

Intra-tumoral IFN- γ -producing Th22 cells correlate with TNM staging and the worst outcomes in pancreatic cancer

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Abstract

PDAC (pancreatic ductal adenocarcinoma) is the fifth leading cause of cancer-related death. The causes of this cancer remain unknown, but increasing evidence indicates a key role of the host immune response and cytokines in human carcinogenesis. Intra-tumoral IL (interleukin)-22 levels have been shown to be elevated in PDAC patients. However, little is known regarding the expression and clinical relevance of Th22 cells in human PDAC and, furthermore, which TILs (tumour-infiltrating lymphocytes) are the main producers of IL-22 is unknown. In the present study, we characterized the functional properties of the different subsets of IL-22-producing TILs and analysed their relationship with the TNM staging system and patient survival. We have demonstrated for the first time that, in PDAC patients, the T-cells co-producing IFN- γ (interferon γ) and exerting perforin-mediated cytotoxicity are the major intra-tumoral source of IL-22. In addition, isolated Th22 cells were able to induce apoptosis, which was antagonized by IL-22. Finally, we observed that the IL-22-producing T-cells were significantly increased in tumour tissue and that this increase was positively correlated with TNM staging of PDAC and poorer patient survival. These novel findings support the dual role of the anti-tumour immune system and that IL-22-producing cells may participate in PDAC pathogenesis. Therefore monitoring Th22 levels could be a good diagnostic parameter, and blocking IL-22 signalling may represent a viable method for anti-PDAC therapies.

Key words: IFN- γ , IL-22, pancreatic cancer, Th22, tumour-infiltrating lymphocytes.

INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer-related death in the developed world, with more than 260000 annual deaths worldwide [1] and with a dismal 5-year survival (5%). The lethality of pancreatic cancer is due to its aggressive nature and its tendency to remain asymptomatic until the tumour reaches an advanced stage, limiting the likelihood of early diagnosis. At the

time of initial presentation, most pancreatic cancers are locally advanced or metastatic and need multimodal therapy [2], hence the need to find factors that may facilitate earlier diagnosis and improve prognosis of this neoplasia.

Pancreatic cancer originates in the ductal epithelium and evolves from a pre-malignant lesion to fully invasive cancer. The causes of pancreatic cancer remain unknown, although some studies have shown that chronic inflammation is a common

Abbreviations: BFA, brefeldin A; FoxP3, forkhead box P3; IFN- γ , interferon γ ; IL, interleukin; IL-10R2, IL-10 receptor 2; IL-22R, IL-22 receptor; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death 1; PDAC, pancreatic ductal adenocarcinoma; Tcc, T-cell clone(s); Th, T helper; TIL, tumour-infiltrating lymphocyte.

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feature of actively growing pancreatic tumours [3]. Moreover, increasing evidence has shown that the host immune response and related inflammatory and immune mediators, such as cytokines, participate in human carcinogenesis [4,5]. IL (interleukin)-22, which belongs to the IL-10 cytokine family, is a T-cell-derived cytokine that mediates epithelial immunity and mucosal tissue repair [6]. IL-22 triggers intracellular signalling by binding to a heterodimeric receptor complex that is composed of IL-22R1 (IL-22 receptor 1) and IL-10R2 (IL-10 receptor 2) [7]. However, the cellular responsiveness of IL-22 is mainly determined by IL-22R1, which is selectively expressed on non-immune cells [8], such as hepatocytes [9], keratinocytes [10], colonic epithelial cells [11] and, particularly, pancreatic cells [12,13].

Principal sources of IL-22 include CD4⁺ Th (T helper) cells, CD8⁺ CTLs (cytotoxic T-cells), $\gamma\delta$ T-cells and ILCs (innate lymphoid cells), encompassing NK (natural killer) cells, LTi (lymphoid tissue inducer)-like cells [14,15] and NKT (natural killer T) cells [16]. Although several IL-22-producing CD4⁺ T-cells also produce other cytokines, such as IL-17 or IFN- γ (interferon γ) [17], some, only secrete IL-22 [18]. These CD4⁺ T-cells that exclusively secrete IL-22 are known as Th22 cells and have been recently identified as the main producers of IL-22 in the peripheral blood [19,20]. Th22 cells play important roles in many inflammatory diseases, such as psoriasis [21], rheumatoid arthritis [22] and multiple sclerosis [23]. Moreover, IL-22 plays a pro-tumour role in cancers of the gastrointestinal tract. Gastric tumours show increased infiltration of Th22, which correlates with tumour stage and poor prognosis [24]. In addition, compared with healthy controls, gastric cancer patients also have higher circulating frequencies of IL-17- and IL-22-producing T-cells, which are positively associated with cancer stage and negatively with patient survival [25]. In colorectal cancer, levels of both IL-17 and IL-22 are positively correlated with the tumour stage [26], and serum IL-22 is elevated in chemoresistant patients [27]. Moreover, IL-22 administration to human colorectal cancer cell lines renders them chemoresistant [27]. In pancreatic cancer, intra-tumoral IL-22 levels and frequency of Th22 cells are elevated compared with the peripheral blood of patients and healthy donors [28]. Similarly, expression of both IL-22 and IL-22R1 is elevated in tissue sections of PDAC (pancreatic ductal adenocarcinoma) tissue [29]. However, little is known regarding the expression and clinical relevance of Th22 in pancreatic cancer and, furthermore, which TILs (tumour-infiltrating lymphocytes) are the main producers of IL-22 is unknown. In the present study, we have investigated the role of IL-22-producing T-cells during PDAC progression. In detail, we have characterized the functional properties of the different subsets of IL-22-producing CD4⁺ and CD8⁺ Tcc (T-cell clones) generated from lymphocytes that infiltrate PDAC tissue, and we analysed their relationship with the TNM staging system and patient survival.

MATERIALS AND METHODS

Patients

Tumours and corresponding adjacent tissues were obtained from 30 PDAC patients (14 males and 16 females), who were classified

according to the TNM classification for pancreatic tumours [30]. The mean age of the PDAC patients was 66 years (range 36–92 years). The clinical characteristics of patients are summarized in Table 1. All patients underwent surgical resection of the primary lesion, but did not receive chemotherapy. Patients with evidence of serious illness, immunosuppression, or autoimmune or infectious diseases were excluded. The study was approved by the local Ethical Committees (Azienda Ospedaliera Universitaria Careggi) and informed consent was obtained from all patients.

Generation of Tcc from TILs of the neoplastic pancreatic tissue

T-cell cultures were performed in RPMI 1640 culture medium (SERO-Med) supplemented with 10% (v/v) HyCloneTM FBS (Gibco Laboratories) and recombinant human IL-2 (EuroCetus). Surgical specimens of PDAC tissue were cultured for 7 days in RPMI 1640 medium supplemented with IL-2 (50 units/ml), in order to expand *in vivo*-activated TILs; in detail, tissue pieces from each patient were obtained from two different sites, namely tumour and surrounding healthy mucosa. Each specimen was then disrupted, and single T-cell blasts were cloned under limiting dilution, as described previously [31]. Briefly, single T-cell blasts were seeded in microwells (0.3 cell/well) in the presence of 2×10^5 irradiated (9000 rad) PBMCs (peripheral blood mononuclear cells), PHA (phytohaemagglutinin) (0.5%) and IL-2 (50 units/ml). At weekly intervals, 2×10^5 irradiated PBMCs and IL-2 were added to each micro-culture to maintain the expansion of growing clones. The Tcc were evaluated for their surface markers and functional properties, namely cytokine profile, B-cell helper activity and cell cytotoxicity.

