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Induction of Th17 Cells in the Tumor Microenvironment Improves Survival in a Murine Model of Pancreatic Cancer

Jennifer L. Gnerlich,* Jonathan B. Mitchem,* Joshua S. Weir,* Narendra V. Sankpal,* Hiroyuki Kashiwagi,* Brian A. Belt,* John M. Herndon,* Timothy J. Eberlein,* † Peter Goedegebuure,* † and David C. Linehan* †

An important mechanism by which pancreatic cancer avoids antitumor immunity is by recruiting regulatory T cells (Tregs) to the tumor microenvironment. Recent studies suggest that suppressor Tregs and effector Th17 cells share a common lineage and differentiate based on the presence of certain cytokines in the microenvironment. Because IL-6 in the presence of TGF-β has been shown to inhibit Treg development and induce Th17 cells, we hypothesized that altering the tumor cytokine environment could induce Th17 and reverse tumor-associated immune suppression. Pan02 murine pancreatic tumor cells that secrete TGF-β were transduced with the gene encoding IL-6. C57BL/6 mice were injected s.c. with wild-type (WT), empty vector (EV), or IL-6-transduced Pan02 cells (IL-6 Pan02) to investigate the impact of IL-6 secretion in the tumor microenvironment. Mice bearing IL-6 Pan02 tumors demonstrated significant delay in tumor growth and better overall median survival compared with mice bearing WT or EV Pan02 tumors. Immunohistochemical analysis demonstrated an increase in Th17 cells (CD4+IL-23R+ cells and CD4+IL-17+ cells) and IL-17+ cells infiltrating lymphocytes was substantiated at the cellular level by flow cytometry and ELISPOT assay and mRNA level for retinoic acid-related orphan receptor γ and IL-23R by RT-PCR. Thus, the addition of IL-6 to the tumor microenvironment skews the balance toward Th17 cells in a murine model of pancreatic cancer. The delayed tumor growth and improved survival suggests that induction of Th17 in the tumor microenvironment produces an antitumor effect. The Journal of Immunology, 2010, 185: 000–000.

The role of CD4+ T cells in tumor immunity is poorly understood. Naïve CD4+ T cells differentiate into mature Th1, Th2, Th17, or T regulatory cells (Tregs). We and others (1–9) have shown that Tregs suppress immune responses and induce tolerance at tumor sites. Treg and Th17 cells share a common lineage, and terminal differentiation of suppressor versus effector cells at the tumor site may tip the balance between tolerance and tumor rejection. Moreover, recent evidence suggests that mature Tregs can be reprogrammed into competent Th17 effector cells, and the plasticity of T cell lineage may be an important mechanism by which immune homeostasis is maintained (10, 11). Efforts to manipulate Treg function to release immune effector cells from immunosuppressive regulatory controls have become of increasing interest to tumor immunologists.

Th17 cell lineage commitment is initiated by TGF-β and IL-6, and the lineage is maintained by IL-23 and amplified by the autoimmune release of IL-21 (12–18). The transcription factors retinoic acid-related orphan receptor (ROR) γt and STAT3 are essential for the differentiation of Th17 cells and IL-17 cytokine expression (19–21). IL-17 is a potent inflammatory cytokine that works on a variety of cell types including fibroblasts, endothelial cells, and epithelial cells to induce the expression of other inflammatory cytokines, such as IL-6, TNF, G-CSF, chemokines, and matrix metalloproteases, all of which coordinate to produce a robust inflammatory response (22–27).

Th17 cells have been implicated in many autoimmune diseases, but their role in cancer has not been fully elucidated and remains controversial (22, 28–33). Increased levels of Th17 cells have been detected in many human cancers, including ovarian, pancreatic, renal cell, and gastric cancer (34, 35). Some studies report that the presence of higher levels of Th17 cells in tumor tissues or peripheral blood correlate with advanced cancer (36). Other studies describe the opposite and suggest that Th17 cells may have a potent antitumor effect, being found in patients with limited disease or long-term survivors (37, 38). In murine models, tumor-specific Th17-polarized cells that recognized an Ag expressed by both normal melanocytes and B16 melanoma eradicated the established melanoma tumors and improved survival (39). Also, inflammatory killing induced in the normal prostate through an IL-6/IL-17–mediated autoimmune response was shown to be effective at rejecting established metastatic murine prostate tumors (40). These studies suggest Th17 cells may play a vital role in tumor rejection based on the intimate relationship between autoimmunity and anti-tumor immunity for tissue-specific Ags.

