Published in final edited form as:

Cancer Immunol Immunother. 2013 February ; 62(2): . doi:10.1007/s00262-012-1324-3.

Rosiglitazone and Gemcitabine in combination reduces immune suppression and modulates T cell populations in pancreatic cancer

Stephanie K. Bunt¹, Ashley M. Mohr¹, Jennifer M. Bailey¹, Paul M. Grandgenett¹, and Michael A. Hollingsworth^{1,2,3}

¹Eppley Institute for Research in Cancer, University of Nebraska Medical Center, 985950 Nebraska Medical Center, Omaha, NE 68198

Abstract

Pancreatic ductal adenocarcinoma (PDA) is a leading cause of cancer mortality with a dismal 2–5% 5-year survival rate. Monotherapy with Gemcitabine has limited success, highlighting the need for additional therapies that enhance the efficacy of current treatments. We evaluated the combination of Gemcitabine and Rosiglitazone, an FDA-approved drug for the treatment of type II diabetes, in an immunocompetent transplantable mouse model of pancreatic cancer. Tumor progression, survival and metastases were evaluated in immunocompetent mice with subcutaneous or orthotopic pancreatic tumors treated with Pioglitazone, Rosiglitazone, Gemcitabine or combinations of these. We characterized the impact of high dose Rosiglitazone and Gemcitabine therapy on immune suppressive mediators, including MDSC and T regulatory cells, and on modulation of peripheral and intra-tumoral T cell populations. Combinations of Rosiglitazone and Gemcitabine significantly reduced tumor progression and metastases, enhanced apoptosis, and significantly extended overall survival compared to Gemcitabine alone. Rosiglitazone altered tumor-associated immune suppressive mediators by limiting early MDSC accumulation and intra-tumoral T regulatory cells. Combination therapy with Rosiglitazone and Gemcitabine modulated T cell populations by enhancing circulating CD8⁺ T cells and intra-tumoral CD4⁺ and CD8⁺ T cells while limiting T regulatory cells. The results suggest that Rosiglitazone, in combination with Gemcitabine, decreases immune suppressive mechanisms in immunocompetent animals and provide pre-clinical data in support of combining Rosiglitazone and Gemcitabine as a clinical therapy for pancreatic cancer.

Keywords

PPAR γ ; Pancreas cancer; Rosiglitazone; T regulatory cells; Gemcitabine

INTRODUCTION

Pancreatic cancer is accompanied by inflammation and the induction of significant immune suppression, through the accumulation of myeloid-derived suppressor cells (MDSC) and T

³This work was supported by grants from the National Institutes of Health (R03 CA149857, R01CA57362, U01CA111294, P50 CA127297), NCI training grant (CA09476) and student assistantship awards from the University of Nebraska and NCI Cancer Center Grant P30CA36727.

²Corresponding author: Dr. M.A. Hollingsworth; Eppley Institute for Research in Cancer, University of Nebraska Medical Center, 985950 Nebraska Medical Center Omaha, NE 68198. Phone: (402) 559-8343; Fax: (402) 559-2813; mahollin@unmc.edu.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

regulatory cells [1, 2], which enables tumor progression and serves as a significant impediment to successful immunotherapy. Pre-clinical studies demonstrate that elimination of mediators of immunosuppression, either directly or by limiting tumor-associated inflammation, significantly improves anti-tumor immune responses and increases apoptosis in a pancreatic cancer model [3]. Although Gemcitabine has been shown to limit MDSC accumulation [4], this reduction is transient and Gemcitabine does not effectively limit T regulatory cells in pancreas cancer [3], highlighting the need for additional therapies that can be effectively combined with Gemcitabine to target inflammation and immune suppression.

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a critical regulator of inflammation and cellular differentiation. Loss of function mutations in the PPAR γ gene are present in some human colon cancers [5] and PPAR $\gamma^{+/-}$ mice have an increased susceptibility to tumor development in several organs [6], suggesting a role for PPAR γ as a tumor suppressor. Activation by synthetic ligands in the thiazolidinediones class, such as Rosiglitazone, limits tumor cell growth in murine and human cancer cell lines, including pancreatic cancer cell lines [7] and limits tumor progression [7–10] and inflammatory conditions *in vivo* [11, 12]. Several epidemiological studies show a decreased incidence of neoplasms in the general population and diabetic patients taking thiazolidinediones [13, 14]. PPAR γ ligands showed growth retardation and differentiation effects on liposarcoma [15] and a stabilization of PSA levels in prostate cancer patients [16].

Despite pre-clinical efficacy, translation of these drugs into clinical trials as single agents has met with limited success, and evidence suggests that these agents may be better as adjuncts to standard therapy. Rosiglitazone enhances Gemcitabine cytotoxicity in pancreas cancer cell lines [17, 18] and fluoracil-induced apoptosis in colon cancer cell lines [19]. Several molecular mechanisms have been proposed for the evident synergy between Rosiglitazone and Gemcitabine; however, regulation of the immune system has not been examined as combination treatments have not been evaluated *in vivo* in an immunocompetent model. The anti-inflammatory effects of PPAR γ , mediated through a reduction in inflammatory cytokines [20], suggested to us that PPAR γ activation may regulate immune suppressive mechanisms, such as T regulatory cells and MDSC, which are induced by tumor-associated inflammation. Additionally, activation of PPAR γ has been shown to promote myeloid differentiation [20] possibly limiting the accumulation of MDSC and evidence shows that PPAR γ agonists can alleviate macrophage suppression of cytotoxic T lymphocytes [21], further suggesting that targeting PPAR γ may diminish tumor-associated suppressive mechanisms.

We evaluated the effects of PPAR γ agonists in the context of Gemcitabine treatment of pancreatic cancer, and found that high doses of Rosiglitazone in combination with Gemcitabine limited tumor progression and invasion and enhanced survival in an immunocompetent murine model of pancreatic cancer. PPAR γ activation limited early accumulation of MDSC and modulated the immune suppressive tumor-microenvironment by decreasing intra-tumoral T regulatory cells and increasing both peripheral CD8⁺ T cells and intra-tumoral CD4⁺ and CD8⁺ T cells.

MATERIALS AND METHODS

Mice, cell lines and reagents

Six to eight week old C57BL6 mice were obtained from the Jackson Laboratories (Bar Harbour, ME) and all animal procedures were approved by the UNMC Institutional Animal Care and Use Committee. The Panc02 carcinoma cell line was maintained in McCoy's 5A medium (Cellgro; Manassas, VA) supplemented with 10% FBS and 1% Antibiotic-Antimycotic solution (Cellgro). Rosiglitazone (Sigma; St. Louis, MO) was resuspended in

DMSO. For animal studies, Rosiglitazone was reconstituted in 100% EtOH and Pioglitazone was reconstituted in DMSO, and both were dissolved at the indicated concentrations in sterile drinking water. Gemcitabine (38 mg/mL; Lilly; Indianapolis, IN), Rosiglitazone Maleate (Avandia; GlaxoSmithKline), and Pioglitazone HCL (Actos, Takeda Pharmaceuticals) were obtained through the UNMC pharmacy. Supernatants from hybridomas expressing an antibody against CD25 (PC61) and control rat IgG antibody (UC7) were purified by the monoclonal facility at UNMC.

