# Specific immune recognition of pancreatic carcinoma by patient-derived CD4 and CD8 T cells and its improvement by interferon-γ

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**Abstract.** Pancreatic carcinoma is a very aggressive disease and little is known about its immunobiology. We here describe the presence in pancreatic cancer patients of spontaneously induced functional CD4 and CD8 memory/effector T cells reactive to autologous tumor cells or to the pancreatic cancer associated antigen, MUC-1. Such specific cells were present in the bone marrow or peripheral blood of most of the 23 tested patients. Low dose stimulation of primary cultures of pancreatic cancer cells with 500 IU/ml IFN-γ for 72 h enhanced HLA-I expression and induced the *de novo* expression of HLA-II molecules. This led to a much better immune recognition by autologous HLA-I restricted and purified CD8 T cells and allowed tumor cell recognition by HLA-II restricted purified CD4 T-helper cells. Thus, interferon-γ appears to be a useful adjuvant cytokine to enhance the immunogenicity of a patients' tumor cells and their recognition by tumor reactive immune cells.

## Introduction

Pancreatic cancer is the 4th leading cause of cancer-related death in the United States (1). In Europe, pancreatic cancer is diagnosed more than 40,000 times a year (2). Once pancreatic cancer is clinically evident, it progresses fast. Approximately 80% of patients present with locally advanced or metastatic disease at diagnosis (3). Despite progress in surgical as well as non-surgical treatments, the prognosis remains poor with a 5-year survival rate of only 3-5% (4). New therapies are therefore required to improve patient prognosis.

Cellular immunotherapy has recently been studied in many cancer entities as a new adjuvant therapy. Because of its target-specific approach, it offers benefits with a limited range of side effects (5). Tumor-specific antigens which can potentially be recognized by T cells are expressed in some pancreatic cancers (6).

In many cancer patients, spontaneous T cell responses against cancer antigens lead to the generation of cancer antigen-specific CD4 and CD8 T cells. These can be isolated from lymphoid organs as well as from peripheral blood (7-9). In pancreatic cancer, such tumor antigen-specific T cells have scarcely been reported. Few studies involving small numbers of tested patients demonstrated the presence of tumor-reactive CTL-precursor cells by repetitive stimulations of T cells *in vitro* (10,11). Functional tumor antigen reactive effector/memory T cells from pancreatic cancer patients have not yet been demonstrated. If present, such specific T cells seem to be unable to infiltrate the patient's tumor and induce cancer regression. One major problem of immune recognition in solid cancers is a loss of immunogenicity due to functional alterations in the expression of major histocompatibility complex MHC molecules (12-14).

Two major classes of polymorphic antigens are encoded by the MHC: class I antigens (human HLA-A, B, and C) and class II antigens (human HLA-DR, DP, and DQ). While MHC class I are expressed in virtually all nucleated cells, MHC class II expression is mainly confined to antigen presenting cells, such as B cells, macrophages and dendritic cells (15) and absent from most cancer cells (16,17).

MHC expression can be modified by the class II cytokine, interferon-γ (23,24). For example, IFN-γ-induced *de novo* MHC class II expression was described in normal and malignant cells such as keratinocytes, human osteogenic sarcoma, colorectal carcinoma, and melanoma cell lines (25-27). Even though the mechanism and the therapeutic value of IFN-γ treatment is not clear, immune-modulatory effects were ascribed to

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modifications of antigen processing and antigen presentation (28-30). Furthermore, an improved T cell-mediated killing of pancreatic carcinoma cells was described after IFN-γ stimulation (31).

Little is known about the possible effects of IFN-γ treatment on MHC expression by pancreatic cancer cells. We tested *ex vivo* isolated T cells from pancreatic cancer patients for the presence of functional anti-tumor effector/memory CD4 and CD8 T cells using short-term Elispot assays and chromium-release cytotoxicity assays and analysed their functional efficiency against autologous viable tumor cells from established primary tumor cell cultures. We also characterized the effects of IFN-γ on expression of MHC molecules by primary pancreatic carcinoma cell cultures and used the patients own repertoire of cancer reactive CD4 and CD8 T cells to evaluate their recognition of and interaction with the cells.

## Patients and methods

*Patients*. The study protocol was approved by the Ethics Committee of the University of Heidelberg, Medical School. Informed consent was obtained from pancreatic cancer patients before the operation. Pieces of tumor tissue, blood and bone marrow samples were collected during operation under general anaesthesia and immediately processed. Patients were staged according to the UICC Classification (6th edition, 2002). The clinical characteristics of the cohort of 23 patients (P1-P23) are summarized in Table I.

*Primary tumor cell culture*. Immediately after resection, tumor samples were mechanically dissected into small pieces of ~1x1 $mm^3$ size and transferred into DMEM (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum and antibiotics (Penicillin/Streptomycin, PAA Laboratories, Pasching, Austria). Cell cultures were supplemented with fresh medium every 3 days. Before use, cultured tumor cells were extensively tested for mycoplasma contamination using a mycoplasma detection kit (Promega, Mannheim, Germany). To verify their tumorigenicity *in vivo*, five different primary cancer cell cultures were successfully transplanted into female 6-week old NOD/SCID mice with $1x10^7$ cells applied s.c. per mouse as previously described (35).

*Cell culture characterization*. After passage 5-10, cultured cells were consecutively characterized by immunohistochemistry to verify their pancreatic cancer cell origin. Cytospins were performed from each cell culture and stained for cytokeratins 7, 18, 19 and 20 (summarized in Table I and supplementary Table I); as well as for HLA-ABC and HLA-DP, DQ, DR surface molecules. The used primary mouse anti-human mAbs were anti-MHC I (W6/32), anti-MHC II (CR3/43), anti-cytokeratin 20 ($K_s$20.8), anti-cytokeratin 18 (DC10), anti-cytokeratin 7 (OV-TL12/30), anti-cytokeratin 19 (RCK108); and mouse $IgG_{2a}$/κ and $IgG_1$/κ as negative controls (Dako, Cambridge, UK). The ABC complex/alkaline phosphatase anti-alkaline phosphatase system (Dako, Glostrup, Denmark) was used for visualization as recommended by the manufacturer. Cytokeratins and MHC I and II surface molecule expression was assessed as the percentage of stained cells among the total number of cells per sight field at x40 magnification under light microscopy. MHC I and II expression was evaluated using a scale as described by Ramal *et al* (36). Flow cytometric analysis was performed with tumor cells to quantify expression levels of MHC molecules using mouse anti-human MHC class I (clone W6/32) and anti-MHC class II (clone CR3/43) followed by staining with secondary goat anti-mouse immunoglobulin-FITC or goat anti-mouse immunoglobulin-PE (Dako). For negative controls, samples were stained with secondary mAbs only.

