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Effector T lymphocyte subsets in human pancreatic cancer: detection of $CD8^+$ $CD18^+$ cells and $CD8^+$ $CD103^+$ cells by multi-epitope imaging

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SUMMARY

Pancreatic cancer is characterized by an increasing incidence and an extremely poor prognosis. It is resistant to most of the conventional treatment modalities. Histomorphologically, it presents with a strong desmoplastic reaction around cancer cells, and lymphocytes are typically localized as aggregates in the fibrotic interstitial tissue. Using the method of multi-epitope imaging with fluorochrome-tagged specific MoAbs which allows the simultaneous localization and characterization of T cells in tissues, we studied phenotypes and distribution of tumour-infiltrating lymphocytes (TIL) in pancreatic cancer. $CD3^+$ T cells comprised up to 90% of the tumour-infiltrating cells which were either $CD4^+$ or $CD8^+$, most of them being memory cells ($CD45RO^+$). In decreasing order of frequency, T lymphocytes carried the markers for CD45RO, CD18, CD103 and TCR $\gamma\delta$. Very few natural killer cells ($CD56^+$) were observed. Twenty percent of $CD8^+$ were labelled with CD103. These $CD8^+$ $CD103^+$ T cells, analogous to the gut intraepithelial lymphocytes (IEL), were found in the fibrous interstitial tissue. Furthermore, an inverse correlation was found between the expression of CD18, the β_2-integrin, which mediates adhesion of activated lymphocytes, and CD45RO in the $CD8^+$ subset of TIL ($P = 0{\cdot}046$). In conclusion, phenotyping of T lymphocytes in pancreatic cancer raises the possibility that pancreatic cancer cells develop several strategies to escape the T cell-induced cytolysis by (i) the aggregation of cytotoxic $CD8^+$ $CD103^+$ T cells in the fibrous tissue distant from the tumour cells, and (ii) the presence of CD18-bearing cells which lack the expression of the activation marker CD45RO.

Keywords CD4 CD8 CD103 pancreatic cancer multi-epitope imaging

INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer death in the Western world, and it is associated with a poor prognosis and a 5-year survival rate of 3% [1]. Clinically, pancreatic cancer is characterized by rapid tumour progression and unresponsiveness to most conventional treatment modalities [1]. The biology of pancreatic cancer and the reasons for its aggressiveness are poorly understood [2]. Pancreatic cancer cells express a variety of growth factors, especially of the epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) family [3–6]. On the molecular genetic level, the most common genetic abnormalities found are *K-ras* gene mutations [7], mutations of the *p53* tumour suppressor gene [8] and deletions of the *DPC-4* tumour suppressor gene [9].

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Failure of the body's immune system to detect or destroy tumour cells may be important in understanding the pathogenesis of cancer. Immunophenotypic studies of various carcinomas have demonstrated that $CD8^+$ (cytotoxic/suppressor) T cells are the predominant T cell subset of tumour-infiltrating lymphocytes (TIL) [10,11]. The presence of other markers, such as CD45RO, indicates that these cells are memory cells and are thus tissue-specific [10,11]. Though studies of the function of TIL indicate that these cells, including $CD8^+$ cells, TCR $\gamma\delta^+$ and $CD56^+$ cells, can be isolated from human pancreatic cancer, further phenotyping of TIL has to date not been undertaken [12–14].

Using the method of multi-epitope imaging with fluorochrome-tagged specific MoAbs, we characterized and analysed the differential distribution of lymphocytes in pancreatic cancer [15,16]. We now report that $CD8^+$ cells form the predominant T cell subset in pancreatic cancer. However, the aggregation of the T cell subset $CD8^+$ $CD103^+$ in the fibrous tissue distant from the cancer cells and the presence of $CD18^+$ cells lacking the activation marker

Table 1. Specificity and dilution of the antibodies [15,17]