Tumor inoculations

For subcutaneous injections, 1×10^6 Panc02 cells were trypsinized, washed, resuspended in 50 μ l of sterile PBS and injected into the flank of C57BL6 mice. Tumor growth was monitored weekly by measuring tumor diameter using a caliper and survival time indicates the number of days until tumors reached 1cm² and mice were euthanized. For orthotopic implantations, 5×10^5 Panc02 cells were prepared as described for subcutaneous injections, resuspended in 30 μ l of sterile PBS and implanted into the pancreas as described previously [22, 23]. For orthotopic experiments, mice were euthanized and tumor volume was calculated when tumors greater than 1 cm³ were palpable or control mice appeared moribund. In the Gemcitabine treated group, mice were injected i.p. with Gemcitabine (60mg/kg) twice a week every other week. Mice treated with Rosiglitazone or Pioglitazone were given drug in the drinking water at final concentrations of 100uM or 1mM. Water consumption studies in adult mice (20–40gm) have consistently shown that individual mice consume 15ml of water/100gm/day. This translates into administration of rosiglitazone at approximately 7.2 and 72 mg/kg/day at concentrations of 100uM and 1mM respectively. Tumor diameters (2/tumor) were measured weekly and tumor volume was calculated ($V = 4/3 * \pi * r^3$). Ascites was evaluated as the presence or absence of fluid in the peritoneal cavity and peritoneal metastases were considered present when >5 nodules were visible.

In vitro growth and invasion assays

For *in vitro* cell proliferation assays, 1×10^5 Panc02 cells were plated in 6 well plates in 3 ml of growth media, serum-free media, or growth media supplemented with Rosiglitazone or Pioglitazone (10 μ M or 100 μ M). Cells were counted microscopically at 24 hrs, 48 hrs, and 72 hrs. For invasion assays, 2×10^4 Panc02 cells were seeded into the upper chamber of a Biocoat Matrigel Invasion Chamber (Becton Dickinson, Bedford, MA) in serum-free media. The lower chamber contained medium alone or supplemented with Rosiglitazone (10 μ M or 100 μ M). The chambers were cultured at 37°C for 24 hours. Cells invading to the bottom of the membrane were fixed and stained with Diff-Quick staining kit (Allegiance) and quantified at 200X magnification by light microscopy as previously described [22].

Western blotting

Panc02 cells, untreated or treated with the indicated drug concentrations, were lysed in RIPA cell lysis buffer (150mM Sodium Chloride, 1% IGEPAL Ca-630, 0.5% Sodium Deoxycholate, 0.1% SDS, 50nM Tris) with protease inhibitors (Complete mini inhibitor cocktail, Roche, Mannheim, Germany) and 1mM PMSF. One hundred micrograms of protein, as determined by Bradford assay, were boiled and reduced with 0.1M DTT and run on a 4–20% Tris/Glycine polyacrylamide gels (Biorad; Hercules, CA) as described previously [24, 25] and transferred to PVDF. Membranes were blocked for at least 1 hr and stained in 5% milk. Anti-TGF β 1 antibody (Santa Cruz) and anti-cleaved caspase 3 (Cell Signaling) were used at 1:100 and 1:500, respectively. All membranes were stripped (0.2M NaOH) and probed with anti- β -actin antibody at 1:5000 (Sigma) as a protein loading control.

Immunohistochemistry and immunofluorescence

Paraffin-embedded primary tumor sections from s.c. and orthotopically inoculated C57BL6 mice, either untreated or treated with Rosiglitazone or Gemcitabine or both, were evaluated by immunohistochemistry and immunofluorescence. For immunohistochemistry, sections were stained with primary antibodies against cleaved caspase 3 (1:50), TGF β (1:20), or COX-2 (1:50) and stained with secondary antibody using the EnVision HRP kit (Dako, Carpinteria, CA) and staining was visualized by DAB. For immunofluorescence staining, directly-coupled FITC or PERCP antibodies against FoxP3 (1:20), CD3 (1:20), CD4 (1:20), and CD8 (1:20) were used and slides were mounted with Fluoromount G (Southern Biotech, Birmingham, AL).

Flow cytometry

White blood cells were collected by submandibular bleeding of C57BL6 tumor-bearing mice and red blood cells were lysed in lysis buffer (154mM NH₄Cl, 10mM KHCO₃, 0.1 mM EDTA) for 10 min at room temperature followed by centrifugation for 5 min at 1200 rpm. Remaining white blood cells were labeled by direct immunofluorescence with antibodies against Gr1, CD11b, CD3, CD4, CD8, and CD25 (BD Pharmingen, San Diego, CA) as previously described [26].

Statistical analyses

All analyses were conducted using analysis of variance after performing a rank transformation on the variables. Post-hoc comparisons and data were analyzed using repeated measures ANOVA with a Tukey adjustment for multiple comparisons. Survival analysis was calculated using the Kaplan and Meier method and outcomes were analyzed using a log-rank test.

RESULTS

Rosiglitazone and Pioglitazone limit cell proliferation in vitro

Thiazolidinediones have been shown to reduce proliferation of several human pancreatic cancer cell lines [10, 27] and Rosiglitazone limits *in vitro* tumor cell growth in several murine and human cell lines (reviewed in [28]). We evaluated the capacity of Rosiglitazone and Pioglitazone to reduce the growth of a murine pancreatic adenocarcinoma cell line, Panc02. Tumor cells were grown in serum-free media, culture media (10% FBS), either alone or supplemented with Rosiglitazone or Pioglitazone at 10uM and 100uM and cells were counted at 24, 48 and 72 hrs (Fig. 1A). A dose-dependent reduction in Panc02 cell proliferation was observed, with a >3.5 fold reduction in growth with Rosiglitazone 100uM treatment. Similarly, Pioglitazone at both 10uM and 100uM also significantly reduced Panc02 cell proliferation demonstrating that both thiazolidinediones can reduce pancreatic cancer cell growth *in vitro*. This reduction in cell growth was not due to increased apoptosis, as no increase in cleaved caspase 3 was detected after Rosiglitazone treatment (10uM and 100uM) (Fig. 1B).

