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TITLE:

Immunophenotyping of Orthotopic Homograft (Syngeneic) of Murine Primary KPC Pancreatic Ductal Adenocarcinoma by Flow Cytometry

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KEYWORDS:

Animal Tumor Model, Immuno-Oncology, Tumor Transplantation, Tumor Tissue Dissociation, FACS, TIL, TAM, PDAC

SUMMARY:

The experimental procedure on the immunophenotyping of murine orthotopic PDAC homografts aims at profiling the tumor immuno-microenvironment. Tumors are orthotopically implanted via surgery. Tumors of 200-600 mm^3 in size were harvested and dissociated to prepare single-cell suspensions, followed by multi-immune marker FACS analysis using different fluorescently-labeled antibodies.

ABSTRACT:

Homograft (syngeneic) tumors are the workhorse of today's immuno-oncology (I/O) preclinical research. The tumor microenvironment (TME), particularly its immune-components, is vital to the prognosis and prediction of treatment outcomes, especially those of immunotherapy. TME immune-components are composed of different subsets of tumor-infiltrating immune cells assessable by multi-color FACS. Pancreatic ductal adenocarcinoma (PDAC) is among the deadliest malignances lacking good treatment options, thus an urgent and unmet medical need. One important reason for its non-responsiveness to various therapies (chemo-, targeted, I/O) has been its abundant TME, consisting of fibroblasts and leukocytes that protect tumor cells from these therapies. Orthotopically implanted PDAC is believed to more accurately recapture the TME of human pancreatic cancers than conventional subcutaneous (SC) models.

Homograft tumors (KPC) are transplants of mouse spontaneous PDAC originating from genetically engineered KPC-mice (**K**ras$^{G12D/+}$/**P**53$^{-/-}$/Pdx1-**C**re) (KPC-GEMM). The primary tumor tissue is cut into small fragments (~2 mm^3) and transplanted subcutaneously (SC) to the syngeneic recipients (C57BL/6, 7-9 weeks old). The homografts were then surgically orthotopically transplanted onto the pancreas of new C57BL/6 mice, along with SC-implantation, which reached tumor volumes of 300-1000 mm^3 by 17 days. Only tumors of 400-600 mm^3 were harvested per approved autopsy procedure and cleaned to remove the adjacent non-tumor

tissues. They were dissociated per protocol using a tissue dissociator into single-cell suspensions, followed by staining with designated panels of fluorescently-labeled antibodies for various markers of different immune cells (lymphoid, myeloid and NK, DCs). The stained samples were analyzed using multi-color FACS to determine numbers of immune cells of different lineages, as well as their relative percentage within tumors. The immune profiles of orthotopic tumors were then compared to those of SC tumors. The preliminary data demonstrated significantly elevated infiltrating TILs/TAMs in tumors over the pancreas, and higher B-cell infiltration into orthotopic rather than SC tumors.

INTRODUCTION:

Pancreatic ductal adenocarcinoma (PDAC) causes nearly half a million mortalities world-wide annually, one of the top 5 cancer killers. There are few effective treatment options and no approved immunotherapies; therefore, new treatments are desperately needed. Cancers are increasingly being recognized as immunological diseases, including PDAC, in addition to the genetic diseases as known today. Immunological and genetic factors would likely determine disease prognosis as well as treatment outcomes. Tumors evade host immune surveillance and eventually advance to cause death. Many of these immune processes occur within the tumor microenvironment (TME) [1-4] where different types of immune cells interact with tumor cells, with each other and with other tumor stromal components, directly or indirectly via cytokines which ultimately determine disease outcome. Therefore, characterization of the tumor immune components of the TME, or tumor immunophenotyping, including subtyping, numeration and localization of different lineages of immune cells, is critical to understanding anti-tumor immunity. In the case of PDAC, it has been proposed that elevated tumor-infiltrating suppressive macrophages (TAM) and B-cells have led to prevention of T-cell infiltration and/or activation and high levels of fibrosis [5,6].