*IFN-γ stimulation*. Ten different primary cultured cell lines were selected for IFN-γ stimulation. After passages 5-10, $5x10^4$ primary cultured neoplastic cells were stimulated with IFN-γ in 6-well plates with escalating doses of 20, 100, 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 U/ml of IFN-γ (Promokine, PromoCell Heidelberg, Germany) for 24 and 72 h. As negative control we co-incubated the neoplastic cells in medium alone. Cytospins and cell suspensions were prepared

Table I. Characteristics of patients and tumors.

| Ident. no. | Gender | Age at OP | Tumor | Lymph node | Metastasis | UICC |
|---|---|---|---|---|---|---|
| P1 | m | 62 | Body | No | Yes | IV |
| P2 | f | 77 | Body | Yes | No | II B |
| P3 | m | 52 | Head | Yes | No | II B |
| P4 | m | 71 | Head | Yes | No | II B |
| P5 | f | 58 | Body | Yes | No | II B |
| P6 | f | 66 | Head | No | No | II A |
| P7 | f | 64 | Body | Yes | Yes | IV |
| P8 | m | 71 | Body | No | No | II A |
| P9 | f | 59 | Body | Yes | Yes | IV |
| P10 | f | 65 | Head | Yes | No | III |
| P11 | f | 65 | Head | No | Yes | IV |
| P12 | m | 69 | Body | Yes | Yes | IV |
| P13 | m | 65 | Body | Yes | Yes | IV |
| P14 | m | 47 | Body | Yes | Yes | IV |
| P15 | m | 63 | Head | No | Yes | IV |
| P16 | m | 55 | Head | x | Yes | IV |
| P17 | f | 61 | Head | x | Yes | IV |
| P18 | f | 59 | Head | x | Yes | IV |
| P19 | m | 50 | Head | x | Yes | IV |
| P20 | m | 54 | Body | x | Yes | IV |
| P21 | m | 51 | Head | No | x | II A |
| P22 | m | 76 | Head | Yes | x | II B |
| P23 | m | 61 | Head | x | Yes | IV |
| P24 | m | 64 | Head | Yes | No | IIB |
| P25 | f | 72 | Tail | Yes | No | IIB |
| P26 | f | 65 | Tail | Yes | Yes | IV |

m, male; f, female; lymph node, tumor invasion into draining lymph nodes; x, not tested. Ident. no., identification number.

from the stimulated and unstimulated cells and characterized by immunocytochemistry or flow cytometry, respectively.

*Generation of dendritic cells and T cells*. Heparinized peripheral blood and bone marrow samples were collected perioperatively during general anaesthesia after informed consent by venipuncture and aspiration from the iliac crest as described (37) and the mononuclear cell population (MNCs) was obtained by Ficoll gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden). Generation of dendritic cells from $CD34^+$ precursor cells of the bone marrow was performed as described (38). Briefly, isolated NMCs from bone marrow were incubated for 14 days with X-Vivo 20 (Cambrex, Verviers, Belgium) supplemented with GM-CSF (50 ng/ml) and IL-4 (1000 U/ml). Obtained DCs were enriched by depletion of contaminating cells using magnetic beads labeled with mAbs against CD3, CD19 and CD56 (Dynal, Oslo, Norway). Alternatively, DCs were generated from peripheral blood samples after incubation of plastic adherent cells for 7 days with X-Vivo 20 supplemented with GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) as described (35). They were subsequently enriched by depletion of contaminating cells as described above. T lymphocytes were obtained from remaining MNCs and incubated in T cell medium (RPMI-1640 supplemented with 10% AB Serum, IL-4 60 U/ml and IL-2 100 U/ml) until use. DCs were pulsed with 200 $\mu$g/ml synthetic $MUC1p_{1\text{-}100}$ polypeptide (synthesized at DKFZ central peptide facility) or the same amount of human immunoglobulin (Venimm-N, Aventis, Stadt, Germany) as a source of negative control antigens overnight in X-Vivo 20 medium without cytokines. Afterwards, DCs were washed and used as APCs in Elispot assays. Enrichment of T cell populations was performed as follows: T cells were enriched by negative depletion of contaminating cells using magnetic beads coupled with mAbs against CD19, CD15, CD14 and CD56 (Dynal) according to the manufacturer's protocol; $CD4^+$ T cells and $CD8^+$ T cells were positively selected using magnetic beads labeled with the respective mAbs (MiniMACS, Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Prior to use in functional assays, T cells were cultured for 24 h in cytokine-free medium.

*Elispot assays*. DCs pulsed for 2 h with TAA or control antigen, pancreatic tumor cells or PBMCs (as negative control targets) were co-incubated for 40 h with $10^5$ autologous T cells or PBMCs per well at a ratio of 1:5 in Elispot plates. These were coated with mAbs against IFN-γ or IL-4 (clone 1-D1K and IL4-1, respectively, Mabtech, Nacka, Sweden) and analyzed for corresponding cytokine secretion according to the manufacturer's protocol. Spot-forming cells were quantified using KS Elispot reader (Zeiss, Jena, Germany) and CTL ImmunoSpot Analyzer and ImmunoSpot software (Cellular Technology, Cleveland, OH). Three to five wells per test group were quantified. Test samples were considered to contain TAA-reactive T cells when spot numbers significantly exceeded those of test wells as calculated by two-sided Student's t-test ($p<0.1$). In some assays, tumor cells were pre-treated for 1 h with 1 $\mu$g/ml mAbs against MHC I or II (Dako, Glostrup, Germany) to block TCR-MHC interactions during the Elispot assay. Prior to their co-culture with autologous T cells, excess mAbs were removed from tumor cells by repetitive washing.