Combination of Rosiglitazone and Gemcitabine limits tumor progression and increases survival

The synergy of some thiazolidinediones with chemotherapeutic agents *in vitro* suggested that Rosiglitazone or Pioglitazone may enhance the efficacy of Gemcitabine *in vivo*. We evaluated the effects of combinations of either Rosiglitazone or Pioglitazone with Gemcitabine on tumor progression in pancreatic cancer. C57BL6 mice were inoculated subcutaneously with Panc02 cells and tumor diameter was measured weekly. Mice were either untreated, treated continuously with Rosiglitazone or Pioglitazone at either 100uM or

1mM in the drinking water, treated with Gemcitabine (60mg/kg; twice weekly, every other week) or combination treatments. Pioglitazone or Rosiglitazone (100uM or 1mM) alone did not significantly affect tumor progression and Pioglitazone at either concentration in combination with Gemcitabine was not more effective than Gemcitabine alone (Pioglitazone at 1mM data not shown), suggesting that Pioglitazone did not have an effect on tumor progression *in vivo*. However, higher doses of Rosiglitazone with Gemcitabine led to significantly reduced tumor growth ($p<0.05$) with the reduction being most significant at 100uM or greater doses of Rosiglitazone (Fig. 1C). The observed decrease in tumor growth was associated with an increase in overall survival (defined as the day post-tumor inoculation when tumors reached 1cm³ and mice were euthanized). Overall survival was significantly extended by the combination treatment compared to untreated and Gemcitabine-treated mice ($p=0.003$) (Fig. 1D), but unchanged by the addition of Pioglitazone (data not shown). These results demonstrate that Rosiglitazone enhanced the *in vivo* anti-tumor effects of Gemcitabine by limiting tumor progression and increasing overall survival.

Combination therapy reduces tumor size and peritoneal invasion, and enhances caspase 3 activation in orthotopically challenged mice

The efficacy of Rosiglitazone and Gemcitabine treatment in mice bearing s.c. tumors led us to evaluate its success in immunocompetent mice bearing orthotopic pancreatic tumors. C57BL6 mice received 5×10^5 Panc02 cells implanted into the body of the pancreas and treatment with Gemcitabine (60mg/kg), Rosiglitazone (100uM or 1mM), or both was initiated 5 days later. All mice were euthanized when the first mouse appeared moribund, and tumor volume was measured. Similar to results observed in the subcutaneous tumor growth experiment, treatment with Gemcitabine resulted in significantly reduced tumor volume ($p=0.03$), whereas treatment with Rosiglitazone alone had no statistically significant effect, although there was a trend for decreased tumor volume in this group (Fig. 2A). However, combination treatment using Rosiglitazone 1uM showed significantly reduced tumor volumes compared to untreated ($p<0.0001$) and Gemcitabine alone ($p=0.009$). In addition to reducing tumor burden, only 80% of the mice treated with Rosiglitazone (1mM) and Gemcitabine had pancreatic tumors compared to 100% for all other groups (data not shown). Rosiglitazone alone had no effect on the percentage of mice with tumor. Since Rosiglitazone and Gemcitabine showed increased caspase 3 expression and apoptosis activity *in vitro* (data not shown), we examined whether the decreased tumor volume was correlated with increased apoptosis. Paraffin-embedded tumor sections from Panc02 tumor-bearing mice treated with single and combination agents were stained by immunohistochemistry for the expression of cleaved caspase 3. Tumors from Gemcitabine-treated mice showed increased numbers of apoptotic cells; however, this increase was not statistically significant (Fig. 2B). Mice treated with Rosiglitazone (100uM) and Gemcitabine had significantly higher numbers of caspase 3 positive apoptotic cells ($p=0.014$).

Tumor growth inhibition by treatment with Rosiglitazone and Gemcitabine was accompanied by a reduction in peritoneal invasion and metastasis as determined by evaluating mice for ascites and numbers of peritoneal nodules. Rosiglitazone alone had no effect on the presence of ascites (Fig. 2C), the development of peritoneal nodules (Fig. 2C) or the percent of mice with splenic invasion (data not shown). Although Gemcitabine alone reduced the percent of mice with ascites by 8 fold, Gemcitabine alone did not have a significant effect on any other parameter evaluated. Addition of Rosiglitazone to Gemcitabine, particularly at the 1mM concentration, reduced the percentage of mice with peritoneal nodules by 50% (Fig. 2C). These results demonstrate that the combination of Rosiglitazone and Gemcitabine limits orthotopic tumor growth and reduces peritoneal invasion and metastasis. We also observed a reduction in invasive properties *in vitro*

following treatment with Rosiglitazone (Fig. 2D). Rosiglitazone treatment led to a dose-dependent reduction in Panc02 cell invasion (a 5–11 fold decrease in invasion with 100uM Rosiglitazone).

Rosiglitazone reduces early levels of CD11b⁺Gr1^{high} MDSC

Pancreatic cancer is accompanied by significant immune suppression, mediated in part by the accumulation of MDSC and T regulatory cells [1, 2]. It is well documented that inflammation, which accompanies pancreatic tumor progression, induces the accumulation of MDSC [29] and T regulatory cells. To establish the anti-inflammatory properties of Rosiglitazone, tumor sections from orthotopic Panc02 tumor-bearing mice untreated or treated with Gemcitabine, Rosiglitazone or a combination of both were stained by immunohistochemistry with antibodies against TGF β and COX-2 (Figure 3A). Immunohistochemical staining of COX-2 and TGF β demonstrates that Rosiglitazone limits TGF β and COX-2 expression in the tumor microenvironment suggesting that the anti-inflammatory properties of PPAR γ may limit inflammation, in turn reducing the expansion of MDSC. Since Gemcitabine has also been shown to limit MDSC *in vivo* [4], we examined whether Rosiglitazone would further reduce the MDSC burden, and whether this reduction contributes to the observed reduction in tumor progression. Orthotopic Panc02 tumor-bearing mice were bled through the submandibular vein at one, three and five weeks after tumor implantation (with the first week being pre-Gemcitabine treatment). Circulating mononuclear cells were stained with antibodies to Gr1 and CD11b to analyze the percent of blood MDSC by flow cytometry. Rosiglitazone significantly decreased blood CD11b⁺Gr1^{high} MDSC levels compared to untreated mice at week 1 and week 3 ($p=0.02$ and $p=0.01$, respectively) and MDSC levels were similar in both Rosiglitazone treated and Gemcitabine treated mice at the early stages of tumor growth (Fig. 3B), although Rosiglitazone appeared more slightly more effective than Gemcitabine at week 3. However, at later stages (week 5), CD11b⁺Gr1^{high} MDSC levels in Rosiglitazone treated mice rebounded to levels comparable to untreated mice, while CD11b⁺Gr1^{high} MDSC levels remained low in Gemcitabine-treated mice. These results suggest that Rosiglitazone can initially suppress the accumulation of CD11b⁺Gr1^{high} MDSC, but as tumors progress CD11b⁺Gr1^{high} MDSC levels expand despite Rosiglitazone treatment and Rosiglitazone does not enhance the Gemcitabine-mediated suppression of MDSC.

