated PBMCs from the same donors (data not shown). These data suggest that functional CEA-specific CTLs can be generated in pancreatic cancer patients and that these cells may have the potential to control tumor growth.

Inhibitory pathways play a role in the modulation of antigen-specific CTL responses in PC patients

Possible explanations for the failure of anti-tumor T cell responses in patients include inadequate T cell priming and insufficient duration of the effector phrase, both of which can be regulated by the co-inhibitory receptors, including CTLA-4, PD-1, TIM3 and LAG-3 (32). For example, PD-1/PD-L1 signaling inhibits T cell function via suppressing TCR-dependent activation of both $CD4^+$ and $CD8^+$ T cells, particularly, through the inhibition of their proliferation and cytokine production, including IFNγ, TNF-α and IL-2 (33). PD-L1 expression by tumor cells has been associated with a poorer prognosis or advanced disease (20, 34-36). About 80% of PC cases express PD-L1, of which 20% have up-regulated expression of PD-L1 (compared to normal pancreatic tissue) and tend to be highly invasive and recurrent (37, 38).

In order to investigate the relationship between CEA691-specific CTL responses and the expression of negative regulatory molecules on T cells, *ex vivo* phenotypic examination of PC patients' peripheral T cells was performed prior to stimulation with CEA691-peptide. As shown in Figure 4A, the frequency of PD-1, TIM-3 and LAG-3 expressing $CD8^+$ T cells was significantly higher in the non-responder group of PC patients than in the healthy controls.

It is known that T cell activation and differentiation status influences the expression of some cell surface markers, such as PD-1. In order to examine the T cell phenotype in more detail, we identified naïve, central memory (TCM), effector memory (TEM), end-stage/effector phenotypes based on CD62L and CD45RO expression (i.e., TCM was defined as $CD62L^+CD45RO^+$ T cells, TEM as $CD62L^-CD45RO^+$T cells, Naïve T cells as $CD62L^+CD45RO^-$, and end-stage/effector T cells as $CD62L^-CD45RO^-$). As shown in Figure 4B, there was no statistically significant difference in

the percentage of gated $CD4^+$ or $CD8^+$ T cells at various differentiation stages between PC responders and non-responders. The percentage of naïve T cells, however, in responders was observed to be higher than in non-responders, but this difference did not achieve statistically difference (p=0.051).

$CD8^+$ T-cell priming and activation take place in draining lymph nodes where, upon interaction with antigen-presenting cells, naïve T cells are primed and differentiate into fully-functional antigen-specific CTLs. We were able to analyse the expression of PD-1, TIM-3 and LAG-3 in matched peripheral- and lymph node-derived $CD8^+$ T cells isolated from three of the PC patients (see Table 1). The proportion of lymph node-derived $CD8^+$ T cells expressing PD-1 and TIM-3 was higher than that observed in peripheral T cells, whereas more LAG-3 positive cells were identified within the peripheral T ell repertoire than in the draining lymph nodes (Figure 4C, left panel). In addition, similar differences were observed when analysing the expression levels of PD-1, TIM-3 and LAG-3, as determined by mean fluorescence intensity, MFI (Figure 4C, right panel).

Blockade of the PD-1/PD-L1 pathway improves tumor antigen-specific CD8+ T-cell responses

To assess whether blockade of the PD-1/PD-L1 negative regulatory pathway enhanced the function of patient derived, self-restricted CEA691-specific CTL, PBMCs from patients with PC (and LN cells from one patient) were cultured for seven days in the presence of pCEA691 or the control peptide, rIL-2, with or without anti-PD-L1 and/or anti-TIM-3 antibodies. In total, the frequency of peptide-specific cytokine producing $CD8^+$ T cells was evaluated in 11 patients with PC (see Table 1 for patient details). In addition, the frequency of CEA691 tetramer positive $CD8^+$ T cells was determined in 8 of the 11 patients (where sufficient samples were available for analysis).

As shown in Figure 5A, a significant increase in the frequency of CEA691 tetramer-binding $CD8^+$ T cells was observed in cells cultured in the presence of anti-PD-L1 antibody (p=0.030, n=8) and the combination of anti-PD-L1+anti-TIM-3 antibodies (p=0.045, n=8), compared to non-treated cells. These findings suggest that PD-L1 and TIM-3 blockade can enhance the proliferation of PC patient derived CEA691-specific CTLs *in vitro*. Similarly, the frequency of IFNγ-producing $CD8^+$ T cells was significantly higher in the cells treated with anti-PD-L1 or anti-TIM-3, alone or in combination (Figures 5A, 5B and 5C). Even though both PD-1/PD-L1 and TIM-3 regulatory pathways were observed to be involved in the modulation of CTL function, our data did not reveal a significant synergistic effect when PD-1/PD-L1 and TIM-3 pathways were blocked at the same time.

To determine the relative function of negative regulatory pathways in the inhibition of antigen-specific CTL responses in the lymph nodes (LN) of patients with PC, MNCs isolated from lymph nodes were cultured with the pCEA691 peptide in the presence or absence of anti-PD-L1 and/or anti-TIM-3 blocking antibodies. Blockade of the PD-1/PD-L1 pathway, but not of the TIM-3 pathway, led to an increase in the frequency of IFNγ producing cells in both circulating and lymph node-derived CTLs, suggesting a role for the PD-1/PD-L1 axis in the modulation of IFNγ responses in patients with PC (Fig. 5C and 5D). We observed that the expression of PD-1 and TIM-3 was higher in lymph node-derived $CD8^+$ T cells than that in peripheral T cells, and blockade of these inhibitory molecules led to a more significant recovery of peptide-specific IFNγ production by $CD8^+$ T cells.

Our findings suggest that the expression level of negative regulatory molecules, in circulating and lymph node-derived CTLs, may influence the magnitude of the CTL mediated anti-tumor response in different tissues.