Antibody	Specificity	Dilution (pH 7·4)
CD4-PE	Clone 13B8.2, IgG1 mouse	1:25
CD8-FITC	Clone B9.11, IgG1 mouse	1:100
CD3-PE	UCHT1, IgG1 mouse	1:100
CD45RO-FITC	UCHL1, IgG2a mouse	1:100
CD56-PE	NCAM, clone B159 IgG1 mouse	1:50
CD64-FITC	Clone 22, FcγRI, IgG1 mouse	1:50
CD18-PE	Integrin β_2, clone 7E4 IgG1 mouse	1:50
TCR γδ-FITC	Pan γ/δ, clone IMMU515 IgG1 mouse	1:25
CD103-FITC	Intestinal lymphocyte (HML-1), clone 2G5 IgG2a mouse	1:25

CD45RO point to cancer cell-dependent strategies which may allow the latter to escape the cytotoxic effects of these TIL.

MATERIALS AND METHODS

Fluorochrome-tagged (FITC or PE) MoAbs against CD3, CD4, CD8, CD18, CD45RO, CD56, CD64, CD103 and TCR γδ were purchased from Coulter Immunotech (Hamburg, Germany). Details of usage and specificities of the employed antibodies are listed in Table 1 and have been previously reported [15,17]. Normal goat serum was obtained from Sigma Pharmaceuticals (Deisenhofen, Germany). All imaging procedures were performed on a Zeiss microscope (Axiophot, Jena, Germany) with a high resolution camera (Photometrics, Munich, Germany) and software from PMIS and IDL (Munich, Germany).

Tissue samples

Pancreatic cancer ($n = 8$; one female, seven male) tissues were obtained from patients undergoing pancreatic surgery. Normal pancreatic tissues were obtained from eight individuals (four male, four female) through an organ donor programme. To assure sampling uniformity, tissue samples from organ donors and pancreatic cancer patients were always obtained from the head of the pancreas. The median ages of the cancer patients and organ donors were 66 years (range 37–74 years) and 38 years (range 14–53 years), respectively.

Immediately following surgical removal, tissue samples were snap-frozen in liquid nitrogen. The cancer samples were classified as pancreatic ductal adenocarcinomas according to the TNM classification for pancreatic tumours [1]. All studies were approved by the Human Subjects Committee of the University of Berne (Berne, Switzerland).

Fluorescent immunohistochemistry

Cryostat sections (5–7 μm) from pancreatic tissue were incubated with fluorochrome-labelled primary antibodies and evaluated by fluorescent microscopy [18]. Non-specific binding was blocked by incubating sections with 10% normal goat serum for 30 min, followed by incubation with the pair of primary antibodies (CD4-PE and CD8-FITC). Details of the specificities and dilutions used are shown in Table 1. CD4 and CD8 T cells observed in 30 high power fields (HPF) were enumerated.