Analysis of cell-surface markers and evaluation of the cytokine profile of isolated Tcc

Fluorochrome-conjugated anti-CD3, -CD4, -CD8 and isotype-matched control mAbs (monoclonal antibodies) were purchased from BD Biosciences. We analysed surface marker (CD3, CD4 and CD8) expression in blasts of single Tcc by flow cytometry, as described previously [31]. To evaluate the amount of secreted cytokines, T-cell blasts of single Tcc were resuspended at a concentration of 10^6 cells/ml of medium and cultured for 36 h in the presence of PMA (10 ng/ml) plus ionomycin (200 ng/ml). Cell-free supernatants were collected and assayed in duplicate for IFN- γ , IL-4, IL-17 and IL-22 content using commercial ELISA kits (Bio-Source International). Supernatants presenting cytokine levels that were 5 S.D. above the mean levels of control supernatants derived from irradiated APCs (antigen-presenting cells) alone were regarded as positive. On the basis of the evaluation of the cytokine profile and the CD4/CD8 expression, we divided the clones into the following groups: Th22/Tc22 (only IL-22), Th22Th1/Tc22Tc1 (IL-22+IFN- γ), Th22Th2/Tc22Tc2 (IL22+IL-4), and Th22Th17/Tc22Tc17 (IL-22+IL-17).

B-cell helper activity

B-cells were prepared using the B-cell isolation kit II (Miltenyi Biotec). A total of 2×10^5 B-cells were co-cultured with 10^5

Table 1 Clinical parameters of patients with pancreatic cancer and Tcc isolated from cancer tissue

Code	Age	Sex	TNM	Tumour size (cm)	Metastasis	Overall Survival (months)	Tcc obtained	IL-22-producing Tcc (%)
01.PDap	50	Male	pT1, N1, Mx	>3	–	11	84	12 (14)
02.PDap	73	Male	pT2, N1, Mx	>3	–	13	26	4 (15)
03.PDap	56	Male	pT3, N0, Mx	>3	++	8	6	4 (67)
04.PDap	36	Male	pT4, N1, M1	≤3	++	7	16	10 (63)
05.PDap	74	Female	pT3, N1, Mx	>3	++	9	91	55 (60)
06.PDap	61	Female	pT4, N1, Mx	>3	++	8	20	10 (50)
07.PDap	63	Male	pT1, N1, Mx	>3	–	12	37	8 (22)
08.PDap	74	Female	pT4, N0, Mx	>3	++	8	74	44 (59)
09.PDap	54	Female	pT2, N0, Mx	>3	–	14	5	1 (20)
10.PDap	92	Male	pT3, N1, Mx	≤3	++	9	3	2 (67)
11.PDap	57	Male	pT2, N1, M1	≤3	+	10	22	8 (36)
12.PDap	51	Female	pT1, N1, Mx	>3	–	11	13	2 (15)
13.PDap	72	Female	pT4, N1, Mx	>3	++	8	22	15 (68)
14.PDap	80	Female	pT2, N1, M1	≤3	–	12	58	11 (19)
15.PDap	58	Male	pT1, N0, Mx	≤3	+	13	28	9 (32)
16.PDap	55	Female	pT1, N0, Mx	≤3	–	13	11	2 (18)
17.PDap	70	Female	pT2, N1, Mx	>3	+	11	32	8 (25)
18.PDap	62	Female	pT3, N0, Mx	>3	++	8	24	14 (58)
19.PDap	42	Female	pT4, N1, M1	>3	++	9	10	6 (60)
20.PDap	58	Male	pT1, N0, Mx	>3	–	11	52	13 (25)
21.PDap	74	Male	pT4, N1, Mx	>3	++	8	31	25 (81)
22.PDap	79	Female	pT4, N1, Mx	≤3	+	10	28	14 (50)
23.PDap	83	Male	pT4, N0, Mx	>3	++	8	22	15 (68)
24.PDap	62	Male	pT1, N0, Mx	>3	–	12	12	1 (8)
25.PDap	85	Female	pT1, N0, Mx	≤3	+	13	8	3 (38)
26.PDap	76	Male	pT2, N1, M1	≤3	+	12	18	8 (44)
27.PDap	71	Male	pT1, N1, Mx	≤3	+	11	7	2 (29)
28.PDap	63	Female	pT3, N1, Mx	>3	++	8	11	7 (64)
29.PDap	66	Female	pT2, N1, M1	≤3	–	14	32	8 (25)
30.PDap	72	Female	pT4, N1, Mx	≤3	+	8	4	2 (50)
All PDAC patients	66	14 male/16 female	Pancreatic ductal adenocarcinoma			10.3	807	323 (40)

blasts of the different Th22/Tc22 Tcc subsets and stimulated with 100 ng/ml anti-CD3 mAb. On day 7, supernatants from each cell culture were collected and evaluated by ELISA for IgM, IgG, IgA and IgE content.

Flow cytometric detection of granzyme A and evaluation of cell cytotoxicity

Granzyme A expression by Tcc was assessed according to the manufacturer's instructions (BD Biosciences). The different subpopulations of IL-22-producing Tcc were tested for their ability to kill P815 target cells upon anti-CD3 mAb activation (5 μ g/ml) by evaluating the percentage of annexin V-binding cells using flow cytometry. The evaluation of apoptosis in Tcc and in L3.6pl tumour cells was performed using the Annexin V kit (BD Biosciences) following the manufacturer's instructions. Cells were

then analysed on a FACSCanto cytofluorimeter (BD Biosciences) using FACSDiva software.

Tumour cell cultures and *in vitro* cytotoxicity assay

Human PDAC L3.6pl cells (cell line kindly provided by Professor Paola Nisticò, Regina Elena National Cancer Institute, Rome, Italy) were cultured in DMEM (Dulbecco's modified Eagle's medium) (Lonza) supplemented with 10% (v/v) FBS (Lonza), L-glutamine (GE Healthcare) and 50 μ g/ml gentamicin (Gentilyn 40 mg/ml, Essex Italia) at 37°C in a 5% CO₂ atmosphere. For the cytotoxicity assay, cells were seeded in 96-well plates at a density of 2×10^4 cells per well, incubated for 4 h and then starved overnight at 37°C. Cells were treated with 100 ng/ml IL-22 or IFN γ , or both, and incubated for 72 h; cell viability was then determined using the MTT assay (Sigma). Briefly, 20 μ l/well of 5 mg/ml MTT solution in PBS was added and incubated for 4 h

at 37°C. Medium was removed and 100 µl of DMSO was added to each well. The formazan salts were quantified by reading the absorbance at a test wavelength of 570 nm.