Tregs, in contrast, maintain immune homeostasis by inhibiting effector T cell proliferation and autoimmune responses (41, 42). Interestingly, Tregs are upregulated in the peripheral blood and tumor microenvironment in many cancers including pancreatic cancer (1–9). High serum levels of TGF-β have also been found in

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Abbreviations used in this paper: EV, empty vector; IL-6 Pan02, IL-6-transduced Pan02 cells; RORγt, retinoic acid-related orphan receptor; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell; WT, wild-type.

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many types of cancer, especially pancreatic cancer, in which elevation of TGF-β in the serum may drive Treg differentiation and is correlated to tumor cell dissemination and poor survival (1, 3, 43). Likewise, an increased prevalence of Tregs correlates with more advanced cancer and is a marker of poor prognosis (5–7). When Tregs are blocked or depleted, a more effective antitumor effect is seen in mouse models of cancer (9, 44–46).

Recent evidence has shown that there exists an intricate and reciprocal regulation between Th17 cells and Tregs (10, 11, 14, 47). IL-6 plays a pivotal role in the CD4+ T cell lineage differentiation. Although TGF-β induces the differentiation of Tregs via Foxp3 transcription, IL-6 inhibits the differentiation of Tregs and, along with TGF-β, drives the differentiation of naïve CD4+ T cells into Th17 cells (12–14, 48–52). Thus, we hypothesized that the addition of IL-6 to the pancreatic tumor microenvironment rich in TGF-β may promote Th17 cell differentiation, which may lead to an improved antitumor response. To test this hypothesis, we genetically engineered the murine pancreatic cancer cell line Pan02, which naturally secretes TGF-β, to also produce IL-6. By changing the cytokine profile in the tumor microenvironment, we aimed to alter the balance of CD4+ T cells in favor of Th17 cells to produce a more effective antitumor response.

### Materials and Methods

**Cloning mIL6 into lentivirus vector: lentivirus generation and transduction**

Human T cell leukemia virus–mIL6 encoding DNA sequence form pORF9-mIL6 (InvivoGen, San Diego, CA) was subcloned into lentiviral vector pSicoR-puro with added XhoI-Nhel restriction sites. For lentivirus generation, All U300 and U300-3′ were transfected into 293T-SicoR-mIL6 and VSVG in Opti-MEM media using Fugene-6 (Roche Diagnostics, Indianapolis, IN). After overnight culture, serum containing media was added, and the incubation was allowed to continue for an additional 48 h. The first and second viral harvests, performed at 48 and 72 h posttransfection, respectively, were used to transduce the murine pancreatic adenocarcinoma Pan02 cell line (also known as Panc-02) (53). Viral supernatants were filtered through a 0.45-μm Millipore filter (Millipore, Billerica, MA) and used directly to infect cells with protamine sulfate (Sigma-Aldrich, St. Louis, MO) at a concentration of 6 μg/ml. Puromycin-resistant cells were selected. Pan02 cells transduced with empty vector (EV) were used as a control. IL-6 transcript was confirmed by quantitative RT-PCR with primers: IL-6 sense: 5′-CCGGAGAGGAGACTTCACAG-3′ and IL-6 antisense: 5′-TCCACGATTTCCCAGAGAAC-3′. Protein expression of IL-6 was confirmed by ELISA.

**Tumor cell lines.** Pan02 is a murine pancreatic adenocarcinoma cell line syngeneic to C57BL/6 and was obtained from the Division of Cancer Treatment Tumor Repository (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). All tumor cell lines, including wild-type (nontransduced) Pan02 cells (WT Pan02), empty control vector Pan02 cells (EV Pan02), and IL-6 transduced Pan02 cells (IL-6 Pan02), were maintained in culture at 37°C in humidified air (5% CO2) in complete media consisting of 1× high glucose DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT), 1% penicillin/streptomycin (Cellgro, Manassas, VA), and 10 mM HEPES buffer (Cellgro). Puromycin was added to the media at a concentration of 2 μg/ml monthly to ensure that transduced cell lines were stable.