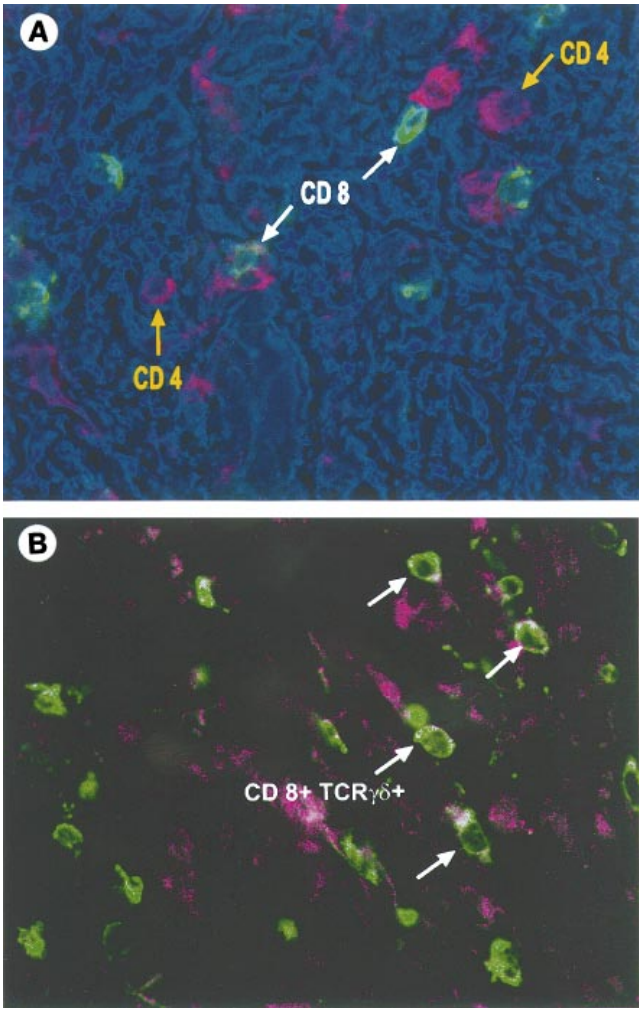

Fig. 1. Fluorescent immunohistochemistry. (A) Localization of $CD4^+$ and $CD8^+$ T cells in the fibrous interstitial tissue in pancreatic cancer by immunofluorescence. $CD4^+$ and $CD8^+$ cells are seen in clusters; there is no distinct pattern noticeable in the distribution of the two T cell subsets. This image was produced by the superimposition of the grey scale fluorescent images obtained for binding with the antibodies against CD4 and CD8, respectively, on the phase contrast image. Each image was assigned a separate colour channel (red, CD4; green, CD8) and the phase contrast image was ascribed the colour blue. (B) Image acquired by superimposition of the fluorescent images obtained on binding with the CD8 (red) and TCR $\gamma\delta^+$ (green) antibodies. White arrows indicate $CD8^+$ TCR $\gamma\delta^+$ cells. Due to the exact superimposition of the two images, the areas of overlap appear white.

Multi-epitope imaging

Sections were treated as mentioned above. Serial incubation with the following primary conjugated antibodies (CD4-PE and CD8-FITC, CD3-PE and TCR γδ-FITC, CD56-PE and CD45RO-FITC, CD64-PE and CD103-FITC, and CD18-PE) was performed as described [15,19]. In short, immunofluorescent sections were examined with a Zeiss microscope equipped with a 50-W mercury lamp and a 40× water immersion objective (Zeiss, Jena, Germany). A region of interest (ROI) of the section with ample T cells was then selected for further analysis. Computerized image acquisition was performed with the PMIS package. All images acquired showed the same cells as seen labelled with different antibodies. Each incubation step was followed by complete bleaching of the fluorochrome conjugated to

