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

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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–secreting CD4+ tumor-infiltrating lymphocytes was substantiated at the cellular level by flow cytometry and ELISPOT assay and mRNA level for retinoic acid-related orphan receptor γt 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. 

many types of cancer, especially pancreatic cancer, in which el-


evocation of TGF-β 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 response is seen in mouse models of cancer (9, 44–46).

Recent evidence has shown that there exists an intricate and re-
ciprocation 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 transcrip-
tion, 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 im-

proved 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-ml6L (InvivoGen, San Diego, CA) was subcloned into lentiviral vector pSicoR-puro with added XhoI-NheI restriction sites. For lentiviru gen-

eration, HEK-293T cells were transfected with pSicoR-mIL6 vector and VSVG in Opti-MEM media using Fugene-6 (Roche Diagnostics, Indiana-

apolis, 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, re-
spectively, were used to transduce the murine pancreatic adenocarcinoma Pan02 cell line (also known as Panc-02) (53). Vivoctransfected cells were filter-
tered 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'-CCGGAGAGGGACCTTCCAGC-3' and IL-6 antisense: 5'-TCCAGGATTTCCAGAAG-3'. Protein expression of IL-6 was confirmed by ELISA.

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**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 con-
trol 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 bimonthly to ensure that transduced cell lines were stable.

**Analysis of in vitro growth kinetics by CFSE staining.** Single-cell sus-
pensions of each of the tumor cell lines were stained with 5 µM CFSE (1 µM × 105 cells) in PBS for 10 min at 37°C. After washing, cells were plated in six-well plates at 2 × 105 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 a time 0 were included in the experiment as control. The samples were analyzed with 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 × 106 or 5 × 106 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. 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 (eBioscience, San Diego, CA) and TGF-β (BD Pharmingen, San Jose, CA) according to the manufacturer’s instructions (BD Biosciences, San Jose, CA) and used for the functional assays.

**Histopathology and Immunohistochemistry.** A section of each murine pancreatic adenocarcinoma was embedded in disposable base molds contain-


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′-CGGCTGAGGGCTTCCAC-3′, reverse: 5′-TGAGGAGTCCGACATTACA-3′, probe: 5′-FAM-AAGGGCTTCTTCCGGCCAGCAGC-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 with 7500 Fast Real-Time System Sequence Detection Software (v1.3.1, Applied Biosystems). Gene expression levels were normalized to GAPDH. Relative RNA 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 × 10^6 or 1.5 × 10^6 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 × 10^3 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^+ 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 two-sided tests were used, 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, $1 \times 10^5$ 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 $5 \times 10^4$ 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](image2.png) **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 $1 \times 10^4$ 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](image3.png) **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 $1 \times 10^4$ 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](image4.png) **FIGURE 4.** Tumor growth is reduced in mice injected with $5 \times 10^4$ 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.
In the tumor microenvironment rich in IL-6 and TGF-β, IL-6 Pan02 tumors expressed significantly higher levels of Th17 cell markers (IL-17, RORγt, and Foxp3) compared with WT Pan02 tumors and EV Pan02 tumors (i.e., no fold difference), as expected. However, expression of IL-17, RORγt, 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, WT Pan02, and EV 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 (Fig. 7). This result again supports the hypothesis that Th17 cells are being induced in the tumor microenvironment rich in IL-6 and TGF-β.

**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
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 IFN-γ secretion. A. There were too few CD8^+ T cells in the suspension from the WT Pan02 tumor to analyze for IFN-γ secretion. B, IL-6 Pan02 TILs had a substantial number of IFN-γ^+CD8^+ T cells in the tumor microenvironment. Pooled samples were used for analysis.

FIGURE 9. 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 IFN-γ secretion. A. There were too few CD8^+ T cells in the suspension from the WT Pan02 tumor to analyze for IFN-γ secretion. B, IL-6 Pan02 TILs had a substantial number of IFN-γ^+CD8^+ T cells in the tumor microenvironment. 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 tumor.
or control EV Pan02 tumors as shown by the increase in Th17 cell markers, especially IL-17 and RORyt. 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 (37). Another study reported a larger percentage of Th17 prostate-infiltrating lymphocytes and Gleason tumor grade in advanced prostate cancer (37). Another study reported a larger percentage of Th17 prostate-infiltrating lymphocytes and Gleason tumor grade in advanced prostate cancer (37).

In conclusion, these findings provide evidence that altering the cytokines in the tumor microenvironment can shift the balance between Treg and Th17 cells in TILs. In this murine model of pancreas cancer, the injection 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

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