

Potential plasticity of T regulatory cells in pancreatic carcinoma in relation to disease progression and outcome

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Received October 25, 2011; Accepted March 26, 2012

DOI: 10.3892/etm.2012.553

Abstract. CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) are understood to maintain peripheral tolerance to self-antigens and inhibit antitumor immune responses. However, compelling evidence suggests that, Tregs provide no anti-inflammatory protection in the tumor microenvironment, but rather contribute to a T helper 17 (Th17)-driven pro-carcinogenic process. Using three-color flow cytometry, we evaluated the percentage of circulating CD4⁺CD25⁺FoxP3⁺ Tregs in the peripheral blood of pancreatic carcinoma patients prior to and after chemotherapy [gemcitabine (GEM) alone, or GEM+oxaliplatin (GEMOX) or bevacizumab+capecitabine+radiotherapy (BEV+CAPE+RT)]. Correlations were sought between Treg counts and plasma levels of cytokines relevant to controlling the Treg/Th17 balance, i.e., interleukin (IL)-23, IL-17A, IL-6 and transforming growth factor β 1 (TGF- β 1), as measured by ELISA and the clinical features of pancreatic cancer. Treg, IL-6 and TGF- β 1 levels were higher in locally advanced and metastatic pancreatic carcinoma patients compared to controls. No parameter was correlated with disease stage except IL-6. IL-17A and TGF- β 1 were significantly associated with increased risk of poor prognosis. IL-17A was positively correlated with IL-23. Treg and IL-6 levels decreased following GEM monochemotherapy, IL-17A levels decreased after GEMOX, and IL-6 levels were reduced subsequent to BEV+CAPE+RT treatment. IL-23, IL-17A and TGF- β 1 levels were significantly lower in patients responding to chemotherapy (partial remission/stable disease) than in non-responders to chemotherapy (progressive disease). These results suggest an impact of the Treg/Th17-balance in pancreatic carcinoma, highlighting the significance of TGF- β 1 and

IL-17A as potential prognostic and predictive indicators. Immunological changes induced by mono and/or combined chemotherapy indicate specific windows of opportunity for introducing integrative interventions on a new target in pancreatic cancer, i.e. IL-17A, possibly improving survival in this highly lethal disease.

Introduction

The typical late presentation of pancreatic adenocarcinoma, its early systemic dissemination and its poor response to chemo- or radiation therapy, render prognosis and chance of a cure under present management poor. The dearth of conventional therapeutic options motivates the search for novel immunotherapeutic approaches to pancreatic cancer, including the development of monoclonal antibodies, cytokines, cellular immunotherapy and vaccines (1).

The risk of developing cancer may be increased by poorly regulated local inflammatory responses to pathogenic challenge. Although the etiology of pancreatic adenocarcinoma is not entirely clear, *in vivo* and *in vitro* studies show that inflammation is important in its carcinogenesis (2). Whereas acute inflammatory responses may be beneficial to the host, as it eliminates cancer cells, if this response becomes chronic it may hinder antitumor immune responses, enhancing tumor growth, angiogenesis, invasion and metastasis.

Natural CD25⁺CD4⁺ regulatory T-cells (Tregs) in the human peripheral bloodstream (identified by high CD25 expression and the IL-2 receptor α chain), alongside transcriptional factor forkhead box P3 (FoxP3) (3), have been implicated in regulating immune responses affecting tumor growth. Normally, Tregs maintain self-tolerance by suppressing immune responses against autoantigens and restore immune homeostasis during chronic inflammatory disorders (4). Although Tregs may reduce the risk of inflammation-associated cancer by downregulating inflammation, it is believed that, in cancer, they mainly function by suppressing the antitumor immune response. However, under poorly regulated pro-inflammatory conditions, Tregs fail to inhibit, and may stimulate, a pro-inflammatory T helper 17 (Th17) response, driving the pro-carcinogenic process (5).

Tregs, which promote tolerance, and Th17 cells, which induce inflammation (6), appear to arise from common naïve

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Key words: T regulatory cells, T helper 17, interleukin-17, interleukin-23, interleukin-6, transforming growth factor β 1, pancreatic carcinoma

CD4⁺ T precursor cells. During activation of human precursor CD4⁺ T cells, interleukin-6 (IL-6) and transforming growth factor β (TGF- β) promote differentiation toward Th17 cells, whereas TGF- β alone promotes differentiation of Tregs and suppresses Th17 (7). Furthermore, IL-23, a heterodimer produced by dendritic cells (DC) comprising the unique p19 subunit plus the p40 subunit in common with IL-12, also contributes to differentiation and maintenance of Th17 cells (8). Moreover, recent data indicate late-stage plasticity of a subpopulation of Tregs, which can be selectively induced to adopt a Th17 phenotype, suggesting that Tregs may do more to modulate cancer than simply block constructive anticancer responses (5).

Tregs and Th17 are involved in opposing immune responses critically involved in modulating inflammation in several diseases, including cancer. IL-6, TGF- β 1, IL-23 and IL-17A are key contributors to the Th17/Treg balance; the present study concentrated on these factors.

