Interferon-stimulated gene 15 is a pro-pancreatic cancer stem cell microenvironmental factor

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Marker expression in primary PDAC FFPE tumors and in vitro cultures. (**A**) IHC analysis of CD68 and CD163 expression in primary patient PDAC FPPE samples. Scale bar = 200 μm. (**B**) Flow cytometry analysis of CD133 cell surface expression on Panc185 and Panc354 cells cultured as adherent monolayers or as spheres.

Figure S2. M2 macrophages increase PDAC CSC tumor growth. Summary of *in vivo* tumor take and growth of 5×10^5 subcutaneously-injected PDAC cells alone or with M1-polarized, M2-polarized or CSC-primed human Mϕ. Data are an average of 2 independent experiments with n = 5 mice/group/experiment. * p value < 0.05, ** p value < 0.01

Figure S3. ISG15 expression in primary patient PDAC tumors. Representative micrographs of ISG15-stained tissues from a TMA containing normal, PanIN (I-III), PDAC, metastases and pancreatitis cores. Scale bars = 200 and 50 μm.

Figure S4. ISG15 expression in primary patient PDAC tumors. Representative micrographs of ISG15-stained tissues from a TMA containing normal PDAC cores. Scale bar = 200 μm.

Figure S5. ISG15 expression in PDAC, breast and prostate cancer patient tumors. (**A**) Confocal immunofluorsecnce micrographs of FFPE PDAC tissue from a selected patient stained for the macrophage marker CD68 (CD68-PGM1-Alexa488; green), ISG15 (Alexa-555, red) and Dapi (blue). Scale bars = 200 and 20 μm. The bottom panels show a higher magnification image of the indicated inset. (**B**) Representative micrographs of ISG15 expression in macrophages of primary human lung tissue (positive control). (**C**) Representative micrographs of ISG15 expression in primary patient FPPE samples of breast and colon cancer. Scale bars = 200 and 50 μm.

Figure S6. Migratory effect of murine PDAC CSCs primed with control media or CM from M2-polarized ISG15+/+ or ISG15-/- murine macrophages. Murine PDAC CSCs primed with control media or CM from M2-polarized ISG15+/+ or ISG15-/- murine macrophages were seeded

as adherent cultures and their migratory capacity assessed in a standard wound healing assay. Representative micrographs (left panel) and quantification of wound size 12 h after wound induction (right panel).

SUPPLEMENTARY MATERIALS AND METHODS

Primary pancreatic cancer cells and macrophages. The use of human material was approved by the local ethics committee of each respective hospital or university, and written informed consent was obtained from all patients. Tissues were processed and subsequently cultured in vitro as previously detailed (1). Murine PDAC cells were established from tumors extracted from K-ras+/LSL-G12D;Trp53LSL-R172H;PDX1-Cre mice (2) at 20-24 weeks of age. Tumors were cut into pieces, and enzymatically digested with collagenase (Stem Cell Technologies, Vancouver, Canada) for 30 minutes at 37°C. Cells were then cultured in RPMI containing 10% fetal bovine serum (FBS) and 50 units/ml penicillin / streptomycin. Epithelial clones were picked, pooled, and further expanded to a heterogeneous cancer cell line. Sygeneic murine PDAC cells were established from a tumor that developed in a KPC mouse backcrossed to C57BL/6 for at least 10 generations. HPDE cells have been previously described (3).

Human blood was obtained from healthy donors with informed consent and in accordance with national regulations for the use of human samples in research. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats following standard protocols, and human macrophage cultures were established under adherent conditions in IMDM supplemented with 10% human AB serum as previously described (4). To generate M1- and M2-polarized human macrophages, 1000U/ml GM-CSF (M2) or M-CSF (M1) was added to the cultures (PeproTech, London, UK) (5). Murine monocytes were isolated from mechanically disrupted spleens, filtered through a 40μm mesh filter, and differentiated into macrophages under adherent conditions on non-tissue culture-treated 100mm dishes in RPMI supplemented with 10% FBS and 10ng/ml of murine M-CSF (PeproTech). To generate M1- and M2-polarized murine macrophages, 10ng/ml of IFN-γ (PeproTech) and LPS (Sigma) (M1) or 10ng/ml IL-4 (M2) (PeproTech) were added to the cultures.

Human PDAC Tissue microarrays (TMAs) and RNA samples. Human TMAs containing quadruplicate 1 mm cores from selected areas of paraffin-embedded pancreatic surgical specimens including acini, pancreatitis, PanIN lesions, PDAC and PDAC metastasis were constructed. A total of 42 tumors were included. The use of human tissue samples for the construction of the TMAs was approved by the Ethics Committee of the Hospital de Madrid Norte Sanchinarro. RNA from 30 flash-frozen primary human PDAC tumors was isolated by the guanidine thiocyanate method using standard protocols (6).