**Analysis of in vitro growth kinetics by CFSE staining.** Single-cell suspensions of each of the tumor cell lines were stained with 5 μM CFSE (1 μl / 10^6 cells) in PBS for 10 min at 37°C. After washing, cells were plated in six-well plates at 2 × 10^5 cells/well in complete media. Cells were incubated at 37°C for 40 h before being washed and fixed. Proliferation was detected by reduction of CFSE fluorescence. Cells stained, washed, and fixed at time 0 were included in the experiment as control. The samples were analyzed using FlowJo 7.2.4 software (Tree Star, Ashland, OR). Triplicate samples were analyzed for each of the independent experiments.

**Tumor growth and survival experiments.** Young (6–8 wk) female C57BL/6 mice were purchased from either the National Cancer Institute (Frederick, MD) or The Jackson Laboratory (Bar Harbor, ME) and maintained in our animal care facility with ad libitum access to water and mouse chow. Per experiment, 30 C57BL/6 mice were divided into three groups of 10 mice each and were given s.c. injections into the right hind leg of either 1 × 10^6 or 5 × 10^5 WT Pan02 cells, EV Pan02 cells, or IL-6 Pan02 cells in a total volume of 0.1 ml. Tumor growth was measured every 5 d, and palpable tumors were measured in two perpendicular axes with a Vernier caliper. Mean tumor size was calculated by multiplying the two size measurements together. Mice were allowed to die spontaneously or sacrificed when tumor size was >2 cm in one direction (in accordance with institutional guidelines) or when there was severe ulceration of the leg from the tumor or affected ambulation. All measurements were performed in a blinded fashion. Tumor growth and survival experiments were repeated independently three times with similar results. All experimental protocols were approved by the institutional Animal Studies Committee, and all murine experiments were conducted in compliance with institutional guidelines for the use of research animals.

**ELISA.** Cytokine amounts in tissue-culture supernatants were assayed with ELISA Ab pairs for IL-6 (Bioscience, San Diego, CA) and TGF-β (BD Pharmingen, San Jose, CA). The results were confirmed by a blinded fashion. Tumor growth and survival experiments were repeated independently three times with similar results. All experimental protocols were approved by the institutional Animal Studies Committee, and all murine experiments were conducted in compliance with institutional guidelines for the use of research animals.

**Histopathology and Immunohistochemistry.** A section of each murine pancreatic adenocarcinoma was embedded in disposable base molds containing Tissue-Tek OCT Compound (Sakura Finetek USA, Torrance, CA), then snap frozen over liquid nitrogen and stored at −80°C. Embedded tumor tissue was sectioned on a Leica CM1900 cryoostat (Leica Microsystems, Bannockburn, IL) at 10 μm, placed on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA), and then stored at −80°C until use.

For CD4+IL-17 and CD4+Foxp3 double staining, frozen tissue sections were fixed for 10 min in 1% paraformaldehyde in PBS. Sections were then permeabilized with 0.05% saponin in distilled H2O for 15 min. For CD4+IL-23R double staining, frozen sections were fixed in cold acetone for 10 min at −20°C and then dried at room temperature. All sections were blocked in Serum-Free Protein Block (Dako, Carpinteria, CA) for 30 min. Primary Abs for IL-17 (Santa Cruz Biotechnology, Santa Cruz, CA), Foxp3 (Abcam, Cambridge, MA), IL-23R (Abcam), or CD4 (eBioscience) were diluted 1:100, 1:250, or 1:30, respectively, in Ab Diluent (Dako) and added for 1 h at room temperature.

For CD4+IL-17 and CD4+Foxp3 staining, Alexa Fluor 647 donkey anti-rabbit IgG secondary Ab (Invitrogen, Carlsbad, CA) were diluted at 1:200 in Ab Diluent and added for 30 min at room temperature in the dark. For CD4+IL-23R staining, Alexa Fluor 555 donkey anti-goat IgG (Invitrogen) and biotinylated rabbit anti-IgG (Vector Laboratories, Burlingame, CA) secondary Abs were diluted 1:200 in Ab Diluent and added for 30 min at room temperature in the dark. Alexa Fluor 647 strepavidin conjugate (Invitrogen) was diluted at 1:200 in Ab Diluent and added for 30 min at room temperature in the dark. Confocal images were taken at ×400 magnification on an Axiosvert 100M microscope equipped with an LSM 510 confocal microscopy system (Zeiss, Jena, Germany).

