Supplemental Materials for:

IFN-γ and CCL2 cooperate to redirect tumor-infiltrating monocytes to degrade fibrosis and enhance chemotherapy efficacy in pancreatic carcinoma

Kristen B. Long[†,1,2], Whitney L. Gladney[†,1,2], Graham M. Tooker[1,2], Kathleen Graham[1,2], Joseph A. Fraietta[1] and Gregory L. Beatty [1,2]*

[1]Abramson Cancer Center; University of Pennsylvania, Philadelphia, PA;

[2]Division of Hematology-Oncology, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA;

[†]These authors contributed equally to this work

* Corresponding author

Supplemental Methods

Antibodies. Antibodies against mouse antigens for flow cytometry were purchased from BD Biosciences, unless otherwise specified: CD11b (BioLegend, M1/70, APC), CD19 (1D3, APC), CD45 (30-F11, PE-Cy7), CCR2 (R&D Systems, clone #475301, PE), F4/80 (eBiosciences, BM8, FITC), Ly6C (AL-21, APC-Cy7), Ly6G (1A8, Percp-Cy5.5). Antibodies against mouse antigens for immunohistochemistry and immunofluorescence microscopy included: Ly6G (clone 1A8, Bioxcell, cat# BE0075-1), Gr-1 (clone RB6-8C5, Bioxcell, cat# BE0075), Ly6C (clone ER-MP20, Abcam, cat# ab15627), type I collagen (polyclonal, Abcam, cat# ab34710), EpCAM (Clone G8.8, eBioscience, cat# 14-5791-85), hyaluronan binding protein (Calbiochem, cat# 385911), F4/80 (Clone BM8, eBioscience, cat# 14-4801-85), MMP13 (polyclonal, Abcam, cat# ab39012), MMP14 (polyclonal, Acris, cat# AP23330PU-N), fibronectin (polyclonal, Abcam,

cat# ab2413), CD31 (clone MEC 13.3, BD Pharmingen, cat# 550274), Ki67 (clone TEC-3, Dako, cat# M7249), and pSTAT1 (Cell Signaling, cat# 9167). Endotoxin-free antibodies for use *in vivo* were purchased from BioXcell including: anti-CD40 (clone FGK45), anti-CCL2 (clone 2H5), anti-Ly6C (clone Monts 1), anti-Ly6G (clone 1A8), anti-Gr-1 (clone RB6-8C5), anti-IFN-γ (clone XMG1.2), rat isotype control (clone 2A3 or HPRN), and hamster isotype control (Hamster IgG).

Chemical reagents. Gemcitabine (GemzarTM, Eli Lilly) pharmaceutical grade powder was purchased through the Hospital of the University of Pennsylvania Pharmacy. Gemcitabine powder was resuspended in sterile PBS (phosphate buffered saline) at 38 mg/mL 2'-deoxy-2',2'-difluorocytidine and administered by intraperitoneal injection at 120 mg/kg in PBS. Clodronate (dichloromethylene diphosphonate) was administered at 10 μL per gram of mouse body weight as previously described (1). Actinonin (CAS 13434-13-4; Santa Cruz, sc-201289), a broad spectrum MMP inhibitor with inhibitory activity against MMP2 (IC_{50} 0.09 μM), MMP7 (IC_{50} 3.5 μM), MMP10 (IC_{50} 0.5 μM), MMP12 (IC_{50} 0.35 μM), and MMP13 (IC_{50} 0.1 μM) (2) was administered by intraperitoneal injection (200 μg/ dose). Selective MMP13 inhibitors, including WAY-170523 (MMP13 IC_{50} 0.017 μM; R&D Systems, 2633/1) (3) and 544678-85-5 (pyrimidine-4,6-dicarboxylic acid, bis-[4-fluoro-3-methyl-benzylamide]; Santa Cruz, sc-205756; MMP13 IC_{50} 0.008 μM) (4, 5), were administered by intraperitoneal injection (20 μg/dose).

Animals. *Kras*$^{LSL\text{-}G12D/+}$, *Trp53*$^{LSL\text{-}R172H/+}$ and *Pdx1-Cre* mice were backcrossed to the C57BL/6 background for more than ten generations. KPC mice on the C57BL/6 background developed pancreatic intraepithelial neoplasia (PanIN) which displayed progression to invasive PDAC with

metastasis that was indistinguishable from the original KPC model developed on a mixed 129Sv and C57BL/6 genetic background (6) . C57BL/6 mice and normal healthy littermate mice (*Trp53*$^{LSL\text{-}R172H/+}$, *Pdx1-Cre*) generated during routine breeding of KPC mice were used in experiments as controls.

