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Tumor derived TGF-Beta mediates conversion of CD4+Foxp3+ regulatory T cells in a murine model of pancreas cancer

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Abstract

$CD4^+25^+$*Foxp3*$^+$ regulatory T cells (Treg) play a critical role in the induction of tolerance to tumor associated antigens and suppression of anti-tumor immunity. How Treg are induced in cancer is poorly understood. We reported previously that Treg are significantly elevated in the peripheral blood of patients with pancreas cancer and that in a murine pancreas cancer model induction of Treg appears to be TGF-Beta dependent. Here we provide additional evidence that Treg are increased locally within the tumor microenvironment by a mechanism that appears dependent on TGF-Beta Receptor expression and the presence of tumor derived TGF-Beta. The murine pancreas cancer cell line Pan02 produces high levels of TGF-Beta both in vitro and in vivo. In contrast, the esophageal murine cancer cell line, Eso2, does not. Immunohistochemical staining of *Foxp3* in explanted tumors shows an identifiable population of Treg in the Pan02 (TGF-Beta positive) tumors but not Eso2 (TGF-Beta negative). Naïve $CD4^+25^-$*Foxp3*$^-$ T cells, when adoptively transferred into *Rag*−/− mice, are converted into *Foxp3*$^+$ Treg in the presence of Pan02 but not Eso2 tumors. Induction of Treg in Pan02 mice is blocked by systemic injection of an anti-TGF-Beta antibody. If *Rag*−/− mice are instead reconstituted with naïve $CD4^+25^-$ T cells expressing a mutated TGF-Beta receptor, induction of *Foxp3*$^+$ Treg in Pan02 bearing mice is blocked. Collectively these observations further support the role of TGF-Beta in the induction of Treg in pancreas adenocarcinoma.

Keywords

Regulatory T cells; Tumor Immunity; Cytokines; Tumor Growth Factor-Beta; Pancreas Cancer; Suppression

Introduction

Adenocarcinoma of the pancreas, a highly invasive and metastatic disease, is the fourth leading cause of cancer mortality in the United States. Like many tumors, pancreas cancer is weakly immunogenic and is associated with a poor anti-tumor immune response [1;2]. This in part is due to the chronically immunosuppressed state that pancreas cancer patients exist.

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Their serum level of the immunosuppressive cytokine, TGF-Beta [3;4], can as much as 4.5 times that of normal healthy subjects; a finding that in one study was associated with a worse overall survival[5]. Furthermore, pancreas cancer patients have reduced number and function of circulating dendritic cells[6;7]. What is interesting is that post-adjuvant treatment dendritic cell numbers and function recover while there also is a reciprocal drop in systemic TGF-Beta levels. Taken together it suggests that logically this state of immunosuppression is detrimental to the host. Adding to this there is now evidence that pancreas cancer patients have elevated numbers of the suppressor T cell, Treg[8-10]. As well, elevated numbers of Treg in ovarian cancer is associated with less favorable outcomes[11]. Yet little is known for how modern medical therapeutics can be used to reverse this immunosuppressive state. To do so, it would be necessary to better understand the mechanisms by which tumors suppress anti-tumor effector responses. The elevation of Treg in cancer suggests one mechanism by which tumors induce immunosuppression.

Regulatory T cells (T_{reg}), identified phenotypically as $CD4^+25^+$*Foxp3*$^+$ T cells, are a unique subset of immunosuppressive T cells which prevent autoimmunity in thymectomized mice [12]. They can be subdivided into two main categories: naturally occurring Treg (nTreg) and peripherally induced Treg (iTreg). Both types express *Foxp3*, a gene encoding the forkhead/winged helix transcription factor that in humans is responsible for Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome and is believed to be a key regulator of T_{reg} development [13]. Where nTreg and iTreg differ is in their origin, antigen specificity, and mechanism of suppression [14]. Natural Treg (nTreg) are thymus derived, constitutively express CD25, and their mechanism of suppression is cell contact dependent [15]. Inducible Treg (iTreg), also known as adaptive Treg, develop in the periphery from $CD4^+25^-$*Foxp3*$^-$ precursor T cells, that variably express CD25, and suppress via a cell contact independent, cytokine dependent mechanism [16;17]. Early evidence shows that TGF-Beta plays an important role in the de novo induction of $CD4^+25^+$*Foxp3*$^+$ T cells[18-20].

Natural and inducible Treg cells can be protective to the host during states of autoimmunity, infection, or transplant rejection. In malignancy, however, Treg play a critical role in progression of tumor growth and suppression of antitumor immune responses[11;21-24]. A few preliminary reports suggest that iTreg play a more significant role in suppressing anti-tumor immunity than nTreg[25-27]. Furthermore human trials that non-selectively neutralized both nTreg and iTreg function using CD25 directed immunotoxins resulted in diseases of autoimmunity and only transient partially reduced populations of Treg[28-30]. The induction of autoimmunity in these hosts likely results from the unintended neutralization of nTreg function as well as the tumor iTreg. What these findings at least suggest is that selective depletion of certain Treg subtypes may be a more efficient and possibly effective way of overcoming Treg suppression in cancer patients. These techniques, however, cannot be developed until we better understand how tumors induce Treg.

In this study, we demonstrate in a murine pancreas cancer model that a high prevalence of tumor induced Treg is associated with elevated systemic levels of TGF-Beta. Using *Rag1*$^{-/-}$ mice reconstituted with naïve $CD4^+25^-$ T cells we demonstrate that tumor-induced Treg conversion is blocked by the systemic use of an anti-TGF-Beta antibody. These tumor-derived Treg have in vitro Treg suppressor function identical to that of nTreg. When tumor bearing mice are reconstituted with naïve T cells expressing a mutated TGF-Beta receptor II (dnTGF-BetaRII) these naïve T cells do not undergo conversion despite the systemically elevated presence of TGF-Beta. Collectively these findings suggest that murine pancreas tumors induce Treg prevalence through a mechanism that appears to be TGF-Beta dependent. Potentially this could represent one mechanism by which iTreg can more

selectively be blocked in an effort to promote anti-tumor immune responses while not disrupting nTreg activity.