**Real-time PCR.** cDNA was isolated from CD4+ tumor-infiltrating lymphocytes (TILs) using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. A total of 500 ng total RNA was reverse transcribed into cDNA in a 20 μl reaction containing the following reagents: (Invitrogen, Carlsbad, CA) 1× First Strand Reaction Buffer, 10 mM DTT, 250 ng random primers, 0.5 mM 2′-deoxynucleoside 5′-triphosphates, and 40 U RNaseOUT. Reactions were performed according to the manufacturer’s instructions.

**Lymphocyte isolation from tumors.** For functional studies, 30 C57BL/6 mice were divided into three groups of 10 mice each and were given s.c. injections into the right hind leg of 5 × 10^5 WT Pan02 cells, EV Pan02 cells, or IL-6 Pan02 cells in a total volume of 0.1 ml. Tumors that were ~1 cm in diameter were harvested after mice had been sacrificed. Tumors were mechanically dissociated into 1 mm3 pieces and then placed into the gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA) with 10 ml enzyme digestion consisting of collagenase DNease (0.01 g/100ml) and hyaluronidase (2.5 U/ml) (all from Sigma-Aldrich) in RPMI 1640 (Sigma-Aldrich) and dissociated on a preset protocol. Tumor digest was then placed on a neutator and incubated at 37°C for 30 min before dissociating in the gentleMACS again (Miltenyi Biotec). The cells were then washed with complete media and passed through 40-μm nylon mesh to obtain single-cell suspensions. CD4+ cells were then isolated using BD Bioscience anti-CD4 magnetic particles according to the manufacturer’s instructions (BD Biosciences, San Jose, CA) and used for the functional assays.
For real-time quantitative PCR, the following predesigned mouse TaqMan Gene Expression Assays from Applied Biosystems (Carlsbad, CA) were used: Foxp3, IL-17, IL-23R, and the endogenous control GAPDH. In addition, the following primers and probe for mouse RORγt were synthesized by Applied Biosystems: forward: 5′-CCGCTGAGGGGCTTCAC-3′, reverse: 5′-TGCAGTGGCACCATTACA-3′, probe: 5′-FAM-AAGGGCTTTCTGCGCCGACGAG-MGBNFQ-3′ (20). For RORγt, a ready-to-use 20× stock primer/probe mixture was made (primers: 18 μM each; probe: 5 μM). Each sample was run in triplicate. Reactions were initially denatured at 95°C for 20 s, then a two-step amplification was performed at 95°C for 3 s and 60°C for 30 s for 40 cycles. For quantitative analysis, relative expression levels were determined using the 2−ΔΔCt method.

**IL-17 ELISPOT assay.** CD4⁺ lymphocytes were harvested from WT Pan02, EV Pan02, or IL-6 Pan02 tumors when the tumor was ∼1 cm in diameter (as described above). The CD4⁺ lymphocytes were assessed for their ability to produce IL-17 using a commercially available murine IL-17A ELISPOT (eBioscience). In precoated wells, 0.75 × 10⁵ or 1.5 × 10⁵ CD4⁺ tumor-derived lymphocytes were plated in duplicate wells with medium containing 50 ng/ml PMA plus 500 ng/ml ionomycin. After ~20 h, the plate was developed according to the manufacturer’s instructions except for the substitution of avidin-alkaline phosphatase for avidin-HRP followed by the alkaline phosphatase substrate BCIP/NBT, and the spots were counted with an ImmunoSpot Series I analyzer (Cellular Technology, Cleveland, OH). Results are presented as the average number of spot-forming cells per 5 × 10⁵ cells plated and corrected for the background medium.

**In vitro stimulation.** Tumors were harvested from both WT and IL-6 tumor-bearing mice when tumors were ∼1 cm in greatest diameter. Tumors were weighed before creating single-cell suspensions using a combination of mechanical and enzymatic digestion per the mPAC tumor protocol provided by Miltenyi Biotec (as described above). Tumor cell suspensions were passed over 70-μm and 40-μm filters in succession in sterile RPMI 1640 before being resuspended in complete RPMI 1640 and counted for absolute number of cells. Cells were then incubated for 6 h at 37°C in complete RPMI 1640 in the presence of 5 ng/ml PMA and 10 μM ionomycin (Invitrogen) with brefeldin A (BioLegend, San Diego, CA). Poststimulation, cells were washed and used for flow cytometry.