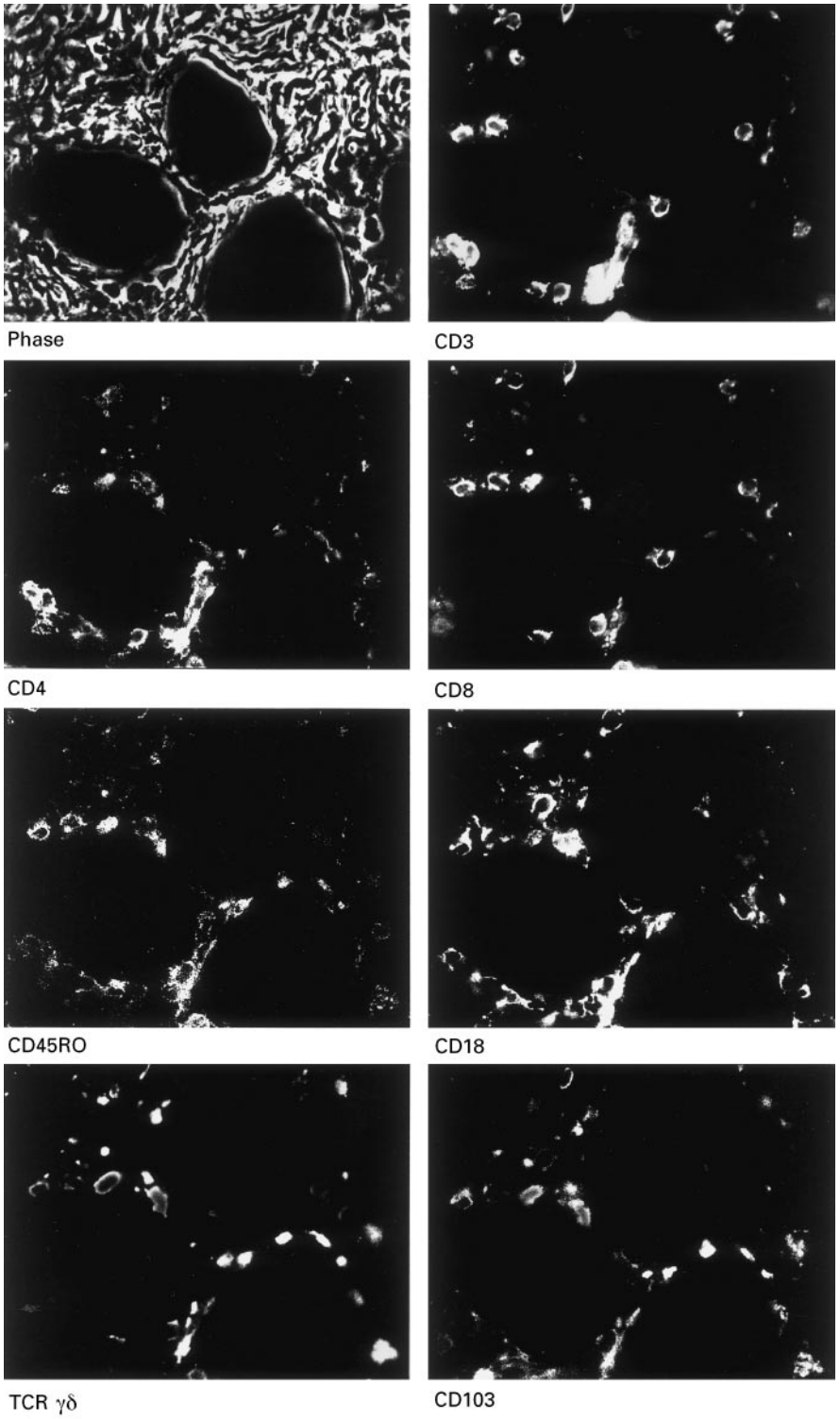

Fig. 2. Multi-epitope-imaging. The phase contrast image gives the orientation of the lymphocytes. All images depict the same high-power field, i.e. the same lymphocytes as seen labelled with different T cell markers. The panels show the grey scale fluorescent images of a section of pancreatic cancer.

the primary antibody. Thus, the ROI was exposed to light of the wavelength required for the excitation of the respective fluorochrome until no more fluorescence was observed, allowing the application of a new set of fluorochrome-tagged primary antibodies without loss in sensitivity or specificity of the fluorescence seen on binding by the next pair of MoAbs to the same cells. Each set of images was digitized and corrected for pixel shift before analysis [16]. As positive controls, we used MoAbs conjugated with fluorochromes different from those