Immunohistochemical analysis

The surgical specimens of PDAC tissue were fixed in 4% paraformaldehyde overnight at 4°C, paraffin-embedded and cut using a rotary microtome (MR2, Boeckeler Instruments). For immunohistochemical staining, 5-µm sections of the blocks were incubated overnight with rabbit anti-human PD-1 (programmed cell death 1) mAb (clone EPR4877, 1:500 dilution; Abcam) or mouse anti-human FoxP3 (forkhead box P3) mAb antibody (clone 22510, 5 µg/ml, 1:200 dilution; Abcam); mouse anti-human CD4 mAb (neat, clone 4B12; Nichirei); mouse-anti-human CD8 mAb (1:20 dilution, Dako Japan) or mouse anti-human CD163 antibody (1:100 dilution, Leica Biosystems). Following antibody incubation, samples were incubated at room temperature for 1 h with anti-rabbit or anti-mouse immunoglobulins conjugated to a horseradish peroxidase-labelled dextran polymer (Simple Staining Kit; Nichirei). After a benzidine reaction, sections were lightly counterstained with haematoxylin.

For analysing the IL-22⁺ T-cells, sections were boiled for 10 min in sodium citrate buffer (10 mM, pH 6.0; Bio-Optica) for antigen retrieval, and incubated in 2 mg/ml glycine (AppLiChem) for 10 min, to quench autofluorescence of the elastic fibres. Non-specific binding was blocked by exposure to 1.5% BSA in PBS for 20 min at room temperature. Sections were immunolabelled overnight at 4°C with mouse anti-CD3 mAb (ready to use; GeneTex) and goat polyclonal anti-IL-22 mAb (1:50 dilution; Abcam). Immunoreactions were visualized by incubation with specific anti-mouse and anti-goat Alexa Fluor® 488- or 568-conjugated IgG (1:300 dilution; Molecular Probes) for 2 h at room temperature. Negative controls were carried out by replacing the primary antibodies with non-immune serum. Digital images were obtained using an epifluorescence Zeiss Axioskop microscope equipped with a ×63 oil-immersion objective.

Staining of peripheral Th22 cells

PBMCs were isolated by density gradient centrifugation from heparinized venous blood of PDAC patients or healthy subjects. PBMC samples were characterized for their expression of IL-22, IL-17 and IFN-γ using intracellular cytokine staining, as described previously [23]. Briefly, fresh or thawed PBMCs were cultured in IMDM (Iscove's modified Dulbecco's medium) (BioWhittaker) supplemented with 10% FBS (Invitrogen) and stimulated for 5 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of BFA (brefeldin A) (10 µg/ml) (all from Sigma-Aldrich). Cells were first stained for the surface antigens CD4 (BD Biosciences), then fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.5% saponin, followed by intracellular staining with anti-IL-22, anti-IL-17 and anti-IFN-γ mAbs (BD Biosciences).

Statistical analysis

Results are expressed as the median (range) or the mean (±S.D.). Comparisons between the two groups were assessed using Stu-

dent's *t* test. Correlations between the parameters were assessed using Pearson's correlation analysis. SPSS statistical software (version 13.0) was used for all statistical analyses.

RESULTS

Isolation of IL-22-producing T-cells from PDAC tissue

To evaluate the intra-tumoral subsets of IL-22-producing (IL-22⁺) T-cells in PDAC patients, we expanded and cloned *in vivo*-activated TILs isolated from two different sites, namely tumour and surrounding healthy pancreas. We isolated Tcc from each patient, and overall we obtained 807 Tcc; of these Tcc, 507 were isolated from the cancer, which was almost double the number of the Tcc isolated from the healthy pancreas (300) (Table 1). Evaluating the surface markers of Tcc, we noticed that 710 (out of 807) (88%) were positive for CD4 and the remaining 97 were positive for CD8 (12%). After mitogen stimulation, we assessed the levels of IL-22 secreted by single Tcc. We observed that 323 Tcc (40%) were able to produce IL-22 and, more precisely, 290 CD4⁺ and 33 CD8⁺ (Figure 1A). Of particular interest, the number of Th22 cells increased at the cancer site; we isolated 215 (74%) Th22 Tcc from the tumour and only 75 (26%) from the healthy mucosa. In addition, we registered similar data on the CD8⁺ population, isolating 25 out of 33 (76%) from the neoplastic tissue and only 8 (24%) from the surrounding healthy pancreatic tissue. Subsequently, we evaluated the IL-22 levels secreted by the Tcc, and we found that the IL-22-producing Tcc (both CD4⁺ and CD8⁺) isolated from the tumour tissue significantly ($P < 0.0001$) produced higher levels of IL-22 compared with the Tcc generated from the healthy pancreas (Figure 1B).

Characterization of the cytokine profile of IL-22-producing Tcc

As different T-cell subsets are able to produce IL-22, we also evaluated which of the 323 IL-22-producing Tcc also produced other cytokines. We observed that 61 (21%) out of 290 CD4⁺ Tcc only secreted IL-22 (pure Th22), whereas 12 (4%) co-secreted IL-4 (Th22/Th2), nine (3%) co-secreted IL-17 (Th22/Th17) and 44% (128/290) of the IL-22-producing Tcc were able to co-secrete IFN-γ (Th22/Th1). The remaining 80 (28%) CD4⁺ Tcc co-produced IFN-γ and IL-17 (Th22/Th1/Th17) (Figure 2A). Out of the 33 IL-22⁺ CD8⁺ Tcc, we found that 19 (58%) Tcc co-produced IFN-γ (Tc22/Tc1), four (11%) synthesized both IFN-γ and IL-17 (Tc22/Tc1/Tc17) and five (15%) also produced IL-4 (Tc22/Tc2). The final five CD8⁺ Tcc (16%) were only able to secrete IL-22 (Tc22) (Figure 2A).

The IL-22-producing Tcc subsets exhibit different functional properties

To define the functional properties of IL-22-producing Tcc, we then evaluated their ability to assist antibody production by B-cells and to display cytotoxic activity, which we then compared between the different IL-22⁺ Tcc. Both Th22/Th2 and