**Flow cytometry.** To determine the frequency of Tregs and Th17 cells within the TIL population, flow cytometry was used to analyze TILs after brief stimulation (as described above). After washing, cells were stained with CD45, CD4, CD8, and NK1.1 (all from BioLegend) for cell-surface analysis. After cell-surface staining, cells were stained for intracellular IFN-γ, Foxp3, and IL-17A (all from eBioscience) using the Foxp3 staining kit from eBioscience per manufacturer protocol. After gating on CD45⁺ cells, cells were gated for either CD4⁺ or CD8⁺ cells. CD4⁺ cells were subsequently analyzed for Foxp3 or IL-17. Tregs were identified as being CD45⁺CD4⁺Foxp3⁺, and Th17 cells were identified as CD45⁺CD4⁺IL-17⁺ cells. CD8⁺ cells were further analyzed for IFN-γ secretion.

To correct for variation in tumor size, cells of interest (i.e., Th17 cells and Tregs) were quantified per gram of tumor tissue (cells/g). The ratio of Th17 and Tregs could then be calculated for both tumor types. All flow cytometry was analyzed using the LSRII (BD Biosciences) and FlowJo analysis software (Tree Star). Pooled samples were used for the analysis.

**Statistical analysis.** Kaplan-Meier survival curves were generated to compare differences in survival probabilities with the log-rank test. Tumor growth over a period of time was analyzed by using linear trend analysis. Data from ELISA and ELISPOTs were analyzed by the Student t test. All tests were two sided, and p < 0.05 was considered statistically significant.

**Results**

**Transduction and characteristics of murine pancreatic cancer cell lines**

WT Pan02 cells were genetically modified to express the mIL6 gene (IL-6 Pan02) or EV Pan02. IL-6 secretion was confirmed by ELISA for the IL-6 Pan02, whereas no IL-6 was produced by WT...
Pan02 or EV Pan02 (Fig. 1A). The transduced IL-6 Pan02 cells retained their ability to produce and secrete IL-6 in vivo as determined by detection of IL-6 in the serum of IL-6 Pan02 tumor-bearing mice (Supplemental Fig. 1) and by in vitro analysis of IL-6 Pan02 tumors grown ex vivo (data not shown). ELISA confirmed that all three cell lines produced similar amounts of TGF-β, ensuring that the gene for TGF-β had not been disrupted by the transduction with the lentiviral vector (Fig. 1B). All three cell lines had nearly identical in vitro growth characteristics (proliferative rate, doubling time) as determined by CFSE fluorescence (Fig. 1C).

**In vivo tumor growth and survival**

To investigate the impact of IL-6 in the TGF-β-rich pancreatic tumor microenvironment, 1 × 10⁴ IL-6 Pan02 cells or WT Pan02 cells were injected s.c. into the right hind leg of C57BL/6 mice. Tumors were measured every 5 d. Mice injected with IL-6 Pan02 developed significantly smaller tumors compared with mice injected with WT Pan02 tumors (p < 0.05; Fig. 2). Of the mice injected with IL-6 Pan02 tumors, 3 of the 10 mice never developed palpable tumor. This experiment was repeated three times with similar results. In contrast, all mice injected with WT Pan02 tumor grew tumors and eventually died of disease. Therefore, the production of IL-6 and TGF-β in the Pan02 tumor microenvironment caused a significant reduction in tumor growth.

To determine if the reduction in tumor growth also translated into a survival advantage, mice were followed until death. Mice bearing IL-6 Pan02 tumors showed a significant survival advantage compared with mice bearing WT Pan02 tumors (Fig. 3). The median survival of mice bearing IL-6 Pan02 tumors was 80 d (range 60–110 d) compared with 55 d (range 40–65 d) for WT Pan02 tumors (p < 0.001). Thus, the secretion of IL-6 in the tumor microenvironment significantly reduced tumor growth and prolonged survival.