Table II. MHC expression of primary pancreatic tumor cell cultures.

| Cell culture | MHC I | MHC II |
|---|---|---|
| P1 | +++ | - |
| P2 | +++ | - |
| P3 | +++ | - |
| P4 | - | - |
| P5 | - | - |
| P6 | +++ | - |
| P7 | +++ | - |
| P8 | +++ | - |
| P9 | +++ | - |
| P10 | +++ | - |
| P11 | +++ | - |
| P12 | +++ | - |
| P13 | +++ | - |
| P14 | +++ | - |
| P15 | +++ | - |
| P16 | +++ | - |
| P17 | +++ | - |
| P18 | +++ | - |
| P19 | +++ | - |

-, ≤ 25% positive tumor cells (negative/low MHC expression); +/-, >25% and ≤ 75% positive tumor cells (heterogeneous MHC expression); +++, >75% positive tumor cells (positive MHC expression).

*Cytotoxicity assays*. Cytotoxicity assays were performed as described (7). Briefly, purified T cells were stimulated with irradiated (100 Gy) autologous pancreatic tumor cells at a ratio of 2:1 for 3 days. Chromium-labelled autologous tumor cells and allogeneic control cell lines were used as targets.

## Results

*MHC I and II expression in primary pancreatic cancer cells*. Between January 2003 and August 2004, blood and tissue samples from 23 different pancreatic adenocarcinoma patients were selected. Table I shows the characteristics of the patients and their cancers. From the cancer samples of 19 patients, primary tumor cell cultures were successfully established. They showed a typical cobblestone-morphology and constant proliferation *in vitro*. Except for P4 and P5, all expressed cytokeratins (Table II and supplementary Table I) and high levels of MHC I (Table II). In contrast, MHC II was completely absent (14/19 cultures) or expressed on very few (1-10%) of the cancer cells (5/19) (Table II). Therefore, all cultures can be considered as MHC II negative according to the scale described by Ramal *et al* (36).