Materials and Methods

Mice and Tumor Cell Lines

All animal study protocols were reviewed and approved by the institutional review committee. Six-week old female C57BL/6 mice and C57BL/6 *Rag*1$^{-/-}$ mice were purchased from Jackson Laboratories. The dnTGF-BetaRII mice were a generous gift from Dr. RA Flavell of Yale University [31]. The mice were housed and bred at Washington University School of Medicine Animal Facilities in specific pathogen-free conditions. Animal care and all procedures were in accordance with the guidelines of the animal care committee. The mouse pancreas adenocarcinoma cell line Pan02 is syngeneic to C57BL/6 and was obtained from DCT Tumor Repository (NCI-Frederick Cancer Research and Development Center, Bethesda, MD). The C57BL/6 syngeneic esophageal tumor cell line, Eso-2 was a gift from Dr. R. Battafarano. Both cell lines were grown in RPMI culture medium (Mediatech, Manassas, VA) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, Carlsbad, CA), and 1% glutamine and antibiotics.

T cell Isolation and Preparation

The spleen and LN from C57BL/6 female mice were harvested and homogenized. The splenocytes were briefly treated with ACK buffer to remove red blood cells. Tumor tissue was digested in a buffer containing 1mg/ml collagenase, 2.5 U/ml hyaluronidase and 0.1mg/ml DNase for 30 minutes. The suspension was then separated from tissue debris by filtration through 40 μm cell strainer and centrifugation on Histopaque®-1083. All cells were then washed twice in PBS containing 0.5% FBS and passed again through 40 micron nylon mesh to obtain single cell suspensions. $CD4^+25^-$ and $CD4^+25^+$ Treg populations were separated using a mouse regulatory T cell isolation kit (Miltenyi Biotech, Auburn, CA) per manufacturer's instructions. Briefly, $CD4^+$ cells were separated from other cells by negative selection with an antibody cocktail provided by the manufacturer. The cells were then passed over a magnetic column and the $CD4^+$ cells were collected. $CD25^+$ cells were then selected by positive selection using anti-CD25 magnetic microbeads and then passed over a magnetic column. The initial flow through was collected as $CD4^+25^-$ T cells and the bound fraction was eluted from the column and collected as the $CD4^+25^+$ T cells. Purity (>93%) of each fraction was checked using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and antibodies against mouse CD4 (Cy-chrome labeled clone L3T4), CD25 (FITC labeled clone 7D4), as well as compatible isotype control antibodies (BD Biosciences, San Jose, CA).

Cell Culture, Cytokine Assays

In vitro conversion of naive T cells to Treg has been described elsewhere [18]. Briefly, freshly isolated C57BL/6 naive $CD4^+25^-$ T cells were cultured in 6 well plates with anti-CD3 (0.5 μg/ml), and either irradiated APCs or soluble anti-CD28 (2ug/ml) in the presence or absence of 0.02, 0.2, 2, or 20 ng/ml of TGF-Beta1 (R&D Systems, Minneapolis, MN). The cells were kept in these culture conditions for 3 days, after which they were washed and subjected to further analysis. Cytokine assay for TGF-Beta production by our tumor cell lines was done using a commercially available ELISA kit (Biosource International, Carlsbad, CA). Pan02 and Eso2 were both plated at a density of 1×10^6 cells per well, and kept in culture for 7 days, after which the supernatant was removed and processed according to manufacture's instructions for TGF-Beta specific ELISA assay. Measurement of TGF-Beta in mice sera was preformed according to the manufacture's instructions (Biosource International, Carlsbad, CA). In brief, C57BL/6 mice were injected with 0.25×10^6 Pan02 or Eso2 cells and

after 3 weeks serum was collected. Samples were stored at 70°C until ready for processing. The sera were diluted to 1/40 for ELISA.

FACS

Freshly isolated tumor infiltrating lymphocytes were labeled using antibodies against mouse CD4 (Cy-chrome labeled clone L3T4), CD25 (FITC labeled clone 7D4) and were analyzed with a FACS Calibur® Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). At least 100,000 live events were collected per sample. The analyses were conducted using Flojo (Tree Star, Ashland, OR).

Real Time PCR

RNA was isolated from 5×10^6 lymphocytes using the RNeasy®Protect Mini Kit (Qiagen, Valencia, CA). Synthesis of cDNA was performed on 100ng of RNA using 8 Oligo (dT)12-18 (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA) per manufacturer's instructions. Real-time PCR for *Foxp3* normalized to hypoxanthine-guanine phosphoribosyl-transferase (HPRT) was performed on a 9600 thermal cycler and analysis of the data was performed using Sequence Detection System 5700 software (Applied Biosystems, Foster City, CA). TaqMan® Universal PCR Master Mix and TaqMan® Primer with non-fluorescent quencher probes for *Foxp3* (Cat # Mm 00475156) and HPRT (Cat # Mm00446968) were purchased from Applied Biosystems.

Regulatory T cell Functional Assays

Cells were cultured in DMEM medium supplemented with 10% FBS (Aleken Biologicals, Nashville, TN) HEPES, non-essential amino acids, sodium pyruvate, 50 μM β-ME, and penicillin/streptomycin/L-glutamine. αThy1.1-PE (clone OX-7) and αCD4-PE-Cy5.5 (clone RM4-5) were purchased from BD Pharmingen (Franklin Lakes, NJ) and Biolegend (San Diego, CA) respectively. For the in vitro suppression assays, 50,000 CFSE-labeled CD4+ CD25−Thy1.1+ T cells were co-cultured in 96-well plates with 200,000 irradiated thy1.2+ splenocytes, 0.1 μg/ml and αCD3, with or without 50,000 CD4+ CD25+ Thy1.2+ cells for 72 hours. Cells were subsequently harvested, stained for Thy1.1 and CD4, and then analyzed by flow cytometry. The gates were set for Thy1.1 and CD4 and a histogram constructed based on CFSE positivity.

Adoptive Transfer Experiments

Six-week old *Rag1*$^{-/-}$ mice received i.p. injections of 200 μl HBSS with or without 5×10^6 freshly isolated $CD4^+25^-$ naive T cells from either normal C567BL/6 mice or dnTGFBetaRII transgenic mice. Two days after reconstitution, the mice received subcutaneous injections in the left outer thigh of 0.5×10^6 Pan02 or Eso-2 tumor cells. Seven weeks later, the mice were sacrificed and the spleen and tumor draining lymph nodes (TDLN) were harvested and analyzed for expression of CD25 and CD4 by FACS, and for *Foxp3* by real-time PCR.