Materials and methods

Patients. The cohort comprised 62 patients (39 males, 23 females; median age 64.5 years, range 31-80) diagnosed with locally advanced or metastatic pancreatic adenocarcinoma at Centro Oncologico Ematologico Subalpino (COES), San Giovanni Battista Hospital, Turin, Italy, between March 2007 and March 2010. None of the patients had undergone anticancer treatment and all provided informed consent prior to entering the study. Study procedures complied with the Helsinki Declaration. Tumors were classified according to the International Union Against Cancer (UICC) Staging System at the start of chemotherapy; Table I presents the clinicopathological features of the patients. Of the 62 patients, 15 underwent radical surgery, only entering chemotherapy at disease progression and seven underwent palliative surgery, entering chemotherapy immediately afterwards. Four patients received no chemotherapy due to rapid progression. Protocols were: gemcitabine (GEM, 1250 mg/m² on days 1 and 8 every 21 days; 28 patients); GEM (1000 mg/m² on day 1) plus oxaliplatin (OX, 100 mg/m² on day 2 every 14 days; 23 patients); bevacizumab (BEV, 5 mg/kg every 14 days) plus capecitabine (CAPE, 825 mg/m²/b.i.d.) and concomitant radiotherapy (RT, 50.4 Gy in 28 cycles, following the study protocol; six patients); 5-fluorouracil (5-FU, 500 mg/m²) plus levofofolinate calcium (250 mg/m² on days 1, 8, and 15 every 28 days; one patient). After the first restaging, responses (by RECIST criteria) were: no complete responses, one partial response (patient with locally advanced carcinoma treated with chemo-radiotherapy in combination with BEV followed by radical surgery), 30 stabilizations and 26 disease progressions (clinical progression in eight cases, not documented radiologically). In two cases the response was not evaluable: one patient succumbed to unrelated causes after a single dose of chemotherapy and one patient discontinued therapy after two cycles due to toxicity and poor compliance. Follow-up continued until death or May 2010. A total of 44 patients died and the median survival time was 7.2 months. Seventeen patients survived to the end of the study: with four being in follow-up and 13 being in chemotherapy. Survival was 34.4% at one year after diagnosis, 11.5% at two years.

Cell isolation and plasma collection. Peripheral blood was collected from the patients in heparinized tubes at admission (time 0, prior to chemotherapy) and at first restaging (after 2-3 months, depending on chemotherapy regime) in parallel with evaluation of clinical course, and from the controls (20 age- and gender-matched volunteers). Peripheral blood mononuclear cells (PBMCs) and plasma were collected at interface and upper phase, respectively, after centrifugation of blood over a Ficoll-Hypaque density gradient. Plasma was stored at -20°C until use.

Flow cytometry. Circulating CD4⁺/CD25^{high}/FoxP3⁺ Tregs were enumerated by three-color flow cytometry. PBMCs were incubated with anti-CD4-FITC (Caltag Laboratories, Burlingame, CA, USA) and anti-CD25-PC5 (Beckman Coulter, Immunotech, France) mAb for 30 min at 4°C. Subsequent to washing with PBS, PBMCs were fixed and permeabilized with fixation/permeabilization buffer for 30 min at 4°C, washed twice with permeabilization buffer and stained with anti-human FoxP3-PE mAb, following the manufacturer's instructions (eBioscience, San Diego, CA, USA). Following a 30-min incubation at 4°C, cells were washed and analyzed by flow cytometry in a Coulter Epics IV Cytometer (Beckman Coulter, Inc, Fullerton, CA, USA) employing Expo32 Software (Beckman Coulter). Cells were gated on viable lymphocytes, following standard forward and sideways scattering parameters. Among cells included in this gate, we evaluated Treg subpopulations as FoxP3⁺ within the CD4⁺/CD25^{bright} subset. The results are expressed as percentages of triple-positive cell values, calculated subtracting the autofluorescence of unstained cells (morphology) from the fluorescence-intensity of antibody-labeled cells. Statistical analyses involved $\geq 30,000$ events gated on the population of interest.

ELISA determination of IL-23, IL-17A, IL-6 and TGF- β 1 plasma levels. IL-23, IL-17A, IL-6 and TGF- β 1 plasma concentrations were determined using commercially available ELISA kits. For TGF- β 1, plasma samples were tested after transient acidification (reducing pH to 1.5 by adding 1 N HCl for 10 min at room temperature and neutralizing with 1.2 N NaOH in 0.5 M HEPES). All evaluations were carried out in duplicate. The lower detection limits of the assays were: 4 pg/ml for IL-17A (eBioscience), 44.7 pg/ml for IL-23 and 4.61 pg/ml for TGF- β 1 (R&D, Minneapolis, MN, USA) and 7 pg/ml for IL-6 (Orgenium Laboratories-Ani Biotech Oy, Vantaa, Finland).