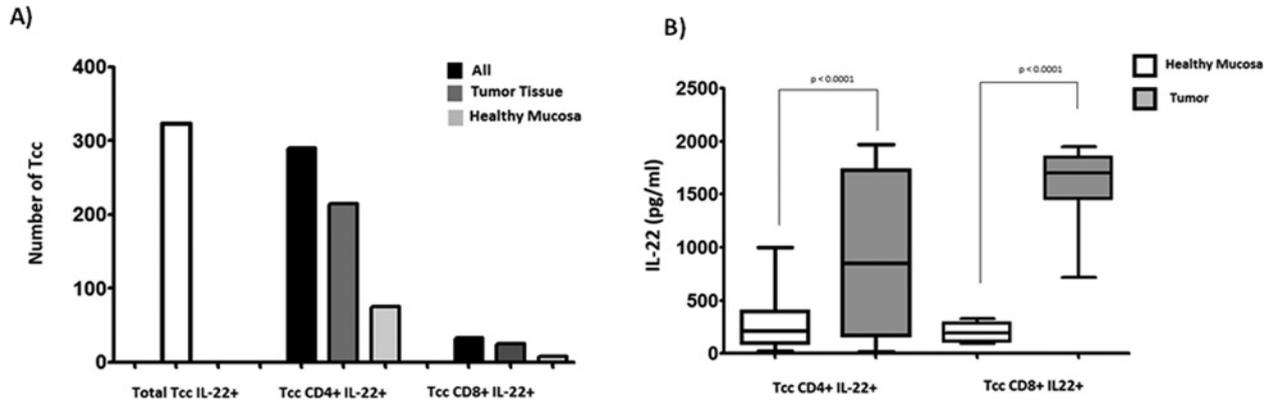


Figure 1 **Repertoire and IL-22 production of Tcc obtained from PDAC patients**
 (A) Total numbers of IL-22⁺ Tcc isolated from tumour tissue and the ratio of CD4⁺/CD8⁺ populations. The number of both CD4⁺ and CD8⁺ IL-22⁺ Tcc is higher in the cancer site. (B) Evaluation and comparison of the amount of IL-22 secreted, after mitogen stimulation, by the Tcc isolated from tumour tissue or surrounding healthy mucosa. The quantity of IL-22 produced by the Tcc (both CD4⁺ and CD8⁺) of tumour tissue is significantly ($P < 0.0001$) higher than that of Th22 cells isolated from healthy mucosa.

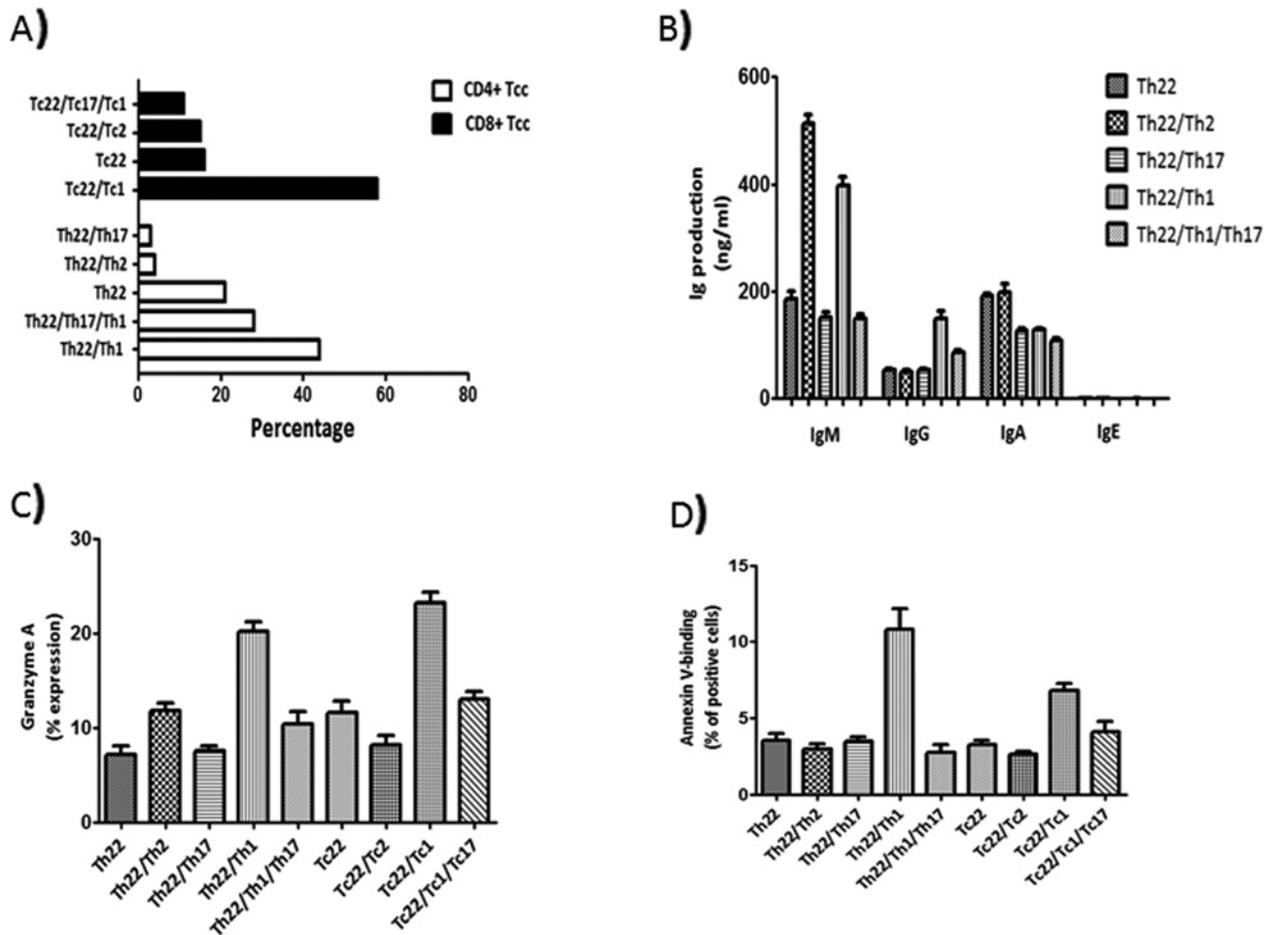


Figure 2 **Cytokine profile and functional profile of intra-tumoral Th22 isolated from PDAC patients**
 (A) Distribution of Th22 subsets. The major representative Tcc subtype is the Th22 with Th1 profile, for both the CD4⁺ population (white column) and the CD8⁺ population (black column). The percentage of Tcc producing only IL-22 (by our definition, pure Th22) is 21 of CD4⁺ Th22 and 16 of CD8⁺ Tc22. (B) To assess the B-cell helper activity, we used autologous B-cells co-cultured with 10^5 blasts of the different Th22/Tc22 Tcc subsets, stimulated with anti-CD3 antibody. The supernatant content of different Ig subclasses was evaluated by commercial ELISA. (C) The granzyme A expression and the percentage of annexin V-binding cells (D) were assessed according to the manufacturer's instructions (BD Biosciences) and analysed on a FACSCanto cytofluorimeter (BD Biosciences) using FACSDiva software.