Immunohistochemistry

Tumors were implanted by subcutaneous injection of 1.0×10^6 PanO2 or ESO2 cells in the hind limb of C57BL/6 mice. At four weeks post implantation the tumors and TDLN were resected and fixed overnight in buffered formalin (Sigma, St. Louis, MO). Prior to staining antigen retrieval was performed by incubating sections in a decloaking chamber (Biocare Medical, Concord, CA) for 10 minutes at 90°C in Reveal™ buffer. Sections were stained with a monoclonal rat anti-mouse-Foxp3 IgG (FJK-16s, 50 μg/ml, eBioscience, San Diego, CA) followed by Alexa Fluor 488 conjugated goat anti-rat antibody IgG (A11029, Invitrogen, Carlsbad, CA). Sections were cover slipped with Vectashield Hard Mounting

Media containing DAPI (Vector Labs, Burlingame, CA). Sections were examined using an Olympus BX61 microscope and captured digitally with an Olympus DP70 camera (Olympus, Center Valley, PA). For the CD4+ and Foxp3+ co-localized tissue sections, Pan02 tumors were section and immediately embedded in base molds (Ted Pella, Redding, CA) containing Tissue-Tek OCT medium (Sakura, McGaw Park, IL) then frozen over liquid nitrogen. Frozen tumor sections were cut at 10μm and mounted on SuperFrost Plus slides (Fisher, Pittsburgh, PA) then stored at −80°C until use. Tumor sections were fixed in 1% paraformaldehyde for 5 minutes at room temperature then washed several times in 1X phosphate-buffered saline (PBS). The slides were blocked for 30 minutes in Serum Free Protein Block (DAKO) at room temperature and then stained with rabbit anti-mouse Foxp3 (Abcam, Cambridge, MA) and rat anti-mouse CD4 (eBioscience, San Diego, CA) primary antibodies. Sections were then stained with AlexaFluor 488 goat anti-rabbit and AlexaFluor 555 goat anti-mouse IgG secondary antibodies (Invitrogen, Carlsbad, CA). Confocal images were visualized on an Axiovert 100M microscope equipped with a LSM 510 confocal system (Axiovert, San Marcos, CA).

TGFBeta Blockade

In vivo blockade of TGF-Beta was achieved using a multi-species monoclonal antibody toward TGF-Beta1,2,3 (Clone 1D11, IgG_1, R&D Biosystems, Minneapolis, MN). On day 0 *Rag1* $^{-/-}$mice were reconstituted with 5×10^6 naive T cells given intraperitoneally. On day 2 the mice were injected subcutaneously with 0.25×10^6 Pan02 cells into the lateral left thigh. After two weeks the mice received intravenous injections via the tail vein of 10μg of anti-TGF-Beta without azide, low endotoxin IgG_1 isotype control (BD Biosciences, San Jose, CA). Treatments were given either weekly (Q7) or every 4 days (Q4). The treatments lasted for a total of four weeks at which time the mice were sacrificed and their TDLN analyzed for expression of Foxp3 by real time PCR. Tumor growth curves were based on weekly measurements taken with calipers, in two separate axes and were confirmed by two independent observers. The *tumor volume* was defined as the multiplication of the diameters taken in two separate axes. *Tumor burden* was defined as the percentage weight of the tumor relative to the total body weight (tumor weight ex vivo ÷ total body weight at sacrifice).

Results

In vitro conversion of naive T cells into Treg

We first studied whether TGF-Beta induced conversion of naive T cells into Treg in vitro. Freshly isolated $CD4^+25^-$ naive T cells were purified from the spleens of normal C57BL/6 mice. The purity of these fractions were found to be >93% by FACS (data not shown). The cells were cultured with soluble anti-CD3 and either anti-CD28 or irradiated antigen presenting cells (APCs) in the presence or absence of TGF-Beta. Cells were then purified from culture after 72 hours and then analyzed for expression of *Foxp3* by real time PCR. In prior studies, we validated that induction of Foxp3 expression is a marker of Treg conversion and function through the use of T cell suppression assays [8]. For this reason functional assays of Treg suppression were not routinely performed. Figure 1 shows that Foxp3 expression, as expected, was significantly higher in freshly isolated regulatory T cells ($CD4^+25^+$) when compared with naive non-regulatory T cells ($CD4^+25^-$). Foxp3 expression was induced in cultures of naive T cells treated with anti-CD3/CD28 and TGF-Beta. The upregulation of Foxp3 by TCR crosslinking did not differ whether costimulation was provided by irradiated APCs or soluble anti-CD28. Neither TGF-Beta nor TCR engagement alone was enough to induce *Foxp3* expression in $CD4^+25^-$ T cells. The effect of TGF-Beta was concentration dependent, where the highest expression of Foxp3 was observed at a TGF-Beta concentration of 2ng/ml.

Pancreas cancer represents a high TGF-Beta environment

Several clinical studies have demonstrated that pancreas cancer patients have significantly elevated levels of the immunosuppressive cytokines TGF-Beta and IL-10 in their systemic circulation [3;4;6]. We investigated the possibility of whether pancreas cancers induce Treg conversion *in vivo* through secretion of TGF-Beta. Pan02, a murine pancreas adenocarcinoma cell line, is a highly tumorigenic cell line derived from 3-methylcholanthrene (3-MCA)-induced tumors in C57BL/6 mice. ELISA studies of culture medium taken from Pan02 cells demonstrated significantly elevated levels of TGF-Beta when compared with media alone (Fig. 2a). An unrelated murine cell line of esophageal adenocarcinoma, Eso2, exhibited no difference in TGF-Beta levels when compared with media alone. Neither cell line produced significant levels of IL-10 (data not shown). Because with successive cultures cell lines can change phenotype, we also analyzed Pan02 and Eso2 production of TGF-Beta *in vivo*. Pan02 and Eso2 tumors were established in the left lateral thigh of C57BL/6 mice and allowed to grow for three weeks. At the time of sacrifice all mice exhibited palpable tumors of relatively similar sizes (data not shown). Serum levels of TGF-Beta in Pan02 mice were significantly elevated above levels measured in non-tumor bearing mice (Fig. 2b). There was no statistically significant difference in TGF-Beta serum levels between Eso2 and non tumor bearing mice.