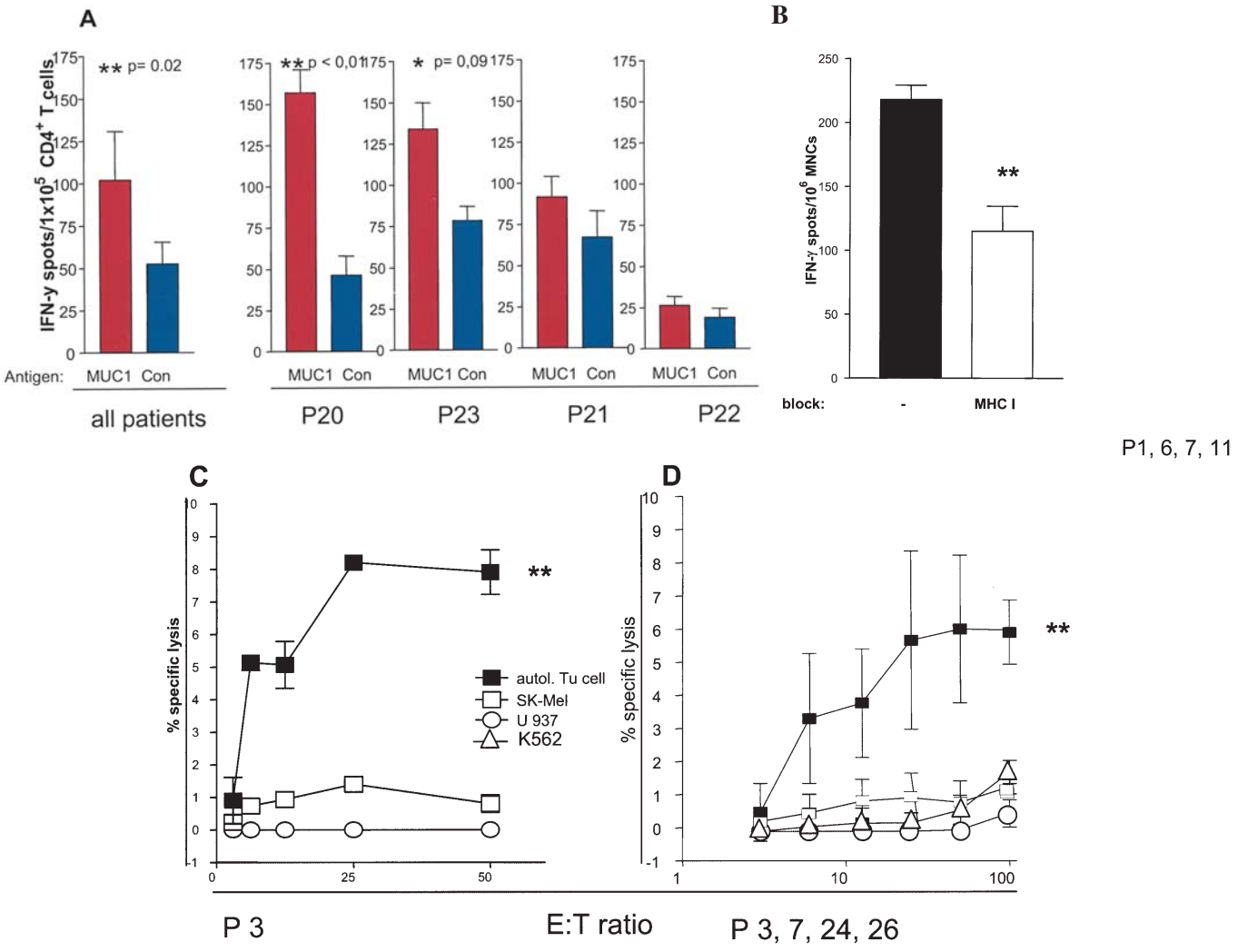



Figure 1. Presence of spontaneously induced tumor-reactive T cells in peripheral blood and bone marrow of pancreatic cancer patients. A, purified CD4 T cells from bone marrow of 4 pancreatic cancer patients (P20-23) were tested for reactivity against $MUC1p_{1-100}$ (red bars) or control antigen (human immunoglobulin; Venimmun, blue bars) in short-term IFN-γ Elispot assay. Mean values and SD of 3-5 wells per group for each sample and cumulative data of all tested samples (all patients) are depicted. Significant differences between test wells and corresponding control wells are depicted by asterisks ($^{*}p<0.1$, $^{**}p<0.05$). B, freshly isolated peripheral blood T cells from 4 pancreatic cancer patients (P1, 6, 7 and 11) were co-cultured with autologous cultured pancreatic tumor cells during 40-h short-term IFN-γ Elispot assay in the absence (black bar), or presence of anti-MHC I (white bar) mAbs. Asterisks depict a significant ($p<0.05$) reduction of spot counts in the presence of anti-MHC I mAbs. C and D, cytotoxic potential of spontaneously generated T cells was assessed in 4-h $^{51}$chromium release assays using purified T cells from peripheral blood of pancreatic cancer patients co-cultured for 3 days with autologous tumor cells and tested against autologous tumor cells (black squares) or the allogeneic irrelevant tumor cell lines SK-Mel (malignant melanoma, white squares), U937 (promonocytic leukemia, white circles) or the NK-sensitive target cell line K562 (white triangle). One original chromium-release assay of a representative patient (C) and means and SD of pooled values from 4 different patients (D) are shown. $^{**}$Significant difference between lysis of autologous tumor cells and lysis of control cell lines. E:T, effector to target ratio.

The two primary cancer cell cultures (P4, P5) which were negative for cytokeratins and MHC molecules were transplanted s.c. into immunodeficient NOD/SCID mice to evaluate if these cultures contained tumor cells. Both cultures induced a rapid outgrowth of human adenocarcinoma in the xeno-transplanted mice, revealing their true cancer cell origin (data not shown).

*Tumor-reactive CD8 and CD4 T cells are spontaneously induced in pancreatic cancer patients.* Recent studies demonstrated an enrichment of spontaneously induced tumor antigen reactive memory T cells in the bone marrow of breast cancer patients, recommending this organ for detection of spontaneous anti-tumor T cell responses (7,8,35). We therefore tested the bone marrow and peripheral blood of pancreatic cancer patients for the presence of tumor reactive CD4 T helper cells and CD8 cytolytic T lymphocytes (CTLs).

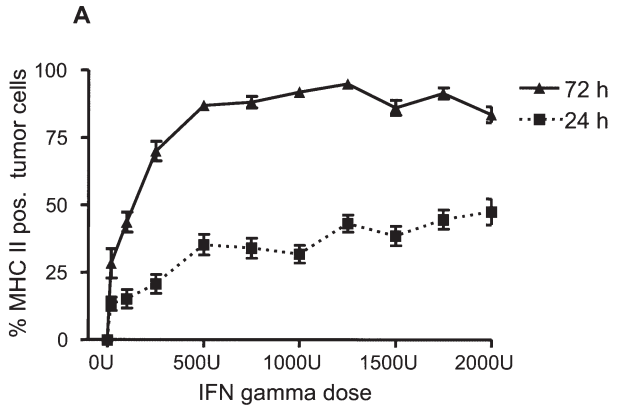
Firstly, we performed short-term IFN-γ Elispot assays, using tumor antigen pulsed autologous dendritic cells as APCs and purified bone marrow-derived CD4 T cells from 4 patients as effector cells. As tumor antigen, we used a synthetic 100mer peptide ($MUC1p_{1-100}$) from the signal sequence of the common cancer antigen, MUC1, which is overexpressed in over 90% of pancreatic cancers. Human immunoglobulin was used as control antigen. We detected a strong MUC1-specific CD4 T cell response in 2 patients whereas, in two other patients, the CD4 response against MUC1 was low (Fig. 1A). In total, the reactivity of freshly isolated CD4 T cells against $MUC1p_{1-100}$

was significant (p=0.02) compared to the control, suggesting the presence of spontaneously induced MUC1-specific CD4 effector cells in the bone marrow of pancreatic cancer patients.

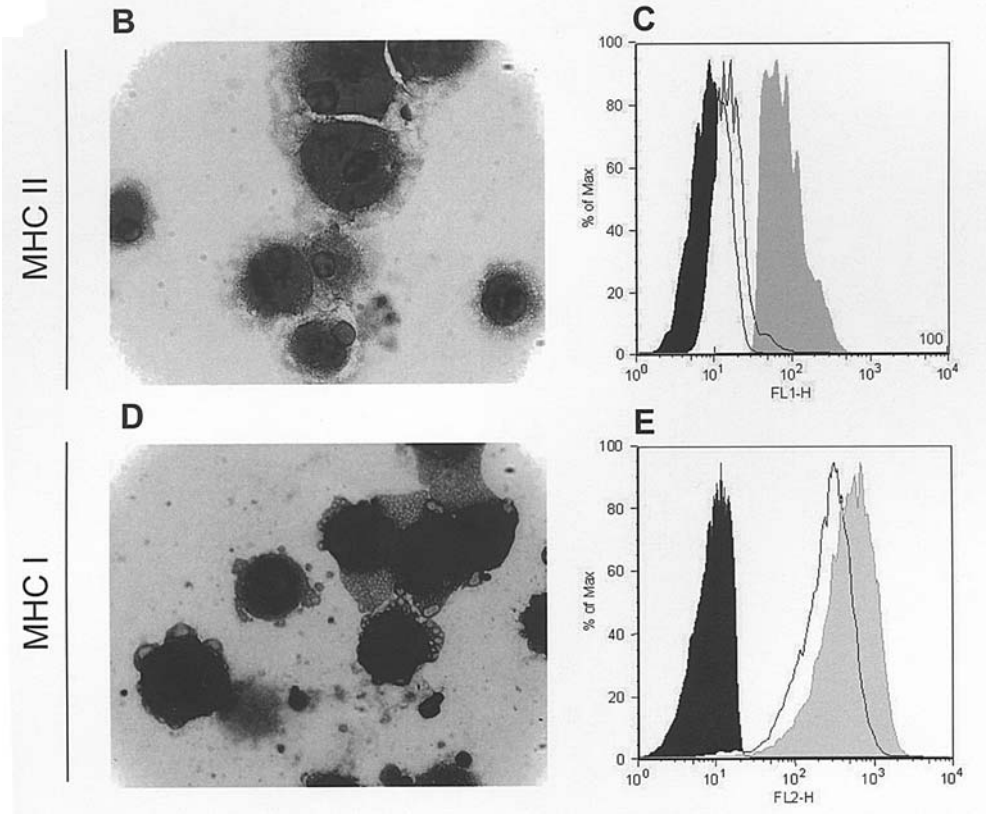
We next tested Elispot responses upon stimulation with live irradiated autologous tumor cells in the absence of APCs. We stimulated peripheral blood mononuclear cells (PBMC) from 4 patients with autologous cancer cells for 40 h in IFN-γ Elispot assays and absence or presence of blocking monoclonal antibodies (mAb) to MHC I molecules. Anti-MHC I mAbs caused partial inhibition of the responses (Fig. 1B), suggesting MHC I restricted recognition of autologous intact tumor cells by MHC I restricted T cells, most likely by CD8 T cells.