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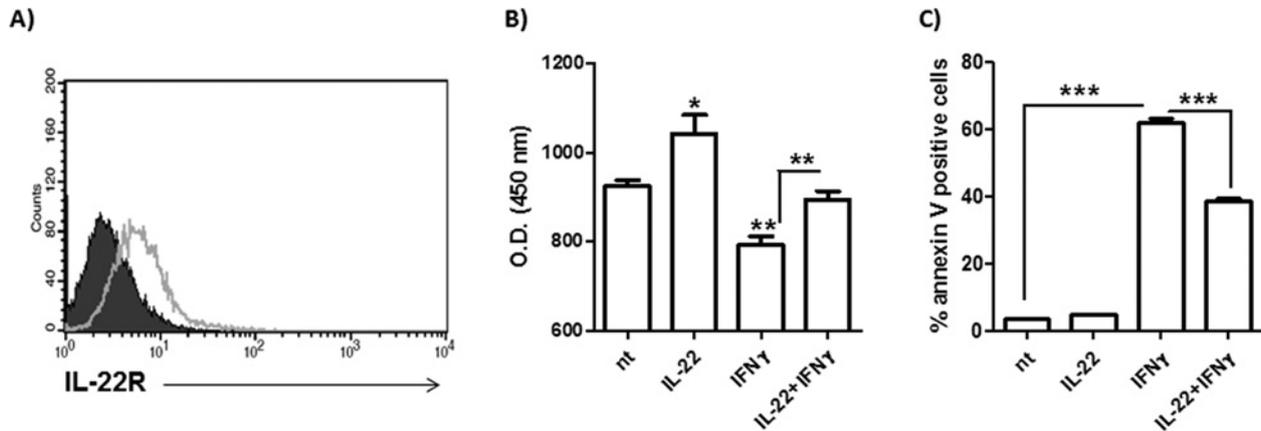


Figure 3 IL-22 reduced IFN- γ -induced apoptosis in the L3.6pl pancreatic tumour cell line (A) Surface expression of IL-22R on L3.6pl cells after incubation with anti-IL-22R mAb (open curve) or isotype control (closed curve). L3.6pl cells were treated with IL-22 (100 ng/ml), IFN- γ (100 ng/ml) or both for 72 h to evaluate cell viability (B) or apoptotic cells (C). Cell viability was determined using the MTT assay and results are mean \pm S.E.M. attenuances ('O.D.') at 450 nm. Apoptotic cells were evaluated as the percentage of annexin V-positive cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (one-way ANOVA).

Th22/Th1 Tcc showed the ability to induce a major B-cell production of IgM compared with the other subsets of IL22⁺ Tcc, whereas the primary inducer of IgG were the Th22/Th1 Tcc. None of the IL-22⁺ subsets was able to induce IgE production, whereas all IL-22-producing Th subsets induced similar amounts of IgA (Figure 2B).

Regarding the cytotoxic potential, all of the different IL22⁺ subsets exhibited low levels of granzyme A expression, as expected, with only the Th22/Th1 and Tc22/Tc1 Tcc displaying granzyme A expression of approximately 20% or slightly more (Figure 2C). We found analogous results regarding the ability to induce apoptosis; all of the IL-22-producing Tcc displayed a low percentage of annexin V-binding cells (<5%). Only the Tcc (as CD4⁺ or CD8⁺) with a Th22/Th1 profile displayed a higher percentage (Figure 2D).

IL-22 reduced IFN- γ -induced apoptosis in pancreatic cells

As the majority of TILs produce IL-22 in combination with IFN- γ , we evaluated the effect of IL-22 alone or in the presence of IFN- γ on the human pancreatic tumour cell line L3.6pl, which is a highly metastatic tumour cell line [32], the cells of which express IL-22 receptor on their membrane (Figure 3A). IL-22 alone increases the cell viability of L3.6pl cells (Figure 3B); as expected, IFN- γ treatment reduced cell viability (Figure 3B) and induced strong apoptosis (Figure 3C) of L3.6pl cells. Interestingly, these cytotoxic effects of IFN- γ were significantly reduced by the presence of IL-22 (Figures 3B and 3C).

Immunohistochemical evaluation of intra-tumoral IL-22⁺ T-cells

To verify whether the results obtained by the *ex vivo* model reflected a real situation, the presence of CD3⁺IL-22⁺ cells infiltrating PDAC tissues was evaluated using immunohistochemistry. Among the infiltrating CD3⁺ T-cells (Figure 4A), CD4⁺ T-cells

were predominant compared with the CD8⁺ T-cells (results not shown). Figures 4(B) and 4(C) only show representative images of the T- (CD4⁺/CD8⁺) cell infiltration. FoxP3⁺ cells (Figure 4E), as well as PD-1⁺ cells (Figure 4F), were more frequently observed, suggesting that regulatory or activated CD4⁺ T-cells had infiltrated the PDAC tissues. Finally, we documented that the number of CD3⁺ T-cells co-expressing IL-22 (Figure 4I) was higher in the neoplastic tissue. Table 2 shows the number of inflammatory cells which we assessed per high power field. Comparing the PDAC tissue with the surrounding healthy mucosa, we noted an increased number of CD4⁺, FoxP3⁺, PD-1⁺ and, especially, IL-22⁺ T-cells in the tumour tissue. Finally, we also documented that the number of IL-22-producing Tcc, isolated in a single patient, significantly correlated ($P = 0.0076$) with the number of tumour-infiltrating IL-22⁺ cells, thus creating an important link between the *in vitro* and *in vivo* data.

Th22 cells increase in the peripheral blood of PDAC patients

Finally, the frequency of Th22 cells was assessed in the peripheral blood of PDAC patients and 30 healthy subjects by flow cytometry. After gating CD4⁺ cells, we identified three different groups of IL-22-producing cells, namely one group producing IL-22 alone (termed Th22 cells), one co-producing IFN- γ and the last co-producing IL-17. The percentage of Th22 cells in the peripheral blood of PDAC patients was significantly higher than in age-matched healthy donors (Figure 5A). The expansion of Th22 cells was accompanied by the increase of IL-22/IFN- γ -co-producing (Figure 5B) and IL-22/IL-17-co-producing (Figure 5C) CD4⁺ T-cells. However, the Th22 cell subset was the highest IL-22-producing group in the peripheral blood.

Clinical relevance of Th22 cells in PDAC patients

The statistical analysis showed that IL-22-producing Tcc positively correlated with TNM staging (Figure 6A) and metastases