Discussion

The prognosis for patients with pancreatic cancer remains extremely poor and novel treatments such as immunotherapy are appealing alternatives to improve survival. However, there is currently little evidence from large clinical trials to demonstrate that pancreatic cancer is sensitive to checkpoint inhibition, and vaccine based immunotherapies have largely failed to generate significant clinical results (10, 17, 18, 24). Meanwhile, some animal experiments have suggested that the combination of checkpoint inhibitors with other immunotherapies, such as therapeutic vaccines, may improve outcomes in pancreatic cancer (24). Our study, has focused on exploring potential epitopes for targeted immunotherapy in pancreatic cancer patients, and has evaluated the influence of blockade of PD-1/PD-L1 pathway or TIM-3 pathway in anti-tumor T cell responses.

Carcinoembryonic antigen (CEA) is an 180 kDa glycosylated membrane protein that is over-expressed in more than 90% of PC cases (16). As a self-antigen, CEA-specific T cells are expected to be subject to immunological tolerance. Although previous studies demonstrated that tolerance to CEA691 is incomplete (30), little is known about the function of CEA691-specific CTL isolated from patients with pancreatic cancer. Here, we identified the most immunogenic CEA-derived epitopes using short-term *in vitro* PBMC stimulation followed by prolonged antigen stimulation to expand primary CEA691-specific T-cell lines. After one round of *in vitro* peptide stimulation we observed that the CEA691 peptide-specific IFNγ producing T cell responses were more readily detectable in PC

patients compared to healthy controls and that responding patients had higher percentages of IFNγ-producing T cells than those stimulated by other peptide epitopes. After a total of 4 rounds of *in vitro* stimulation, the CEA691-specific CTLs from PC patients were also able to produce TNFα and kill relevant target cells.

Despite the small sample size we were able to demonstrate that T cells isolated from patients presenting with more advanced disease were less likely to generate effective antigen-specific cytokine-producing responses. It is possible that prolonged TAA exposure in the tumor microenvironment may induce exhaustion of antigen-specific T cell responses. Unfortunately, we were unable to test PBMCs at different time points over the course of PC progression for individual patients. Thus, the evolution of phenotypic and functional changes in the anti-CEA691 T cell responses in our patients may be related to multiple factors including prior treatment, lymphopenia in advanced patients, co-morbidities including age and/or the use of immunosuppressive drugs. Similarly, PC patients without demonstrable CEA691-specific T cell responses may have presented with more aggressive and/or quickly progressing cancers, hence having more advanced disease at diagnosis. To address these issues, a prospective longitudinal study with a larger sample size is needed.

Interestingly, our preliminary findings suggest that surgical treatment, which had occurred prior to patients being evaluated in the study (and therefore prior to T cell isolation), may also modulate $CD8^+$ T cell responses, suggesting different modes of treatment received may also impact on TAA-specific immune responses in PC. For example, the influence of medical interventions on T cell response was observed in our previous study, in which embolization improved AFP-specific $CD4^+$ T cell responses in patients with hepatocellular carcinoma (39). Again, to confirm these findings a much larger study would be required to control for all the relevant variables.

Activation of the inhibitory PD-1/PD-L1 pathway has been linked to the poor prognosis of PC (40). Our data has also shown that patients not responding to pCEA691 had larger numbers of PD-1 expressing T cells in their peripheral T cell compartment, compared to the T cells isolated from responding patients or healthy controls. Also, T cells isolated from the tumor draining LNs were enriched for PD-1 and TIM-3 expressing cells compared to T cells isolated from the peripheral blood. Not surprisingly, these findings support the existence of an immunosuppressive tumor microenvironment in PC patients. It is therefore likely that combination therapies with checkpoint inhibitors and antigen specific T cells may be required to optimize immunotherapeutic approaches to PC.

Recently, the efficacy of anti-CTLA-4 and anti-PD1/PDL1 antibodies in treating several types of cancers has been demonstrated in early phase clinical trials (41). However, these checkpoint inhibitors are considered most effective in treating cancers with high mutational loads, e.g., melanoma and lung cancer, because such cancers typically generate more neoantigens and checkpoint inhibitors reverse the inhibition of tumor infiltrating neoantigen-specific T cells (42). On average, the neoantigen repertoire of pancreatic cancer is 1 mutation per megabase, which is much less than that observed in melanoma and lung cancer (with more than 10 mutations per megabase on average) (43). In theory, therefore, pancreatic cancer should not be very sensitive to checkpoint blockade. To date there have been a number of clinical trials assessing the safety and efficacy of checkpoint inhibitors in PC patients (44), but no positive results have yet been published.

However, our *in vitro* results suggest that the PD-1/PD-L1 pathway is an important factor in the inhibition of anti-tumor functions of $CD8^+$ T cells from PC patients. Following the addition of PD-L1 blocking antibodies, antigen-specific T cell proliferation and cytokine production (IFNγ and TNFα) was improved, with a greater effect observed on T cells isolated from the tumor draining LNs than from the peripheral blood. In addition, the percentage of T cells expressing PD-1 in LNs was higher than that observed in the peripheral blood. Thus, the limited impact to date of checkpoint inhibition in PC may be due to the relatively small neoantigen repertoire, and/or that TAA-specific T cells less readily enter the tumor microenvironment due to associated desmoplasia. Finally, we did not observe a synergistic effect when combining PD-L1 blockade with TIM-3 blockade. This may have been because TIM-3 levels were generally low in freshly isolated T cells. Little is known about the expression and role of TIM-3 (45, 46) and LAG-3 (47) in T cells from pancreatic cancer patients. In our study, the percentage of TIM-3 and LAG-3 positive $CD4^+$ and $CD8^+$ T cells was higher in pancreatic cancer patients, compared to healthy donors.