Pan02 recruits regulatory T cells within the tumor microenvironment

In prior studies, we have shown that regulatory T cells are elevated in the peripheral blood and TDLN of patients and mice with pancreas cancer [8;21]. Based on these observations we hypothesized that Treg were actively being recruited to the tumor microenvironment. To test this we took tumors explanted from Pan02 and Eso2 bearing mice and stained them for Foxp3 using immunofluorescence. Hematoxylin and Eosin (H&E) staining of Pan02 and ES02 demonstrated standard morphology of murine pancreas and esophageal adenocarcinoma (Fig. 3*A,B*). Staining for Foxp3 in Pan02 tumors demonstrated a high number of $Foxp3^+$ cells within the Pan02 tumors (Fig. 3*C*). These finding are consistent with our real-time PCR and FACS analysis of TDLN taken from Pan02 bearing mice. Tumors established with the non-TGF-Beta secreting Eso2 cell-line showed no tumor infiltrating $Foxp3^+$ cells, suggesting that the increased prevalence of Treg in Pan02 tumors is possibly related to tumor dependent expression of TGF-Beta (Fig. 3*D*). To further phenotype the Foxp3+ tumor infiltrating lymphocytes we did co-localization of Foxp3 and CD4. Figure B demonstrates the Pan02 tumors have strong positivity for CD4+ tumor infiltrating lymphocytes, some of which co-localize with Foxp3. Taken together, this suggests that populations of tumor infiltrating lymphocytes in Pan02 are CD4+Foxp3+ T cells.

The induced populations of Treg in Pan02 tumors were found to have functional Treg suppressor activity similar to that of natural Treg (Figure 4). To demonstrate this, we labeled freshly naïve CD4+CD25−*Thy1.1*+ effector cells (Teff) with CFSE and cultured them in the presence of CD4+25+*Thy1.2*+ regulatory T cells (Treg) derived from the spleen and tumors of Pan02 challenged mice. FACS was used to analyze CFSE staining with gating for CD4+Thy1.1 Teff cells. These experiments demonstrate that tumor induced Treg are capable of suppressing effector T cell proliferation, a function that likely impairs the anti-tumor immune response within the tumor microenvironment.

Tumors induce conversion of naive T cells into Treg through TGF-Beta

An increased prevalence of Treg has been demonstrated within the peripheral blood, TDLN, malignant ascites, and effusions of cancer patients [8;9;11;32;33]. It is unclear whether this is mediated by recruitment or expansion of existing Treg, or whether the tumor induces de novo generation of Treg from peripherally circulating naive $CD4^+25^-$ T cells. We

established an adoptive transfer model using *Rag1*-knockout (*Rag1*$^{-/-}$) who because of a defect in the recombinase gene have no mature T cells. Here we reconstituted *Rag1*$^{-/-}$ with an intraperitoneal injection of 5×10^6 syngeneic naive $CD4^+25^-$ T cells followed two days later by a subcutaneous injection of either 0.25×10^6 Pan02 or Eso2 in the left medial thigh. The tumors became palpable after approximately two weeks and reached their maximum allowable size by six weeks. Once the mice were sacrificed the spleen and TDLN were harvested and analyzed for expression of Foxp3. There was low expression of *Foxp3* in the freshly isolated $CD4^+25^-$ T cells used to reconstitute *Rag1*$^{-/-}$ mice and in TDLN of mice that were not reconstituted (Figure 5). By contrast, $CD4^+CD25^-$ reconstituted mice challenged with Pan02 had marked upregulation of Foxp3 expression within the TDLN compared to those challenged with ES02. Additionally, Foxp3 induction within the Pan02 tumor-bearing mice was restricted to the TDLN as it was not seen in the harvested splenocytes.

To study the importance of TGF-Beta in tumor induced conversion, we utilized the same *Rag1*$^{-/-}$ adoptive transfer model and studied the effects of systemic TGF-Beta blockade on Treg prevalence. Four weeks following tumor challenge, reconstituted *Rag1*$^{-/-}$ mice all exhibited palpable tumors with an average size $40mm^2$ (Fig. 6*A*). Starting at Week 4 mice received either no treatment, anti-TGF-Beta antibody (10 μ.g., i.v.), or IgG1 isotype-matched control. The decision to treat mice with established tumors at the fourth week was based on prior studies that indicated the maximal increase in prevalence of Treg numbers occurred at four weeks following tumor challenge [33]. The mice were treated on a schedule of either every four days (Q4) or weekly (Q7) for a total of four weeks. Tumor volume was recorded as the measured product of diameters taken in two separate axes. The growth curves in Figure 6*A* show that there was no difference in tumor size between treatment groups at the start of treatment. However over time mice treated with anti-TGF-Beta demonstrated a statistically significant delay in tumor growth when compared to untreated and isotype controls. FACS analysis of total cells purified from the TDLN of these mice showed that mice receiving a Q7 schedule of anti-TGF-Beta had more than a fifty percent reduction in the percentage of $CD4^+25^+$ T cells when compared with the no treatment controls (Fig. 6*B*). (FACS analysis of the no tumor group was not preformed due to insufficient number of cells isolated from normal LN). Analysis of Foxp3 expression by real-time PCR demonstrated that untreated tumor bearing mice had a 2.5 fold induction in Foxp3 expression compared with the non-tumor bearing control (Fig. 6*C*). Treatment with anti-TGF-Beta given on a Q7 schedule resulted in a significant reduction in Foxp3 expression whereas treatment with isotype or antibody given more frequently than Q7 did not reduce tumor mediated induction of Foxp3. We speculate that the Q4 frequency was less effective at blockade given that the tail veins by often the 4^{th} treatment and mice were unlikely receiving an appropriate dose of the antibody.