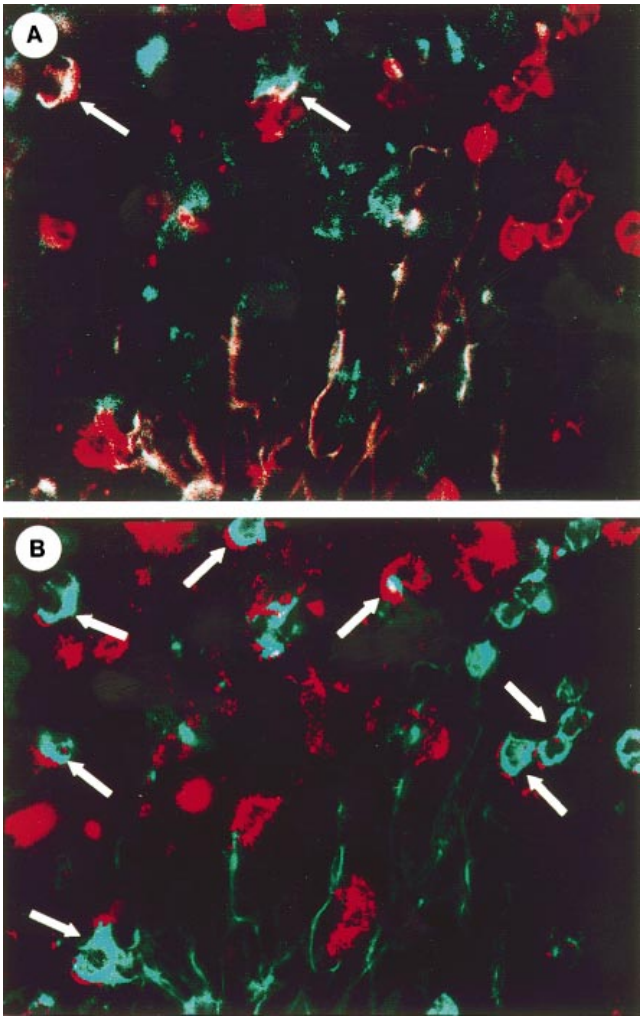

Fig. 3. Multi-epitope-imaging. (A) Superimposition of the fluorescent images obtained on binding with the CD8 (red) and CD103 (green) antibodies. White arrows indicate $CD8^+$ $CD103^+$ cells. (B) Superimposition of the fluorescent images obtained on binding with the CD8 (red) and CD18 (green) antibodies. The white arrows indicate $CD8^+$ $CD18^+$ cells.

used in the original experiment, e.g. we used a PE-labelled CD4 MoAb and as a control CD4-FITC was employed, which gave the same binding pattern. Further, we repeated incubations with sections from the same specimen after interchanging the pairs of primary antibodies. As negative controls, fluorochrome-tagged IgG mouse MoAbs were employed which did not show any specific binding (data not shown). The specificity of the antibodies was tested by using peripheral blood smears from healthy donors subjected to the same experimental conditions as the pancreatic tissue (data not shown).

Statistical analysis

Statistical analysis was performed using the SPSS package (Version 6.0.1; SPSS Inc., Chicago, IL). All results are expressed as mean with s.e.m. Student's *t*-test was used for statistical analysis, with $P < 0{\cdot}05$ taken as the level of significance. Pearson's test for examining correlations was employed [20].

RESULTS

Few $CD4^+$ or $CD8^+$ T lymphocytes were found in sections of normal pancreatic tissue. Due to the small numbers of T cells found in normal pancreatic tissue, no further characterization of these cells was performed. In sections of pancreatic cancer, aggregates of T cells were observed in the fibrotic interstitial tissue (Fig. 1A), few lymphocytes were seen in the periductal regions and intraepithelial lymphocytes (IEL) intercalating between the epithelial cells were rarely observed. There were wide variations in the numbers of $CD4^+$ and $CD8^+$ cells in sections of pancreatic cancer, ranging from 24 to 141 $CD4^+$ cells ($77{\cdot}5 \pm 16$ (mean $\pm$ s.e.m.)) and 22 to 151 $CD8^+$ cells ($79{\cdot}9 \pm 17$) observed in a total of 30 HPF. In five of eight cases examined, $CD8^+$ formed the predominant T cell subset (Fig. 1B).

In pancreatic cancer, the $CD3^+$ marker was found on 73% of $CD4^+$ T cells and 82% of $CD8^+$ cells, respectively. Eighty-four percent of the $CD4^+$ and 74% of the $CD8^+$ subset of T lymphocytes expressed CD45RO (memory/activated marker) (Fig. 2). The human mucosal lymphocyte marker, $CD103^+$, also known as HML-1, $\alpha^E\beta_7$ integrin and E-cadherin ligand, was present on 8% of $CD4^+$ and 20% of $CD8^+$ cells. Wide variations were observed in the distribution of CD103 among the $CD8^+$ lymphocytes, varying from 9% to 32% ($20{\cdot}1 \pm 7{\cdot}2$%). This T cell subset was localized only in the fibrous interstitial tissue and was not found periductally or intraepithelially (Fig. 3A). Overall, 24·5% of the T cells were $CD18^+$. This subset of $CD8^+$ $CD18^+$ T cells was also located in aggregates of the TIL in the fibrous stroma (Fig. 3B). Furthermore, CD18 was expressed on cells that did not express markers typical of T cells. Statistical analysis using Pearson's coefficient of correlation showed an inverse correlation between the expression of the memory/activation marker (CD45RO) and β_2-integrin CD18 in the CD8 subset ($P < 0{\cdot}046$).