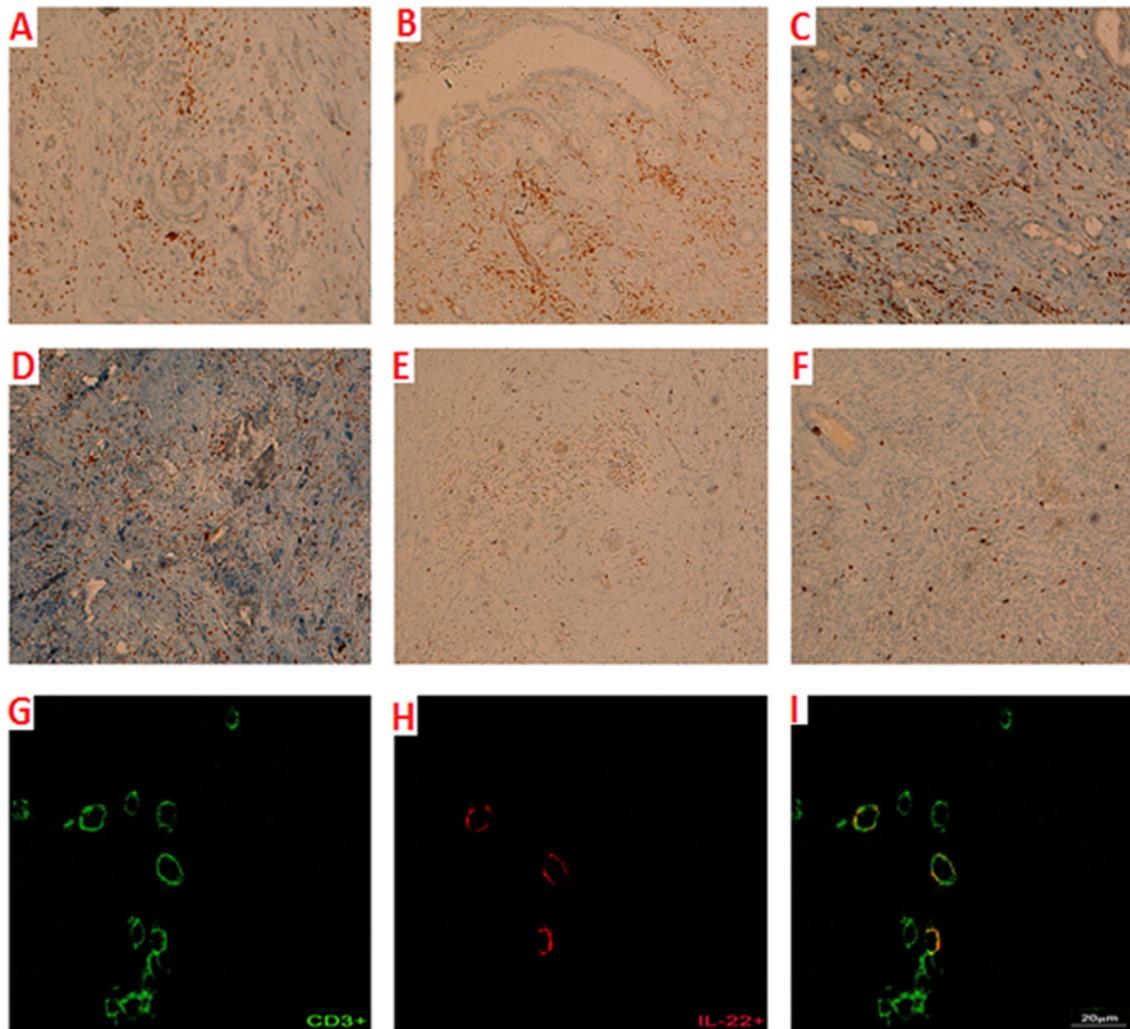


Figure 4 Immunohistochemical analysis of PDAC tissues

Surgically resected PDAC tissues were immunohistochemically stained. CD3 (A): lymphoid aggregation around PDAC was mainly composed of CD3⁺ T-cells. CD4 (B): the majority of lymphoid aggregation was CD4⁺ T-cells. CD8 (C): the number of CD8⁺ T-cells was small compared with that of CD4⁺ T-cells shown in (B). Most T-cells were IL-22-positive (D), some were FoxP3-positive (E) (nuclear expression) and some were PD-1-positive (F). Magnification $\times 10$ high power field. In the lower panels are digital images, obtained using an epifluorescence Zeiss Axioskop microscope equipped with a $\times 63$ oil-immersion objective, of CD3⁺ T-cells (G), IL-22⁺ cells (H) and CD3⁺ IL-22⁺ T-cells (I). The images are from tissue of different patients and are representative images of high/low-expressing samples for each marker.

(Figure 6B), but were not related to the sex or tumour size (results not shown). Taken together, these results suggest that IL-22 expression by T-cells is closely associated with PDAC progression and with the patient outcome. To test this hypothesis, we evaluated whether the number of Th22 Tcc negatively correlated with the survival of PDAC patients. The patients were divided into two groups on the basis of the percentage of intra-tumoral IL-22-producing Tcc, either (i) $< 50\%$ or (ii) $\geq 50\%$ (Table 1). Kaplan–Meier analysis revealed that the patients of group (i) showed a better survival compared with group (ii) ($P < 0.0001$), suggesting a negative correlation between the percentage of IL-22-secreting Tcc and a better outcome of the disease (Figure 6C). In other words, elevated expression of intra-tumoral Th22 correlates with PDAC progression and is a predictor of poor patient survival.

DISCUSSION

IL-22 is a member of the IL-10 family of cytokines produced by T-cells and innate lymphoid cells [33]. The distribution of the two IL-22 receptors, IL-22R1 and IL-10R2, indicates that the most important target cells of IL-22 reside in the skin, digestive system (pancreas and liver), lungs, and kidney. In general, the IL-22 signalling pathway orchestrates mucosal immune responses and tissue regeneration through pleiotropic effects, including pro-survival signalling, cell migration, dysplasia and angiogenesis [34]. Although these functions can prevent the initial establishment of tumours, they can also be exploited by aggressive cancers to enhance tumour growth and metastasis. In fact, as well as being implicated in the pathogenesis of many autoimmune and

Table 2 Inflammatory features of PDAC tissue and surrounding healthy pancreas
The number of each inflammatory cells was assessed per high power field.

Patient	Degree of inflammation	CD4 tumour/healthy mucosa	FoxP3 tumour/healthy mucosa	PD-1 tumour/healthy mucosa	IL-22 tumour/healthy mucosa
01.PDAp	Mild	>100/>100	<10/<10	<5/<5	38/27
02.PDAp	Moderate	54/62	58/22	14/6	45/31
03.PDAp	Severe	83/80	<10/<10	41/27	62/48
04.PDAp	Severe	>100/>100	24/<10	10/<5	73/49
05.PDAp	Severe	99/62	32	55/33	77/59
06.PDAp	Severe	88/72	<10/<10	<5/<5	65/51
07.PDAp	Mild	>100/>100	37/20	<5/<5	18/8
08.PDAp	Severe	98/82	25/15	44/25	63/48
09.PDAp	Mild	>100/>100	15/<10	21/8	72/54
10.PDAp	Severe	80/63	<10/<10	12/<5	61/53
11.PDAp	Moderate	>100/>100	22/10	8/<5	61/49
12.PDAp	Mild	71/82	13/<10	12/<5	38/29
13.PDAp	Severe	>100/82	22/15	15/6	70/56
14.PDAp	Mild	52/72	48/34	<5/<5	55/46
15.PDAp	Moderate	>100/>100	28/17	19/8	47/38
16.PDAp	Mild	63/75	11/<10	22/15	22/15
17.PDAp	Moderate	>100/>100	32/20	38/24	45/36
18.PDAp	Severe	88/63	<10/<10	<5/<5	65/54
19.PDAp	Severe	99/74	18/11	16/6	79/57
20.PDAp	Mild	>100/>100	35/21	13/<5	68/51
21.PDAp	Severe	>100/>100	<10/<10	25/16	72/49
22.PDAp	Moderate	>100/>100	28/21	14/8	76/51
23.PDAp	Severe	88/>100	22/12	15/7	67/53
24.PDAp	Mild	72/85	12/<10	<5/<5	54/42
25.PDAp	Moderate	>100/>100	<10/<10	31/20	19/8
26.PDAp	Moderate	84/>100	18/<10	28/16	23/15
27.PDAp	Moderate	>100/>100	27/18	12/7	75/52
28.PDAp	Severe	>100/>100	<10/<10	<5/<5	78/49
29.PDAp	Mild	>100/>100	<10/<10	18/6	49/31
30.PDAp	Moderate	68/78	14/<10	23/10	71/59