In conclusion, here we describe, the *in vitro* stimulation and expansion of self-restricted, autologous, functional CEA691-specific T cells, isolated from the peripheral blood and draining LNs of patients with pancreatic adenocarcinoma. This raises the possibility that expansion and infusion of autologous CEA691-specific T cells may be an effective immunotherapeutic approach to PC. Whilst alternative CEA-based immunotherapies are still being designed and optimised for PC (12, 48, 49), including CEA-specific engineered T cells, which have been shown to eradicate PC tumors without inducing toxicity in mice (50, 51), in another study the administration of autologous T

lymphocytes genetically engineered to express a murine TCR specific for CEA691 to colorectal cancer patients was shown to induce severe transient colitis (52) resulting in suspension of the clinical trial, raising questions about the suitability of this particular epitope for the design of novel TCR-based therapeutic approaches. Compared to the CEA691 specific high-affinity TCR generated by others (52-54), our CEA691 specific T cell lines were isolated from patients with pancreatic cancer and it is possible that their anti-tumor efficacy can be improved by the in vivo reversal of dysfunction mediated by increased expression of PD-1 and TIM-3.

AUTHOR CONTRIBUTIONS

YC, SB, SP and ECM analysed the data and wrote the manuscript. SP consented patients, collected PBMC samples and provided clinical care of the patients. YC, XAX and SB performed the laboratory experiments. GM provided PC cell lines.

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TABLE

Table 1 Patients demographic information

PBMC samples, N=18					
ID of PC patients	**Age (year)**	**Gender**	**Histological diagnosis**	**Stage**	**Treatment**
CA01	51	M	AC[a]	IV	Untreated
CA02	37	M	PDAC[b]	IV	Untreated
CA03	86	F	AC	III	Untreated
CA04	56	F	Poorly differentiated AC	IV	Resected + FOLFOX[c]
CA05	67	M	AC	IV	GemCap[d]
CA06	66	M	Moderately differentiated PDAC	IIB	Resected, Pre-chem[e]
CA07	60	M	Ductal AC	IIA	Pre-chem
CA08	73	F	Mucinous carcinoma	IIA	Gemcitabine
CA09	45	M	AC	IIB	FOLFIRINOX[f]
CA10	72	F	AC	IIB	Resected + Gemcitabine
CA11	68	F	Moderately differentiated AC	IIB	Resected +Gemcitabine
CA12	69	F	Adenocarcinoma	IIB	Resected + GemCap
CA13	60	M	Adenocarcinoma	IIB	Post-operation
CA14	46	M	Adenocarcinoma	III	Untreated
CA15	52	M	AC	IIB	Gemcitabine
CA16	60	M	AC	IV	Resected + GemCap
CA17	45	M	Adenocarcinoma	IIB	FOLFIRINOX
CA18	55	M	Moderately differentiated AC	III	Resected, pre-chem
LN samples, N=3					
ID of PC patients	**Age (year)**	**Gender**	**Histological diagnosis**	**Stage**	**HLA-A2**
CA13	60	M	Adenocarcinoma	IIB	+
CA19	52	M	Moderately differentiated AC	IIB	-
CA20	51	F	Adenocarcinoma	IIB	-

a.AC: adenocarcinoma. b.PDAC: pancreatic ductal adenocarcinoma. c. FOLFOX: Folinic acid, Fluorouracil and Oxaliplatin. d. GemCap: Gemcitabine and capecitabine. e.chem: chemotherapy. f.FOLFIRINOX: FOLFOX+ Irinotecan

FIGURES:

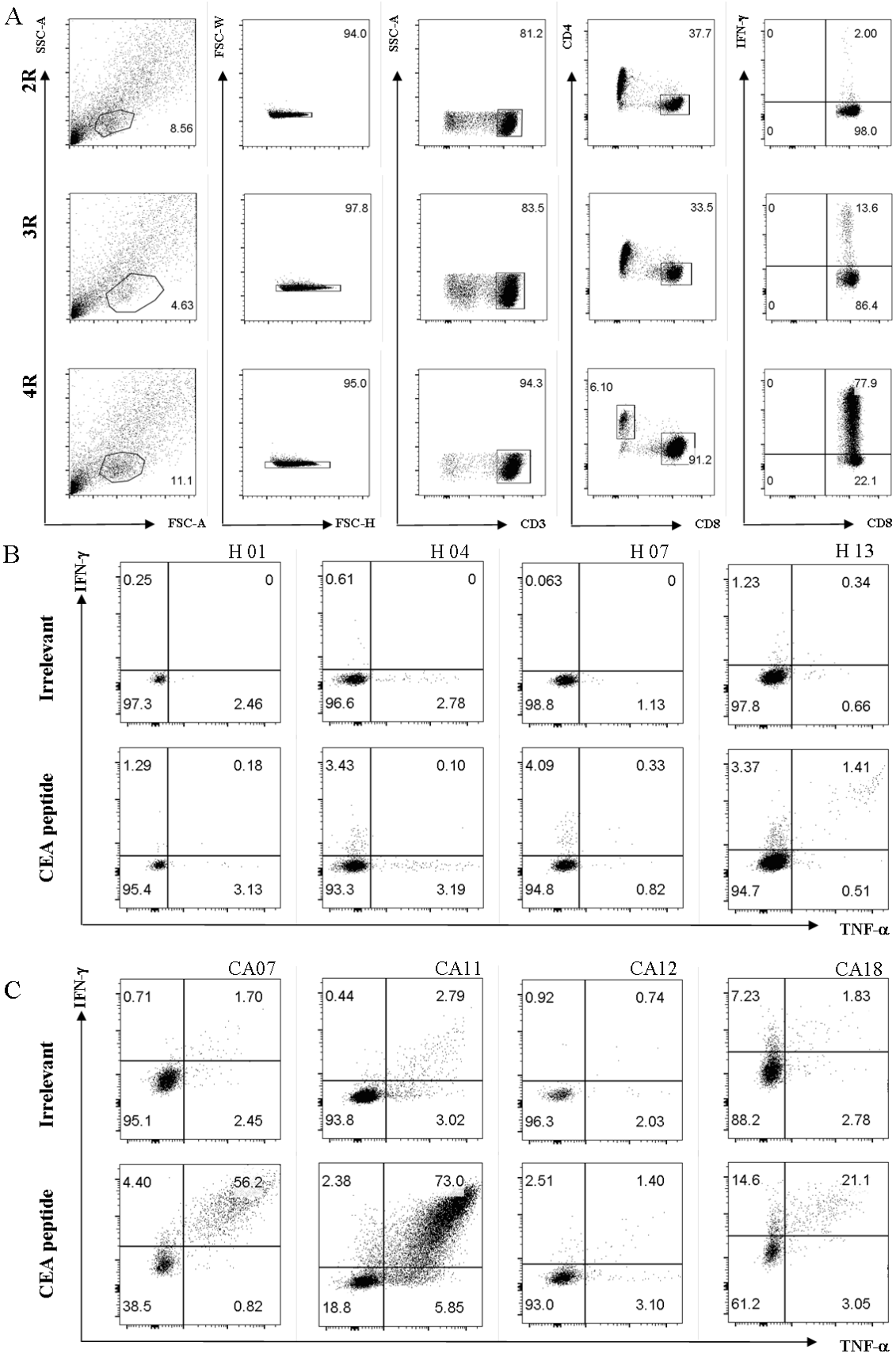


Figure 1. CEA691 specific T cells isolated form pancreatic cancer patients and healthy controls produce type 1 cytokines.

(A) A typical example of T cells expanded during stimulation with pCEA691 loaded T2 cells. Between the second (2R) and fourth round (4R) of stimulation, the percentage of INF-γ producing CD8+ T cells gradually increased. By the end of the 4th round stimulation, 77.9% of CD8+ T cells were INF-γ secreting, compared to 2% after 2 rounds.

Dot blots representing IFN-γ and TNF-α producing CD8 T cells cultured with CEA691 pulsed T2 cells and control peptide-loaded T2 cells after 4 rounds of stimulation (Gated on CD8 T cells) in four out of 15 healthy controls (B) and four out of 18 cancer patients (C) are shown. The percentages of IFN-γ+TNF-α+ CD8 T cells are shown in the upper-right quadrants.

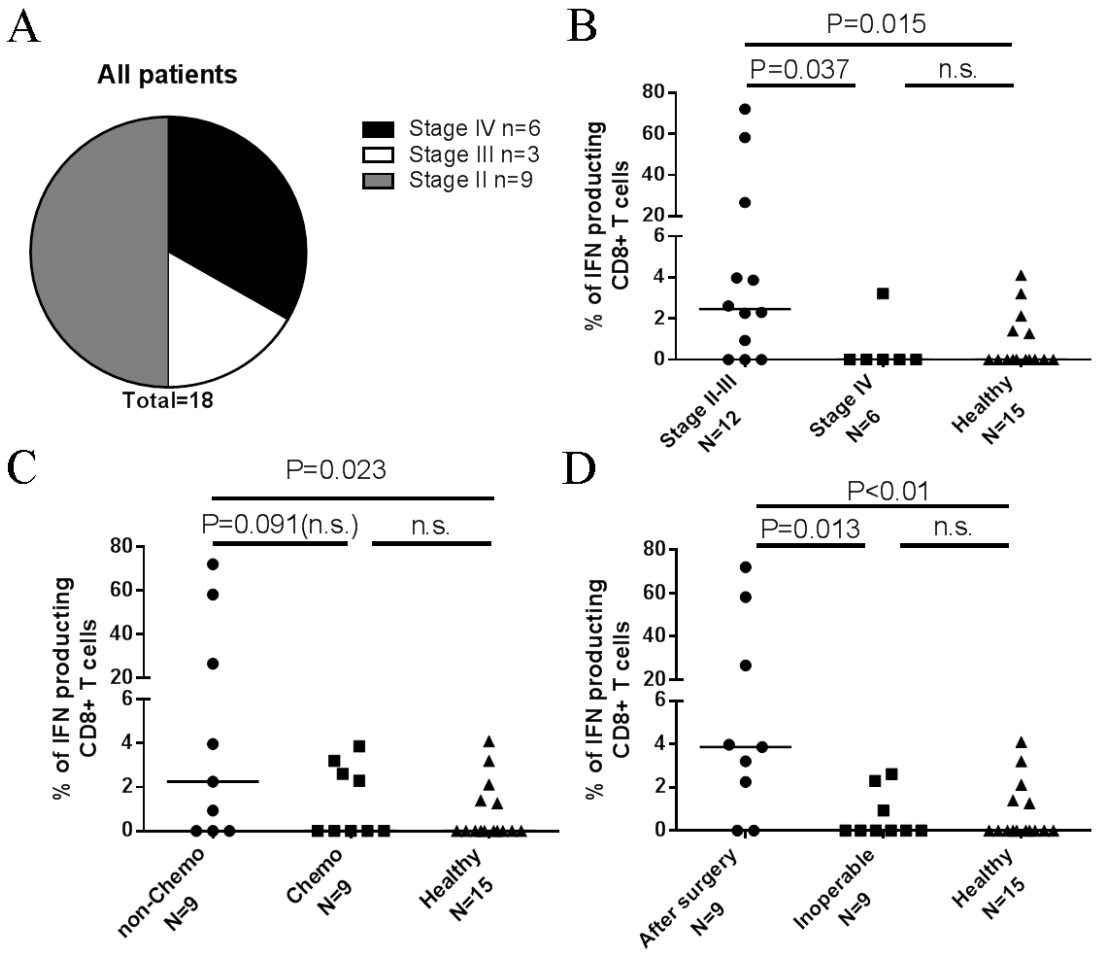


Figure 2. Higher frequencies of CEA691-specific CD8+ T-cell responses are detected in earlier stages of pancreatic cancer.