Intact TGFBetaRII signaling is necessary for Pan02 mediated conversion of $CD4^+25^-$ naive T cells into Treg

TGF-Beta plays an important role in the induction and maintence of immune tolerance. Mice expressing a dominant negative TGF-Beta receptor II (dnTGF-BetaRII) transgene have a mutation that prevents downstream signaling via the receptor and renders T cells insensate to TGF-Beta [31]. To study the effect of TGF-Beta receptor signaling on Pan02 mediated conversion of naive T cells, we isolated naive $CD4^+25^-$ T cells from dnTGFBetaRII transgenic mice, adoptively transferred them into *Rag1*$^{-/-}$ mice, and subsequently challenged the mice with Pan02. Mice that were reconstituted with wildtype $CD4^+CD25^-$ naive T cells and then challenged with tumor expectedly showed a marked induction of Foxp3 expression, demonstrating that these naive T cells had undergone conversion (Fig. 7*)*. In contrast, when tumor-challenged *Rag1*$^{-/-}$ mice were instead

reconstituted with dnTGFBetaRII $CD4^{+}25^{-}$ naive T cell there was no observed induction of Foxp3 expression. This suggests that intact TGF-Beta receptor signaling is necessary for tumor induced conversion of naive T cells into Treg. Interestingly, there was no statistically significant difference in the extent of tumor burden in mice reconstituted with wildtype versus those transgenic $CD4^{+}25^{-}$ naive T cells (Fig. 8*A*). By contrast, if the transgenic line of mice themselves were challenged with tumor versus the wildtype controls, the transgenic mice had statistically less tumor burden than their wildtype controls (Fig. 8*B*). Previously published studies with dnTGFBetaRII transgenic mouse had shown that these mice were resistant to tumor challenge with thymoma and melanoma cell lines [34].

Discussion

Cancers have evolved numerous mechanisms by which they evade immune recognition. Evidence suggests that regulatory T cells (Treg) play an important role in tumor induced immunosuppression [35]. Published works due in fact show that non-selective removal of host Treg with either an anti-CD25 antibody or CD25-directed immunotoxin results in improved anti-tumor effector responses. This effect however is transient and in some cases comes at a cost to the host. This is because non-selective neutralization of both nTreg and iTreg puts the host at risk of developing autoimmunity[22;28-30]. In some cancer models this could mean only clinical manifestations of vitiligo whereas in a model for pancreas cancer it could result in treatment induced diabetes or an autoimmune pancreatitis. Therefore our group set out to better understand the specific mechanisms by which Treg are induced in the tumor microenvironment, and hopefully allow for the development of more selective targets to block tumor mediated immunosuppression. The premise for the current study was taken from four observations in the literature: 1) pancreas cancer patients are chronically immunosuppressed and have elevated levels of serum TGF-Beta [4], 2) Treg numbers are elevated in the periphery and tumor microenvironment of patients with pancreas cancer[8], 3) TGF-Beta induces *in vitro* conversion of naïve T cells into Treg[18], and 4) preliminary evidence suggests induced Treg within the tumor microenvironment may play a more significant role than thymically derived nTreg in suppressing anti-tumor immunity[25-27] .

Other studies have elucidated in both cancer and non-cancer models that increased prevalence of Treg can occur via three different mechanisms: recruitment, expansion, and conversion. Treg recruitment has been evidenced in an ovarian cancer model, where the release of a Treg specific chemokine, CCL22, resulted in active recruitment of circulating Treg to the tumor microenvironment [11]. Expansion of resident Treg was exhibited in a diabetic mouse model where pancreatic islet cells engineered to transiently produce TGF-Beta via a tetracycline inducible promoter resulted an increased number of resident “intra-islet” Treg [36]. Induction of Treg function was shown early on *in vitro* through the costimulation of naïve CD4+25− T cells in the presence of TGF-Beta[18]. The works presented here are an extension of our previous studies where we introduced the role of TGF-Beta in tumor induction of Treg in pancreas cancer. The unique angle of the current study is that it: 1) provides both qualitative and functional data for tumor induced Treg; 2) confirms the presence of a “high TGF-Beta” dependent environment by which pancreas cancer induces Treg; 3) introduces the role of TGF beta receptor signaling as specific mediator in tumor induction of Treg; and 4) places focus on the role of conversion in tumor induced Treg prevalence.

We show here that the pancreas tumor cell line, Pan02, produces elevated TGF-Beta levels *in vitro* and *in vivo*. By contrast, Eso2 is an esophageal cancer cell line that does not produce significant levels of TGF-Beta *in vitro* or *in vivo*. Presence of TGF-Beta production correlated with increased immunohistochemical staining for the Treg marker, *Foxp3*, in Pan02 tumors. Not surprisingly, the Eso2 tumors showed no evidence of *Foxp3* staining

within the tumor sections. To further phenotype the *Foxp3*+ cells found in the Pan02 tumors we co-stained the cells for CD4 and *Foxp3*. In separate experiments not shown here, we also confirmed that of the CD4+ T cells present within the tumor 35% of them were CD4+*Foxp3*+. In the adoptive transfer model naïve T cells were only converted in Treg while in the presence Pan02 and not Eso2. These mice at baseline do not express functional levels of $Fop3^{+}$ Treg cells. Therefore once these mice were reconstituted with purified wildtype syngeneic $CD^{+}25^{-}$ naive T cells any induction of Foxp3 expression is occurring exclusively within our adoptively transferred T cell population.

An obvious limitation of this study design is that we are comparing the ability of a tumor to induce Treg in two morphologically different cell lines. Thus, it is appropriate to state that the two cell lines possibly exhibit other differences in their tumor biology besides their ability to produce TGF-Beta. Preferably we would utilize an Eso2 cells line transfected with TGF-Beta or alternatively, a Pan02 cells harboring a “knock-out” element for TGF-Beta. Indeed we attempted to engineer such an Eso2 line and were able to maintain a stably TGF-Beta transfected clone in culture, but found by ELISA analysis that our vector expressed an “inactive” form of TGF-Beta that did not allow for release from the latent TGF-Beta binding protein. In light of this limitation, the focus of our study was to show how “tumor-derived” TGF-Beta was accountable for inducing Treg conversion. Thus, utilizing as a “negative-control” a tumor cell line that did not produce elevated levels of TGF-beta both *in vitro* and *in vivo*, we felt this was a near comparable model that demonstrated i*n vivo* conversion of Treg required a “high TGF-Beta” environment. The tumors implanted subcutaneously likely do induce local inflammation from their invasive nature and an acute desmoplastic reaction that we know is TGF-Beta driven. This likely was the case with both Pan02 and Eso2. However, it doesn’t appear this minute elevation of TGF-Beta is enough to induce in vivo conversion of naïve T cells into Treg locally within the tumor microenvironment.