All TCR $\gamma\delta^+$ cells were $CD8^+$ (Fig. 1B). TCR $\gamma\delta^+$ cells formed only a small proportion of the $CD8^+$ T lymphocytes (mean 14·4%).

Other T cell markers such as CD103 and CD18 were also expressed by $CD8^+$ TCR $\gamma\delta^+$ lymphocytes, but no statistical correlation was found between the expression of this T cell receptor and other T lymphocyte markers used in this study.

Only one or two natural killer (NK) cells ($CD56^+$) were observed per section in pancreatic cancer. While most of the macrophages ($CD64^+$) did not carry T cell markers, a few macrophages were also $CD4^+$; these cells were uniformly $CD3^-$.

DISCUSSION

Predominance of the $CD8^+$ T cell subset in TIL is a common feature of many human cancers, including those of the gastrointestinal tract, liver and breast [10,11,21]. A preponderance of CD8 cells was observed in gastric cancer [10] as well as in primary liver carcinoma [11]. Our observation that $CD8^+$ T cells, which are cytotoxic or suppressor cells, are the predominant T lymphocyte subset in pancreatic cancer is in agreement with prior functional studies of TIL in pancreatic cancer [13,14]. Despite the *in vitro* findings of cytotoxicity of T cells towards pancreatic cell lines [13], the nature of the interaction of $CD8^+$ cells with cancer cells *in vivo* is unclear.

We have examined, among other T cell markers, the expression of the integrin HML-1 ($\alpha^E\beta_7$) in this study, to elucidate the nature of the cross-talk between tumour cells and $CD8^+$ cells. HML-1, also known as $\alpha^E\beta_7$ integrin, was found to be expressed on at least 85% of intestinal IEL (iIEL) and 40% of lamina propria lymphocytes, and only on 2% of peripheral blood lymphocytes (PBL) in

healthy individuals [22]. As this integrin is mainly associated with $CD8^+$ cells [23], the $CD8^+$ $CD103^+$ cells observed in our study are analogous to iIEL. HML-1 mediates the interaction between epithelial cells and the IEL [17]. The functions of these cells in the gastrointestinal tract appear to be manifold and are distinct from those of PBL. IEL have been demonstrated to possess spontaneous cytotoxicity against epithelial tumour cell lines, which is distinct from that of NK cells [24]. This finding that IEL-induced cytotoxicity is target cell-restricted would suggest a role in recognizing and destroying transformed epithelial cells [24]. Furthermore, the integrin HML-1 mediates the adhesion of iIEL to epithelial cells [25] and colon carcinoma cells [17]. The ligand for $\alpha^E\beta_7$ is E-cadherin, which is expressed by pancreatic epithelial cells [26,27]. However, E-cadherin expression is decreased in pancreatic tumours compared with normal pancreas. Furthermore, aberrant expression of E-cadherin in the cytoplasm rather than at intercellular junctions in pancreatic carcinoma has been reported [28]. These observations, taken together with our finding that $CD8^+$ cells are mostly located within the interstitial tissue, imply that tumour cells may avoid cell-to-cell contact with cytolytic $CD8^+$ $CD103^+$ cells via down-regulation of E-cadherin, the ligand for CD103.