To perform mechanistic studies, mice were inoculated with a greater number of tumor cells so that sufficient numbers of CD4+ TILs could be obtained from both IL-6 Pan02 tumors and control tumors. Tumor growth analysis in mice injected with 5 × 10⁴ tumor cells showed that there was a significant reduction in growth of IL-6 Pan02 tumors compared with either EV Pan02 tumors or WT Pan02 tumors (p < 0.05; Fig. 4). Analysis of tumor weights at 5 wk also confirmed that IL-6 Pan02 tumors were smaller than either WT or EV Pan02 tumors. IL-6 Pan02 tumors had an average weight of 0.94 g compared with WT Pan02 tumors with an average weight of 1.74 g or EV Pan02 tumors with an average weight of 2.12 g (p < 0.05 for IL-6 tumor weights compare with WT or EV tumor weights).

**Immunohistochemical analysis of the CD4⁺ tumor lymphocyte infiltrate**

By modifying the tumor cytokine environment with the addition of IL-6 and showing an antitumor effect, we hypothesized that there was either a downregulation of Tregs or an induction of Th17 cells. Immunohistochemistry was initially performed to analyze the tumor-immune infiltrate when tumor sizes were, on average, 1 cm

**FIGURE 2.** Tumor growth is reduced in mice injected with IL-6 Pan02 compared with WT Pan02 tumor cells. Mice (n = 10/group) were injected s.c. with 1 × 10⁴ IL-6 Pan02 or WT Pan02 tumor cells. Tumors were measured every 5 d. The mice bearing the IL-6-transduced Pan02 cells demonstrated a statistically significant reduction in tumor growth compared with WT Pan02 controls. Three mice receiving the IL-6 Pan02 tumor cells did not grow palpable tumors and are not included in the tumor growth curve. This experiment was repeated independently three times with similar results. p < 0.05 for the separation of the tumor growth curves.

**FIGURE 3.** Mice injected with IL-6 Pan02 have a survival advantage over mice receiving WT Pan02 tumor cells. Mice (n = 10/group) were injected s.c. with 1 × 10⁴ IL-6 Pan02 or WT Pan02 tumor cells and were allowed to die spontaneously or were sacrificed when tumor size was >2 cm or there was severe ulceration of the leg from the tumor that affected ambulation. Either death event was used to generate Kaplan-Meier survival estimates. Mice bearing IL-6 Pan02 tumors showed a significant survival advantage compared with mice bearing WT Pan02 tumors (p < 0.001). This experiment was repeated independently three times with similar results.

**FIGURE 4.** Tumor growth is reduced in mice injected with 5 × 10⁴ IL-6 Pan02 compared with EV Pan02 or WT Pan02 tumor cells. Ten mice were included in each group, and tumors were measured every 5 d. The mice bearing IL-6 Pan02 tumors demonstrated a statistically significant reduction in tumor growth (p < 0.05). All mice grew palpable tumors. This experiment was repeated and the data confirmed three independent times.
Pan02 tumors again demonstrated more CD4+IL-17+ cells, indicative of Th17 cells, in the lymphocytic infiltrate compared with the WT Pan02 tumors. Green: compared with WT Pan02 tumors. See Material and Methods for immuno-histochemical staining. Confocal images were taken at ×400 magnification. In diameter. IL-6 Pan02 tumors qualitatively showed increased staining for the cell surface IL-23R and intracellular IL-17 (both Th17 markers) when compared with WT Pan02 tumors (Fig. 5A, 5B). Of note, there also appeared to be a greater CD4+ infiltrate in the IL-6 Pan02 tumors. Intracellular staining for Foxp3 was observed in both the IL-6 Pan02 tumors and WT Pan02 tumors (Fig. 5C). Thus, it appeared that there was an induction of Th17 cells in the tumors secreting IL-6 and TGF-β.