Mice. NU-Foxn1nu nude mice were purchased from Charles Rivers (L'Arbresle, France). Wild-type $ISG15^{+/+}$ and $ISG15^{-/-}$ mice (C57BL/6 background) were obtained from Klaus-Peter Knobeloch (Universitäts Klinikum, Freiburg, Germany). Mice were housed according to institutional guidelines and all experiments were approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain) and performed in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals as stated in The International Guiding Principles for Biomedical Research involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS).

Antibodies and recombinant proteins. The antibodies used in this study are detailed in **Table S1**. Recombinant ISG15 (rISG15) was purchased from Abcam and resuspended to a concentration of 1 µg/µl in water. Unless otherwise indicated, rISG15 was used at a concentration of 100 ng/ml and replenished every 48-72 h. The concentrations used are in line with previously published reports (7) and are only marginally higher than the physiological levels of circulating ISG15 found in healthy individuals (1 ng/ml serum) (8). Recombinant human and mouse M-CSF and GM-CSF were purchased from PeproTech (London, UK) and diluted to 1 µg/µl in water. Type I IFN was purchased from PBL Assay Science (Piscataway, NJ) and resuspended to a concentration of 10^4 IU/µl in 1X PBS/10% FBS.

Flow cytometry. Primary human macrophage cultures were resuspended in Sorting buffer [1X PBS; 3% FBS (v/v); 3mM EDTA (v/v)] before analysis with a FACS Canto II instrument (BD, Heidelberg, Germany). For cell surface marker expression, refer to primary and secondary antibodies listed in **Table S1**. DAPI was used for exclusion of dead cells. Data were analyzed with FlowJo 9.3 software (Tree Star Inc., Ashland, OR).

Microarray analysis. Microarray assays were performed using an Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray according to the manufacturer's protocols. RNA samples were labeled using a two-color (Cy3 and Cy5) and dye-swapping protocol. Microarray slides were scanned using an Agilent microarray scanner G2505C (Agilent technology) and microarray images were automatically analyzed using Feature extraction™ software, version 10.7 (Agilent technology).

ISG15 ELISA. Free ISG15 was quantified using an in-house sandwich ELISA. Briefly, Nunc-Immuno™ MicroWell™ 96 well ELISA plates were coated overnight at 4°C with 50 µl/well of 10ng/µl affinity-purified rabbit IgG capturing anti-ISG15 antibody (ProteinTech). The following day, wells were washed three times with 1X PBS and blocked for 2 h at room temperature with 3% BSA in PBS. Wells were washed three times with 1X PBS and incubated for 2 h at room temperature with diluted supernatants from experimental samples or serially diluted rISG15 (standard curve). Following antigen capture, wells were washed three times with 1X PBS and incubated with 50 µl/well affinity-purified mouse IgG capturing anti-ISG15 antibody (4ng/ml, Abnova) for 2 h at room temperature. Subsequently wells were washed three times with PBS containing 0.05% (v/v) Tween-20 (PBS-T) and detection was performed with an HRP-conjugated secondary antibody (1.3ng/ml) for 1.5 hr at room temperature, followed by washes with PBS-T. Development was performed for 30 min at room temperature in the dark using an ELISA substrate reagent pack (R&D Systems) according to the manufacturer's instructions. All incubations were performed on an orbital microplate shaker. Free ISG15 levels (ng/ml) were

compared to a standard curve of serially diluted rISG15 and normalized to total intracellular protein levels.

VSV antiviral assay. Vero cells left either untreated or pre-treated with either IFN-α (1000 U ml^{-1}, 16 h) or with the supernatant obtained from MVA-infected macrophages from 3 independent donors were infected with VSV (0.1 PFU/cell). VSV titers were quantified by plaque assay on Vero Cells as previously described (9).