Rosiglitazone reduces TGF β production and limits intra-tumoral infiltration of T regulatory cells

The anti-inflammatory effects of PPAR γ activation result in part from the transrepression of NF κ B leading to a decrease in a wide array of inflammatory cytokines, including TGF β [20]. Rosiglitazone treatment decreased *in vitro* TGF β production by Panc02 cells in a dose-dependent manner, as seen by the reduction in expression of the 12 kDa monomer (Fig. 3C). To determine whether this decrease was associated with alterations in the accumulation of intratumoral T regulatory cells *in vivo*, paraffin-embedded sections of orthotopic Panc02 tumors from mice either untreated, or treated with Rosiglitazone, Gemcitabine or both were stained by immunofluorescence with FITC labeled FoxP3, a T regulatory cell marker (Fig. 3D). Treatment of tumor-bearing mice with Rosiglitazone alone significantly decreased the number of intra-tumoral FoxP3⁺ T regulatory cells ($p=0.003$), while Gemcitabine had no effect (Fig. 3D). These results demonstrate that Rosiglitazone treatment limited TGF β production from Panc02 cells and reduced the intra-tumoral infiltration of immune suppressive T regulatory cells.

In vivo depletion of T regulatory cells reduces tumor progression but does not enhance the effects of Rosiglitazone and Gemcitabine therapy

Since the reduction in tumor progression in Rosiglitazone and Gemcitabine treated mice is accompanied by a decrease in the number of infiltrating T regulatory cells, we asked whether depletion of T regulatory cells would further enhance this effect. Panc02 cells were orthotopically implanted into the pancreas of C57BL6 mice and the following treatments were started one week post-implantations: a control rat IgG antibody or an antibody against CD25 (PC61) to deplete T regulatory cells; Gemcitabine with or without PC61 or rat IgG; Rosiglitazone and Gemcitabine with or without PC61 or rat IgG. Mice were euthanized when control mice became moribund. PC61 and rat IgG antibody (0.5mg) were given on day -4, -2, +1, and every two weeks for the duration of the experiment. Leukocytes obtained from weekly bleeds were double-stained with antibodies against CD4⁺ and CD25⁺ to ensure T regulatory cell depletion in PC61 treated mice. Compared to rat IgG treated mice, PC61 treated mice had a significant reduction in circulating CD4⁺CD25⁺ T regulatory cells (Fig. 4A). Treatment with PC61 significantly reduced tumor volume compared to rat IgG treated mice ($p<0.0001$); however, tumors were not significantly smaller than Gemcitabine alone and tumor volumes were significantly larger than Rosiglitazone and Gemcitabine treated mice ($p=0.003$) (Fig. 4B). Addition of PC61 treatment to Rosiglitazone and Gemcitabine did not reduce tumor volumes compared to control Rosiglitazone and Gemcitabine treated mice, suggesting that targeted CD4⁺CD25⁺ T regulatory cell depletion did not further enhance the effects of this combination treatment, and supporting our conclusion that the inhibition of tumor progression we observed was due in part to blocking of T regulatory activity.

Rosiglitazone and Gemcitabine treatment increases both peripheral CD3⁺CD8⁺ T cells and intra-tumoral infiltration of CD4⁺ and CD8⁺ T cells

The reduction in tumor progression observed in mice treated with Rosiglitazone and Gemcitabine may result from enhanced T cell responses, either due to a decrease in the presence of T regulatory cells in Rosiglitazone and Gemcitabine treated mice, or as a result of enhanced proliferation and recruitment of effector T cell populations. We evaluated the effects of depletion of T regulatory cells on numbers of peripheral CD3⁺CD8⁺ T cells. Gemcitabine, Rosiglitazone, and PC61 treatment did not alter the percentage of circulating CD3⁺CD8⁺ T cells, which decreased significantly after week 1 when tumors became established (Fig. 4C). Only Rosiglitazone and Gemcitabine combination-treated mice showed a significant recovery of peripheral CD3⁺CD8⁺ T cells compared to rat IgG treated mice as tumors progressed ($p=0.03$). We also evaluated treated mice for tumor infiltration by T cells. Paraffin-embedded tumor sections from the same orthotopic treated mice were stained by immunofluorescence to FITC-labeled CD4⁺, CD8⁺ and FoxP3⁺. As discussed previously, Rosiglitazone treatment reduced the number of tumor infiltrating FoxP3⁺ T regulatory cells and this reduction was equivalent to the reduction seen with PC61 treatment (Fig. 4D) suggesting that Rosiglitazone effectively depletes T regulatory cells from the tumor milieu. Although Rosiglitazone alone did not alter the percent of peripheral CD8⁺ T cells (Fig. 4C), there was a significant increase in the number of CD8⁺ tumor infiltrating T cells ($p=0.05$) (Fig. 4D). The combination of Rosiglitazone and Gemcitabine led to a significant increase in both CD4⁺ and CD8⁺ T cell infiltration and a significant decrease in FoxP3⁺ infiltration. These results suggest that Rosiglitazone and Gemcitabine significantly alter the intra-tumoral lymphocyte populations and skew towards more favorable immune effector populations.

DISCUSSION

Pre-clinical [7–10] and epidemiological [13, 14] evidence suggest that activation of PPAR γ results in synergy with chemotherapeutic agents that may reduce tumor burden in pancreatic cancer. Despite this preliminary evidence, PPAR γ agonists have not been assessed in the context of chemotherapy for pancreatic cancer. These combination therapies have been examined mostly *in vitro* and a few *in vivo* studies to date have been performed in immune deficient animal models, which would not detect effects on tumor immunity. We report here that PPAR γ agonist, Rosiglitazone, when combined with Gemcitabine limited pancreatic tumor progression and extended survival in a mouse model in part by modulating immune suppressive cellular mediators, such as MDSC and T regs, and altering peripheral and intra-tumoral T cell populations.