Statistics. Patient features were presented using mean and median for continuous variables and absolute and percentage frequency for categorical variables. To compare the Treg count and cytokine levels of patients with those of the healthy donors, we used the non-parametric Mann-Whitney two-sample statistic. Correlations among Tregs, IL-23, IL-17A, IL-6 and TGF- β 1 were assessed by Spearman's correlation coefficient. Overall survival was defined as the interval from diagnosis to death or last follow-up. Hazard ratios were estimated using the semi-parametric Cox model. The Wilcoxon matched-pairs signed-ranks test was used to compare Treg counts and cytokine levels pre- and post-treatment. Statistical analysis was carried out with Stata 9.2 (StataCorp LP, College Station, TX, USA) and SigmaStat 3.1 (Jandel Scientific, San Rafael, CA, USA).

Table I. Clinicopathological features of the patients.

Feature	Group A (n=27)	Group B (n=35)	Total (n=62)
Gender [n (%)]			
Female	11 (40.74)	12 (34.29)	23 (37.1)
Male	16 (59.26)	23 (65.71)	39 (62.9)
Age [mean (median)], in years	63.81 (66)	64.69 (62)	64.30 (64.5)
Disease stage at start of chemotherapy [n (%)]			
II	2 (7.41)	5 (14.29)	7 (11.29)
III	3 (11.11)	12 (34.29)	15 (24.19)
IV	22 (81.48)	18 (51.43)	40 (64.52)
Surgery [n (%)]			
None	20 (74.07)	20 (57.14)	40 (64.52)
Palliative	1 (3.7)	6 (17.14)	7 (11.29)
Radical	6 (22.22)	9 (25.71)	15 (24.19)
Metastases [n (%)]			
No	5 (18.52)	17 (48.57)	22 (35.48)
Yes	22 (81.48)	18 (51.43)	40 (64.52)
Metastatic site [n (%)]			
Liver	13 (59.09)	7 (38.89)	20 (50)
Peritoneum	4 (18.18)	4 (22.22)	8 (20)
Lung	2 (9.09)	2 (11.11)	4 (10)
Liver, peritoneum	1 (4.55)	4 (22.22)	5 (12.5)
Liver, lung	1 (4.55)	0 (0)	1 (12.5)
Lung, peritoneum	1 (4.55)	1 (5.56)	2 (5)
Chemotherapy [n (%)]			
No	4 (14.81)	0 (0)	4 (6.45)
Yes	23 (85.19)	35 (100)	58 (93.55)
Type of chemotherapy [n (%)]			
GEM	13 (56.52)	15 (42.86)	28 (48.28)
GEMOX	9 (39.13)	14 (40)	23 (39.66)
BEV+CAPE+RT	0 (0)	6 (17.14)	6 (10.34)
5-FU+levofofinate calcium	1 (4.35)	0 (0)	1 (1.72)
Response [n (%)]			
Complete remission (CR)	0 (0)	0 (0)	0 (0)
Partial remission (PR)	0 (0)	1 (2.86)	1 (1.72)
Stable disease (SD)	8 (34.78)	22 (62.86)	30 (51.72)
Disease progression (DP)	14 (60.87)	12 (34.29)	26 (44.83)

Group A, patients evaluated only on the day of admission prior to chemotherapy (time 0); group B, patients evaluated on the day of admission (time 0) (prior to chemotherapy) and at first restaging (after an interval of 2-3 months depending on the chemotherapy regime) in parallel with evaluation of the clinical course. GEM, gemcitabine; OX, oxaliplatin; BEV, bevacizumab; CAPE, capecitabine; RT, radiotherapy; 5-FU, 5-fluorouracil.

Results

Pre-chemotherapy circulating Treg counts in pancreatic cancer patients versus healthy donors. Tregs were characterized by flow cytometry, based on coexpression of CD4/CD25^{high}/FoxP3, in PBMCs from 62 patients with locally advanced or metastatic pancreatic cancer prior to chemo-

therapy, and from 20 healthy donors. Patients had a statistically significantly higher percentage of Tregs than controls [median (range): 4.72 (0.34-16.7) vs. 2.13 (0.2-7.4), $p=0.004$] (Fig. 1).

Pre-chemotherapy plasma levels of IL-23, IL-17A, IL-6 and TGF- β 1 in pancreatic cancer patients versus healthy donors. To investigate whether the tumor microenvironment perturbs

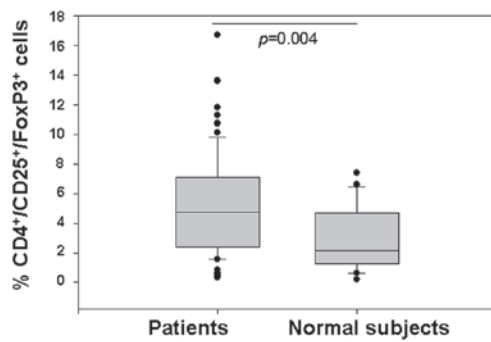


Figure 1. Basal Treg counts determined by flow cytometry in the peripheral blood of patients with locally advanced or metastatic pancreatic carcinoma and that of normal donors. p-values were obtained using the Mann-Whitney U test.