(A) Pie chart illustrates the frequency of pancreatic cancer patients at different disease stages (II-IV). Frequencies of IFN-γ+ cells within the CD8+ T-cell population are shown for patients stratified according to (B) disease stage, (C) prior administration of chemotherapy, and (D) submission to surgery. Each symbol represents one individual and horizontal bars represent median. P values <0.5 were considered statistically significant and are shown in the graphs.

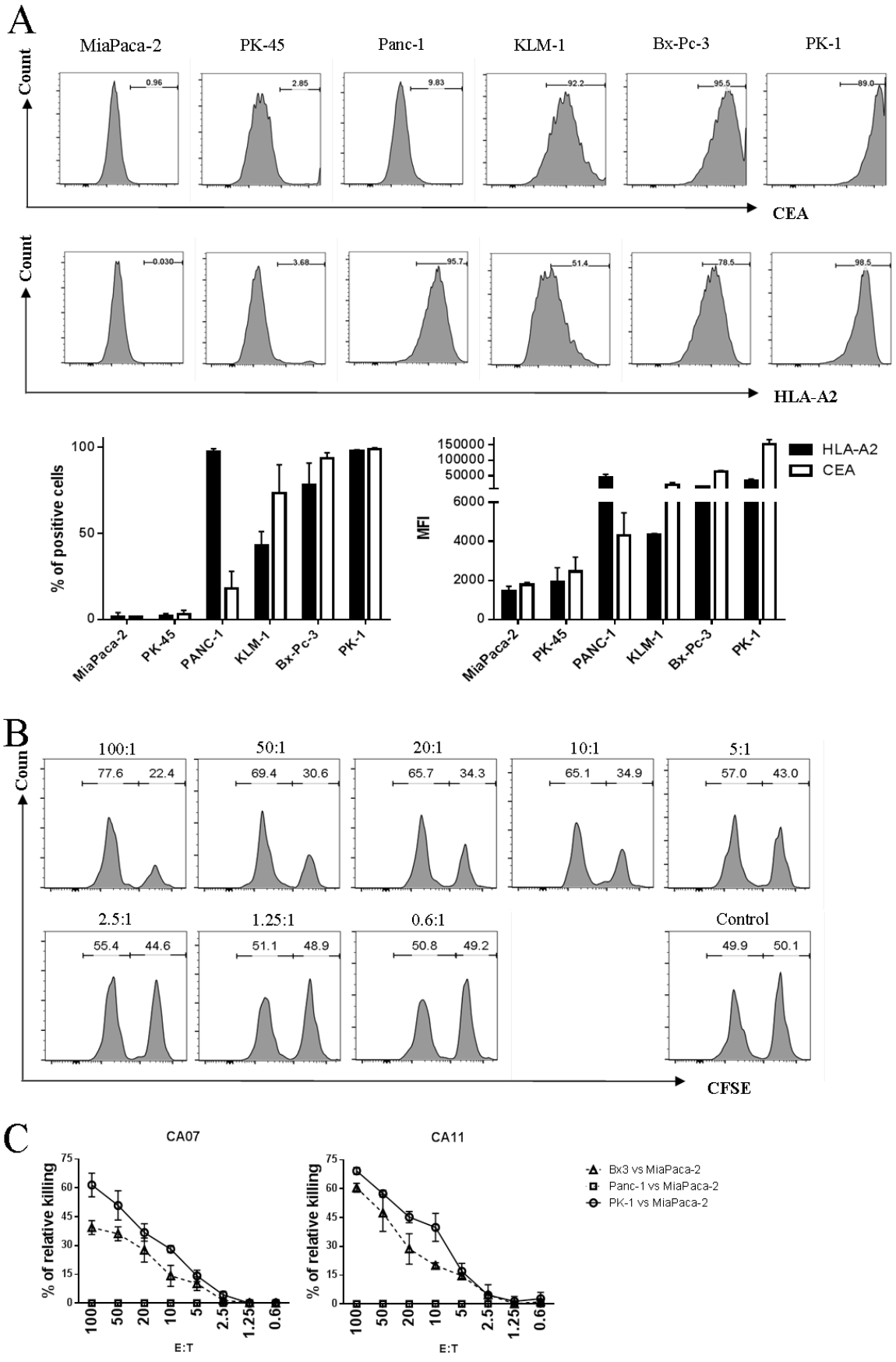


Figure 3 Cytotoxic activity of CTL lines against pancreatic cancer cell lines.

(A) CEA and HLA-A2 expression of 6 pancreatic cancer cell lines. The results were analysed by FACS and presented in

histogram (upper). Isotype antibodies were used to determine the background. Percentage and MFI of HLA-A2/CEA expressions by different PC cell lines was also shown (bottom). N.B. The unstained cells were gated out. (B) FACS analysis of CA11 T cells killing activity in response to recognise and kill PK-1 cell line (HLA-A2+, CEA+, labelled with high dose CFSE), and MiaPaca-2 cell line (HLA-A2-, CEA-, labelled with low dose CFSE), at different E:T ratios. (C) The percentage of relative killing of PK-1, Panc-1 and Bx-Pc-3 by CTLs from CA07 or CA11, compared to MiaPaca-2, at different E:T ratios. All the experiments were repeated twice and the mean of the results was shown in the figure.