Previous evidence of synergy between Rosiglitazone and Gemcitabine is derived primarily from *in vitro* work; hence, our studies are among the first to evaluate this synergy in the context an active anti-tumor immune response. It is established that Rosiglitazone enhances Gemcitabine-mediated apoptosis through several possible mechanisms, such as negative regulation of the AKT/PTEN pathway [30, 31], induction of cancer cell differentiation [15] and/or suppression of angiogenesis [32]. Similarly, our *in vivo* data supports the hypothesis that an increase in apoptosis may result from the combination of these two agents, although the observed increase in apoptosis *in vivo* as measured by caspase 3 activation may also be a result of the increased intra-tumoral infiltration of T cells and the reduction in immune suppressive T regulatory cells observed in the combination-treated group. Our data demonstrate a significant increase in apoptosis in the primary tumor in the presence of Rosiglitazone alone, suggesting that this increase is not due completely to synergy between Rosiglitazone and Gemcitabine. Additionally, in the Rosiglitazone-treated animals, we observed a decrease in T regulatory cell infiltration and an increase in CD8⁺ T cell infiltration, suggesting that the increase in apoptosis seen in this group may be a result of the altered immune cell infiltrates. Although a trend for reduced tumor size was observed in the Rosiglitazone-treated group, these changes did not result in a significant reduction in tumor progression in the absence of Gemcitabine, highlighting the potential synergy between these two agents.

It is well documented that inflammation promotes and accompanies tumor progression in pancreatic cancer and is a significant impediment to therapy. Reducing inflammation through the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been linked to a decrease in pancreatic cancer risk [33], a decrease in pancreatic cancer cell proliferation *in vitro* [34, 35], and good clinical benefit in a Phase II trial evaluating NSAID use in combination with Gemcitabine [36], supporting the concept that a reduction in inflammation in the context of chemotherapy may enhance current treatment options. In addition to enhancing angiogenesis and the growth factor milieu, inflammatory mechanisms promote tumor-associated immune suppression, through the induction of MDSC [29, 37] and T regulatory cells [38], further limiting anti-tumor immunity. A major function of PPAR γ activation is to limit the inflammatory environment through several pathways, including transrepression of NF κ B and STATs, thereby significantly modulating the tumor microenvironment [20, 39]. PPAR γ activation has been shown to limit inflammation in a model of inflammatory bowel disease (IBD), supporting the use of PPAR γ ligands as anti-inflammatory agents [12]. Rosiglitazone has been shown to limit the production of inflammatory prostaglandin E2 (PGE2) [40], a major inducer of both MDSC [37] and T regulatory cells [41]. Our data demonstrate that at the early stages of tumor growth (up to week 3), Rosiglitazone limits blood accumulation of CD11b⁺Gr1^{high} MDSC to levels similar to those seen with Gemcitabine treatment. This reduction in peripheral MDSC may be due to a reduction in inflammatory mediators or a maturation of this immature cell

population, as PPAR γ activation leads to maturation of myeloid lineage cells [20]. As tumors progress into late stages of growth (week 5), CD11b⁺Gr1^{high} MDSC expansion was equivalent to that seen in untreated mice; however, this delay in MDSC expansion may enhance the capacity of early anti-tumor immunity. Both Rosiglitazone and Gemcitabine can limit early CD11b⁺Gr1^{high} MDSC; however, combination therapy does not further limit accumulation of these cells suggesting that maximal inhibition of MDSC has been achieved with either agent alone. Additionally, we show that Rosiglitazone treatment limits T regulatory cell accumulation in the primary tumor which may also boost anti-tumor functions. Since MDSC can induce T regulatory cells through the production of TGF β , the reduction in MDSC by Rosiglitazone may also lead to the observed reduction in T regulatory cells. The reduction in both of these suppressive populations may enhance anti-tumor immunity by limiting peripheral and intra-tumoral T cell suppression leading to a reduction in tumor progression.

We observed that T regulatory cell depletion with PC61 or combination therapy with Gemcitabine and Rosiglitazone significantly limited tumor growth; however, the triple combination of these treatments was not additive, but rather less effective in reducing tumor burden. A possible explanation for this finding is that treatment with PC61 or Rosiglitazone and Gemcitabine were shown to specifically reduce intra-tumoral FOXP3⁺ T regulatory cells and to increase peripheral and intra-tumoral CD8⁺ T cells; however, because PC61 specifically targets CD25⁺ T cells, PC61 may also be depleting CD25⁺ effector T cells. These data suggest that a reduction in T regulatory cells and a recovery of CD8⁺ T cells are essential components to the anti-tumor effect of Rosiglitazone and Gemcitabine therapy.

Our *in vivo* data in the subcutaneous model suggested that Rosiglitazone alone was ineffective as a single agent and that both concentrations of Rosiglitazone (100 μ M and 1mM) were effective in combination with Gemcitabine. Rosiglitazone alone did not significantly alter tumor size in the orthotopic model; however, there was a trend towards reduced tumor size and the 1mM Rosiglitazone concentration was significantly more effective in combination with Gemcitabine. In this context it is important to consider the dose and schedule of Rosiglitazone we utilized, as this differs from that of current clinical practice. Rosiglitazone is administered at 4–8 mg/day as a single or divided dose to humans, which corresponds to 0.04–0.15 mg/kg. Given that mice consume 15ml of water/100gm/day, we delivered Rosiglitazone to mice at approximately 7.2 and 72 mg/kg/day at concentrations of 100 μ M and 1mM in drinking water, respectively. These significantly higher doses (50–500 times that used in humans) were achieved without toxicity, presumably in part because the continuous administration in drinking water allowed for higher daily dose. The dose and schedule of administration may explain in part our results, although confirmation of this hypothesis should be obtained by careful pharmacokinetic analyses and additional studies of dose and schedule. It is possible that the prolonged administration of drug in drinking water affected the immune cells or tumor cells through continuous activation of PPAR γ and that the net effect was different from exposure to a bolus of drug from a single administration. Moreover, drug delivery in pancreatic cancer is a significant impediment to chemotherapy, and there are differences in vascular content and stromal content in subcutaneous and orthotopic animal models that impact the ability of these agents to reach the growing tumor and synergize effectively. Although the administration of Rosiglitazone and Gemcitabine were identical in both subcutaneous and orthotopic models, the delivery of these drugs to tumor undoubtedly varies based on tumor location and stromal content, which may explain why higher doses were more effective at the orthotopic site. It will be important in the future to investigate this drug combination in appropriate genetically engineered models of pancreatic cancer, which may better reflect typical poorly vascularized conditions encountered in human pancreatic cancer.