Immunohistochemistry and immunofluoresecnce. For histopathological analysis, formalin-fixed paraffin-embedded (FFPE) blocks were serially sectioned (3 µm thick) and stained with hematoxylin and eosin (H&E). Additional serial sections were used for immunohistochemical (IHC) studies. Primary antibodies, secondary antibodies and dilutions are detailed in **Table S1**. For IHC, antigens were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+). Counterstaining was performed with hematoxylin. Histological quantification of digitalized slides was performed using Pannoramic Viewer (3DHistech, Budapest, Hungary). Deparafinized FFPE sections were blocked for 1 h with 1X PBS containing 3% (w/v) bovine serum albumin (BSA) (Sigma) and 10% (v/v) FBS and 20% anti-goat serum. Details regarding primary and secondary antibodies and dilutions used can be found in Table S1. Cell nuclei were stained by DAPI dye. Bound antibodies were visualized via confocal microscopy (40X, Leica TCS-SP2-AOBS-UV, Germany) and compared to negative control samples stained with irrelevant mouse or rabbit IgG control antibody (Santa Cruz Biotechnology) and appropriate Alexa-555- or Alexa-488-conjugated secondary antibody. Images were analyzed using Image J software (National Institutes of Health, Bethesda, MD) and brightness and contrast were adjusted using Adobe® Photoshop® (San Jose, CA).

RNA preparation and RTqPCR. Total RNA was isolated by the guanidine thiocyanate method using standard protocols (6). RNA from normal pancreas was purchased from Life Technologies. One µg of purified RNA was used for cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Barcelona, Spain), followed by SYBR green RTqPCR using an

Applied Biosystems 7500 real-time thermocycler (Applied Biosystems). Thermal cycling consisted of an initial 10 minute denaturation step at 95 °C followed by 40 cycles of denaturation (15 sec at 95 °C) and annealing/extension (1 min at 60 °C). mRNA copy numbers were determined relative to standard curves comprised of serial dilutions of plasmids containing the coding sequences of each target gene and normalized to β-actin levels. Primers used are listed in **Table S2**.

Western blot analysis. Cells were harvested in RIPA buffer (Sigma) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Twenty to fifty micrograms of protein was resolved by SDS-PAGE and transferred to PVDF membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 1X TBS containing 5% BSA (w/v), 1% chicken albumin (w/v) and 0.1% Tween20 (v/v), incubated with a 1:1000 dilution of indicated antibodies (**see Table S1**) overnight at 4ºC, washed 3 times with 1X PBS containing 0.05% Tween20 (v/v), incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Sigma, St. Louis, MO), and washed again to remove unbound antibody. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Amersham).

Sphere formation assay. Pancreatic cancer spheres were generated by culturing primary pancreatic cancer cells (10,000 cells/ml) in anchorage-independent suspension conditions for 7 days as previously described (10). For serial passaging, 1st generation PDAC spheres were harvested by clearance through a 50 μM web mesh, dissociated into single cells by trypsinization, and then re-cultured for an additional 7 days. A CASY Cell Counter (Roche Applied Sciences, Mannheim, Germany) was used to quantify spheres >40 microns.