Treg generation or function via soluble factors, for the first time for pancreatic cancer, we simultaneously analyzed the profile of all the circulating cytokines involved in these complex regulatory mechanisms. Patient plasma concentrations of IL-23, IL-17A, IL-6 and TGF- β 1 were measured by ELISA prior to chemotherapy and compared with corresponding healthy levels. There was no significant difference between patients and controls for levels of IL-23 [median (range) pg/ml: 18.72 (0-154.4) vs. 29.54 (0-75.67), $p=0.340$]. Levels of IL-6 [median (range) pg/ml: 43.42 (4.54-470.58) vs. 20.41 (1.34-48.1), $p<0.001$] and TGF- β 1 [median (range) pg/ml: 1762.90 (907.60-5986.40) vs. 1247.70 (847.20-2290.00), $p=0.003$] were higher in cancer patients than those in the controls (Fig. 2). The patient-control difference in IL-17A was at the limit of statistical significance [median (range) pg/ml: 3.60 (0-130.61) vs. 0 (0-15.94), $p=0.075$].

Correlation among Tregs, IL-23, IL-17A, IL-6 and TGF- β 1. To assess any correlation between Tregs and cytokines, we looked at the relationship between pre-chemotherapy Treg counts and the plasma concentrations of IL-23, IL-17A, IL-6 and TGF- β 1. There was no correlation between Treg counts and levels of IL-23, IL-17A, IL-6 or TGF- β 1 ($r=0.128$, $p=0.322$; $r=0.099$, $p=0.444$; $r=-0.066$, $p=0.609$; $r=0.072$, $p=0.578$, respectively). Concerning relationships among cytokines, there was no correlation between IL-23 and IL-6 levels ($r=-0.031$, $p=0.809$) nor between IL-23 and TGF- β 1 ($r=0.232$, $p=0.070$), IL-17A and IL-6 ($r=0.242$, $p=0.058$), IL-17A and TGF- β 1 ($r=0.211$, $p=0.099$) nor IL-6 and TGF- β 1 ($r=0.023$, $p=0.856$). However, there was a statistically-significant positive correlation between IL-23 and IL-17A ($r=0.261$, $p=0.041$).

Correlation between disease stage, Treg count and levels of plasma IL-23, IL-17A, IL-6 and TGF- β 1. To clarify the clinical significance of these findings in locally advanced or metastatic pancreatic cancer, pre-chemotherapy levels of circulating Tregs and cytokines were compared between patients categorized as being at stages II/III ($n=22$) and stage IV ($n=40$). Levels of peripheral Tregs did not differ significantly between early (II/III) and late (IV) stage disease [median (range) %: 5.13 (0.41-16.7) vs. 3.86 (0.34-13.60), $p=0.162$]. Furthermore, circulating levels of IL-23, IL-17A or TGF- β 1 did not differ significantly between early and late stage disease [median

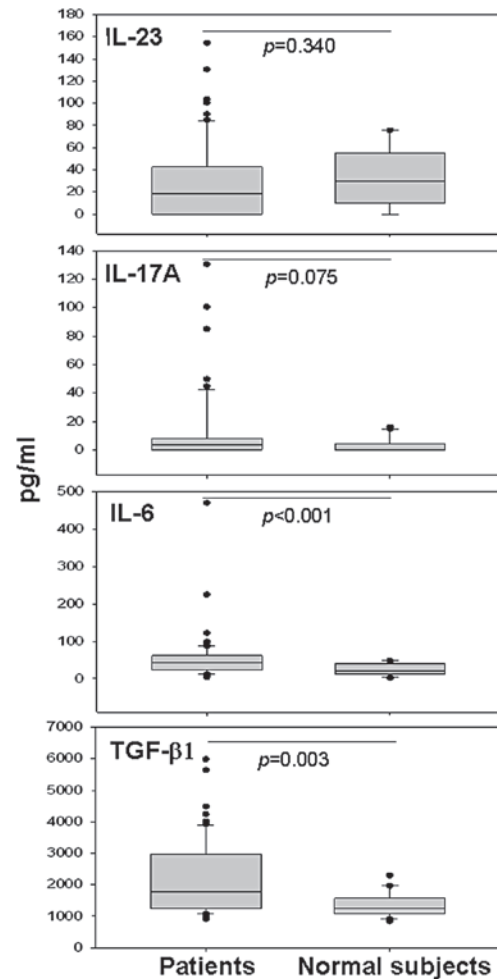


Figure 2. IL-23, IL-17A, IL-6 and TGF- β 1 levels determined by ELISA in the plasma of patients with locally advanced or metastatic pancreatic carcinoma compared to normal donors. p-values were obtained using the Mann-Whitney U test.

(range) IL-23 pg/ml: 15.44 (0-130.61) vs. 18.72 (0-154.40), $p=0.881$; median (range) IL-17A pg/ml: 0.40 (0-130.61) vs. 4.35 (0-49.76), $p=0.465$; median (range) TGF- β 1 pg/ml: 1580.80 (943.60-5643.50) vs. 1842.30 (907.60-5986.40), $p=0.431$]. Conversely, early-stage patients had significantly lower median levels of IL-6 vs. late-stage patients [median (range) pg/ml: 23.72 (10.68-225.07) vs. 52.61 (4.54-470.58), $p=0.008$].

Overall survival. The Cox model, adjusting for age, radical surgery, metastasis and gender, showed that the Treg counts, IL-23, and IL-6 plasma levels were not prognostic for overall survival ($p=0.75$, $p=0.133$, $p=0.246$, respectively). However, IL-17A, and especially TGF- β 1, were significantly associated with increased risk of poor prognosis ($p=0.050$ and $p=0.001$, respectively) (Table II).