The link between pancreatic cancer and diabetes is not well understood, although it is estimated that 80% of pancreatic cancer patients are intolerant to glucose or have diabetes [42]. Insulin and glucose production have been shown to promote cancer cell growth and contribute to chemoresistance, suggesting that management of these diabetic symptoms may enhance the efficacy of chemotherapy regimens [17]. Epidemiological data suggests that the survival of diabetic cancer patients may vary based on the antidiabetic treatments and certain treatments, such as Rosiglitazone, have been shown to enhance Gemcitabine apoptosis and overcome chemoresistance *in vitro* while other antidiabetic drugs do not exhibit the same therapeutic effects [17]. We demonstrate here that both Pioglitazone and Rosiglitazone were effective at similar concentrations in limiting cell proliferation *in vitro*; however, only Rosiglitazone in combination with Gemcitabine had a therapeutic effect *in vivo*. The differences between these two agents of the same thiazolidinedione class and their ability to synergize with Gemcitabine are currently unclear. Reports examining these two drugs in diabetic patients note differential effects on serum lipoprotein profile; however, to our knowledge other parameters that may invoke anti-tumor effects have not been examined. One possibility for distinct effects *in vivo* may be due to potency, as Pioglitazone has been shown to be ten times less potent in PPAR γ activation than Rosiglitazone and less selective as it also enhances PPAR α activity [43]. In our model Pioglitazone and Rosiglitazone alone at the 1mM concentration had similar effects on tumor growth rate. The different specificities for the PPAR receptors may also result in different vascular effects as Pioglitazone has been shown to increase VEGF and angiogenin, which stimulate new vasculature formation, compared to Rosiglitazone [44]. Pioglitazone has been shown to increase TGF β production [45], which is in contrast to our data demonstrating that Rosiglitazone limits TGF β production. It is notable that there are low-level toxicities associated with prolonged use of Rosiglitazone, which has led to a substitution of Pioglitazone in clinical applications. In the context of treating pancreatic cancer, the toxicities associated with high doses of Rosiglitazone should be considered, but it remains possible that these toxicities will show a degree of specificity for modulating immunosuppression and pancreatic tumor growth, in which case they may be clinically useful. It may be possible to minimize undesirable toxicities from higher total doses by use of an appropriate schedule of administration. Moreover, if a major beneficial effect of Rosiglitazone in combination with Gemcitabine is a modulation of immunosuppression, then it might be possible and desirable to combine appropriate doses and schedules of this strategy with targeted immunotherapies.

In conclusion, this study examined the potential therapeutic effect of PPAR γ activation, through Rosiglitazone, in combination with Gemcitabine in the treatment of pancreatic cancer. To our knowledge, this is the first report to evaluate this combination therapy in an immune competent animal model of pancreatic cancer and to characterize the effects of these agents on immune suppressive mechanisms. Our study demonstrates that Rosiglitazone, an FDA-approved antidiabetic agent, in combination with Gemcitabine, resulted in a significant downregulation of immune suppressive mechanisms and modulation of the T cell tumor microenvironment to a more favorable cellular content for tumor rejection, and that this slightly prolonged survival of immunocompetent mice challenged with pancreatic cancer. There is merit in further exploring the efficacy of this combination in the preclinical and perhaps the clinical setting for the treatment of pancreatic cancer.

Acknowledgments

The authors thank the UNMC Comparative Medicine for their excellent care of our mice, the UNMC Histology Facility, and the UNMC Monoclonal Antibody facility. We also would like to thank Dr. Jane Meza for her help with our statistical analyses.

REFERENCES

1. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res.* 2006; 12:5423–5434. [PubMed: 17000676]
2. Zhao F, Obermann S, von Wasielewski R, Haile L, Manns MP, Korangy F, Greten T. Increase in frequency of myeloid-derived suppressor cells in mice with spontaneous pancreatic carcinoma. *Immunology.* 2009; 128:141–149. [PubMed: 19689743]
3. Mukherjee P, Basu GD, Tinder TL, Subramani DB, Bradley JM, Arefayene M, Skaar T, De petris G. Progression of pancreatic adenocarcinoma is significantly impeded with a combination of vaccine and COX-2 inhibition. *J Immunol.* 2009; 182:216–224. [PubMed: 19109152]
4. Le HK, Graham L, Cha E, Morales JK, Manjili MH, Bear HD. Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. *Int Immunopharmacol.* 2009; 9:900–909. [PubMed: 19336265]
5. Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Fletcher C, Spiegelman BM. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. *Nat Med.* 1998; 4:1046–1052. [PubMed: 9734398]
6. Koeffler HP. Peroxisome proliferator-activated receptor gamma and cancers. *Clin Cancer Res.* 2003; 9:1–9. [PubMed: 12538445]
7. Itami A, Watanabe G, Shimada Y, Hashimoto Y, Kawamura J, Kato M, et al. Ligands for peroxisome proliferator-activated receptor gamma inhibit growth of pancreatic cancers both in vitro and in vivo. *Int J Cancer.* 2001; 94:370–376. [PubMed: 11745416]
8. Grommes C, Landreth GE, Sastre M, Beck M, Feinstein DL, Jacobs AH, Schiegel U, Heneka MT. Inhibition of in vivo glioma growth and invasion by peroxisome proliferator-activated receptor gamma agonist treatment. *Mol Pharmacol.* 2006; 70:1524–1533. [PubMed: 16887936]
9. Kassouf W, Chintharlapalli S, Abdelrahim M, Nelkin G, Safe S, Kamat AM. Inhibition of bladder tumor growth by 1,1-bis[3'-indolyl]-1-[p-substitutedphenyl]methanes: a new class of peroxisome proliferator-activated receptor gamma agonists. *Cancer Res.* 2006; 66:412–418. [PubMed: 16397256]
10. Sawai H, Liu J, Reber HA, Hines OJ, Eibl G. Activation of peroxisome proliferators-activated receptor-gamma decreases pancreatic cancer cell invasion through modulation of the plasminogen activator system. *Mol Cancer Res.* 2006; 4:159–167. [PubMed: 16547153]
11. Necela BM, Su W, Thompson EA. Toll-like receptor 4 mediates cross-talk between peroxisome proliferator-activated receptor gamma and nuclear factor-kappaB in macrophages. *Immunology.* 2008; 125:344–358. [PubMed: 18422969]
12. Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanagan A, Murthy S, Lazar MA, Wu GD. A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. *J Clin Invest.* 1999; 104:383–389. [PubMed: 10449430]
13. Govindarajan R, Ratnasinghe L, Simmons DL, Siegel ER, Midathada MV, Kim L, Kim PJ, Owens RJ, Lang NP. Thiazolidinediones and the risk of lung, prostate, and colon cancer in patients with diabetes. *J Clin Oncol.* 2007; 25:1476–1481. [PubMed: 17442990]
14. Monami M, Lamanna C, Marchionni N, Mannucci E. Rosiglitazone and risk of cancer: a meta-analysis of randomized clinical trials. *Diabetes Care.* 2008; 31:1455–1460. [PubMed: 18375416]
15. Demetri GD, Fletcher CD, Mueller E, Sarraf P, Naujoks R, Campbell N, Spiegelman BM, Singer S. Induction of solid tumor differentiation by the peroxisome proliferators-activated receptor-gamma ligand troglitazone in patients with liposarcoma. *Proc Natl Acad Sci U S A.* 1999; 96:3951–3956. [PubMed: 10097144]
16. Hisatake JI, Ikezoe T, Carey M, Holden S, Tomoyasu S, Koeffler HP. Down-Regulation of prostate-specific antigen expression by ligands for peroxisome proliferator-activated receptor gamma in human prostate cancer. *Cancer Res.* 2000; 60:5494–5498. [PubMed: 11034093]
17. Feng YH, Velazquez-Torres G, Gully C, Chen J, Lee MH, Yeung SC. The impact of type 2 diabetes and antidiabetic drugs on cancer cell growth. *J Cell Mol Med.* 2011; 15:825–836. [PubMed: 20455996]