The β_2-integrin, CD18, comprises one subunit of the leucocyte function-associated antigen 1 (LFA-1), which is expressed by monocytes, T cells and polymorphonuclear leucocytes. This integrin mediates adhesion required for T lymphocyte target cell lysis, T cell proliferation and natural killing [29,30]. The interaction via LFA-1 is mediated by the direct interaction of LFA-1 with intercellular adhesion molecule-1 (ICAM-1), which is over-expressed in pancreatic cancer [31]. However, although ICAM-1 is over-expressed in pancreatic cancer, adhesion as mediated by CD18 is regulated by the state of activation of the T cell expressing this integrin as well [32]. Thus, the inverse correlation between the activation/memory molecule, CD45RO, and CD18 in the $CD8^+$ subset, which we found in this study, could imply a defect in the cytotoxic activity of this $CD8^+$ T cell subset in pancreatic cancer.

TGF-β plays a central role in the mediation of the interaction between pancreatic tumour cells and T lymphocytes. Pancreatic cancer cells have been demonstrated to over-express TGF-β1, TGF-β2 and TGF-β3 [6]. *In vitro* studies have shown that TGF-β1 can induce the expression of $\alpha^E\beta_7$ on PBL [33] and can up-regulate this integrin in lymphocytes destined to become IEL [34,35]. However, it also mediates the down-regulation of E-cadherin expression on epithelial cells, as well as suppresses the outgrowth of $CD4^+$ $CD45RO^+$ T cells *in vitro* [36]. Moreover its antagonistic effect is reflected by the up-regulation of HML-1 and down-regulation of LFA-1 in the IEL microenvironment [35]. TGF-β1 is also instrumental in the stimulation of fibroblast proliferation and formation of the extracellular matrix [37]. Taken together, these findings raise the possibility that TGF-β1 may prevent TIL from coming into direct contact with tumour cells and hence avoid the cytolysis of these cells.

Inasmuch as therapeutical options are limited in pancreatic cancer, new approaches are crucial for the future management of patients suffering from advanced pancreatic cancer [1]. Enhancing the activity of cytotoxic effector cells by induction with IL-2 has been reported recently [38]. Following activation with IL-2 *in vitro* and *in vivo* enhanced lymphokine-activated killer (LAK) cell activity is mediated in part by $CD3^-$ $CD56^+$ NK cells and $CD3^+$ $CD56^+$ cytotoxic T lymphocytes. Our analysis revealed the presence of only few $CD56^+$ cells in pancreatic carcinoma. Moreover, IL-2 is also instrumental in the induction of HML-1 on PBL destined to become IEL [23]. Thus, in summary, in order to enhance the infiltration of pancreatic cancers by cytotoxic effector cells, the administration of IL-2 in clinical settings may seem beneficial in these patients. In addition, the regulation of the expression of E-cadherin and ICAM-1 on pancreatic cancer cells may present an alternative approach aimed at enhancing the interaction of epithelial cells and lymphocytes in pancreatic cancers *in vivo*. Thus, several factors, including interferon-gamma (IFN-γ) and IL-2, have been reported to enhance the direct interaction of ICAM-1 and LFA-1 [17]. Furthermore, E-cadherin expression can be induced by IFN-γ, tamoxifen and retinoic acids [39].

In conclusion, our study demonstrates that mainly $CD45RO^+$ T cells of the $CD4^+$ and $CD8^+$ lymphocyte subsets infiltrate the pancreas in human pancreatic cancer, with the $CD8^+$ phenotype representing the predominant T lymphocyte subset. However, a T cell phenotype, analogous to gut intraepithelial lymphocytes bearing markers typical for IEL ($CD8^+$ $\alpha^E\beta_7{}^+$) is found mainly in the fibrous stroma distant from the cancer cells. These findings, taken together with reports of down-regulation of the adhesion molecule ligand, E-cadherin, on intercellular junctions as well as the over-expression of TGF-β by pancreatic cancer cells, raise the hypothesis that pancreatic cancer cells may mediate the aggregation of cytotoxic T cells in the fibrous tissue and may thus escape their cytolytic effects. Furthermore, the down-regulation of activation markers on cytotoxic $CD8^+$ $CD18^+$ T lymphocytes could result in decreased cytotoxic activity towards pancreatic tumour cells.

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