To further address this limitation, we chose to then investigate whether neutralization of TGF-Beta made by our Pan02 tumor blocked conversion of naïve T cells into Treg in our adoptive transfer model. This was done in place of giving systemic recombinant TGF-Beta to our Eso2 mice because it was felt to be an artificial rendering of the Pan02 system for the reasons stated above and suggestions in the literature claimed that TGF-Beta given systemically is often neutralized by factors in the serum. Indeed, use of an anti-TGF-Beta antibody showed that reconstituted mice no longer exhibited induction of Tregs while in the presence of Pan02. As a side observation we also noted that mice treated with TGF-Beta blockade also had significantly smaller tumors than the untreated group. Given that these experiments were conducted in immunocompromised models that have no functional T cells we do not assume that this “treatment effect” is secondary to removal of Treg suppressive function and subsequent enhanced anti-tumor T cell immunity. It is more likely that the smaller tumors in our treatment group were because of blocking the tumor growth promoting properties of TGF-Beta, such as tumor cell proliferation, angiogenesis, and evasion of apoptosis[37] while also possibly promoting natural killer cell activity[38;39].

The observation that antibody neutralization of TGF-Beta blocks tumor induced conversion of naïve T cells into Treg suggests that TGF-Beta receptor signaling plays an important role. To test this hypothesis we again utilized our adoptive transfer model but instead reconstituted them with naïve T cells expressing a mutated TGF-Beta receptor. These mice despite Pan02 implantation did not demonstrate conversion of naïve T cells into Treg. Logically because the adoptively transferred cells were unable to transducer the effects of the “high TGF-beta” environment produced by the tumor. As a side observation we did note that tumors in *Rag1*−/−mice reconstituted with mutated naïve T cells did not have smaller tumors, which is contrary to what we saw in the anti-TGF-Beta treated model. It is technical limitation of our *Rag1*−/−adoptive transfer model. Treatment effects would better be

demonstrated in an immunocompetent model. Indeed, when dnTGFBetaRII transgenic mice were challenged with Pan02 they developed tumors that were significantly smaller than those in wildtype mice. This observation mirrors what is found in the literature, where these transgenics have been shown to be resistant to tumor challenge and have enhanced anti-tumor immunity. The key focus of our study, however, is not to show treatment effect but instead further understand the process of Treg conversion in the absence of competing mechanisms such as expansion and recruitment. A question that still remains is what percentage of Treg prevalence is the result of increased recruitment by tumor, expansion of resident Treg, or conversion of naïve T cells. Our current study aims only at better understanding the mechanism of tumor induced conversion. For future directions with targeted immunotherapy this would be important to know, since inhibiting only one of the three mechanisms potentially would result in suboptimal blockade of Treg mediated tumor immunosuppression and an infective anti-tumor response.

Since our works began, a few publications have come out suggesting a similar role of TGF-Beta in Treg conversion[40;41]. Petty et al in a 2008 study of Treg in an AIDS feline model showed the importance of TGF-Beta Receptor signaling as a mechanism for Treg conversion. Another paper recently published by Liu et al constructed a model similar to ours by using prostate cell line in vitro and renal cancer cell line in vivo to look at tumor mediated induction of Treg via TGF-Beta production. They too, utilized antibody directed neutralization of TGF-beta to demonstrate blockade of tumor induced Treg conversion. They show that mice receiving 1D11 had reduced tumor burden and number of CD4+CD25+ T cells within isolated lung metastases. Based on these findings they comment that TGF-Beta neutralization “abolished the conversion” of naïve T cells into Treg. These works support our hypothesis that Treg prevalence in the tumor microenvironment is reduced by blocking TGF-Beta. However, because Treg prevalence in the tumor microenvironment is likely influenced by a triad of expansion, conversion, and recruitment, their studies do not clearly elucidate whether TGF-Beta blockade abolished conversion, recruitment, induction or all three. This is because they used an immunocompetent model that has circulating natural Treg and host tissue Treg that may have expanded or been recruited from the periphery. We show in an immunodeficient *Rag*−/− model replete with *ex vivo* isolated naïve T cells that Treg are induced by the presence of TGF-Beta secreting tumor cells and that indeed neutralization of TGF-Beta results in blockade of this induction. Additionally, our study differs from this model in that we show Treg prevalence in both the tumor draining lymph nodes and the tumors themselves which helps demonstrate on what levels the induction of Treg is occurring. These works along with those previously published hopefully will lead to the discovery of more specific immunoregulatory targets that help harness anti-tumor immune effector responses in a cancer greatly in need of more effective therapeutic options.

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Abbreviations

(Treg)	Regulatory T cells
(nTreg)	natural Treg

(iTreg) induced Treg

(TDLN) Tumor draining lymph node

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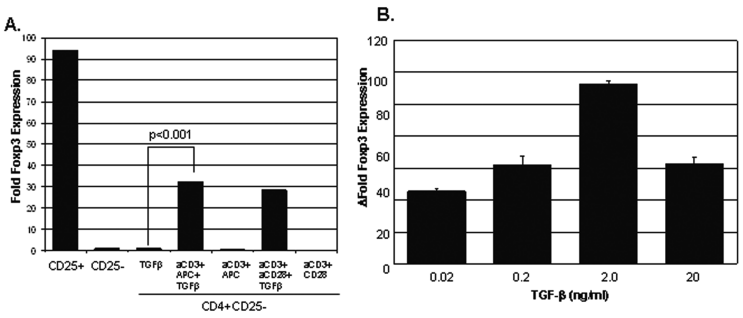

Figure 1.

In vitro conversion of naive $CD4^{+}25^{-}Foxp3^{-}$ T cells into $Foxp3^{+}$ T cells. *A*, Purified $CD4^{+}25^{-}$ T cells were co-cultured in the presence or absence of TGF-Beta, irradiated splenocytes (APC), anti-CD3, or anti-CD28. The data shown is representative of three experiments. The expression of Foxp3 in $CD4^{+}25^{-}$ T cells was normalized to 1.0. *B*, Induction of Foxp3 by TGF-Beta is concentration dependent. $CD4^{+}25^{-}$ T cells were stimulated with anti-CD3 $^{+}$ anti-CD28 in the presence of various concentrations of TGF-Beta. Foxp3 expression in cells cultured without TGF-Beta was normalized to 1.00. The data represents the average of 3 experiments shown as fold change in expression relative to negative control.