Quantification of mRNA for Th17 and Treg markers from CD4+ tumor lymphocytes

Freshly isolated CD4+ TILs from tumors ~1 cm in diameter were lysed, and mRNA was purified. cDNA was synthesized from this mRNA and used in a real-time PCR assay as described in the Materials and Methods section. RT-PCR for all of the markers of interest was first normalized to the internal control GAPDH. mRNA from CD4+ TILs of WT Pan02 tumor cells was used as a reference to compare EV and IL-6 Pan02 CD4+ TIL marker profiles, expressed as a fold difference. Markers of interest included IL-17, RORyt, IL-23R (for Th17 cells), and Foxp3 (for Tregs). Expression levels of all markers were similar between the WT Pan02 tumors and EV Pan02 tumors (i.e., no fold difference), as expected. However, expression of IL-17, RORyt, and IL-23R in CD4+ TIL from IL-6 Pan02 tumors was significantly higher than in CD4+ TIL from either WT Pan02 tumors or EV Pan02 tumors, further demonstrating that Th17 cells are upregulated in the tumor microenvironment of tumors secreting IL-6 and TGF-β (Fig. 6). A small increase in expression of Foxp3 was also noted in the CD4+ TIL from the IL-6 Pan02 tumors. Enhanced secretion of IL-17 by CD4+ TILs from IL-6 Pan02 tumors

To obtain further confirmation that there was an upregulation of Th17 cells in the tumor microenvironment of mice bearing IL-6 Pan02 tumors, ELISPOT assay was used to detect IL-17 secreting CD4+ TILs. CD4+ TILs were isolated from IL-6 Pan02 tumors and stimulated with PMA and ionomycin in IL-17 Ab-coated ELISPOT plates for ~20 h. The ELISPOT assay depicted a significantly higher number of IL-17–secreting CD4+ TILs from the IL-6 Pan02 tumors compared with the EV Pan02 or WT Pan02 CD4+ TILs. Shown is the average number of spot-forming cells per 5 × 105 cells. Error bars indicate SE of mean. Experiments run in duplicate. *p < 0.05.

IL-6 in the tumor microenvironment induces Th17

To assess if the Treg/Th17 ratio was altered by the addition of IL-6 in the tumor microenvironment, flow cytometry was used to quantify the Th17 and Treg infiltration in both tumors (as described in
the Materials and Methods). Both the WT and IL-6 Pan02 tumors contained similar absolute number of cells per gram of tumor tissue (3.1 × 10^7 cells/g versus 3.0 × 10^7 cells/g, respectively), but the ratio of Th17 cells to Tregs were different between the two tumors. The WT Pan02 tumor contained 5.0 × 10^4 Tregs/g and 8.5 × 10^4 Th17 cells/g. The ratio of Th17 cells to Tregs in the TIL population of the WT tumor was 1.7. In contrast, the IL-6 Pan02 tumor contained 3.0 × 10^4 Tregs/g and 2.5 × 10^5 Th17 cells/g. So, for the IL-6 Pan02 tumor, the ratio of Th17 cells to Tregs was 8.3. In addition, the absolute number of Tregs in IL-6 Pan02 tumors was 1.7 times lower than in the WT Pan02 tumors, whereas the number of Th17 cells was 2.9 times higher in IL-6 tumors compared with WT tumors, suggesting that Th17 cells are most likely induced from naive CD4^+ T cells. Gated CD4^+CD4^+ cells and percentage of Foxp3^+ and IL-17^+ cells for the WT and IL-6 Pan02 tumor are depicted in Fig. 8A and 8B. Thus, the addition of IL-6 to the tumor microenvironment induced Th17 cells and altered the balance of Th17 and Tregs in the tumor.

Greater amount of IFN-γ^+ CD8^+ T cells demonstrated in the TILs from IL-6 Pan02 tumors compared with WT Pan02 tumors. Gated CD4^+CD8^+ cells from stimulated single tumor cell suspensions were analyzed for Foxp3 (Tregs) and IL-17 (Th17 cells). Calculations were performed using the absolute number of cells per gram of tumor tissue to increase accuracy. A. Tumor suspensions from the WT Pan02 showed a similar ratio of Th17 cells and Tregs. The ratio of Th17 cells to Tregs was 1.7. B. Tumor suspensions from the IL-6 Pan02 tumor showed a predominance of Th17 cells compared with Tregs with a ratio of 8.3. Pooled samples were used for analysis.

Discussion
In this study, we showed that induction of Th17 and altering the Treg/Th17 balance in the tumor microenvironment improves survival. Previous research has shown that Tregs are increased in the tumor microenvironment of patients and mice with pancreatic cancer (1, 6–9). By genetically engineering the murine Pan02 pancreatic adenocarcinoma cell line to express IL-6 cytokine, a Th17 cell population was induced compared with the WT Pan02...
or control EV Pan02 tumors as shown by the increase in Th17 cell markers, especially IL-17 and RORγt. Mice injected with the IL-6–expressing Pan02 tumor cells demonstrated significantly delayed tumor growth and improved survival compared with mice bearing WT or EV Pan02 tumors. The different in vivo proliferation was not a direct effect of the IL-6 transduction because in vitro proliferation kinetics were the same among the WT, EV, and IL-6–transduced Pan02 tumors. Thus, the induction of Th17 cells in the tumor microenvironment appeared to mediate an antitumor response to the weakly immunogenic Pan02 pancreatic adenocarcinoma.