Effect of different treatment protocols on the percentage of Tregs and plasma levels of IL-23, IL-17A, IL-6 and TGF- β 1. Percentages of circulating Tregs pre- (Time 0) and post-chemotherapy were analyzed comparatively at first restaging (Time 1) in 15 patients treated with GEM, 14 with GEMOX and 6 treated with BEV+CAPE+RT. As Table III shows, following the stratification of patients by treatment protocol,

Table II. Hazard ratio estimated by Cox model.

Variables	Hazard ratio ^a	95% CI	P-value
Tregs	1.015	0.320-1.110	0.75
IL-23	1.007	0.998-1.015	0.133
IL-17A	1.019	0.998-1.040	0.050
IL-6	1.002	0.998-1.007	0.246
TGF-β1	1.050	1.021-1.079	0.001

^aAdjusted for age, radical surgery, metastasis and gender.

there was no significant pre- or post-treatment difference in the percentages of peripheral Tregs in those who received GEMOX and BEV+CAPE+RT treatment. However, those receiving GEM alone showed a statistically significant reduction in Tregs (p=0.021).

Pre- and post-therapy plasma levels of IL-23 and TGF-β1 did not differ significantly with GEM or BEV+CAPE+RT. Only GEMOX significantly reduced levels of IL-17A (p=0.046) and IL-23 levels but only at the limit of statistical significance (p=0.059). IL-6 was significantly reduced by treatment with GEM or BEV+CAPE+RT (p=0.001, p=0.028, respectively).

Among the patients who passed first restaging after pharmacological treatment (n=57), 55.6% of those receiving GEM had stable disease (SD) with median (range) survival 457 (106-2005) days, whereas 44.4% had disease progression

(DP) with median (range) survival 158.5 (43-601) days; of patients receiving GEMOX, 43.5% showed SD with median (range) survival 375 (112-836) days, whereas 56.5% had DP, with median (range) survival 118 (73-957) days; 83.4% of patients receiving BEV+CAPE+RT (n=5) had SD with median (range) survival 406 (126-770) days and 16.6% (n=1) were in partial remission (PR) with median survival of 728 days.

Objective tumor response rate, percentage of Tregs, plasma levels of IL-23, IL-17A, IL-6 and TGF-β1. Overall chemotherapy response rate was 54.3% (31 responders comprising 1 PR and 30 SD, and 26 non-responders with DP). No statistically significant responder/non-responder difference was found between pre-treatment Treg percentages and IL-6 plasma levels [median (range) % Treg: 5.06 (0.34-16.70) vs. 4.10 (1.60-13.60), p=0.773; median (range) IL-6 pg/ml: 40.7 (4.54-225.07) vs. 50.73 (4.54-470.57), p=0.178]. However, IL-23, IL-17A and TGF-β1 levels were significantly lower in responders than non-responders [median (range) IL-23 pg/ml: 4.68 (0-103.66) vs. 33.17 (0-130.61), p=0.030; median (range) IL-17A pg/ml: 0 (0-85) vs. 5.65 (0-130.61), p=0.040; median (range) TGF-β1 pg/ml: 1469.8 (907.6-5643.5) vs. 1912.85 (934.7-5986.4), p=0.032].

Discussion

Tregs are significant in tumor immune evasion, operating both by blocking generation of immunity to tumor antigens in the periphery and by neutralizing tumor-infiltrating effector

Table III. Treg and plasma levels of IL-23, IL-17A, IL-6 and TGF-β1 pre- and post-treatment.

Chemotherapy	% Treg ^a	IL-23	IL-17A	IL-6	TGF-β1
	median (range)	pg/ml ^b median (range)			
GEM (n=15)					
Time 0 ^c	4.90 (0.87-16.7)	34.21 (0-85)	0 (0-85)	40.70 (10.68-225.07)	1691.80 (907.6-5643.5)
Time 1 ^d	2.80 (0.33-7.9)	24.88 (0-120.24)	0 (0-6.2)	17.19 (3.24-160.21)	1469.80 (690.3-3256.3)
p-value	0.021	0.842	0.161	0.001	0.100
GEMOX (n=14)					
Time 0	3.95 (0.57-11.8)	31.10 (0-103.61)	0 (0-130.61)	29.67 (4.54-470.58)	1502.90 (1127.4-5986.4)
Time 1	3.6 (0.4-10)	21.77 (0-80.85)	0 (0-50.7)	20.58 (3.24-230.91)	1855.65 (1074.8-3753.3)
p-value	0.638	0.059	0.046	0.088	0.683
BEV+CAPE+RT (n=6)					
Time 0	5.10 (0.41-11.3)	4.15 (0-81.89)	0 (0-2.07)	24.08 (20.27-37.62)	1548.85 (1069.4-3269.2)
Time 1	6.36 (1.5-13)	23.32 (0-40.43)	0 (0-0)	12.70 (4.54-25.60)	1964.95 (1056-3134.8)
p-value	0.753	0.518	0.159	0.028	0.463

^aPercentage of CD4⁺/CD25⁺/FoxP3⁺ cells obtained by flow cytometry; ^bplasma levels measured by ELISA. ^cPre-chemotherapy; ^dpost-chemotherapy (first restaging).