We therefore tested for the presence of functional cancer-specific CTL in the blood of 4 patients. We stimulated freshly isolated T cells for 72 h with autologous cancer cells and, directly afterwards, measured cancer cell-specific cytotoxicity in a 4-h $^{51}$chromium release assay, using autologous cancer cells as test targets and various unrelated cancer cell lines as control targets (Fig. 1C and D). We detected a low but significant autologous cancer cell lysis in all tested samples.

*IFN-γ augments MHC I/II expression in primary pancreatic cancer cells*. We next tested whether stimulation of primary pancreatic cancer cell cultures with low doses of IFN-γ might enhance their expression of MHC I and II molecules. Stimulation of primary tumor cell cultures with up to 2000 IU/ml IFN-γ for 24-72 h caused a dose- and time-dependent induction of MHC class II in all but 2 (P4, P5) of the tumor cell cultures (Fig. 2A-C). Low dose IFN-γ stimulation with 500 IU/ml for 72 h resulted in an upregulation of MHC class II in 90% of the cells in 17 of 19 cell cultures (Table III). Such low-dose IFN-γ stimulation also enhanced the expression level of MHC I in all tested cultures by a factor of 2 as shown by flow cytometry (Fig. 2D and E).




P1-3, 6




P16

Figure 2. Induction of MHC molecules on primary pancreatic tumor cell cultures by IFN-γ treatment. A, proportion of MHC II-positive tumor cells in primary pancreatic tumor cell cultures after treatment with various amounts of IFN-γ for 24 or 72 h. One representative out of xy experiments is shown. B-E, MHC II (B and C) or MHC I (D and E) expression of pancreatic tumor cell cultures after treatment with 500 U IFN-γ for 72 h as demonstrated by immunocytochemistry (B and D) or flow cytometry (C and E) using MHC II- or MHC I-specific mAbs, respectively. C and E, IFN-γ-stimulated tumor cells stained with isotype antibodies were used as negative control (black histogram), unstimulated tumor cells stained for MHC expression are depicted as white histograms and IFN-γ-stimulated tumor cells stained for MHC expression as shaded histograms.

Table III. MHC-expression of primary pancreatic tumor cell cultures after low dose IFN-γ stimulation.

| Cell culture | Without IFN-γ | | 500 U IFN-γ/ml/72 h | |
|---|---|---|---|---|
| | MHC I | MHC II | MHC I | MHC II |
| P1 | +++ | - | +++ | +++ |
| P2 | +++ | - | +++ | +++ |
| P3 | +++ | - | +++ | +/- |
| P4 | - | - | - | - |
| P5 | - | - | - | - |
| P6 | +++ | - | +++ | +++ |
| P7 | +++ | - | +++ | +++ |
| P8 | +++ | - | +++ | +/- |
| P9 | +++ | - | +++ | +/- |
| P10 | +++ | - | +++ | +++ |
| P11 | +++ | - | +++ | +/- |
| P12 | +++ | - | +++ | +/- |
| P13 | +++ | - | +++ | +++ |
| P14 | +++ | - | +++ | +++ |
| P15 | +++ | - | +++ | +++ |
| P16 | +++ | - | +++ | +++ |
| P17 | +++ | - | +++ | +++ |
| P18 | +++ | - | +++ | +++ |
| P19 | +++ | - | +++ | +++ |

-, ≤ 25% positive tumor cells (negative/low MHC expression); +/-, >25% and ≤ 75% positive tumor cells (heterogeneous MHC expression); +++, >75% positive tumor cells (positive MHC expression).

*IFN-γ stimulation of pancreatic cancer cells enhances their recognition by autologous cancer reactive CD4 and CD8 T cells.* To test whether the observed effects of IFN-γ stimulation on pancreatic cancer cells (upregulation of MHC I and II) might enhance the capacity of spontaneously induced autologous cancer-specific T cells to directly recognize their targets, we performed comparative short-term Elispot assays. Freshly isolated autologous peripheral blood derived T cells from five patients were tested for reactivity upon stimulation with IFN-γ pre-treated or not pre-treated autologous tumor cells. T cell stimulation was evaluated with regard to secretion of two cytokines, IFN-γ and IL-4, the latter being predominantly secreted by CD4 T helper cells. As shown in Fig. 3A, IFN-γ-treated cancer cells stimulated significantly more autologous patient-derived T cells than non-treated cancer cells. These results were consistent regarding IFN-γ (Fig. 3A) and IL-4 (Fig. 3B) secretion. T cell stimulation was MHC-restricted, since it could be nearly completely blocked by specific mAbs against MHC I and II (Fig. 3B), and tumor antigen specific, since T cells co-cultured with IFN-γ-stimulated autologous PBMCs were not activated (Fig. 3C). Selective blockage of MHC I molecules in this experiment caused a strong reduction of IFN-γ secreting cells in 4 out of 5 tested patients (Fig. 4A). Interestingly, IFN-γ as well as IL-4 secretion was inhibited to a similar extent upon selective blocking of MHC II (Fig. 4B), suggesting the possibility of direct recognition by CD4 T cells of IFN-γ-treated tumor cells (in case of IL-4 secretion) as well as a role of CD4 T cells in stimulating CD8 T cells.