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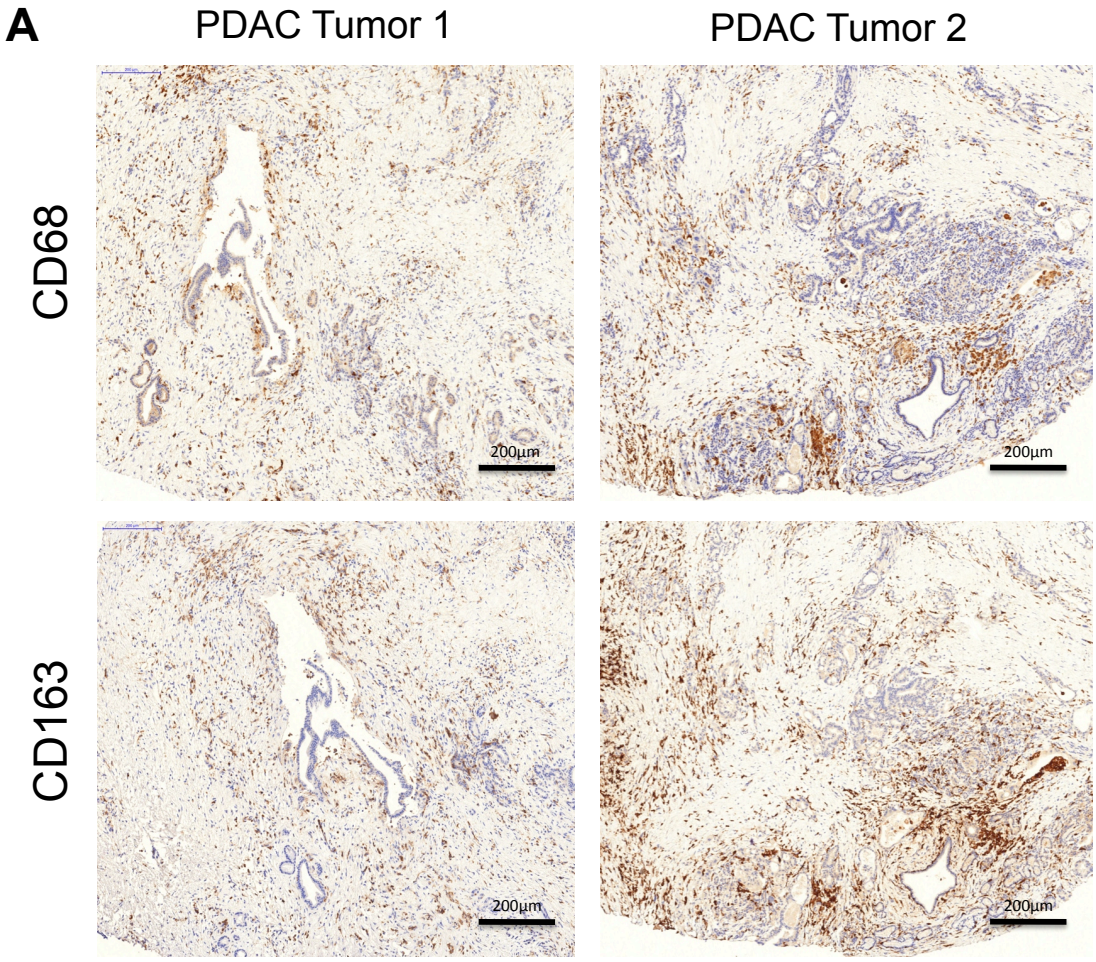
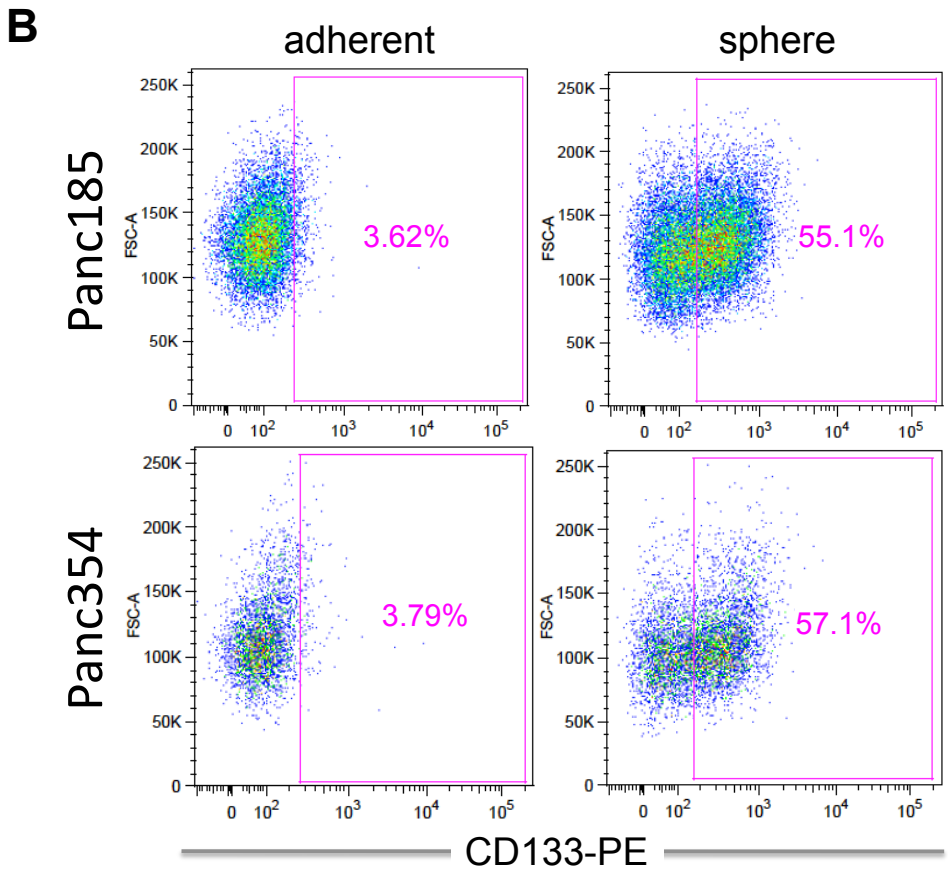