Animal experiments. KPC mice were monitored weekly for the presence of spontaneous pancreatic tumor development. Tumors were detected by palpation and confirmed by ultrasonography as previously described. Mice with tumors measuring between 50 and 200 mm^3 were block randomized and enrolled into studies. Depleting antibodies were administered on days -1, 0, 1 and 3 of treatment with anti-CD40 antibodies. Clodronate encapsulated liposomes (CEL) were administered as previously described. MMPSense 645 Fast (PerkinElmer, Inc., Boston, MA) was reconstituted according to manufacturer instructions and administered by intravenous injection (100 μL/mouse) at 3 hours prior to anti-CD40 treatment. MMP inhibitors were administered 30 minutes prior to anti-CD40 and repeated twice daily on day +1 and once daily on day +3. In some experiments, low-passage PDAC cell lines derived from KPC mice were implanted subcutaneously into syngeneic C57BL/6 mice and allowed to develop over 14-17 days to approximately 5 mm in diameter before mice were enrolled into treatment studies. Change in tumor volume was monitored by calipers 2-3 times per week with tumor volume calculated as $V=(L \times S^2)/2$ by measuring the long (L) and short (S) axis of tumors.

Flow cytometry. Peripheral blood samples were obtained from the tail vein of mice as previously described (1). Bone marrow derived cells were obtained from the long bones of the hind legs by flushing with ice-cold DMEM. Both peripheral blood and bone marrow samples

were centrifuged and red blood cells lysed using ACK Lysis Buffer (Cambrex/BioWhittaker). Cell surface molecule staining was performed at 4^{o}C for 15-30 minutes in PBS containing 0.2 mM EDTA with 2% FCS. Samples were acquired on a FACSCanto flow cytometer (BD Biosciences). MMPsense-positive cellular subsets were identified by flow cytometry with excitation laser at 633nm. Collected data were analyzed using BD FACSDiva software (BD Biosciences Immunocytochemistry) or FlowJo (Tree Star Inc).

For flow cytometric detection of phosphorylated STAT1, primary human monocytes from healthy volunteers were resuspended in IMDM with 10% human AB serum. Monocytes were incubated for 30 minutes at 37C with serially-matched plasma samples (diluted 1:2 in IMDM) collected from a PDAC patient treated with CP-870,893 (1, 7). For pSTAT1 detection in mouse myeloid cells, peripheral blood was collected one day after treatment with anti-CD40 (clone FGK45) or isotype control (clone 2A3). For both mouse and human pSTAT1 detection, cells were fixed with Phosflow Lyse/Fix Buffer (BD Biosciences) for 12 minutes at 37C. Cell surface molecules were then detected on mouse leukocytes by antibody staining at 4C for 30 minutes. Cells were then permeabilized on ice for 30 minutes using Phosflow Perm Buffer III (BD Biosciences). Intracellular staining was performed for 60 minutes at room temperature using a human/mouse cross-reactive PE anti-STAT1 (pY701) antibody (clone 4a, BD Biosciences) at the manufacturer's recommended concentration. Samples were immediately acquired on a FACSCanto flow cytometer (BD Biosciences). Collected data were analyzed using BD FACSDiva software (BD Biosciences Immunocytochemistry) or FlowJo (Tree Star, Inc). Mean fluorescence intensity (MFI) of pSTAT1 expression was determined for select cell populations as indicated.

Cytokine analysis. Peripheral blood was collected by intracardiac puncture at the time of euthanasia and allowed to coagulate at room temperature for 15 minutes. Serum was collected by centrifugation at 13,000 rpm x 15 minutes and analyzed by cytometric bead array (CBA; BD Biosciences) for the presence of murine cytokines and chemokines as previously described (1). For tissue cytokine detection, tissues were excised, weighted and minced into small fragments with scissors. Tissue supernatant was collected by adding 10 μL of PBS per mg of tissue and then homogenizing tumor fragments with a syringe plunger. Tissue homogenate was centrifuged at 13,000 rpm x 12 minutes at room temperature and supernatant collected for analysis by cytometric bead array.

Histology, immunohistochemistry and immunofluorescence analysis. For immunofluorescence staining, frozen sections were fixed with 3% formaldehyde, then blocked with 10% normal goat serum in PBS + 0.1% Tween-20 (PBST). For detection of intracellular antigens, tissues were fixed with 3% formaldehyde then 100% ice-cold methanol; tissues were then permeabilized in blocking solution with 0.3% Triton X-100. Tissues were stained with primary antibody in blocking buffer for 1 hour at room temperature or overnight at 4^{o}C. Sections were washed in PBS and then incubated with Alexa488- or Alexa568-conjugated goat anti-rabbit or goat anti-rat IgG (Life Technologies, Eugene, OR) for 1 hour at room temperature to visualize the antigen of interest. For detection of MMP14, immunofluorescence by TSA amplification was performed on frozen tissue sections fixed in 3% formaldehyde as previously described (8) . Nuclei were stained with DAPI.