T cells. They are therefore a significant obstacle to successful tumor immunotherapy (9). However, it has recently emerged that, in the tumor microenvironment, Treg cells can undergo aberrant conversion, producing the pro-inflammatory IL-17 instead of the immunosuppressive IL-10 (5). Thus, the role of Tregs may be dual, helping the tumor to survive and expand – Tregs fail to inhibit, and may contribute to, a Th17-driven pro-carcinogenic process.

Concurrently, alongside DC activation, local and systemic alterations of the effector T cell response have been reported in pancreatic cancer patients (10,11). However, in pancreatic cancer little is known regarding the effects of host Treg switch from protecting to suppressing the antitumor immune response.

This study analyzed the frequency of circulating Tregs and, for the first time, concomitant levels of IL-23, IL-17A, IL-6 and TGF- β 1, cytokines implicated in the complex mutual regulation mechanism of Treg/Th17 subpopulations. Patients with locally advanced or metastatic pancreatic carcinoma were evaluated prior and subsequent to palliative chemotherapy. Three-color flow cytometry was employed for Tregs, and ELISA was carried out to assess levels of cytokines. The results were compared with those of matched controls, investigating correlation with clinical features.

Treg levels were approximately twice the normal level in pancreatic carcinoma patients, consistent with other studies reporting increased Treg levels in various cancer patients (12-15), including pancreatic carcinoma (16).

According to the physiological-steady-state theory, Tregs in tumor patients may increase to the highest level compatible with prolonged tumor-host interaction, allowing the tumor to escape immune-mediated rejection, while protecting the host from excessive and indiscriminate immunosuppression. However, this equilibrium is temporary, and the host eventually surrenders to the overwhelming tumor burden. Animal studies have demonstrated that during tumor progression, the initial concomitant immunity is progressively lost as suppressor CD4⁺ T cells are generated (17). *In vitro* studies have revealed that suppressor T cells generated in tumor-draining lymph nodes to abrogate antitumor reactivity possess the same functional properties and *FoxP3* expression levels as Tregs naturally present in the thymus in order to maintain self-tolerance (18). Tumor cells may actively recruit, activate, and expand Tregs by directly or indirectly presenting self-antigenic peptides for their recognition. Although they are refractory to T cell receptor stimulation *in vitro*, Tregs are able to proliferate in response to self-antigens *in vivo* in the presence of IL-2 (19). Moreover, suppressive cytokines such as IL-10 and TGF- β and chemokines such as CCL22, all secreted by tumor cells, tumor-infiltrating macrophages, myeloid suppressor cells and by DC, not only recruit Tregs to tumor sites, but also favor the conversion of non-suppressive T cells into suppressive-function Tregs (20).

Since the percentage of Tregs was not correlated with disease stage in our series of advanced pancreatic carcinoma patients, it is tempting to speculate that pancreatic tumor-induced Treg expansion occurs early in tumorigenesis and, once established, remains constant during the late phase of tumor progression. This has been reported to occur in ovarian, breast, head and neck cancers and gastrointestinal carcinomas (16,21-24). However, due to late presentation and delayed

diagnosis, the timing of Treg expansion in pancreatic cancer is difficult to assess. Moreover, in contrast to findings in ovarian carcinoma and breast cancer (21,22), Treg frequency appears not to be correlated with poor survival in pancreatic carcinoma patients. However, since our study only investigated patients with locally advanced or metastatic cancer, the significance of Tregs as potential prognostic and predictive indicators must be evaluated prospectively, in a large cohort of patients with operable tumors.

There is mounting evidence that the composition of CD4⁺ cell functional subsets is dynamic and is controlled by several key cytokine networks, which play pivotal roles in promoting T regulatory or inflammatory responses upon microenvironment request. Naturally occurring CD4⁺CD25⁺FoxP3⁺ suppressor cells (nTregs), which govern self-tolerance, derive from the thymus, but CD4⁺FoxP3⁺ immunoregulatory cells (iTregs) related to IL-10 and/or to TGF- β can be induced in the periphery during immune response.

The conversion of CD4⁺FoxP3⁻ to CD4⁺FoxP3⁺ Treg occurs at immune-privileged sites and has been shown *in vivo* to depend on TGF- β (25). Tumor cells themselves, including pancreatic cancer cells (26), and tumor-educated immune cells, can locally produce abundant TGF- β . It is therefore likely that the Treg pool in tumor bearers includes newly generated Tregs, alongside proliferating nTregs. However, it has recently been shown that TGF- β , under inflammatory conditions, is important in inducing Treg and IL-17-producing cells (Th17) from T CD4⁺ naïve cells, firmly linking these two subsets, which possess opposing functions (27). A T-cell activation state, which produces IL-17 as principle effector cytokine, can be promoted by the combined action of TGF- β and IL-6 (7); IL-23, a cytokine produced by myeloid DC and activated macrophages, is required for their expansion and maintenance (8).

