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Carcinoembryonic Antigen-Specific but Not Antiviral CD4+ T Cell Immunity Is Impaired in Pancreatic Carcinoma Patients

Elena Tassi,* Francesca Gavazzi,†# Luca Albarello,¶ Vladimir Senyukov,* Renato Longhi,** Paolo Dellabona,§ Claudio Doglioni,¶ Marco Braga,‡# Valerio Di Carlo,‡# and Maria Pia Protti*||

Pancreatic carcinoma is a very aggressive disease with dismal prognosis. Although evidences for tumor-specific T cell immunity exist, factors related to tumor microenvironment and the presence of immunosuppressive cytokines in patients’ sera have been related to its aggressive behavior. Carcinoembryonic Ag (CEA) is overexpressed in 80–90% of pancreatic carcinomas and contains epitopes recognized by CD4+ T cells. The aim of this study was to evaluate the extent of cancer-immune surveillance and immune suppression in pancreatic carcinoma patients by comparing the anti-CEA and antiviral CD4+ T cell immunity. CD4+ T cells from 23 normal donors and 44 patients undergoing surgical resection were tested for recognition of peptides corresponding to CEA and viral naturally processed promiscuous epitopes by proliferation and IFN-γ production.

Importantly, whereas CD4+ T cells from normal donors produced mainly GM-CSF and IFN-γ, CD4+ T cells from the patients produced mainly IL-5, demonstrating a skew toward a Th2 type. On the contrary, the extent of antiviral CD4+ T cell immunity was comparable between the two groups and showed a Th1 type. The immunohistochemical analysis of tumor-infiltrating lymphocytes showed a significantly higher number of GATA-3+ compared with T-bet+ lymphoid cells, supporting a Th2 skew also at the tumor site. Collectively, these results demonstrate that Th2-immune deviation in pancreatic cancer is not generalized but tumor related and suggests that the skew might be possibly due to factor(s) present at the tumor site.

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2 Address correspondence and reprint requests to Dr. Maria Pia Protti, Tumor Immunology Unit, Cancer Immunotherapy and Gene Therapy Program, DIBIT, Scientific Institute H. San Raffaele, Via Olgettina 58, 20132 Milan. E-mail address: m.protti@hsr.it

3 Abbreviations used in this paper: Tr, T regulatory; CEA, carcinoembryonic Ag; EBNA2, Epstein-Barr nuclear Ag 2; HA, influenza hemagglutinin.
Others (35–37) and we (38, 39) previously reported that CEA contains naturally processed epitopes recognized by CD4 T cells from normal donors and colon and lung cancer patients. In the present study, we addressed the questions whether in pancreatic cancer patients tumor Ag-specific CD4 T cells are indeed polarized toward a Th2 type and whether this is generalized or related to local mechanisms by comparing the anti-CEA with antiviral CD4 T cell immunity.

CEA peptides corresponding to the identified epitopes were used to study the spontaneous anti-CEA response in normal donors and pancreatic carcinoma patients as compared with CD4 T cell responses specific for influenza and EBV-derived peptides. We found that the patients compared with normal donors have impaired quantitative (i.e., reduced number of responders) and qualitative (i.e., Th2 skew) anti-CEA CD4 T cell responses. This impairment is CEA specific because quantitative and qualitative CD4 T immunity against viral proteins was preserved, suggesting that local more than systemic immunosuppressive/regulatory mechanisms are active in pancreatic cancer patients. In agreement, immunohistochemical analysis at the tumor site showed the presence of a significantly higher number of GATA-3 compared with T-bet infiltrating lymphoid cells.

### Materials and Methods

#### Subjects and tumor samples

PBMC were obtained from the blood of 24 healthy subjects (i.e., normal donors) of the H. San Raffaele Blood Bank and 46 consecutive patients undergoing surgical resection for suspected pancreatic cancer. Patients’ blood was obtained at the time of surgery. Tumor samples were obtained from surgical specimens, cut into small pieces, and frozen for RNA extraction. The Institutional Ethics Committee had approved the study protocol and informed consent was obtained from all donors before blood sampling and surgical resection.
Selection and synthesis of peptides

Sequences CEA \(_{99-111}\), CEA \(_{117-129}\), CEA \(_{177-189/355-367}\), CEA \(_{425-437}\), CEA \(_{568-582}\), CEA \(_{652-666}\), and CEA \(_{666-678}\) were selected based on the TEPITOPE algorithm (40) as promiscuous HLA-DR binders (i.e., able to bind to several alleles) and previously shown to contain naturally processed epitopes (35, 37–39). Sequence 307–319 from influenza hemagglutinin (HA307–319) (41) and sequence 280–294 from Epstein-Barr nuclear Ag 2 (EBNA2280–294) (42) were selected to test antiviral immunity. Peptides corresponding to selected sequences were synthesized by the stepwise solid-phase method as described previously (43). The peptides were lyophilized, reconstituted in DMSO at 10 mg/ml, and diluted in RPMI 1640 (BioWhittaker) as needed.