For immunohistochemistry, frozen tissues were fixed as described above and endogenous peroxidases were quenched in 0.3% H_2O_2 in water for 10 minutes and then blocked with 10%

normal goat serum in PBST. Primary antibody in blocking buffer was applied to tissues for 1 hour at room temperature or overnight at 4^{o}C. Sections were washed in PBS and then incubated with goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) or anti-rat biotinylated IgG (BD Biosciences, San Jose, CA). Staining was detected using Vectastain ABC kit (Vector Labs, Burlingame, CA) and slides were counterstained with hematoxylin.

For *in situ* hybridization, tissue were fixed in 10% formalin and paraffin embedded. The protocol was performed according to manufacturer instructions, using the RNA scope probe mM-Mmp13 (300031; Advanced Cell Diagnostic, INC, Hayward, CA) by the Pathology Core at Children's Hospital of Philadelphia.

Masson's trichrome stain was used to visualize extracellular matrix deposition. Sections were fixed in heated Bouin's solution and staining performed according to manufacturer instructions.

The relative area within tumor tissue of necrosis, detected on H&E imaging, and extracellular matrix deposition, detected by type I collagen or fibronectin immunofluorescence imaging or Masson's trichrome staining, was determined using Image J software (http://rsbweb.nih.gov/ij/). The percentage of extracellular matrix protein per standardized field of view (20x or 40x magnification) and the percentage of tumor necrosis across all fields of view were calculated. Quantification of immunostains was determined by counting positive cells per hpf using at least five fields per view.

Quantification of Evan's Blue leakage into tumors. Mice with implanted PDAC tumors were treated with anti-CD40 or isotype control antibody. At defined time points after treatment, vascular leakage into tumors was quantified using Evan's Blue (0.5% sterile solution in PBS)

administered at 200 μL/mouse by i.v. injection. Twenty minutes later, mice were euthanized and kidney and tumor harvested, weighed, and placed in 500 μL formamide at 55C for 24 hours. Tissue Evan's Blue content was then calculated by measuring absorbance at 610 nm with comparison to a standard curve for Evan's Blue in formamide/PBS. The ratio of Evan's Blue content within tumor to kidney is reported to control for variability in i.v. injection.

RNA and gene expression array. For analysis, RNA from three mice per treatment group was pooled and cDNA synthesized from 1 μg of total RNA using a RT^2 First Strand kit (SABioscience) according to manufacturer instructions. The expression level of extracellular matrix proteins and adhesion molecules were evaluated using a Mouse Extracellular Matrix & Adhesion Molecule RT^2 Profiler PCR Array (pamm-013zc-2, SABioscience) relative to the average of five housekeeping genes (beta-actin, beta-2 microglobulin, glyceraldehyde-3-phosphate dehydrogenase, beta-glucuronidase, and heat shock protein 90 alpha, class B member 1). The array was run according to manufacturer protocol using the ABI ViiA7 machine. The fold increase or decrease (ΔΔCT) of transcript levels from treated mice (ΔCT) was calculated relative to transcript levels from control mice (ΔCT).

Quantitative real-time PCR (qRT-PCR). cDNA was synthesized from 1 μg of RNA per sample using MultiScribe Reverse Transcriptase and random hexamers (Applied Biosystems, Foster City, CA). Primers for qRT-PCR were designed using the Primer 3 online program (http://frodo-wi.mit.edu) and synthesized by Integrated DNA Technologies. Relative quantification of all products was measured using SYBR Green chemistry (Applied Biosystems). Expression was normalized to beta-actin and relative expression of each gene was calculated

using the ΔCT formula. The fold increase or decrease in expression for samples obtained from treated mice was calculated as a ratio over the expression observed in samples obtained from control mice (ΔΔCT). Primer sequences are shown in Supplemental Table 1.

***In vitro* MMP detection assay.** Murine bone marrow derived F4/80^{+} myeloid cells were obtained from the femurs of C57BL/6 mice and cultured for 6 days in the presence of M-CSF in DMEM supplemented with 10% FCS, 1% L-glutamine, and gentamicin. Myeloid cells were stimulated with IFN-γ or PBS as control for 48 hours and then plated in wells containing a matrix of type I collagen (rat tail, BD Biosciences) with human BxPC3 pancreatic tumor cells. After 24 hours, cells were harvested, RNA collected, and transcript levels for specific *Mmp*s were evaluated by qRT-PCR. Murine specific primers (Supplementary Table 1) were used to amplify only murine *Mmp* transcripts. This approach allowed for distinguishing murine and human *MMP* transcripts that would be produced by myeloid and tumor cells, respectively. RNA from wells containing only matrix with BxPC3 tumor cells was used as a species specific control.

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