We addressed the relationship between Tregs and the third arm of the CD4⁺ T-cell effectors, Th17, in pancreatic cancer patients, simultaneously investigating plasma levels of IL-23, IL-17A, IL-6 and TGF- β 1, the crucial soluble factors orchestrating the Treg/Th17 balance (28). We found a significant elevation in the levels TGF- β 1 and IL-6 and slightly elevated IL-17A levels in advanced pancreatic carcinoma patients compared to normal donors; levels of IL-23 did not differ significantly. Circulating cytokine levels were not correlated with the percentage of Tregs. However, the finding that IL-6 levels reflect disease status suggests that its overexpression may be crucial, not only in determining tumor growth, invasion, angiogenesis and cachexia, but also in controlling the Th17/Treg balance in this tumor type. IL-6 participates in differentiation of both T-cell subsets, whereas it induces Th17 differentiation, acting jointly with TGF- β , and inhibits TGF- β -induced Treg differentiation (28).

Notably, among the cytokines studied, TGF- β 1 and IL-17A were significantly associated with poor prognosis. In the case of IL-17A, this is the first report of such an association in cancer. Whereas, during early tumor formation, TGF- β can function as a tumor suppressor and tends to prevent tumorigenesis, in established tumors overproduction of TGF- β is often associated with poor prognosis (29). TGF- β can suppress or alter the activation, maturation, and differentiation of innate and adaptive immune cells, including natural killer (NK)

cells, DC, macrophages, neutrophils, and CD4⁺ and CD8⁺ T effector cells (29), thus promoting an overall tolerogenic state. However, depending on the cytokine milieu, TGF- β can also control the ratio between Tregs and Th17 cells in the tumor microenvironment (30). Since IL-17A was an independent factor that negatively affected prognosis in our patients, and since Th17 cells are the sole cellular source of IL-17 in the human tumor microenvironment, we may speculate that, at a certain point during tumor development, Tregs are converted to Th17, stimulated by high levels of IL-6 and TGF- β 1; this contributes to tumor progression in advanced pancreatic carcinoma. A similar event has been reported in hepatocellular carcinoma and non-small cell lung carcinoma patients (31,32), whereas in ovarian carcinoma, high levels of IL-17 predict improved patient survival (33). This contradiction may be accounted for by the intensity and nature of IL-17-producing cells and the other inflammatory and immune cells infiltrated in the tumor microenvironment. The precise situations in which IL-17 has pro-tumor or antitumor activity remain to be fully explored. However, increasing evidence points to the ability of IL-17 to induce IL-6 and promote chemotaxis of inflammatory cells, angiogenesis and invasion in cancer (34). Importantly, we found a significant association between IL-23 and IL-17A plasma concentrations in support of the hypothesis that, in advanced pancreatic carcinoma, Th17-polarizing (TGF- β , IL-6) and/or stabilizing (IL-23) cytokines are important molecular links between tumor-promoting pro-inflammatory processes and the failure of adaptive immune surveillance to infiltrate tumors. In chemical carcinogenesis and transplantable tumor models, and through genetic deletion in mice, IL-23 has been shown to directly participate in triggering a pro-inflammatory cytokine cascade and sustaining proliferation and/or survival of IL-17-producing Th17 cells (35), but reducing CD8⁺ T-cell infiltration, activation and function (36). Significant upregulation of IL-23 mRNA has been found in the overwhelming majority of carcinoma types, as has expression of IL-17 transcript (37) as the result of the tumor-induced pro-inflammatory environment.

Like IL-12, IL-23 is efficiently produced by activated macrophages and DC; secretion of these cytokines is reciprocally regulated and affects the nature and evolution of the initial innate and immune responses. It was recently reported that Stat3 signaling, operative in the tumor microenvironment, shifts inflammation from an antitumor IL-12 program to a tumor-enhancing IL-23 program, suggesting that preponderant production of IL-23 over IL-12 by antigen-presenting cells in tumor-draining lymph nodes ultimately skews the differentiation of tumor-specific T cells, possibly leading to dominance of Treg and Th17 responses over antitumor Th1 responses (38).

Further investigation is needed, as information concerning the relevance of the IL-23/IL-17A axis in cancer biology is scant and contradictory. However, since it has been shown that IL-18 participates in the differentiation of Th17 cells in synergy with IL-23 (39), and since elevated levels of free active IL-18 can play a paradoxically detrimental role in pancreatic carcinoma (40), it is tempting to hypothesize a functional link with the present findings, envisaging a new important mechanism whereby the Th17 effector function is augmented in cancer.

As curative surgery is only practicable in a small group of pancreatic carcinoma patients, systemic palliative chemo-

therapy remains the standard-of-care for patients with locally advanced or metastatic cancer. This study employed standard single-therapy (GEM), two-drug combination therapy (GEMOX), or a newer therapeutic approach consisting of BEV+CAPE+RT. At the time of first restaging, GEM administered alone significantly reduced the percentage of Treg cells, as well as IL-6 levels. By contrast, GEMOX significantly reduced IL-17A plasma levels and induced downregulation of IL-23. Combined administration of BEV+CAPE+RT produced no change in the frequency of Treg, nor IL-23 or IL-17A levels, but caused a statistically significant decrease in IL-6.