The few recently published studies on Th17 cells and cancer did not clearly define a protumor or antitumor role for Th17 cells. One study found an increased prevalence of Th17 cells in ovarian, renal, and pancreatic cancer, and another study found there was a higher percentage of Th17 cells in more advanced gastric cancer (34–36). More specifically, Zhang et al. (36) noted an increased prevalence of Th17 cells in tumor draining lymph nodes of patients with advanced gastric cancer and reported an association between higher gastric cancer stages and increasing percentages of Th17 cells in tumor tissues and peripheral blood. Although this may suggest an association between advancing cancer and Th17 cells, it does not take into account the ratio of Tregs to Th17 cells. It is not surprising that the cytokines in the tumor microenvironment would support the differentiation of both Th17 cells and Tregs, especially because high levels of both IL-6 and TGF-β are found in late stage tumors (54). Kryczek et al. (34) noted that most of the Th17 cell differentiation in advanced cancers occurred within the tumor and not within the tumor draining lymph nodes, suggesting the importance of cytokine expression in the tumor microenvironment for immune cell differentiation. Also, Kryczek et al. (34) showed that the ratio of Tregs to Th17 cells was significantly higher throughout murine B16 melanoma growth. Finally, the increased prevalence of Th17 cells in advanced cancers does not clarify Th17 cell function or how the larger percentage of Tregs may influence Th17 cell function. Thus, it may be more important to look at Treg to Th17 cell ratio in tumors to better elucidate the role of Th17 cells and how their function may be compromised by Tregs.

There are also recent studies that have shown an antitumor effect for Th17 cells. One study showed an inverse correlation between Th17 prostate-infiltrating lymphocytes and Gleason tumor grade in prostate cancer (37). Another study reported a larger percentage of Th17 prostate-infiltrating lymphocytes and Gleason tumor grade in advanced prostate cancer (38)–(36). More specifically, Kryczek et al. (34) noted an association between higher gastric cancer stages and increasing percentages of Th17 cells in tumor tissues and peripheral blood. Although this may suggest an association between advancing cancer and Th17 cells, it does not take into account the ratio of Tregs to Th17 cells. It is not surprising that the cytokines in the tumor microenvironment would support the differentiation of both Th17 cells and Tregs, especially because high levels of both IL-6 and TGF-β are found in late stage tumors (54). Kryczek et al. (34) noted that most of the Th17 cell differentiation in advanced cancers occurred within the tumor and not within the tumor draining lymph nodes, suggesting the importance of cytokine expression in the tumor microenvironment for immune cell differentiation. Also, Kryczek et al. (34) showed that the ratio of Tregs to Th17 cells was significantly higher throughout murine B16 melanoma growth. Finally, the increased prevalence of Th17 cells in advanced cancers does not clarify Th17 cell function or how the larger percentage of Tregs may influence Th17 cell function. Thus, it may be more important to look at Treg to Th17 cell ratio in tumors to better elucidate the role of Th17 cells and how their function may be compromised by Tregs.

In conclusion, these findings provide evidence that altering the cytokines in the tumor microenvironment may shift the balance between Treg and Th17 cells in TILs. In this murine model of pancreatic cancer, the addition of IL-6 to a TGF-β-rich tumor microenvironment allowed for the differentiation of Th17 cells and a greater population of IFN-γ +CD8 + T cells in the TILs of the IL-6 Pan02 tumor, support that thought. Finally, there may also be a neutrophil or NK cell recruitment to the tumor site that cooperates with other effector cells for a tumor-specific immune response. However, NK cells were not a large part of the lymphocyte infiltrate in either the WT or IL-6 Pan02 tumor. Therefore, the downstream effects of Th17 cells and the cytokines secreted need to be more fully investigated to illuminate the antitumor effects.

Disclosures

The authors have no financial conflicts of interest.

References