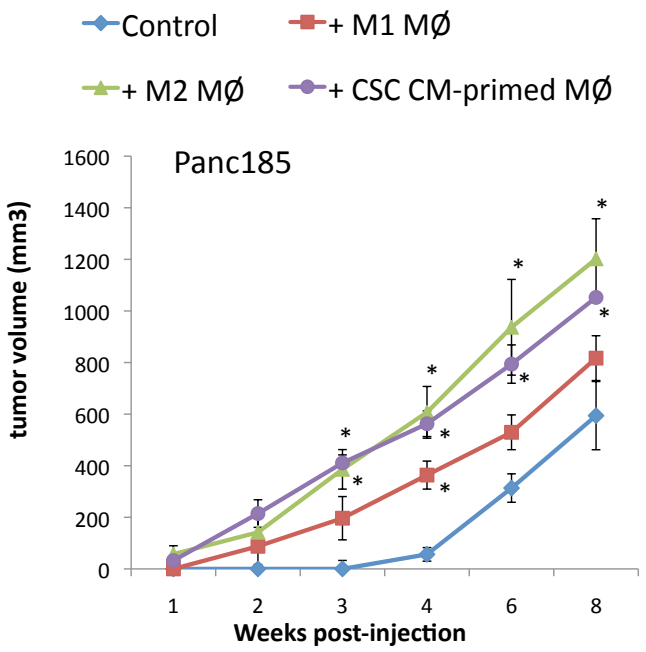
Supplementary Figure 1 – Marker expression in primary PDAC FFPE tumors and in vitro cultures