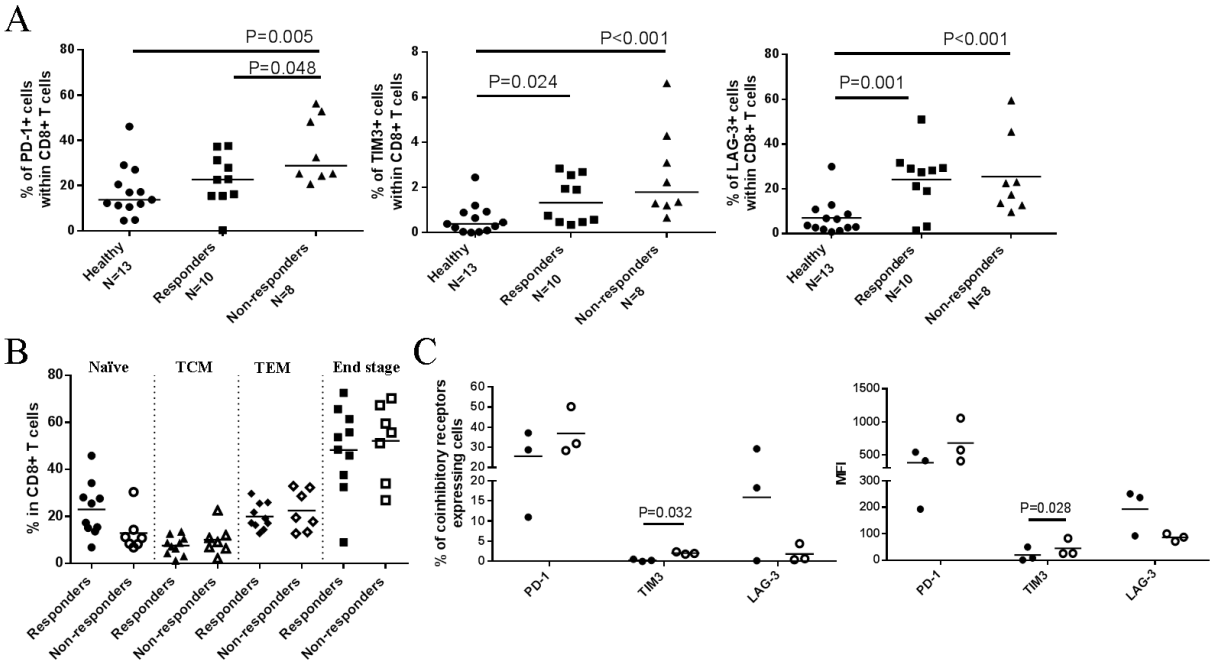


Figure 4. Expression of negative regulatory molecules in pancreatic cancer.

PBMC were isolated from pancreatic cancer (PC) patients and healthy controls, and surface stained to assess (A) the expression of PD1, TIM-3 and LAG-3 molecules; as well as (B) the relative proportions of naïve/memory subsets (based on the expression of CD45RO and CD62L), within the CD8+ T-cell population. Pancreatic cancer patients were stratified according to their ability to mount CEA691-specific CD8+ T-cell responses (responders vs. non-responders). Each symbol represents one individual and horizontal bars represent mean. P values <0.5 were considered statistically significant and are shown in the graphs. (C) T cells were obtained from matching samples of peripheral blood and lymph nodes from three pancreatic cancer patients. Graphs illustrate the levels of negative regulatory molecule expression within the CD8+ population, both in terms of frequency and number of molecules per cell, expressed as mean fluorescence intensity (MFI). Each symbol represents one individual and horizontal bars represent mean. P values <0.5 were considered statistically significant and are shown in the graph.