Short-term culture and cytokine release assay

The experiments were performed as described previously (31, 44). Briefly, CD4\(^+\) T cells were purified from total PBMC by using magnetic beads (Miltenyi Biotec) and cultured in 96-well plates at \(3 \times 10^4\) cells/well in five replicates for each condition in 200 µl/well X-VIVO 15 (BioWhittaker) supplemented with penicillin (100 U/ml; BioWhittaker), streptomycin (50 mg/ml; BioWhittaker), and 3% heat-inactivated pooled human serum (tissue culture medium; BioWhittaker) in the presence of irradiated CD4\(^+\) depleted PBMC as APC, at a CD4\(^+\):APC ratio of 1:3. Stimuli were: PHA (10 µg/ml; Sigma-Aldrich), as positive control; CD4\(^+\) T cells in the presence of the APC only, as baseline (blank); and each single peptide (10 µg/ml). At day 7, half medium from each well was removed and replenished with fresh tissue culture medium containing IL-2 (25 IU/ml; R&D Systems) without any further Ag stimulation. At day 13, 100 µl of supernatant was collected from each well for cytokine detection and the cultures were pulsed for 16 h with \([^{3}H]\)TdR (1 Ci/well, 6.7 Ci/mol; Amersham Biosciences). The cells were collected with a FilterMate Universal Harvester (Packard Instrument) in specific plates (Unifilter GF/C; Packard Instrument) and the thymidine incorporated was measured in a liquid scintillation counter (TopCount NXT; Packard Instrument). Supernatants

FIGURE 1. CD4\(^+\) T cells against CEA in normal donors produce mainly GM-CSF and IFN-γ. CD4\(^+\) T cells from 23 normal donors were tested as described in Materials and Methods with the CEA peptides. Results for the 15 responders are shown. For \([^{3}H]\)thymidine incorporation assays, all data from the five replicates in the absence (CD4 + APC) or in the presence of the peptides are reported. For cytokine release assays, supernatants from the five wells were pooled and determination was performed in duplicate. Samples from peptide-stimulated cells were considered positive if at least double the values from unstimulated cells and above 50 pg/ml. The mean value of the unstimulated cells was subtracted from each value of peptide-stimulated cells and data reported are means of duplicate determinations ± SD. Responses significantly higher than the blanks (i.e., CD4 + APC) are indicated as *, \(p < 0.05\); **, 0.001 < \(p < 0.05\); and ***, \(p < 0.001\) (determined by unpaired, one-tailed Student’s \(t\) test). n.t., Peptide not tested.

FIGURE 2. CD4\(^+\) T cells against CEA in pancreatic cancer patients produce mainly IL-5. CD4\(^+\) T cells from 44 pancreatic cancer patients were tested as described in Materials and Methods with the CEA peptides. Results for the 13 responders are shown. For \([^{3}H]\)thymidine incorporation assays, all data from the five replicates in the absence (CD4 + APC) or in the presence of the peptides are reported. For cytokine release assays, supernatants from the five wells were pooled and determination was performed in duplicate. Samples from peptide-stimulated cells were considered positive if at least double the values from unstimulated cells and above 50 pg/ml. The mean value of the unstimulated cells was subtracted from each value of peptide-stimulated cells and data reported are means of duplicate determinations ± SD. Responses significantly higher than the blanks (i.e., CD4 + APC) are indicated as *, \(p < 0.05\); **, 0.001 < \(p < 0.05\); and ***, \(p < 0.001\) (determined by unpaired, one-tailed Student’s \(t\) test). n.t., Peptide not tested.
collected from each well of the five replicates of each condition were pooled and used for the detection by ELISA, according to the manufacturers’ instructions, of the following cytokines: GM-CSF, IFN-γ, and TGF-β (BioSource International); IL-5, IL-10, and IL-13 (Mabtech); and IL-17 (eBioscience).

**RNA extraction and RT-PCR**

Tumor samples (50–100 mg) were homogenized in the presence of 1 ml of TRIzol Reagent (Invitrogen) and total RNA was extracted following the manufacturer’s instruction. Single-strand cDNA was synthesized from 2 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). RT-PCR was performed with TaqDNA polymerase (Promega) by using CEA-specific oligonucleotides: CEA left, 5′-AACCCA GAACCCAGTGAGTG-3′ and CEA right, 5′-ATTGCTGGAAAGTCCCATC-3′. PCR conditions were: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, and a final elongation step of 10 min at 72°C.