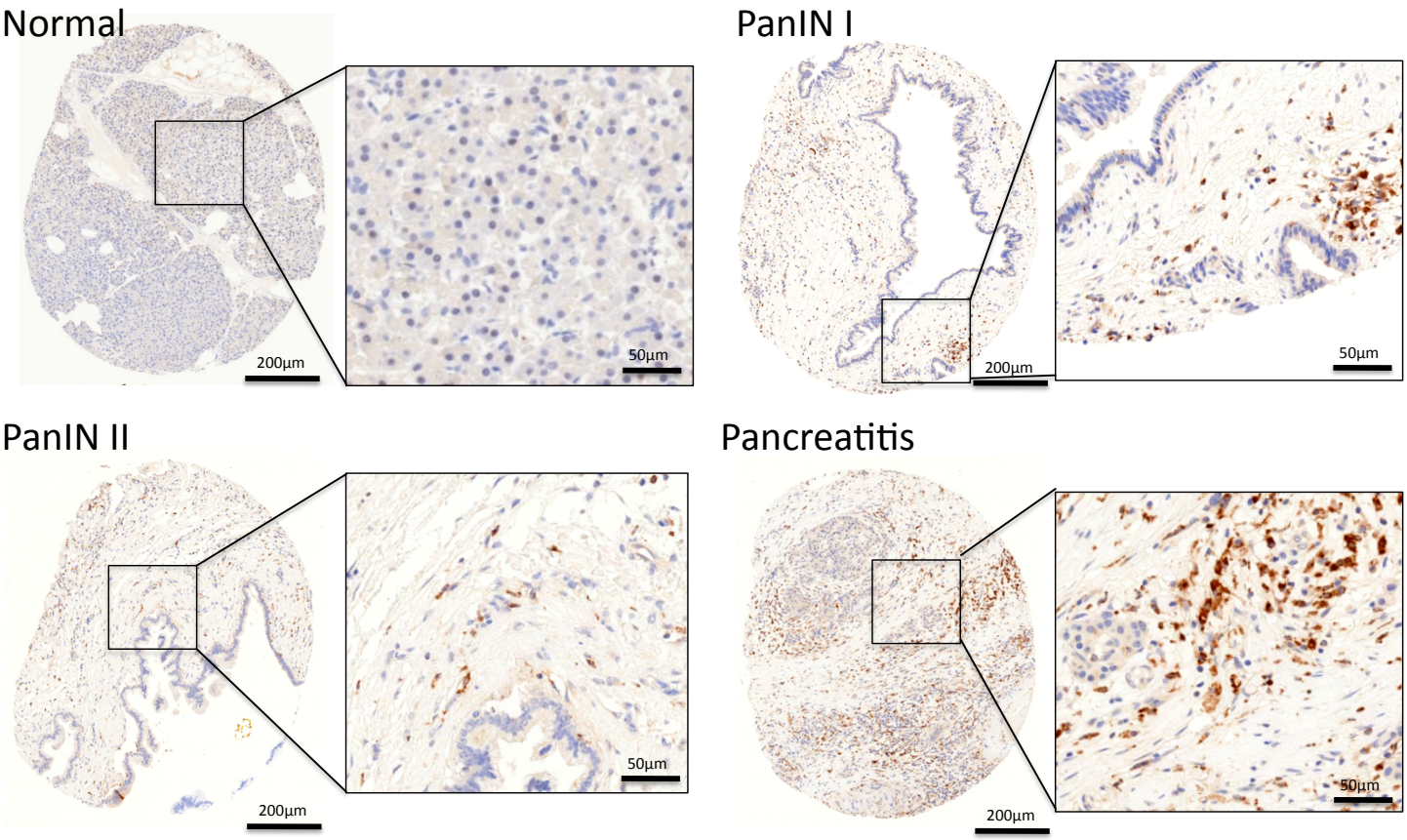




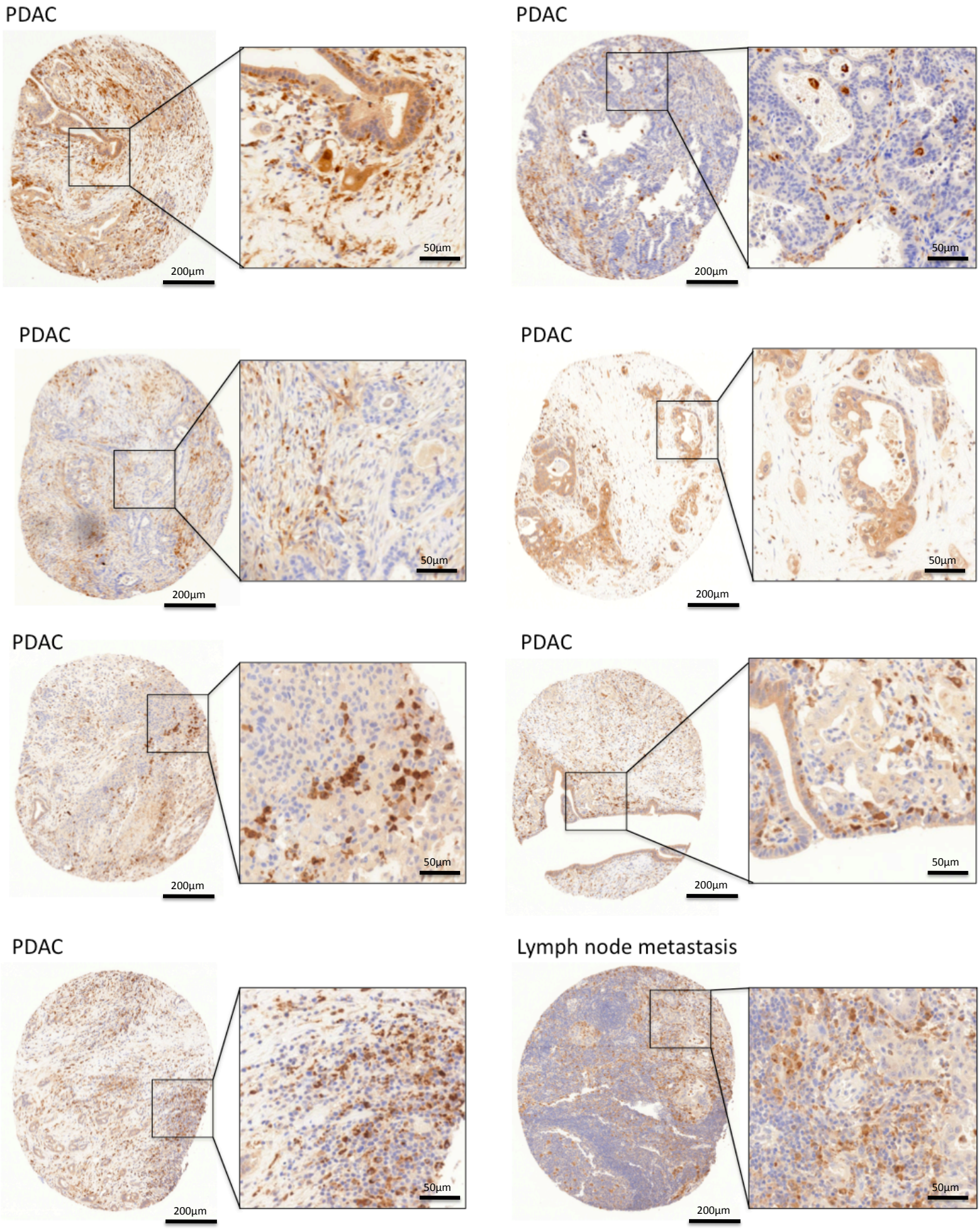
Supplementary Figure 2 – M2 macrophage increase PDAC CSC tumor growth