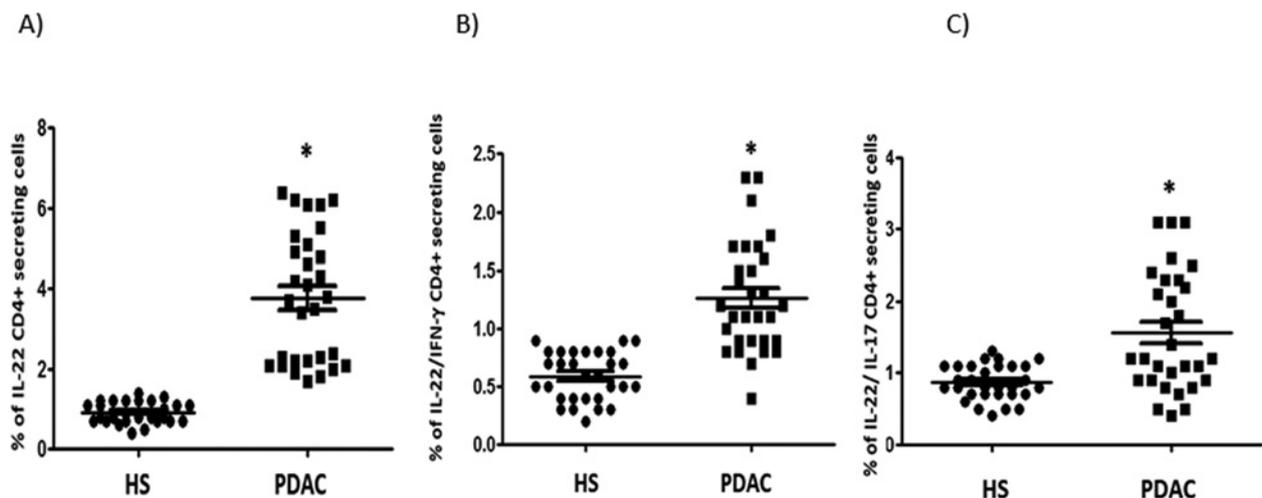


Figure 5 IL-22-producing cells in the peripheral blood of PDAC
Ex vivo FACS analysis of cytokine production from CD4⁺ T-cells in healthy subjects (HS) and in PDAC patients stimulated for 5 h with PMA and ionomycin in the presence of BFA. Results are the percentages of IL-22-producing CD4⁺ cells in the peripheral blood. **P* < 0.001.

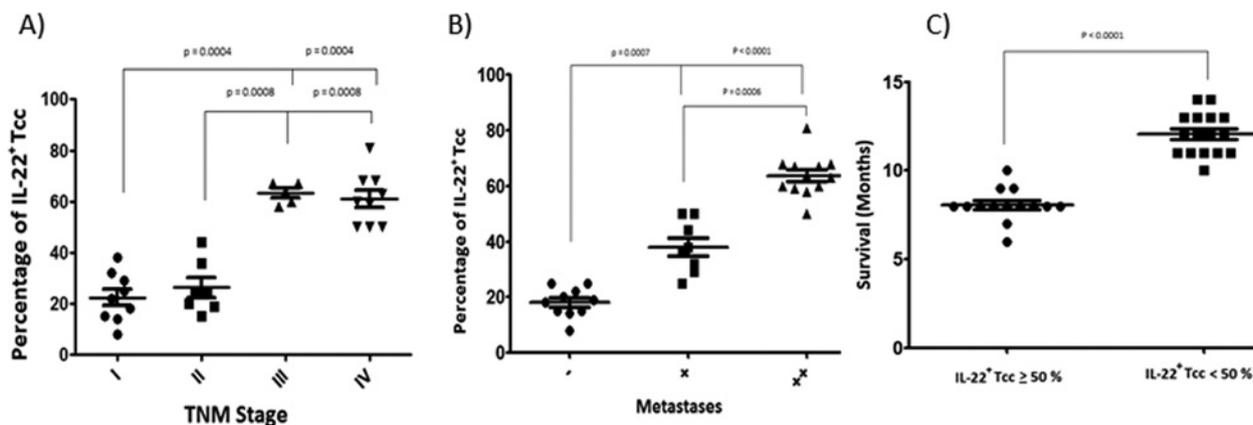


Figure 6 Correlation between clinical parameters of patients and number of Th22 Tcc

Correlation between the percentages of Th22 cells with TNM stage (A) and presence of metastases (B). The increased number of IL-22⁺ TILs positively and significantly correlates with TNM PDAC staging and number of metastases: -, no metastases, +, metastases in one site, ++, multisite metastases. (C) To correlate the survival of PDAC patients with the number of Th22 Tcc, we divided the patients into two groups: (i) patients with Th22 Tcc ($\geq 50\%$), and (ii) the patients with Th22 Tcc ($< 50\%$). Correlating the two groups by the Kaplan-Meier curve, we observed that group (ii) showed significantly ($P < 0.0001$) better survival compared with group (i).

inflammatory diseases, such as psoriasis [35] and rheumatoid arthritis [22], IL-22 has been associated with cancer progression, including hepatocellular carcinoma [36] and gastric cancer [24]. However, the potential role of IL-22 and especially Th22 (the major source of IL-22) in PDAC is far from clear.

In line with immunohistochemical analysis, which revealed the presence of CD3⁺IL-22⁺ cells in neoplastic tissue, we demonstrated that the number of IL-22-producing CD4⁺ and CD8⁺ Tcc isolated from PDAC patients was significantly elevated in PDAC tissues compared with the surrounding healthy pancreas and, of note, that the number of IL-22-producing Tcc significantly correlated with the number of tumour-infiltrating IL-22⁺ T-cells. In other words, the number of IL-22⁺ Tcc reflects the *in vivo* situation. In addition, we demonstrated, for the first time, that the IL-22-producing Tcc (as CD4⁺ or CD8⁺) isolated from PDAC tissue significantly produced higher IL-22 levels compared with IL-22⁺ Tcc isolated from the healthy pancreas. This finding suggests that the high number of IL-22-producing T-cells can be associated with PDAC progression and, in accordance with other studies [28,29], Th22 Tcc positively correlated with TNM staging and metastases. IL-22 improves the invasive ability of pancreatic cancer cell lines by increasing MMP9 (matrix metalloproteinase 9), and the association between IL-22 and poor prognosis is attributable to the increased cancer cell mobility and metastatic potential [29].