18. Salesiotis AN, Laguinde L, Ling S, Marshall J, Jessup JM. Rosiglitazone works synergistically with Gemcitabine to induce cell death in pancreas cancer cell lines. *Clinical Pharmacology and therapeutics*. 2004; 75:P61.
19. Zhang YQ, Tang XQ, Sun L, Dong L, Qin Y, Liu HQ, Xia H, Cao JG. Rosiglitazone enhances fluorouracil-induced apoptosis of HT-29 cells by activating peroxisome proliferator-activated receptor gamma. *World J Gastroenterol*. 2007; 13:1534–1540. [PubMed: 17461445]
20. Daynes RA, Jones DC. Emerging roles of PPARs in inflammation and immunity. *Nat Rev Immunol*. 2002; 2:748–759. [PubMed: 12360213]
21. Van Ginderachter JA, Meerschaut S, Liu Y, Brys L, De Groeve K, Hassanzadeh Ghassabeh G, Geert R, De Baetselier P. Peroxisome proliferator-activated receptor gamma [PPARgamma] ligands reverse CTL suppression by alternatively activated [M2] macrophages in cancer. *Blood*. 2006; 108:525–535. [PubMed: 16527895]
22. Bailey JM, Mohr AM, Hollingsworth MA. Sonic hedgehog paracrine signaling regulates metastasis and lymphangiogenesis in pancreatic cancer. *Oncogene*. 2009; 28:3513–3525. [PubMed: 19633682]
23. Tsutsumida H, Swanson BJ, Singh PK, Caffrey TC, Kitajima S, Goto M, Yonezawa S, Hollingsworth MA. RNA interference suppression of MUC1 reduces the growth rate and metastatic phenotype of human pancreatic cancer cells. *Clin Cancer Res*. 2006; 12:2976–2987. [PubMed: 16707592]
24. Behrens ME, Grandgenett PM, Bailey JM, Singh PK, Yi CH, Yu F, Hollingsworth MA. The reactive tumor microenvironment: MUC1 signaling directly reprograms transcription of CTGF. *Oncogene*. 2010; 29:5667–5677. [PubMed: 20697347]
25. Singh PK, Behrens ME, Eggers JP, Cerny RL, Bailey JM, Shanmugam K, Gendler SJ, Bennett EP, Hollingsworth MA. Phosphorylation of MUC1 by Met modulates interaction with p53 and MMP1 expression. *J Biol Chem*. 2008; 283:26985–26995. [PubMed: 18625714]
26. Kusmartsev SA, Li Y, Chen SH. Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J Immunol*. 2000; 165:779–785. [PubMed: 10878351]
27. Galli A, Ceni E, Crabb DW, Mello T, Salzano R, Grappone C, Milani S, Surrenti E, Surrenti C, Casini A. Antidiabetic thiazolidinediones inhibit invasiveness of pancreatic cancer cells via PPARgamma independent mechanisms. *Gut*. 2004; 53:1688–1697. [PubMed: 15479693]
28. Michalik L, Desvergne B, Wahli W. Peroxisome-proliferator-activated receptors and cancers: complex stories. *Nat Rev Cancer*. 2004; 4:61–70. [PubMed: 14708026]
29. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J Immunol*. 2006; 176:284–290. [PubMed: 16365420]
30. Farrow B, Sugiyama Y, Chen A, Uffort E, Nealon W, Mark Evers B. Inflammatory mechanisms contributing to pancreatic cancer development. *Ann Surg*. 2004; 239:763–769. discussion 9–71. [PubMed: 15166955]
31. Han S, Roman J. Rosiglitazone suppresses human lung carcinoma cell growth through PPARgamma-dependent and PPARgamma-independent signal pathways. *Mol Cancer Ther*. 2006; 5:430–437. [PubMed: 16505118]
32. Panigrahy D, Singer S, Shen LQ, Butterfield CE, Freedman DA, Chen EJ, Moses MA, Kilroy S, Duensing S, Fletcher C, Fletcher JA, Hlatky L, et al. PPARgamma ligands inhibit primary tumor growth and metastasis by inhibiting angiogenesis. *J Clin Invest*. 2002; 110:923–932. [PubMed: 12370270]
33. Bradley MC, Hughes CM, Cantwell MM, Napolitano G, Murray LJ. Non-steroidal anti-inflammatory drugs and pancreatic cancer risk: a nested case-control study. *Br J Cancer*. 2010; 102:1415–1421. [PubMed: 20372155]
34. Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML, Sinicrope FA. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res*. 1999; 59:4356–4362. [PubMed: 10485483]
35. Yip-Schneider MT, Wu H, Njoku V, Ralstin M, Holcomb B, Crooks PA, Neelakantan S, Sweeney CJ, Schmidt CM. Effect of celecoxib and the novel anti-cancer agent, dimethylamino-