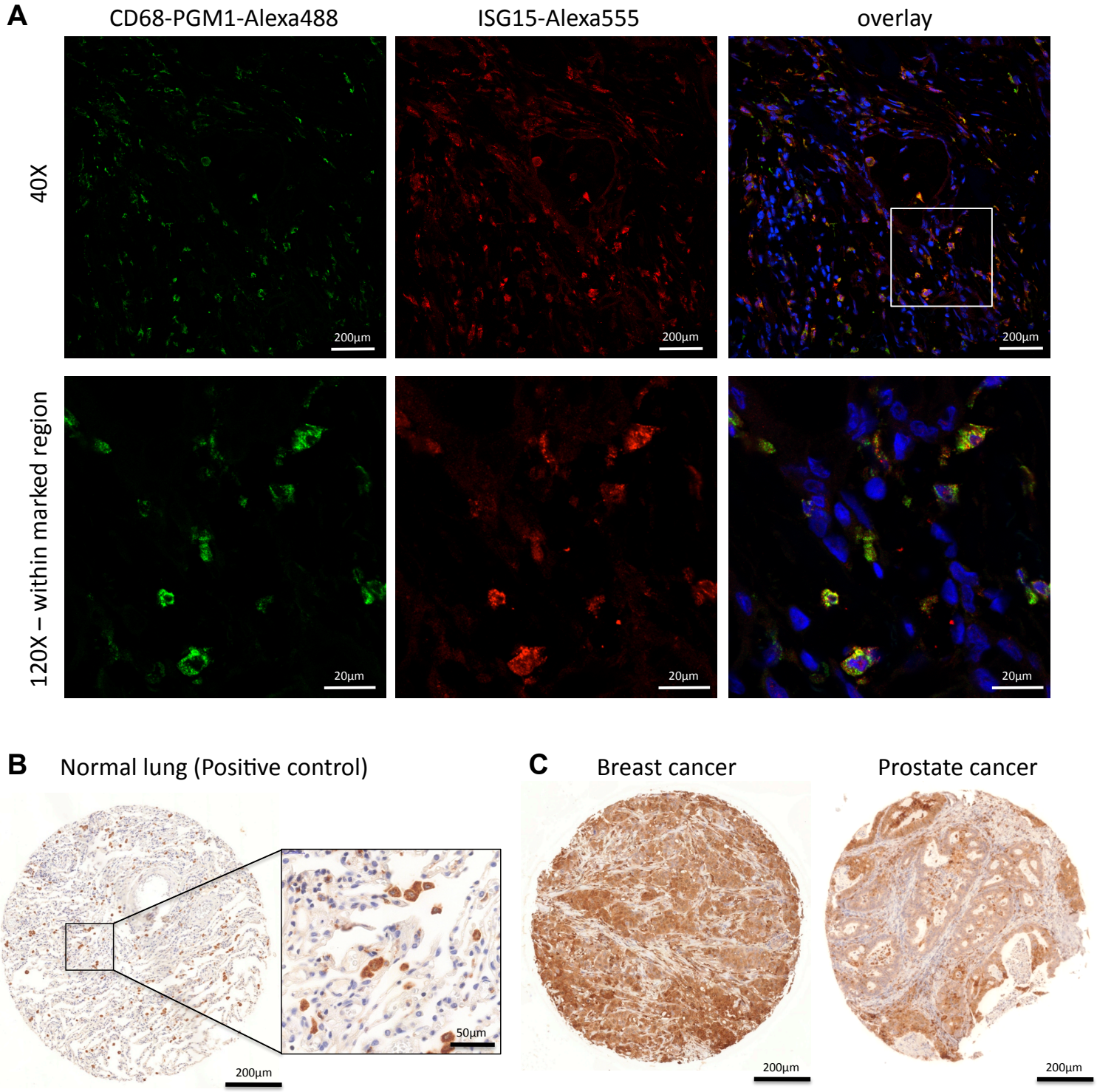
Supplementary Figure 3 – ISG15 expression in primary patient PDAC tumors



Supplementary Figure 4 – ISG15 expression in primary patient PDAC tumors



Supplementary Figure 5 – ISG15 expression in PDAC, breast and prostate cancer patient tumors



Supplementary Figure 6 – Migratory capacity of murine PDAC CSCs primed with control media or CM from M2-polarized ISG15$^{+/+}$ or ISG15$^{-/-}$ murine macrophages