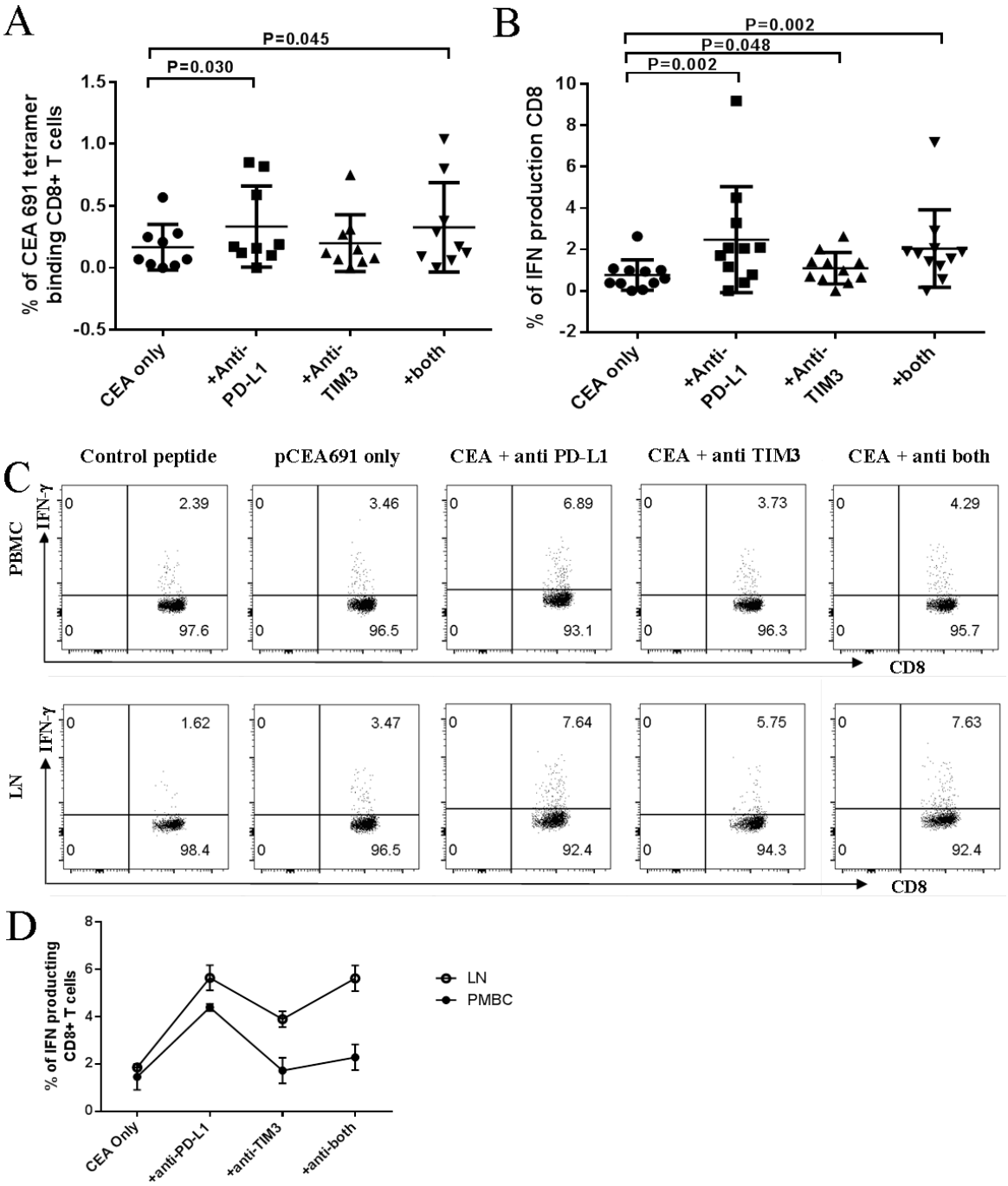


Figure 5. PD-1/PD-L1 and TIM-3 blockade in pancreatic cancer.

(A) The percentage of CEA tetramer binding CD8+ T cells after seven days of culture with CEA691 in the presence or absence of anti-PD-L1 and/or anti-TIM-3 blocking antibodies. (B) CEA691 CD8+ T cells from 11 pancreatic cancer (PC) patients were expanded for seven days with or without anti-PD-L1 and/or anti-TIM-3 blocking antibodies, and intracellular stained to assess the levels of IFN-γ production. The graph shows frequencies of IFN-γ+ cells within the

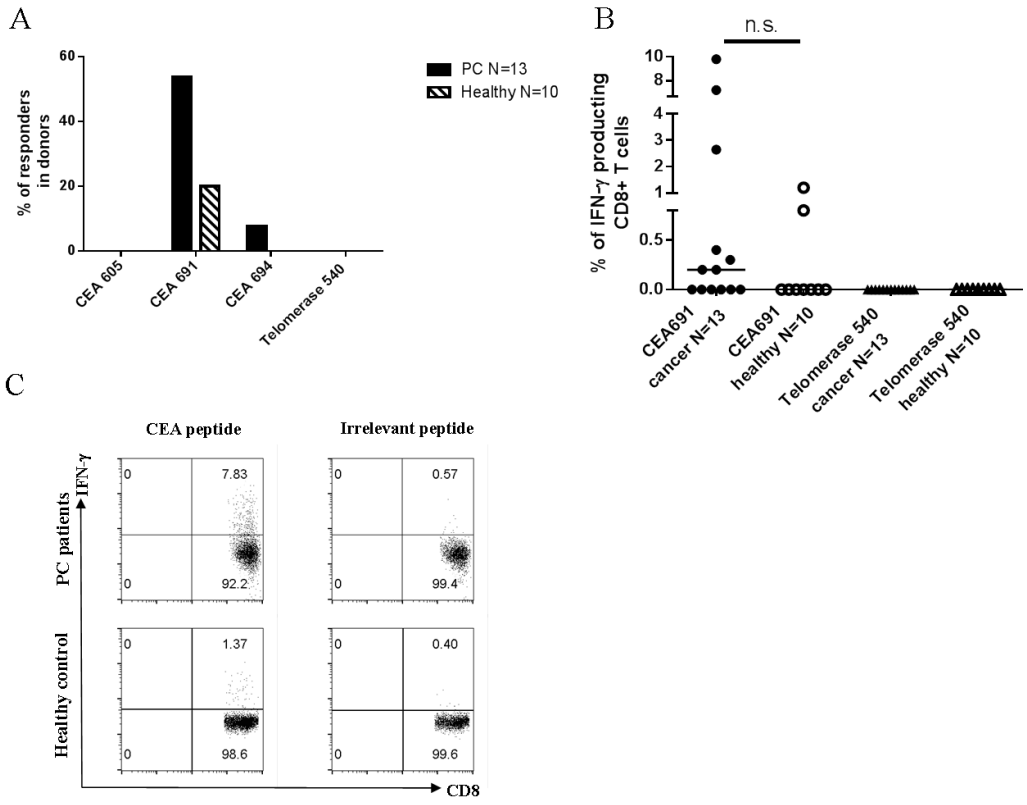
CD8+ T-cell population. Each symbol represents one individual and horizontal bars represent mean. P values <0.5 were considered statistically significant and are shown in the graph. PBMCs and MNCs isolated from lymph nodes of (CA13) were also stimulated for seven days by pCEA691 with or without anti-PD-L1 and/or anti-TIM-3 blocking antibodies, and assessed for levels of IFN-γ production. A representative example of IFN-γ production is shown in the dot-plots (C). (D) The graph shows the frequency of IFN-γ+ cells within the CD8+ T-cell population. Experiments are duplicated and symbols indicate mean and SD.

Supplemental information:

Supplemental Table S1 Patients demographic information for short term stimulation