To verify direct tumor cell recognition by CD4 T cells, we co-cultured purified CD4 T cells with IFN-γ-stimulated autologous cancer cells in a 40-h IL-4 Elispot assay. This revealed significant induction of IL-4 secretion in all tested patients when compared to non-stimulated tumor cells as controls. The responses could be blocked by MHC II-specific antibodies (Fig. 5A). Thus, CD4 T cells are capable to recognize MHC II expressing pancreatic cancer cells via their T cell receptor. In contrast to CD4 T cells, purified CD8 T cells showed a lower potential to respond to autologous cancer cells: significant MHC I restricted induction of IFN-γ was detected in only 2 out of 4 patients, pointing to a potential need of CD4 T cell help for adequate stimulation of cancer-specific CD8 T cells (Fig. 5B).

We also tested whether IFN-γ pre-treatment of cancer cells could enhance T cell-mediated cytotoxicity. We stimulated T cells from 3 pancreatic cancer patients for 3 days with autologous IFN-γ pre-treated cancer cells and tested them afterwards in a 4-h $^{51}$chromium release assay with IFN-γ-treated or untreated autologous cancer cells as targets. As shown in Fig. 5C a marked increase of cancer-specific cytotoxicity was observed when target cells had been pre-treated with IFN-γ.

## Discussion

Immunotherapies for pancreatic cancer are potentially limited by tumor immune-escape mechanisms that may prevent immune activation, effector function and cancer cell recognition (39). These mechanisms comprise the secretion of immunosuppressive cytokines, such as TGF-ß and IL-10 in the cancer stroma (39), upregulation of Fas-ligand as a means of tumor counter-attack (40) and, most importantly, down-regulation of MHC expression (41). Therefore, strategies to alter the adverse micro-environment of pancreatic cancers with regard to dominant cytokines and expression of MHC molecules appear necessary to improve future immunotherapeutic approaches, such as vaccination protocols.

In the past, systemic cytokines, such as IL-2, were administered systemically for the treatment of various types of cancer in order to enhance or induce spontaneous cancer-specific T cell responses (42). IFN-γ was used for the treatment of melanoma and ovarian cancer (43,44). *In vitro* studies with a variety of cancers demonstrated the ability of IFN-γ to induce MHC class I upregulation, MHC class II expression and generation of the immunoproteasome that allows presentation of a broader range of antigenic peptides by cancer cells (44). IFN-γ induces pro-inflammatory type 1 macrophages and, as the main TH1 effector cytokine, inhibits TH2 responses (45). Thus, IFN-γ appears to be a potential candidate to shift the balance between immune-suppressive and immune-stimulatory effects in the cancer microenvironment.

We report for the first time that treatment of primary pancreatic adenocarcinoma cell cultures with low doses of IFN-γ causes an upregulation of MHC I and induces *de novo*

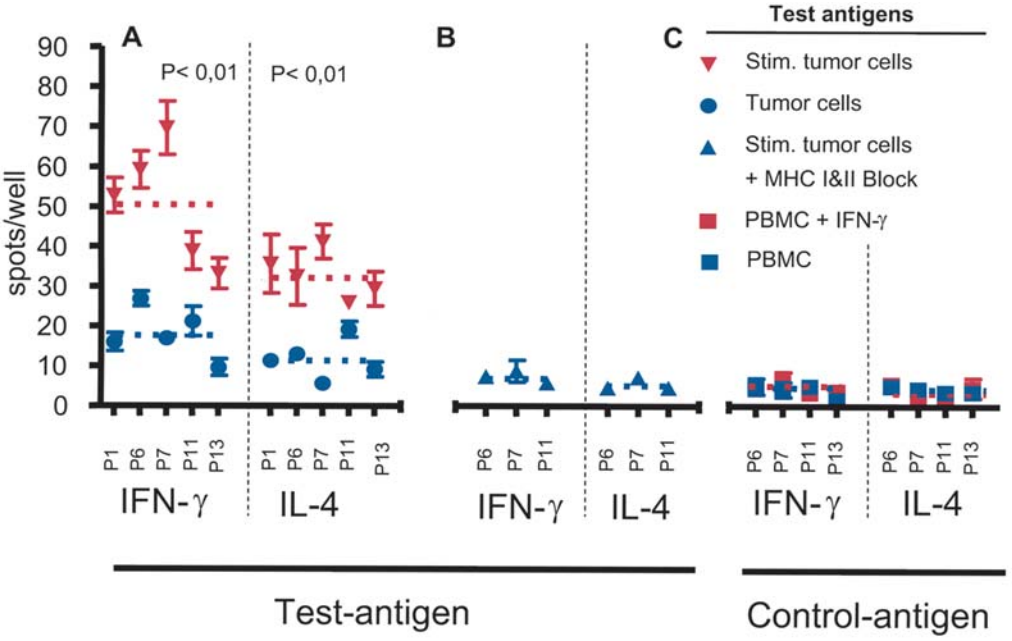



Figure 3. Enhanced immunogenicity of IFN-γ-stimulated pancreatic tumor cells. Purified patient-derived peripheral blood T cells were co-incubated in 40-h IFN-γ or IL-4 Elispot assays with autologous, low dose (500 U/72 h) IFN-γ-treated tumor cells (A, red inverted triangles), non-stimulated tumor cells (A, blue circles), low dose IFN-γ-stimulated tumor cells blocked with anti-MHC I and MHC II mAbs (B, blue triangles) or, as control target cells, with low dose IFN-γ-stimulated autologous mononuclear cells (MNC) (C, red squares) or unstimulated autologous MNC (C, blue squares) at a ratio of 5:1. Mean spots and SD per triplicate wells are depicted. P-values indicate the overall difference between Elispot reactivities against stimulated and unstimulated tumor cells.