**Immunohistochemical analysis**

Surgical specimens were fixed in buffered formalin and embedded in paraffin. Immunohistochemistry was performed on 5-μm tissue sections. All cases were immunostained with a sensitive nonbiotin detection system (NovoLink polymer; Novocastra), with diaminobenzidine development. Heat-induced Ag retrieval was performed using 0.01 M citrate buffer (pH 6.0) or Tris-EDTA (pH 9.0) in a water bath for 30 min. The following Abs were used: anti-CD3 mAb (clone PS1; Novocastra), anti-CD4 mAb (clone 4B12; Novocastra), anti-CD8 mAb (clone 1A5; Novocastra), anti-T-bet mAb (clone 4B10; Santa Cruz Biotechnology), anti-GATA3 polyclonal Ab.

**FIGURE 3.** CD4+ T cells against HA and EBNA2 in pancreatic cancer patients. CD4+ T cells from 21 pancreatic cancer patients were tested as described in Materials and Methods with the HA and EBNA2 peptides. Results for the 17 responders are shown. For [3H]thymidine incorporation assays, all data from the five replicates in the absence (CD4 + APC) or in the presence of the peptides are reported. For cytokine release assays, supernatants from the five wells were pooled and determination was performed in duplicate. Samples from peptide-stimulated cells were considered positive if at least double the values from unstimulated cells and above 50 pg/ml. The mean value of the unstimulated cells was subtracted from each value of peptide-stimulated cells and data reported are means of duplicate determinations ± SD. Responses significantly higher than the blanks (i.e., CD4 + APC) are indicated as *, p < 0.05; **, 0.001 < p < 0.05; and ***, p < 0.001 (determined by unpaired, one-tailed Student’s t test).

**FIGURE 4.** CD4+ T cells against HA and EBNA2 in normal donors. CD4+ T cells from 12 normal donors were tested as described in Materials and Methods with the HA and EBNA2 peptides. Results for the nine respondents are shown. For [3H]thymidine incorporation assays, all data from the five replicates in the absence (CD4 + APC) or in the presence of the peptides are reported. For cytokine release assays, supernatants from the five wells were pooled and determination was performed in duplicate. Samples from peptide-stimulated cells were considered positive if at least double the values from unstimulated cells and above 50 pg/ml. The mean value of the unstimulated cells was subtracted from each value of peptide-stimulated cells and data reported are means of duplicate determinations ± SD. Responses significantly higher than the blanks (i.e., CD4 + APC) are indicated as *, p < 0.05; **, 0.001 < p < 0.05; and ***, p < 0.001 (determined by unpaired, one-tailed Student’s t test). n.t., Peptide not tested; n.d., cytokine not determined.
with specific reactivity of CD4⁺ T cells in the presence of one or more CEA peptides in either one or both assays. CEA-specific CD4⁺ T cells were present in the blood of 65.2 and 29.5% of normal donors and pancreatic carcinoma patients, respectively. The two percentages are significantly different as from the χ² test (p = 0.0108).

To evaluate the functional activity of CEA-specific CD4⁺ T cells, culture supernatants were tested for cytokines released (i.e., GM-CSF, IFN-γ, and IL-5). IFN-γ and IL-5 were chosen as prototypic cytokines released by Th1 and Th2 cells, respectively. GM-CSF was tested to detect unpolarized cells. IL-4 was not tested because of potential consumption by CD4⁺ T cells in culture in the assay used (44). IL-10 and IL-13 were tested in a limited number of subjects to confirm production of Th2 cytokines. Fig. 1 shows the results obtained with normal donors. CD4⁺ T cells produced mostly GM-CSF and/or IFN-γ. IL-5 was produced in the majority of cases in association with the other two cytokines. On the contrary, CD4⁺ T cells from most pancreatic carcinoma patients produced IL-5 alone or in association with GM-CSF and/or IFN-γ (Fig. 2). IL-10 was rarely produced while IL-13 paralleled IL-5 production (data not shown). Collectively, these results suggest an impairment of the anti-CEA CD4⁺ T cell immunity in pancreatic cancer.

To rule out the presence of Th17 and Th3/Tr1 CD4⁺ T cells, IL-17 and TGF-β release were assayed in the culture supernatant...
of 12 patients (subjects 11, 29, and 37–46) and 2 normal donors (subjects 14 and 21). The two cytokines were not detected in any sample (data not shown).