- parthenolide, in a developmental model of pancreatic cancer. *Pancreas*. 2008; 37:e45–e53. [PubMed: 18815538]
36. Ferrari V, Valcamonico F, Amoroso V, Simoncini E, Vassalli L, Marpicati P, Pangoni G, Grisanti S, Tiberio GA, Nodari F, Strina C, Marini G. Gemcitabine plus celecoxib [GECO] in advanced pancreatic cancer: a phase II trial. *Cancer Chemother Pharmacol*. 2006; 57:185–190. [PubMed: 16151811]
  37. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res*. 2007; 67:4507–4513. [PubMed: 17483367]
  38. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001; 357:539–545. [PubMed: 11229684]
  39. Cuzzocrea S, Mazzon E, Di Paola R, Peli A, Bonato A, Britti D, Genovese T, Mula C, Crisafulli C, Caputi AP. The role of the peroxisome proliferator-activated receptor- α [PPAR- α] in the regulation of acute inflammation. *J Leukoc Biol*. 2006; 79:999–1010. [PubMed: 16501055]
  40. Hazra S, Batra RK, Tai HH, Sharma S, Cui X, Dubinett SM. Pioglitazone and rosiglitazone decrease prostaglandin E2 in non-small-cell lung cancer cells by up-regulating 15-hydroxyprostaglandin dehydrogenase. *Mol Pharmacol*. 2007; 71:1715–1720. [PubMed: 17412838]
  41. Lee SY, Choi HK, Lee KJ, Jung JY, Hur GY, Jung KH, Kim JH, Shin C, Shim JJ, In KH, Kang KH, Yoo SH. The immune tolerance of cancer is mediated by IDO that is inhibited by COX-2 inhibitors through regulatory T cells. *J Immunother*. 2009; 32:22–28. [PubMed: 19307990]
  42. Wang F, Herrington M, Larsson J, Permert J. The relationship between diabetes and pancreatic cancer. *Mol Cancer*. 2003; 2:4. [PubMed: 12556242]
  43. Hirakata M, Tozawa R, Imura Y, Sugiyama Y. Comparison of the effects of pioglitazone and rosiglitazone on macrophage foam cell formation. *Biochem Biophys Res Commun*. 2004; 323:782–788. [PubMed: 15381068]
  44. Vijay SK, Mishra M, Kumar H, Tripathi K. Effect of pioglitazone and rosiglitazone on mediators of endothelial dysfunction, markers of angiogenesis and inflammatory cytokines in type-2 diabetes. *Acta Diabetol*. 2009; 46:27–33. [PubMed: 18758684]
  45. Redondo S, Ruiz E, Santos-Gallego CG, Padilla E, Tejerina T. Pioglitazone induces vascular smooth muscle cell apoptosis through a peroxisome proliferator-activated receptor- γ , transforming growth factor- β 1, and a Smad2-dependent mechanism. *Diabetes*. 2005; 54:811–817. [PubMed: 15734860]

FIGURE 1.

Rosiglitazone and Gemcitabine combination therapy limit tumor growth and extend survival in a subcutaneous model of pancreatic cancer. (A) 1×10^5 Panc02 cells were plated in 6 well plates in 3 ml of growth media (DMEM with 10% FBS), serum-free DMEM, or growth media supplemented with Rosiglitazone or Pioglitazone (10 μ M or 100 μ M). Cells were counted microscopically at 24 hrs, 48 hrs, and 72 hrs. Data is representative of triplicate experiments. (B) Western blots of whole cell lysates from 2×10^6 Panc02 cells treated for 48 hrs with the indicated concentrations of Rosiglitazone and Gemcitabine were probed with anti-cleaved caspase 3 and anti-p-actin. (C and D) C57BL6 mice were inoculated s.c. in the flank with 1×10^6 Panc02 cells and mice were either untreated (vehicle only) or treated with Gemcitabine (60mg/kg), Rosiglitazone (100uM or 1mM), Pioglitazone (100uM) or a combination of Gemcitabine and either Rosiglitazone or Pioglitazone. Data are shown as means of values from 10 mice per group. (C) Tumor volume was measured weekly by

caliper (* $p < 0.05$). (D) Survival was evaluated and defined as the day post-tumor inoculation when tumors reached 1 cm³ and mice were euthanized (* $p = 0.003$).

FIGURE 2.

Rosiglitazone and Gemcitabine limits tumor progression, enhances apoptosis, and reduces invasion in an orthotopic pancreatic cancer model. (A) C57BL6 mice (10 mice per group) were implanted into the body of the pancreas with 5×10^5 Panc02 cells. Five days post-implantation, mice were either untreated (vehicle only), or treated with Gemcitabine (60mg/kg), Rosiglitazone (100uM or 1mM), or both Gemcitabine and Rosiglitazone. Animals were euthanized when control mice appeared moribund, and tumor volumes were measured by caliper. (B) Immunohistochemistry with an antibody against cleaved caspase 3 was performed on paraffin-embedded tumor sections from untreated and treated mice as a measure of *in vivo* apoptosis. The number of CC3 positive cells was counted per 200X field and data is shown as averages of 4 fields/tumor section for 5 tumors/group. (C) The percentage of mice in [A] with the presence of ascites and peritoneal metastases were analyzed. (D) The invasion of untreated or Rosiglitazone treated (10uM or 100uM) 1×10^4 Panc02 cells through matrigel was quantified after 24 hrs.

FIGURE 3.

Rosiglitazone limits early MDSC accumulation and intra-tumoral T regulatory cell infiltration. (A) Paraffin-embedded tumor sections from mice with orthotopic tumors that received the indicated treatments were stained by immunohistochemistry with antibodies against TGFβ and COX-2. Brown stain indicates positive staining. (B) C57BL/6 mice with orthotopic Panc02 tumors, either untreated or treated with Gemcitabine, Rosiglitazone or a combination, were bled every other week, and the percentage of Gr1⁺CD11b⁺ MDSC were quantified by FACS analysis (week 1 analysis was performed two days post Rosiglitazone administration and before Gemcitabine injections). Data shown are averages of 5 mice per group. (C) Reduced whole cell lysates from 1×10⁶ Panc02 cells untreated or treated for 48

hrs with Rosiglitazone at the indicated concentrations were probed with an antibody against TGF β and control β -actin. (D) Paraffin-embedded tumor sections from orthotopic mice with the indicated treatments were stained by immunofluorescence with a directly-coupled FITC antibody against FoxP3. The number of FoxP3⁺ cells per 40X field was counted and data shown are averages of 4 fields/tumor for 5 tumors/group.

FIGURE 4.

Rosiglitazone and Gemcitabine therapy limits increase peripheral CD3⁺CD8⁺ T cells and intra-tumoral CD4⁺ and CD8⁺ T cells while reducing CD4⁺CD25⁺ T regulatory cells. (A–D) C57BL/6 mice (5/group) were orthotopically challenged with 5×10⁵ Panc02 cells into the body of the pancreas and were untreated or treated with: a control rat IgG antibody or an antibody against CD25 (PC61) to deplete T regulatory cells; Rosiglitazone; Gemcitabine with or without PC61 or rat IgG; Rosiglitazone and Gemcitabine with or without PC61 or rat IgG. (A) Untreated and PC61 treated mice were bled weekly and the percentage of CD4⁺CD25⁺ cells were quantified by FACS analysis. (B) Tumor volume was measured by caliper at the time of euthanasia when the first mouse appeared moribund. (C) Mice were bled every other week and the percentage of CD3⁺CD8⁺ T cells was quantified by two-color FACS analysis using directly-coupled antibodies (# p=0.0005). (D) Paraffin-embedded tumor sections were stained by immunohistochemistry with directly-coupled FITC antibodies against CD4, CD8 and FoxP3 and the number of FITC positive cells/200X field were counted (# p<0.05; ++ p<0.0009; * p<0.0001; + p<0.0001). Data are averages of 5 mice/group.