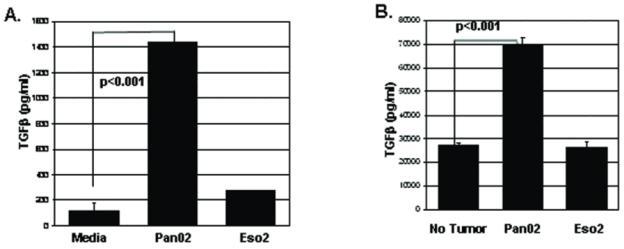

Figure 2.

Pan02 pancreas adenocarcinoma produces elevated levels of TGF-Beta both in vitro and in vivo. *A*, Measured levels of TGF-Beta in the culture medium of Pan02 tumor cells. Pan02 and Eso2, a murine esophageal cancer line, were plated at 1.0×10^6 in RPMI/10% FBS and cultured for seven days. TGF-Beta levels were measured in the supernatant by ELISA. *B*, Elevated levels of TGF-Beta in the serum of Pan02 bearing mice. C57BL/6 mice were implanted with 0.25×106 Pan02 or Eso2 tumor cells. The tumors were allowed to grow for four weeks at which time the mice were sacrificed and the serum collected. Data is presented as the average of three experiments. Statistical significance is expressed as a p-value<0.001 as compared to the negative control.

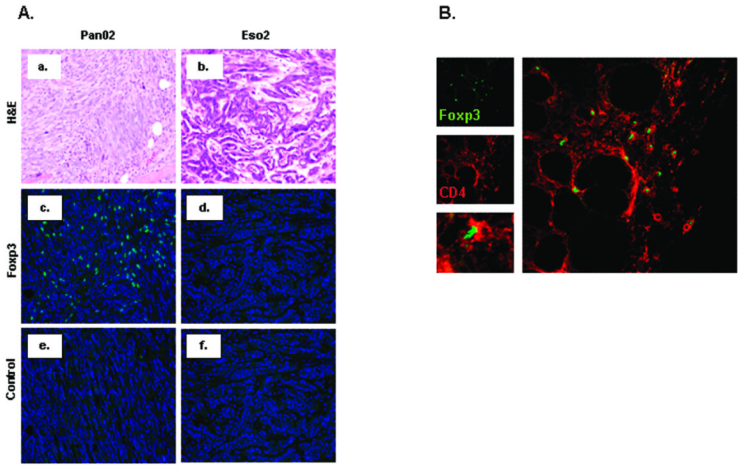

Figure 3.

Foxp3^{+} T cells are prevalent in the Pan02 but not Eso2 tumor microenvironment. Immunohistochemical staining of Pan02 and Eso2 tumors grown in C57BL/6 mice for four weeks. In A, *a and b*, Hematoxylin and Eosin (H&E) staining of Pan02 and Eso2. In *c and d*, Immunohistochemical staining (Alexa Fluor 488) for Foxp3 in Pan02 and Eso2 tumors. In *e* and *f*, negative control sections processed identically to the test sections but stained without primary antibody. In B, the Pan02 tumors were stained for co-localization of Foxp3 and CD4 to further phenotype the tumor infiltrating lymphocytes.

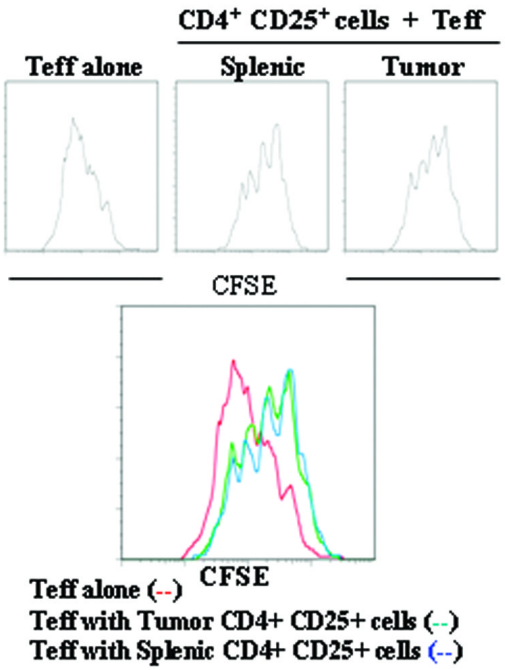

Figure 4.

Tumor-derived CD4+ CD25+ cells are suppressive in vitro. To examine the suppressive capacity of tumor-infiltrating Treg, CD4+ CD25+ cells were isolated from the spleen and tumor of C57BL/6 (thy1.2+) mice 5 weeks after inoculation with Pan02. These tumor-induced CD4+25+ T cells were then placed in co-culture with CFSE-labeled thy1.1+ CD4+ CD25− T effector cells under activating conditions. Figure is representative FACS analysis and gating for CD4+ Thy1.1+ T cells.

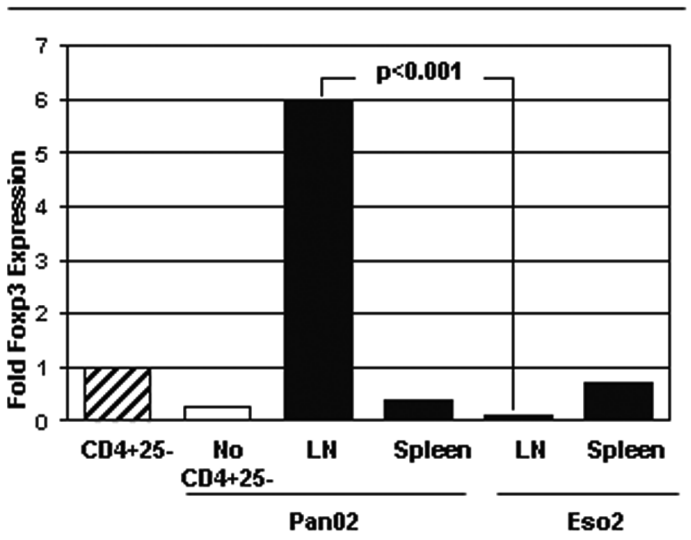

Figure 5.

In vivo induction of Foxp3 in $CD4^{+}25^{-}Foxp3^{-}$ naive T cells by Pan02. All but control *Rag1*$^{-/-}$ mice were reconstituted with a purified population of $CD4^{+}25^{-}Foxp3^{-}$ T cells on day -2. On day 0, all mice were either challenged with 0.25×10^{6} Pan02 or Eso2. After four weeks the mice were sacrificed and the tumor draining lymph node (TDLN) and spleen removed. *Foxp3* expression was measured by real-time PCR and is expressed as fold increase normalized to the initial $CD4^{+}25^{-}$ naive T cell population.