Comparison between anti-CEA and antiviral CD4+ T cell immunity

To verify whether the Th2 skew in anti-CEA CD4+ T cell immunity observed in pancreatic carcinoma patients was generalized or CEA specific, we tested the reactivity of CD4+ T cells against the promiscuous viral sequences HA307–319 and EBNA2280–294 in 21 patients and 12 normal donors. The results are reported in detail in Figs. 3 and 4 and summarized in Fig. 5. In normal donors, the percentages of responders to HA and EBNA2 were almost comparable to the percentage of responders to CEA (Fig. 5A, left panel). On the contrary, in pancreatic cancer patients, while the percentage of responders to CEA was lower than in normal donors the percentages of responders to HA and EBNA2 were even higher (Fig. 5A, right panel), although not significantly different (p = 0.9183 and p = 0.3022, respectively, as from χ2 test).

Most importantly, although CEA-specific CD4+ T cells in patients produced mostly IL-5 (Fig. 2), HA- and EBNA2-specific CD4+ T cells produced mostly IFN-γ and/or GM-CSF (Figs. 3 and 5B, right panel). The pattern of cytokine produced by CD4+ T cells against the three different Ags did not change substantially in normal donors (Figs. 1, 4, and 5B, left panel). When comparing Th1

![Immunohistochemical analysis of immune infiltrates](image)

**FIGURE 6.** Immunohistochemical analysis of immune infiltrates. Representative staining of tumor samples from patient 4 (left panels), patient 15 (middle panels), and patient 39 (right panels) showing CD8+ (A–C)-, CD4+ (D–F)-, T-bet (G–I)-, GATA-3 (J–L)-, and FoxP3 (M–Q)-positive lymphoid cells within cancer cell stroma (original magnification, ×200). Cytoplasmic and nuclear GATA-3 staining are also present in neoplastic epithelial cells. The arrows in the T-bet and FoxP3 staining help the reader by indicating the positive cells in panels with a low/moderate number of positive cells.

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Table II. Immunohistochemical analysis of immune infiltrates

Note: Positive cells were enumerated as described in Materials and Methods. Patients with CEA-specific CD4+ T cells in the blood are highlighted in bold.

\* n.d., Not determined.
(IFN-γ production), Th2 (IL-5 production) and mixed Th1/Th2 (IFN-γ and IL-5 production) responses in normal donors and pancreatic cancer patients, we found that normal donors had a statistically significant higher percentage of Th1 CEA-specific CD4+ T cells responses than patients (p = 0.003, determined by a one-tailed z test) and patients showed a statistically significant higher percentage of Th2 CEA-specific CD4+ T cell responses than normal donors (p = 0.005, determined by a one-tailed z test; Fig. 5C, upper panel). The percentages of Th1, Th2, and Th1/Th2 responses toward HA and EBNA2 between normal donors and patients did not differ substantially (Fig. 5C, middle and lower panels). As expected, CD4+ T cells in the presence of the positive control (i.e., PHA) produced all three cytokines in the majority of the cases in both normal donors and patients (Fig. 5B, lower panels).

GATA-3+ lymphoid cells predominate within immune infiltrates in pancreatic cancer tissues

Immunohistochemical analysis was performed to characterize the immune infiltrate in 13 pancreatic cancer tissues (Table II). We determined the presence of CD8+ and CD4+ T cells and the expression by lymphoid cells of transcription factors T-bet and GATA-3 to distinguish between Th1 and Th2 cells, respectively (45). FoxP3 expression was also evaluated to identify T/recently activated effector T cells (46). We found immune infiltrates in all cases, mostly within the tumor stroma; although, as shown in Table II, the intensity differed among samples. Both CD8+ and CD4+ T cells were represented without a significant predominance of one or the other subset. When considering the Th1/Th2 polarization of tumor-infiltrating lymphocytes, we found that the number of GATA-3+ was significantly higher than the number of T-bet+ lymphoid cells (p < 0.001, determined by unpaired, one-tailed Student’s t test). FoxP3+ T cells were also present and their number was significantly higher than the number of T-bet+ cells (p < 0.02) but lower than GATA-3+ cells (p < 0.03). Interestingly, patients 39, 41, and 43, who had CEA-specific CD4+ T cells in the blood that produced discrete amounts of IFN-γ, showed the highest number of T-bet+ in their infiltrate. Fig. 6 shows representative examples of immunohistochemical analyses in patients with an intense (patient 4, left panels) or a moderate infiltrate (patient 15, middle panels), and in patients with the highest IFN-γ production in the blood (patient 39, right panels). Accordingly, in these last patients (subjects 39, 41, and 42), the number of T-bet+ was comparable or higher than the number of GATA-3+ cells (Table II). Cytoplasmic and nuclear GATA-3 staining was present in neoplastic epithelial cells, as also previously described (47); although in contrast to the report of Kalthoff and colleagues (48), we did not observe any FoxP3 staining in tumor cells.