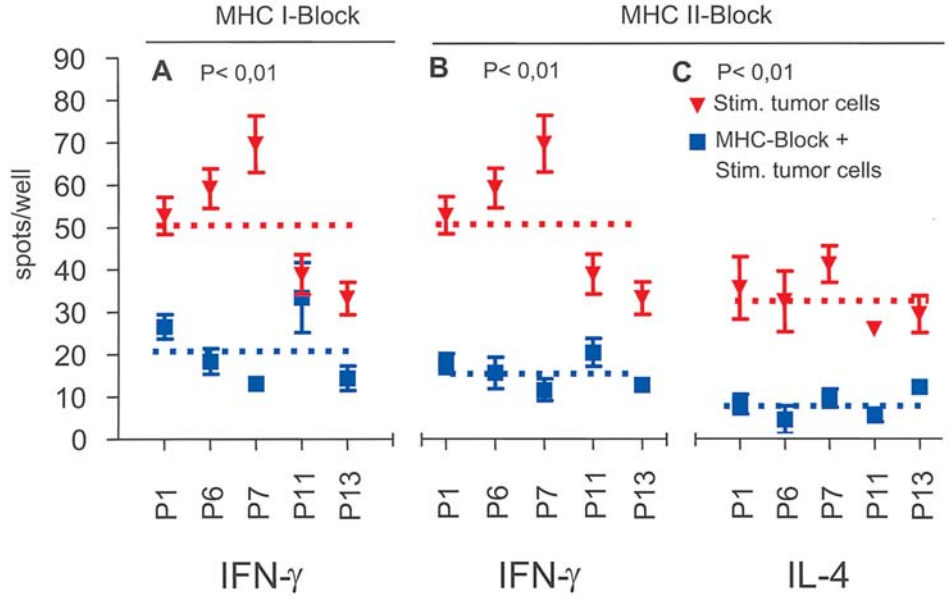
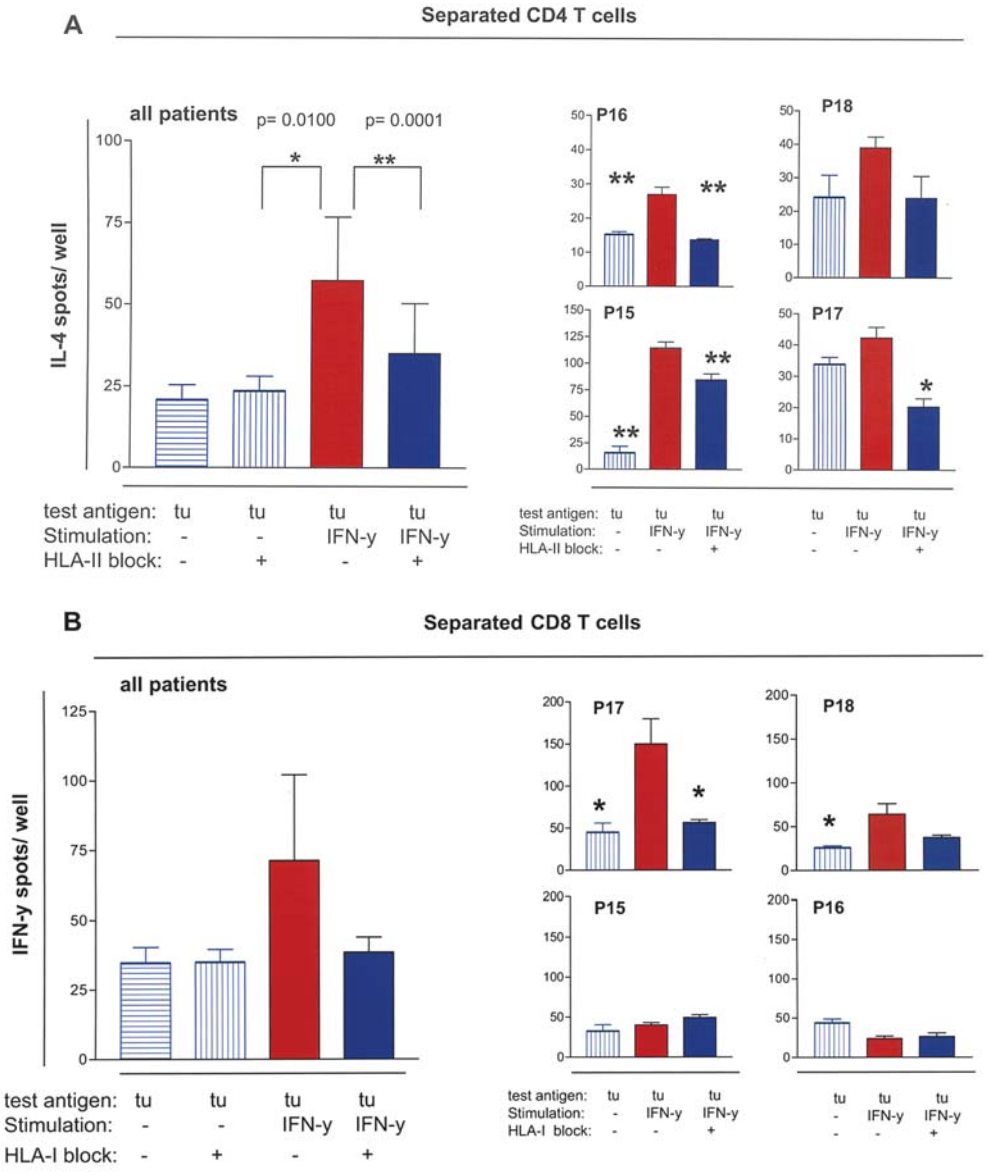



Figure 4. MHC restriction of T cell responses against IFN-γ-stimulated pancreatic tumor cells. Purified patient-derived peripheral blood T cells were co-incubated in 40-h IFN-γ (A and B) or IL-4 (C) Elispot assays with autologous, low dose (500 U/72 h) IFN-γ-treated tumor cells blocked with anti-MHC I (A, blue squares), anti-MHC II (B and C, blue squares) or without MHC blocking (A-C, red inverted triangles) at a ratio of 5:1. Mean spot numbers and SD per triplicate wells are shown. P-values indicate the overall difference between Elispot reactivities against MHC blocked and unblocked tumor cells.

expression of MHC II molecules. Our work corroborates the observations of Scupoli *et al* on 3 commercial pancreatic adenocarcinoma cell lines (41). Presentation of major histocompatibility complex (MHC) class I-restricted epitopes involves processing by cytosolic proteasomes, whereas MHC class II-restricted epitopes are generated by endosomal proteases. In addition, recent studies demonstrated generation and presentation of MHC class II-restricted epitopes by proteasome- and TAP-dependent and -independent pathways in antigen presenting cells and in MHC II expressing cancer cell lines, indicating the possibility that cytosolic antigens can also be presented via MHC II (18,19).