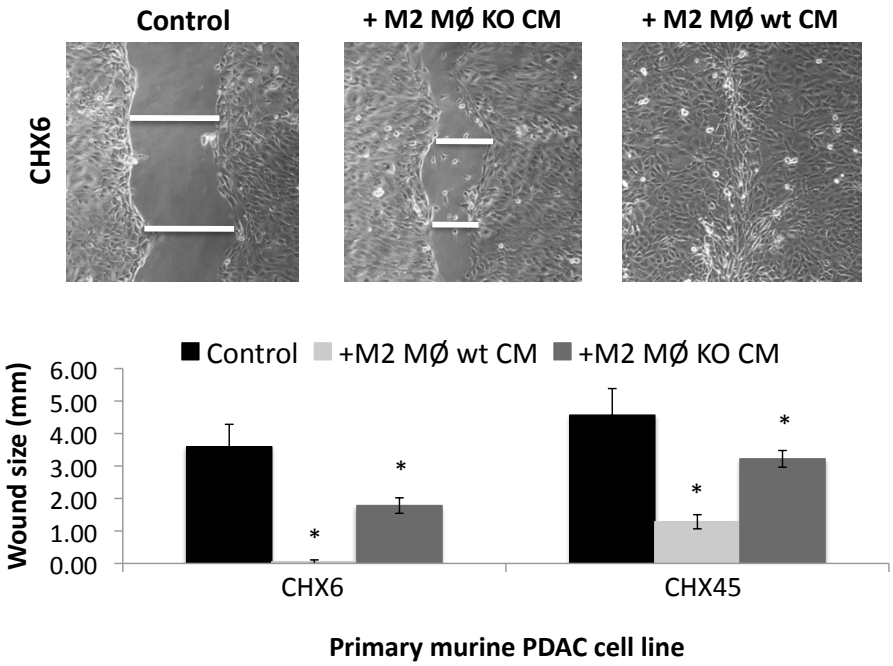


Table S1 – Antibodies

Primary Abs - Epitope	Source	Dilution	Application	Manufacturer
ISG15	Rabbit polyclonal	1:300 1:500	IHC, WB, IF	ProteinTech
ISG15	Mouse polyclonal	1:500	ELISA	Abnova
CD163-PE	Mouse monoclonal	1:20	Flow cytometry	BD Biosystems
CD68-PGM1	Mouse monoclonal	1:200	IF	Dako
p-ERK1/2 (T202/Y204)	Rabbit	1:500	WB	Cell Signaling
Total-ERK1/2	Rabbit	1:500	WB	Cell Signaling
Tubulin	Mouse	1:5,000	WB	Sigma

Secondary Abs	Source	Dilution	Application	Manufacturer
Anti-rabbit-HRP	Goat	1:50	IHC	Dako
Anti-rabbit-HRP	Donkey	1:5,000	WB	Amersham
Anti-mouse-Alexa-488	Donkey	1:1,000	IF	Life technologies
Anti-mouse-Alexa-555	Donkey	1:1,000	IF	Life technologies

IHC = Immunohistochemistry, ELISA = Enzyme-linked immunosorbent assay, WB = Western blot, IF = immunfluorescence

Table S2 – RTqPCR primer sequences

Gene	Primer sense 5’ to 3’	Primer antisense 5’ to 3’
hu ISG15	CACAGCCATGGGCTGGGACCTG	GCACGCCGATCTTCTGGGTGA
hu USP18	GAAAACGAAAGCTGGGCGGGG	GCCAGGCACGATGGAATCTCTCAA
hu RIG-I	GACTGGACGTGGCAAAACAA	TTGAATGCATCCAATATACACTTCTG
hu Klf4	ACCCACACAGGTGAGAAACC	ATGTGTAAGGCGAGGTGGTC
hu Sox2	AGAACCCCAAGATGCACAAC	CGGGGCCGGTATTTATAATC
hu Oct3/4	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGTGGGTTTCGGGCA
hu Nanog	TGAACCTCAGCTACAAACAGGTG	AACTGCATGCAGGACTGCAGAG
hu E-cadherin	TGCCCAGAAAATGAAAAAGG	GGATGACACAGCGTGAGAGA
hu Vimentin	GACAATGCGTCTCTGGCACGTCTT	TCCTCCGCCTCCTGCAGGTTCTT
hu Zeb-1	TTCACAGATCGAGCCTGCGTGC	GGGGCCATCCGCCATGATCG
hu β-actin	GCGAGCACAGAGCCTCGCCTT	CATCATCCATGGTGAGCTGGCGG
mu Klf4	GCGAACTCACACAGGCGAGAAACC	TCGCTTCCTCTTCCTCCGACACA
mu Hes1	TGCCAGCTGATATAATGGAGAA	CCATGATAGGCTTTGATGACTTT
mu Oct3/4	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGCGGACATGGGGAGATCC
mu LRG5	GACTTTAACTGGAGCAAAGATCTCA	CGAGTAGGTTGTAAGACAAATCTAGC
mu GAPDH	TCTGGAAAGCTGTGGCGTG	CCAGTGAGCTTCCCGTTCAG