The apparently contradictory effect of GEM as a single agent and GEM combined with OX is unsurprising, since metabolic and biological interactions between drugs are possible and may result in synergistic, additive, or antagonistic actions. Elsewhere we report an antagonistic interaction between GEM and 5-FU in some pancreatic cancer cases (41).

Very few data exist regarding the effects of the chemotherapeutics used here on the levels of circulating Tregs, or on plasma levels of IL-23, IL-17A, IL-6 and TGF- β 1. In colon carcinoma patients, GEM in association with other chemotherapeutics, including OX, has been shown to reduce Tregs in the peripheral blood (42). However, these analyses were performed at early time points, whereas ours were after 2-3 months, depending on the chemotherapy regime; this difference may account for the discrepancy in findings. GEM has, moreover, been found to cause a significant decrease in the number of myeloid suppressor cells, one of the major sources of TGF- β 1 in the cancer microenvironment, present in the spleens of tumor-bearing animals (43). CAPE and/or RT were probably responsible for the effects observed here, since these two treatments have been shown to decrease circulating IL-6 and TGF- β 1 levels in human and in animal models (44,45), whereas BEV is a humanized neutralizing monoclonal antibody that acts against vascular endothelial growth factor A.

Considering the relation of immunological parameters to clinical response, irrespective of the chemotherapeutic regimen, responders (PR/SD) had lower IL-23, IL-17A, and TGF- β 1 levels than non-responders, indirectly suggesting that inflammatory status may play a role in inducing chemotherapy resistance. Potential limitations of these findings could be the different types of chemotherapy employed and the limited cohort. However, some preliminary conclusions may be drawn. GEM as a single agent and the combined treatment BEV+CAPE+RT reduced plasma levels of IL-6 (a cytokine that, in association with TGF- β 1, induces pro-Th17 development) and produced more favorable effects in terms of overall survival (BEV+CAPE+RT: 100% of patients with SD, median overall survival 406 days; GEM: 69% with SD, 31% with DP, median overall survival 360 days) than did GEMOX (42% with SD, 31% with DP, median overall survival 186.5 days).

Current theories indicate a role of chronic inflammation in generating conditions that contribute to malignant transformation, e.g., the progressive accumulation of genetic mutations consequent on repeated DNA damage and cell regeneration in an environment favoring proliferation and neovascularization. The present data provide novel insight into the significance of the pro-inflammatory response in human pancreatic carcinoma progression. We may speculate that, under pathophysiological

conditions, the persistence of a still-unidentified inflammatory trigger in a particular context, may transform potentially protective cytokines, such as IL-17A, IL-18 and IL-23 into pro-cancer cytokines.

These observations support our hypothesis that, in pancreatic carcinoma, during chronic inflammatory processes linked to tumor development, Tregs may augment rather than suppress Th17-mediated immune responses. This pro-inflammatory switch may play a crucial role in determining tumor progression, and paradoxically appears to be associated with a less favorable outcome. We may assume that the tumor microenvironment converts inflammation, from an IL-12-type program with strong antitumor effects, due to activation of NK cells and cytotoxic CD8⁺ T lymphocytes, to a tumor-promoting IL-23-type program. It is known that the prevailing production of IL-23 (rather than IL-12) by DC and tumor antigen-stimulated macrophages in tumor-draining lymph nodes and microenvironment, respectively, may result in subversion of tumor-specific T-cell differentiation, with a prevalence of Treg and Th17-type responses over the antitumor Th1 response (35). Moreover, IL-23, acting in an autocrine/paracrine manner on DC and activated macrophages, induces production of IL-1, IL-6 and TNF- α , responsible for the marked cachexia in patients with advanced pancreatic carcinoma (36).

Aberrant Tregs have been reported in polyp-ridden mice. By promoting inflammation and suppressing Th functions, these provide a dual advantage for tumor growth (46). The discovery of the two opposing cellular and signal networks (Treg/Th17, IL-23/IL-17), critically involved in modulating inflammation, induced not only by autoimmunity or bacterial infection but also by the tumor microenvironment, has dramatically changed the view of cell-mediated immune responses. Importantly, and for the first time, our results show the impact of the Treg/Th17 balance in pancreatic carcinoma in a new perspective, highlighting the significance of IL-17A as a potential prognostic and predictive indicator and showing that chemotherapy, if appropriately associated with immunotherapy, may amplify antitumor effects.

Due to its control of tumor-promoting inflammatory and angiogenic pathways and its immunoregulatory effects on tumor immune surveillance, IL-17A may be an attractive target for tumor treatment. As the underlying mechanism regulating the Th17/Treg balance is better understood, it may lead to the development of effective pancreatic cancer vaccine strategies that circumvent these tumor-related mechanisms.

Acknowledgements

We thank Dr Manuela Ceccarelli and Dr Anna Castiglione [Unit of Cancer Epidemiology (CPO-Piemonte), Azienda Ospedaliera Universitaria San Giovanni Battista, Turin, Italy] for their assistance with statistical analysis and for critical reading of the manuscript. This study was supported by research funds from the Piedmont Regional Government (Regione Piemonte, Italy) to G.B.

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