A major aim of MHC class II transduction of cancer cells for vaccination purposes is direct cancer cell recognition by tumor antigen-specific CD4 T cells. We demonstrate for the first time the presence in the peripheral blood of pancreatic



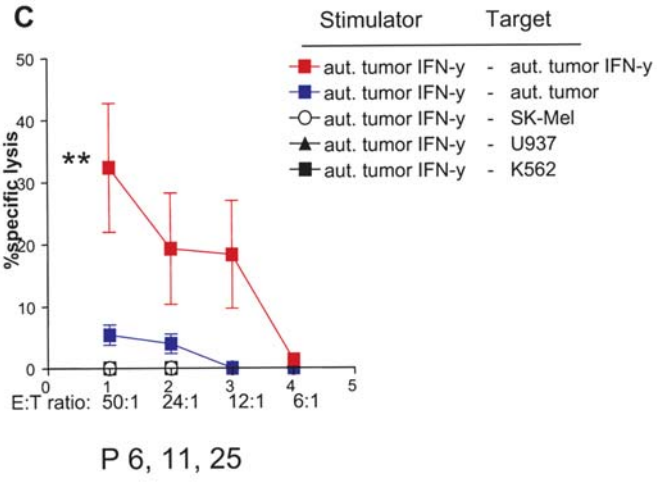




Figure 5. Direct recognition by purified patient-derived CD4 and CD8 T cells of IFN-γ-stimulated autologous pancreatic cancer cells. A, purified peripheral blood CD4 T cells from 4 pancreatic cancer patients were incubated during 40-h IFN-γ Elispot assay together with autologous tumor cells which were treated with low-dose (500 U/72 h) IFN-γ (red and full blue bars) and/or anti-MHC II mAb (full blue and vertically striped bars) or left untreated (horizontally striped bars) at a ratio of 5:1. Mean spot numbers and SD per triplicate wells are shown for accumulative data of all 4 patients (all patients) and for each tested patient separately. Respective control groups significantly differ from test groups (red bars) with $^{**}p<0.05$ or $^{*}p<0.1$. B, purified peripheral blood CD8 T cells from 4 pancreatic cancer patients were incubated during 40-h IFN-γ Elispot assay together with autologous tumor cells which were treated with low-dose (500 U/72 h) IFN-γ (red and full blue bars) and/or anti-MHC I mAb (full blue and vertically striped bars) or left untreated (horizontally striped bars) at a ratio of 5:1. Mean spot numbers and SD per triplicate wells are shown for accumulative data of all 4 patients (all patients) and for each tested patient separately. Respective control groups differ from test groups (red bars) with $^{*}p<0.1$. C, cytotoxic potential of spontaneously generated T cells assessed in 4-h $^{51}$chromium release assays using purified T cells from peripheral blood of cancer patients co-cultured for 3 days with autologous, IFN-γ-stimulated tumor cells and tested against IFN-γ-stimulated (red squares) or non-stimulated (blue squares) autologous tumor cells or the allogeneic irrelevant tumor cell lines, SK-Mel (malignant melanoma, white squares) or U937 (promonocytic leukemia, black triangles), or the NK-sensitive target cell line, K562 (black squares). Cummulative means and SD of 4 chromium-release assays from 3 different patients are shown. $^{**}$Significant difference between lysis of autologous, IFN-γ-stimulated and unstimulated autologous tumor cells ($p<0.05$). E:T, effector to target ratio.

cancer patients of spontaneously induced, functional CD4 T helper cells specific for epitopes of the well-defined pancreatic cancer associated antigen, MUC1. These cells were able to recognize autologous cancer cells when the latter had been treated with IFN-γ. The recognition required IFN-γ mediated MHC II expression, since it was blocked by MHC II specific antibodies. Similarly, IFN-γ treatment enhanced MHC I restricted recognition of tumor cells by CD8 T cells as demonstrated by blocking with MHC I-specific antibodies. IFN-γ exposure caused a substantial increase of cancer cell directed effector function, such as IFN-γ secretion and cancer cell lysis. Interestingly, full activation of CD8 T cells also required to a considerable extent the simultaneous activation of CD4 T cells, since it could be reverted by antibody mediated MHC II blocking. Such requirement of direct T cell help at the site of CD8 effector function has previously been demonstrated in a mouse lymphoma model (46).

While transduction of cancer cells with MHC II molecules is therapeutically limited to cancer cell vaccinations, systemic or local administration of low dose IFN-γ could be suitable to substantially enhance the immunogenicity of primary cancer or metastases. Through upregulation of MHC I and II expression and through activation of the immunoproteasome, IFN-γ affects the tumor cell's immunogenicity, immunosensitivity and its microenvironment. Such changes might be suitable to enhance the activity of spontaneous cancer reactive T cells and to support the effectiveness of additional vaccination protocols.

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Supplementary Table I. Cytokeratin-expression of primary pancreatic tumor cell cultures.

| Cell culture | CK 7 | CK 18 | CK 19 | CK 20 |
|---|---|---|---|---|
| P1 | ++ | +++ | + | - |
| P2 | ++ | ++ | + | + |
| P3 | +++ | +++ | ++ | + |
| P4 | - | - | - | - |
| P5 | - | - | - | - |
| P6 | +++ | +++ | ++ | + |
| P7 | +++ | +++ | ++ | + |
| P8 | +++ | +++ | + | + |
| P9 | +++ | +++ | + | - |
| P10 | + | +++ | ++ | - |
| P11 | ++ | +++ | ++ | + |
| P12 | ++ | +++ | + | + |
| P13 | +++ | +++ | +++ | - |
| P14 | +++ | +++ | +++ | +++ |
| P15 | ++ | +++ | + | - |
| P16 | +++ | +++ | ++ | - |
| P17 | +++ | +++ | + | + |
| P18 | +++ | +++ | + | - |
| P19 | ++ | +++ | ++ | - |

-, ≤ 1% positive tumor cell (no cytokeratin expression); +, >1% and ≤ 25% positive tumor cells (faint cytokeratin expression); ++, >25% and ≤ 50% positive tumor cells (moderate cytokeratin expression); +++, >50% positive tumor cells (strong cytokeratin expression).