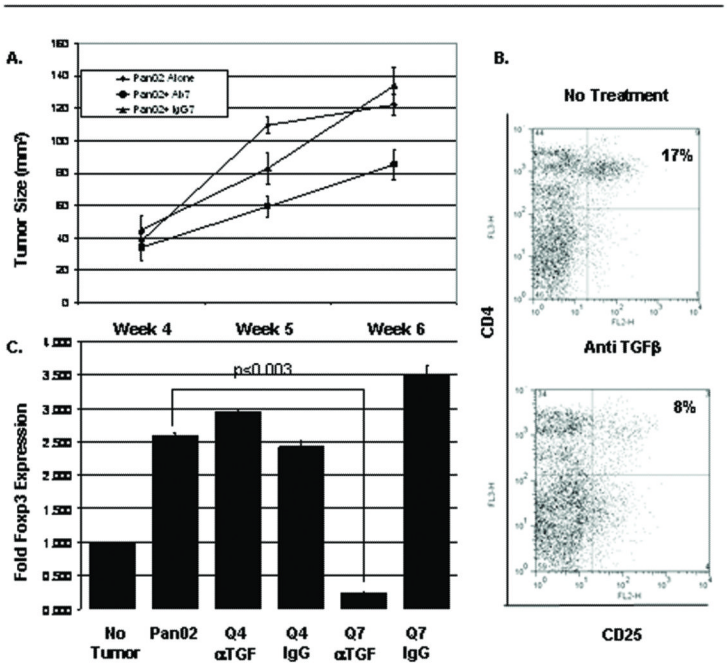

Figure 6.

Blockade of TGF-Beta prevents induction of Foxp3 by Pan02 *Rag1*$^{-/-}$ mice were reconstituted on day -2 with CD4^{+}25^{-} T cells and challenged with tumor on day 0. The tumors were allowed to grow for four weeks after which the mice underwent Q7 injection with intravenous anti-TGF-Beta antibody (Ab7) or IgG isotype control antibody (IgG7). At six weeks the mice were sacrificed and the tumors and TDLN analyzed. *A*, Tumor-bearing mice that were treated with anti-TGF-Beta antibody had significantly smaller tumors. Tumor size is given as the multiplication of the diameter of the tumor in two dimensions which approximates the volume of the tumor. *B*, FACS analysis of TDLN for CD4 and CD25. The percentage CD4^{+}25^{+} is indicated in each plot. *C*, Foxp3 expression in TDLN determined by real-time PCR. The expression in untreated Pan02-bearing mice was normalized to 1.0. The expression of Foxp3 after treatment with anti-TGF-Beta antibody was significantly lower than in untreated controls ($p<0.001$)

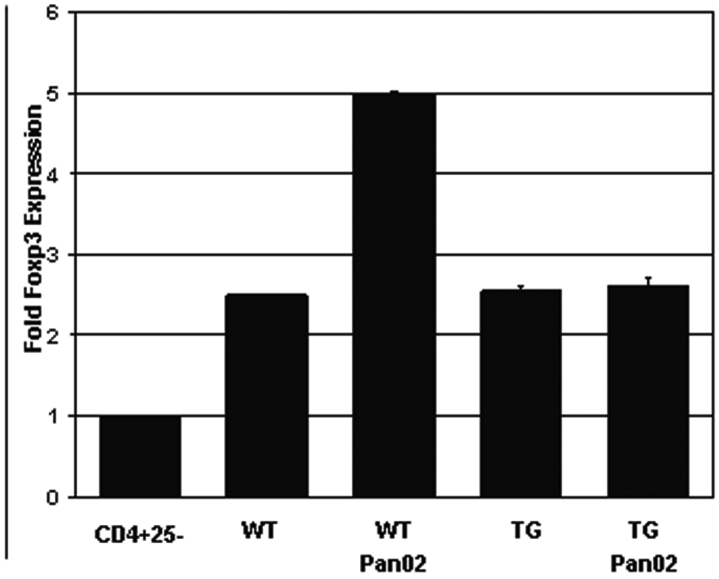

Figure 7.

Pan02 does not induce Foxp3 in dnTGFBetaRII $CD4^{+}25^{-}$ T cells . *A*, *Rag1*$^{-/-}$ mice were reconstituted with $CD4^{+}25^{-}Foxp3^{-}$ T cells taken from either CD57BL/6 (WT) or dnTGFBetaRII (TG) mice that cannot signal through the TGF-Beta receptor. At two days the mice were challenged with tumor and sacrificed after four weeks. The tumors were measured and TDLN were analyzed for expression of Foxp3 by real-time PCR.

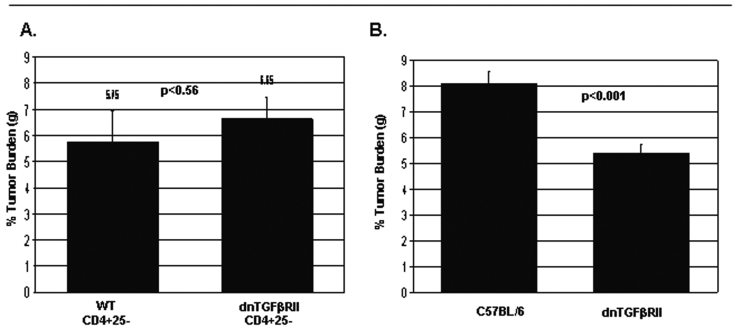

Figure 8.

Tumor burden in reconstituted mice is not affected by TGF-Beta receptor signaling. *A*, Tumor burden of *Rag1*$^{-/-}$ mice reconstituted with WT versus dnTGFBetaRII CD4^{+}25^{-} naive T cells and subsequently challenged with Pan02 tumor. There was no statistically significant difference between the two groups. *B*, Pan02 tumor burden in normal C57BL/6 versus dnTGFBetaRII mice. Tumors in dnTGFBetaRII mice were significantly smaller than in wild type mice ($p<0.02$). The tumor burden is calculated as the tumor weight divided by the mouse total body weight expressed as a percentage.