Supplemental Material

Ostapoff et al., Neutralizing the activity of murine TGF-β receptor 2 promotes a differentiated tumor cell phenotype and inhibits pancreatic cancer metastasis

Supplemental Methods

mPLR cell line isolation

Mouse pancreata from 5 week-old *LSL-Kras^{G12D}; Cdkn2a$^{lox/lox}$; p48^{Cre}* (*KIC*) mice were minced and subjected to digestion with 1% collagense type 1, DME, 10 mM Hepes, 1% FBS and PBS at 37^{o}C until a single cell suspension was obtained. Cell suspensions were centrifuged at low speed, resuspended, passed through a 70 µm strainer and plated on 10 cm dish coated with collagen type 1. Cloning rings were used to isolate single colonies. Cells were confirmed to be tumor cells by immunocytochemistry and PCR. Clones mPLR 2C, mPLR 2D and mPLR 3B were used in subsequent experiments.

Cell Viability Assays

mPLR 2C, 2D and 3B cells and Pan02 were plated in 10% FBS DMEM overnight. The following day there were serum starved and treated with Rat IgG (mac 48, 30 ng/mL), TGFβ1 (10 ng/mL), 2G8 (30 ng/mL) or TGF-β+2G8 for 72 hours. Viability was assessed by using Promega Cell Titer Blue Cell Viability Assay (G8081). Human tumor cells were plated overnight in serum containing conditions. After 24 hours, cells were serum starved for 8 hours and then

treated with conditioned media from either RAW 264.7 or 3T3 cells for 24 hours. Cell viability was then assessed as above.

Conditioned Media Experiments

Conditioned Media (CM) from RAW 264.7 and NIH 3T3 cells treated with media alone, Rat IgG or 2G8 (30 ng/mL) for 24 hours were collected. Cytokine secretion was assessed using SA Biosciences: Mouse Autoimmune Response Multi-Analyte ELISArray Kit (MEM-005A). For anchorage independent growth assays, cells ($1x10^3$/well) were suspended in 0.375% SemKem agar (FMC Bioproducts) in conditioned media and plated in quadruplicate over a layer of 0.75% agar base medium in 12-well plates. Cells were treated with serum containing media (control) or conditioned media from RAW 264.7 (or NIH 3T3) treated cells with media alone, Rat IgG (mac48) or 2G8. Conditioned media was replaced twice weekly. 3-4 weeks post initial plating, colonies were visualized with 0.05% crystal violet and counted.

Transwell Migration Assay

RAW 264.7 or NIH 3T3 cells were plated and allowed to adhere overnight in serum containing media in lower wells. Serum containing media was removed and cells were placed in serum-free media (SFM) containing, Rat IgG (mac48, 30 ng/mL) or 2G8 (30 ng/mL) for 24 hours. The stimulatory media was removed and replaced with SFM for 6 hours prior to addition of upper inserts. Human tumor cells were added to 8 µm inserts and allowed to migrate overnight. Inserts were fixed and stained according to Diff-Quick manufacturer's instructions (Dade Behring, B4132-1A) and cells counted per high powered field.

Immunocytochemistry

Tumor cells were plated on chamber slides and maintained overnight in serum containing conditions. For baseline Tgfβr2 expression, cells were stained immediately (Santa Cruz, sc-220). For EMT experiments, after overnight incubation media was changed to reduced serum conditions and cells were treated with Control IgG (mac48, 30 ng/mL), 2G8 (30 ng/mL), TGF-β (10 ng/mL Peprotech 100-21) or the combination of 2G8 + TGF-β for 24-72 hours. Cells were stained for E-cadherin (Santa Cruz, sc-7870), vimentin (PhosphoSolution, 2105-VIM), NCAD (Abcam, ab12221) and Zeb1 (Santa Cruz, sc-25388).

Macrophage phenotype was determined by plating RAW 264.7 cells on chamber slides. The following day, cells were treated with media, Control IgG (30 ng/mL) or 2G8 (30 ng/mL) for 24 hours and stained with the following antibodies: iNOS (Cell Signaling, 2977), Hes1 (Novus, NBP 1-19029), MCP-1 (Santa Cruz, sc1304) and CD206-MMR FITC-conjugated (Biolegend, 123006).

Flow Cytometry

Tumor lysates were prepared at the time of sacrifice from Colo357 tumor bearing mice and minced in DMEM (Cellgro, 10-013-CV) and digested for 45 minutes in collagenase (Sigma) and then homogenate was filtered through 40 μm filters. Single cell suspension was then labeled with primary antibody for 30 minutes. Primary antibodies included F4/80-APC conjugated (Biolegend, 122614) and CD206-FITC conjugated (Biolegend, 123006). Flow cytometry was done on FACScaliber (BD Biosciences). Propidium iodide (Sigma) positive cells were excluded and gates were adjusted on negative control. Data was analyzed using FloJo software (Tree Star Inc).

Cell lysates and Western Blot Analysis

Cell lysates were prepared and Tgfβr2 expression (Santa Cruz, sc-220) was determined by Western Blot. For cell signaling experiments, cells were plated on 6 well plates and serum starved or incubated in Optimem overnight at 60% confluency. Rat IgG (mac48 30 ng/mL) or 2G8 (30 ng/mL) were added for 1h and cells stimulated for 5 minutes with TGF-β1 (10 ng/mL) alone or in the presence of 2G8. Cell lysates were prepared, proteins separated by SDS-PAGE and subjected to Western blot analysis with the following antibodies: psmad2 (Ser465/467) (Millipore, #AB3849), tsmad2 (L16D3) (Cell Signaling, 3103), Actin (Sigma, A2066), phospho-ERK1/2 (Cell Signaling, 9101), total ERK 1/2 (Cell Signaling, 9102), phospho-AKT (Cell Signaling, 9271), total AKT (Cell Signaling, 9272), phospho-p38 (Cell Signaling, 9211) and total p38 (Cell Signaling, 9212). Binding was detected with standard conditions.

Phospho-smad2/total smad2 ELISA

Cells were plated in 10 cm dishes at ~90% confluence then serum-starved overnight. Cells were washed twice in PBS then treated with 2G8 or isotype control antibody at 37°C for one hour at the indicated concentration. After two washes in PBS, cells were stimulated with 10 ng/ml TGFβ1 (Invitrogen, HG9240) at 37°C for one hour. Cells were washed twice then lysed in cell lysis buffer (Cell Signaling, 9803) containing Phospho-Stop (Roche, 04906837001). Total protein was quantitated using the BCA method (Pierce, C23228). Extracted proteins were used for pSMAD2/tSMAD2 analysis by ELISA using commercial kits as per the manufacturer's instructions (Cell Signaling, 7348, 7244). pSMAD2 was normalized to tSMAD2 using the formula pSMAD2 = pSMAD2/SQRT(tSMAD2). IC_{50}s were determined using GraphPad Prism software.

Cell Viability Assays

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