IL-22 can be produced by different T-cell subpopulations, in particular by the CD4⁺ T-cells: Th22, Th17 and Th1 [37]. Therefore, in order to evaluate the nature of the different IL-22-producing Tcc that have infiltrated the neoplastic tissue, we characterized the cytokine profile of each single IL-22-producing Tcc. We observed that some CD4⁺ Tcc only produced IL-22, but the majority of them co-produced IFN- γ alone or with IL-17, and similarly only five out of the 33 CD8⁺ Tcc, secreted IL-

22 alone, whereas many co-produced IFN- γ or both IFN- γ and IL-17. In conclusion, we have shown, for the first time, that in PDAC patients, the major intra-tumoral source of IL-22 is the T-cell subset with a Th1 profile.

Notably, as further confirmation of these *ex vivo* data, we also documented a similar trend *in vivo*; in detail, we showed that expansion of Th22 cells in the peripheral blood of PDAC patients was accompanied by an increase in IL-22/IFN- γ -co-producing and IL-22/IL-17-co-producing CD4⁺ T-cells.

As most of the IFN- γ -producing Tcc are able to exert perforin-mediated cytotoxicity, we assessed the cytolytic potential of IL-22⁺ Tcc and, as expected, only the Th22/Th1 Tcc showed a satisfactory expression of granzyme A, whereas the remaining different IL-22-producing Tcc subsets exhibited low levels of granzyme A. In addition, we registered analogous data about the ability to induce apoptosis: of all IL-22⁺ Tcc, only those co-producing IFN- γ (Th22/Th1) displayed a significant percentage of annexin V-binding cells.

Indeed, as we have shown that PDAC cells express IL-22R, they are responsible for the anti-apoptotic effect of IL-22. We have shown that IFN- γ induced the apoptosis of PDAC cells and that this apoptosis was antagonized by the addition of IL-22.

Taken together, these data support the hypothesis that IL-22 favours PDAC progression by inhibiting IFN- γ -induced apoptosis. The IL-22 production by intra-tumoral Th1/Th22 cells during PDAC progression may therefore neutralize the anti-tumour effect of Th1-polarized T-cells, protecting the cancer cells by the pro-apoptotic effect of IFN- γ [38].

Finally, to complete the functional characterization of intra-tumoral IL-22⁺ Tcc, we evaluated their ability to assist antibody production by B-cells. In agreement with previous studies [8], all of the different Th22 subsets of Tcc were unable to induce assistance for the production of IgE, but had

a similar ability to induce IgA. In addition, both Th22/Th2 and Th22/Th1 Tcc induced IgM production, whereas only the Th22 with a Th1 profile displayed an ability to induce IgG. In other words, IL-22 does not seem to affect the isotype switch of antibodies.

In summary, our results highlight the well-established dual role of the anti-tumour immune response in PDAC patients. Indeed, Th1 lymphocytes usually play an effector role against tumour cells by assaulting them and then blocking tumour growth [31,39,40]. Instead, in this context, the same Th1 cells, which produce IL-22, could promote tumour progression, as demonstrated recently in the pathogenesis of arthritis [41]. It is well known that the pro-survival and proliferative effects of IL-22 are utilized by many cancers; in particular, lung and liver cancers appear to be particularly sensitive to these effects [36,42,43]. In addition, IL-22 can promote carcinogenesis by favouring angiogenesis [44,45] or enhancing immune evasion by tumours [45]. PDAC cells themselves secrete VEGF (vascular endothelial growth factor) and TGF- β (transforming growth factor β) and IL-10 in response to IL-22 stimulation [45].

Finally, most importantly, our findings also shed light on the clinical relevance of Th22 cells in PDAC. We found that an increased frequency of Th22 cells correlated with TNM staging and with an unfavourable outcome of the disease, thus the number of IL-22-producing Tcc negatively correlated with PDAC patient survival.

Pancreatic cancer has a dismal 5-year survival rate of <5%, partly because most patients are diagnosed when the disease is already at an advanced stage, and partly because only 20% of patients have local resectable tumours [46,47]. Increased responsiveness to IL-22 may be the tipping point which separates resectable tumours from incurable and metastasized cancers (often already present at diagnosis). One study showed that the expression of both IL-22 and IL-22R1 was elevated in sections of PDAC tissue and predicted poorer patient survival [29]. However, a different group found that IL-22R1 was more poorly expressed in patients with PDAC compared with normal controls [48]. Primary cells and metastases of oral squamous cell carcinomas showed intense staining for IL-22R α 1 [49]. Nonetheless, a chromosomal loss of the region IL-22R α 1 was also found in a cohort of Sudanese patients, but not in Norwegian patients of the same study [50], mirroring the discrepancy between the results in immunohistochemical observation and array comprehensive genomic hybridization for pancreatic cancer. These data reveal how differences in genotypes between ethnic groups may affect the significance of IL-22 in a given cancer. IL-22R1 may also be more affected specifically in buccal oral squamous cell carcinoma, which the majority of Sudanese patients had, but was absent from the Norwegian cohort.

In conclusion, our data demonstrate that all IL-22-producing T-cells are significantly increased in pancreatic cancer. Furthermore, these increases positively correlated with TNM staging of PDAC and poorer patient survival. These novel findings suggest that Th22 cells may participate in PDAC pathogenesis, that the monitoring of Th22 can be a good diagnostic parameter and, finally, that blockade of IL-22 signalling may represent a viable method for anti-PDAC therapies.

CLINICAL PERSPECTIVES

- The principal sources of IL-22 are Th22 cells, which secrete only IL-22 or other cytokines such as IL-17 or IFN- γ . Intra-tumoral IL-22 levels were elevated in PDAC (pancreatic ductal adenocarcinoma). However, the nature, role and clinical relevance of Th22 remain unknown.
- We have shown that in PDAC patients, cytotoxic IFN- γ + Th22 cells are the major intra-tumoral source of IL-22, which induce apoptosis, and are antagonized by IL-22. The increased levels of Th22 positively correlated with TNM and poorer patient survival.
- Elucidation of how IL-22 regulates the pathogenesis of PDAC is important, and may help to identify a new target for developing innovative PDAC treatments.

AUTHOR CONTRIBUTION

Elena Niccolai, Amedeo Amedei and Francesco Novelli conceived and designed the study, and drafted the paper. Elena Niccolai, Simona Rolla, Francesca Castiglione, Federica Ricci, Marisa Benagiano and Alessandro Pini acquired experimental data. Antonio Taddei, Paolo Bechi, Lapo Bencini, Maria Novella Ringressi, Andrea Coratti, Fabio Cianchi, Daniele Giordano and Maria Antonietta Satolli were involved in enrolment and obtaining clinical data of patients. Elena Niccolai, Mario Milco D'Elios, Daniele Bani, Simona Rolla, Francesco Novelli and Amedeo Amedei analysed and interpreted data. Amedeo Amedei, Francesco Novelli and Domenico Prisco critically revised the paper.

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