Discussion

In this study, we compare the spontaneous antiviral and anti-CEA CD4+ T cell immunity in pancreatic carcinoma patients and normal donors. We found Th1-type antiviral immunity present in both patients and normal donors. On the contrary, in patients compared with normal donors, we found quantitative (i.e., reduced number of responders) and qualitative impairment (i.e., Th2 type) of anti-CEA immunity. In agreement, as shown by immunohistochemical analysis, the number of GATA-3+ lymphoid cells was predominant among tumor-infiltrating lymphocytes. These results show for the first time that pancreatic cancer patients have a Th2 skew in CD4+ T cells specifically directed against a tumor-associated Ag, suggesting that in pancreatic cancer local more than systemic immune-subversive factors are responsible for the observed immune deviation.

Previous studies (6–10) have addressed the presence of spontaneous immune responses against tumor-associated Ags in pancreatic carcinoma patients. CD8+ and CD4+ T cells specific for SART1–3 and ART4 (7), wild-type and mutated ras (6, 8, 10), MUC-1 (9), and the autologous tumors (9) were found in variable percentages. In most cases, effectors were shown to produce IFN-γ in response to their cognate Ags, suggesting a Th1 polarization. However, in the majority of reports, the release of Th2 cytokines by Ag-specific T cells was not investigated or was limited to IL-4 and IL-10. We also found 10% of patients with IFN-γ-producing CD4+ T cells and do not exclude the possibility that a Th1 along with a Th2 repertoire against the same or different tumor Ags co-exist in the patients. Beckhove and colleagues (9) reported higher frequencies of T cells specific for the autologous tumor in the bone marrow than in peripheral blood. They explained this discrepancy with the presence of a suppressive microenvironment at the tumor site, which might negatively affect the antitumor response measurable in the blood. On the contrary, the presence of low amounts of pancreatic tumor cells in the bone marrow could explain priming/restimulation of tumor-reactive T cells in a more favorable environment. It would be interesting to verify whether CEA-specific CD4+ T cells are also present in the bone marrow and whether these cells are endowed with the same or different effector functions compared with the ones detected in the blood.

A distinctive feature of our results is the observed impairment of the anti-CEA CD4+ T cell immunity in the patients compared with normal donors: this impairment being both quantitative and qualitative. With the experimental system used, we found proliferation and/or cytokine released by CEA-reactive CD4+ T cells in ~65% of normal donors. We previously (39) failed to detect spontaneous anti-CEA CD4+ T cells in a small cohort of seven normal subjects: important differences between the two studies are the number of subjects tested and the culture conditions used. The importance of the culture conditions in monitoring of immune responses for both background and low frequency detection has been recently reported (49). A high number of normal donors with CEA-reactive CD4+ T cells was also recently reported (50). It has been shown (51, 52) that CEA is expressed in the thymus with potential deletion of CEA-specific T cells. Nonetheless, it has been reported that negative selection in the thymus is incomplete, causing T cells with intermediate to low avidity escape into the periphery (53). These autoreactive T cells may then be kept under control by regulatory cells or by avoiding contact with the Ag, which is expressed at a low level in normal adult tissues. This second hypothesis is further supported by the possibility that CEA expressed in the thymus and in the tumor might have different posttranslational modifications, as it has been shown for MUC-1 (51). It is interesting to note that, although we confirmed CEA177-186/135-145 as immunodominant (38), other sequences (CEA196-201 and CEA560-565) were preferentially recognized by a larger number of normal donors or patients, respectively (Figs. 1 and 2). Indeed, de novo priming might have occurred in the patients with a different form of CEA and/or in the presence of different Ag-processing machineries, explaining the tendency to a preferential repertoire of the epitopes recognized within the two populations.

A Th2-immune deviation in CD4+ T cell responses against tumor-associated Ags may be due to either generalized or local factors. The intact repertoire of Th1 antiviral CD4+ T cells found here points to factor(s) present at the tumor site. Due to the likely existence of a preexisting repertoire of anti-CEA Th1 CD4+ T cells in pancreatic cancer patients, it may be speculated that during cancer development Th1 cells are recruited to the tumor site and exhausted/deleted after having exerted their effector function. At the