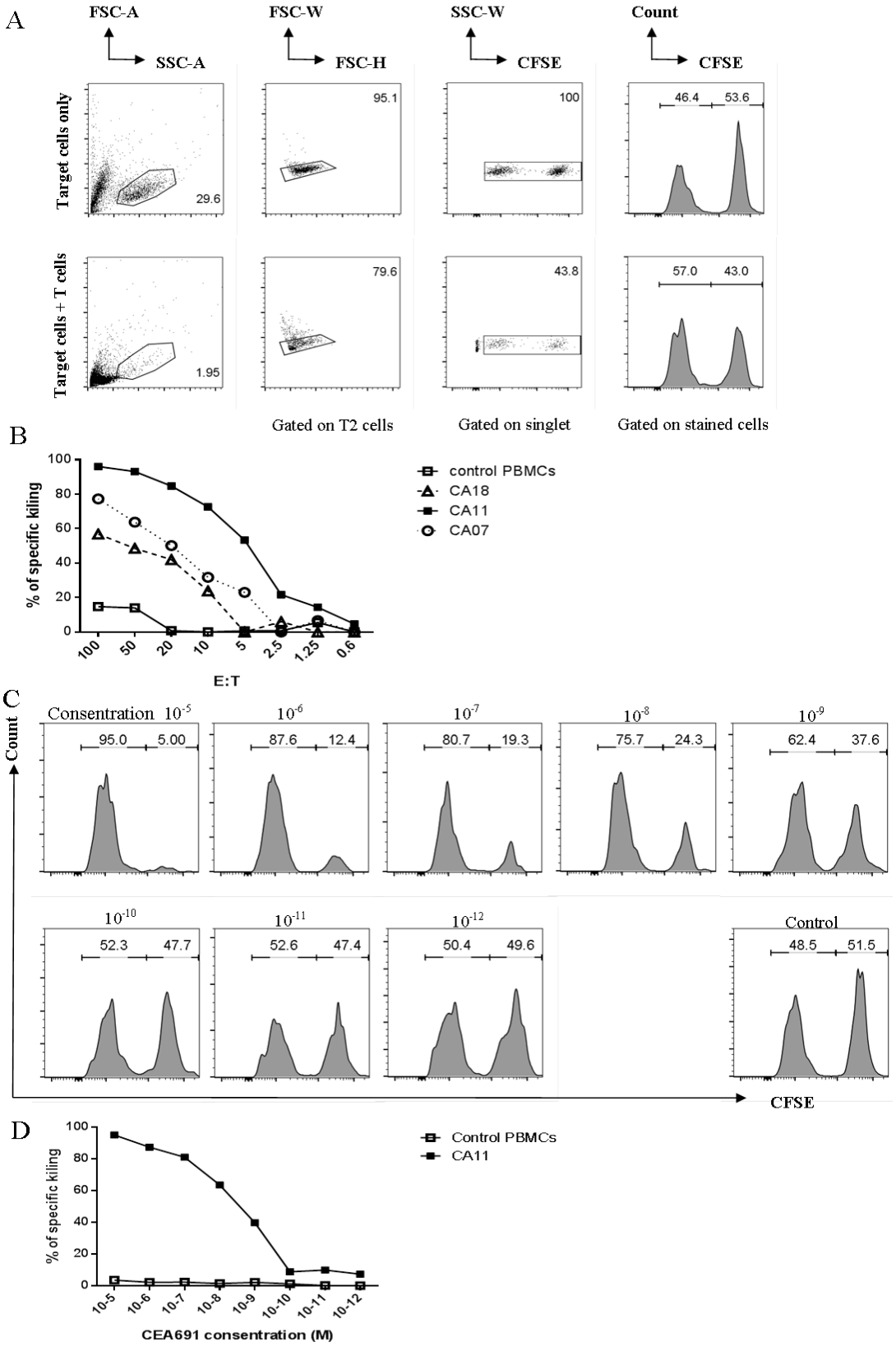
ID of PC patients	Age (year)	Gender	Histological diagnosis	Stage
PC01	63	F	Adenocarcinoma	IV
PC02	77	M	Adenocarcinoma with squamous differentiation	IV
PC03	47	M	PDAC	IIB
PC05	66	F	PDAC	n.a.
PC08	54	F	Poorly differentiated adenocarcinoma	IIB
PC10	71	F	Morderately differentiated AD	IIA
PC11	86	F	Adenocarcinoma	III
PC12	83	F	Adenocacinoma	IIA
PC14	60	F	Adenocarcinoma	IIB
PC16	74	M	PDAC	IV
PC17	78	M	PDAC	IV
PC20	76	M	PDAC	III
PC24	41	M	Poorly differentiated ampullarycancer	IV

Supplemental Figures:



Supplementary Figure 1. Antigen-specific CD8+ T-cell responses in pancreatic cancer patients.

PBMC isolated from 13 pancreatic cancer (PC) patients and 10 healthy controls were cultured for nine days in the presence of each one of a library of 3 different CEA-derived antigens. After stimulation, cells were harvested and re-challenged with either the respective peptides or irrelevant peptides, as controls, for 5 hours, in the presence of Brefeldin A. Intracellular staining was then performed to assess the levels of IFN-γ production by CD8+ T cells. Antigen-specific response was defined as a frequency of IFN-γ+ cells within the CD8+ T-cell population at least two-fold higher than that obtained upon re-challenge with an irrelevant peptide control. **(A)** Frequency of pancreatic cancer patients and healthy controls who were able to mount CEA-specific responses upon stimulation with different pancreatic CEA peptides (Responders). Bars indicate frequencies referring to either the number of pancreatic patients out of a total of 13, or the number of healthy controls out of a total of 10, who were found to be able to mount specific CD8+ T cell responses. **(B)** IFN-γ production by CD8+ T cells of pancreatic cancer patients and healthy controls after stimulation with either CEA691 or Telomerase540 (control peptide). Each symbol represents one individual and horizontal lines represent the median. **(C)** Representative dot-plots of CD8+ T-cell IFN-γ production upon stimulation with either CEA691 or Mucin12 in pancreatic cancer patients (top dot-plots) and healthy controls (bottom dot-plots). The irrelevant peptides used to address CEA691-specific responses were CEA605, respectively.



Supplementary Figure 2. CEA691-specific cytotoxic activity of CTL lines isolated from PC patients.

A. T cell line from CA11 were measured for their capability to recognise and kill T2 cells coated with 1mM (10-6 M) of CEA691, compared to control peptide, in 4-h CFSE cytotoxicity assay . **B.** The percentage specific cytotoxicity of CEA

691 loaded T2 cells (1mM peptide) at various E:T ratio, using CTL lines from 3 PC patients (CA07, 11, and 18). **C.** The cytotoxicity of T cells from CA11 against T2 cells coated with 10^{-5} to 10^{-12} M CEA691 peptide and control peptide, at E:T ratio 20:1 (left). The cytotoxicity curve was summarised on the bottom **(D).**