The common approach to investigating immune TMEs experimentally would be using surrogate tumor preclinical animal models, mainly relevant mouse tumor models [7], particularly mouse syngeneic (homograft) or genetically engineered mouse models (GEMM) of cancers, on the assumed similarity of mouse and human for tumors and immunity [8,9]. It is understood in reality that there are inherent differences between the two species [10,11].

Transplanted mouse tumors have significant operational advantages over spontaneous tumors [7], namely synchronized tumor development, in contrast to the parental GEMM spontaneous tumor development. Homografts of spontaneous murine tumors are considered primary tumors having never been manipulated *in vitro*, and mirroring original mouse tumor histo-/molecular pathology [7], as well as possible immune profiles. These murine homografts are often considered to be "a mouse version of patient-derived xenografts (PDXs)". They therefore likely have a better translatability than conventional syngeneic cell line-derived mouse tumors [12]. In particular, many homografts are derived from specific GEMM where specific human disease mechanisms, *e.g.* oncogenic driver mutations, are engineered, and these homografts should therefore have advantages to their clinical relevance. In particular, KPC GEMM develop mouse PDAC within 15-20 weeks of age, which morphologically recapitulates human disease with predominately well- to moderately-differentiated glandular architecture and highly enriched stroma. This model also

recapitulates the most common genetic features of human PDAC, namely Kras activating mutation and P53 loss-of-function, which occur in 90% and 75% of human PDAC, respectively [5,6].

Sites of transplantation have also been suggested to play a role in model translatability. The specific surrounding tissue environment, such as a corresponding orthotopic environment, could be a niche for specific tumors to progress, as opposed to the uniform subcutaneous (SC) environments for commonly transplanted tumors. It would be of particular interest if, and/or, what difference exists between the two transplantation sites, in terms of immune-microenvironment, and the relevance to human cancer, *e.g*. in the case of PDAC.

One of the most important aspects of immune profiling, or immunophenotyping, is to determine tumor-infiltrating immune cells of different lineages, the numbers, relative percentage within tumors, as well as their activation states, and locations. This includes tumor-infiltratrating lymphoctyes (TILs, both T- and B-), tumor-infiltrating macrophages (TAMs), tumor-infiltrating natural killer cells (NKs) and tumor-resident dendritic cells [3,13-17], and the subcellular localization of certain cells [18-20], *etc*. Fluorescence Activated Cell Sorting (FACS) or flow cytometry is a single-cell detection technology that is commonly used to measure the specific parameters of a cell. Multi-color flow cytometry measures multiple markers on a single cell [3,4,21] and is the most commonly used method to determine the numbers and relative percentage of different subsets of immune cells, including those within tumors.

This report describes procedures for profiling tumor-infiltrating immune cells: 1) Implantation of orthotopic PDAC mouse tumor homografts, along with SC implantation; 2) tumor tissue harvest and single cell preparation via tumor dissociation; 3) flow cytometry analysis of all of the cells derived from tumors as a baseline; 4) comparison of baseline profiles of both transplantation approaches.

PROTOCOL:

All the protocols and amendment(s) or procedures involving the care and use of animals will be reviewed and approved by the Crown Bioscience Institutional Animal Care and Use Committee (IACUC) prior to the conduct of studies. The care and use of animals will usually be conducted in accordance with AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International guidelines as reported in the Guide for the Care and Use of Laboratory Animals, National Research Council (2011). All animal experimental procedures will be under sterile conditions at SPF (specific pathogen-free) facilities and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals from different government institutions (*e.g.* The National Institutes of Health). The protocol will need to be approved by the Committee on the Ethics of Animal Experiments at the facility institution (*e.g.* institutional IACUC Committee).

1. Preparation for Tumor Transplantation

1.1. Animal Housing

1.1.1. Obtain generic C57BL/6 mice from a commercial breeding vendor.

1.1.2. House mice (5) in individual ventilated cages at the following conditions: Temperature: 20~26 °C; humidity 30-70%; lighting cycle: 12-h light and 12-h dark.

1.1.3. Use corn cob bedding that is changed weekly.

1.1.4. For diet, provide irradiation sterilized dry granule food during the entire study period.

1.1.5. For water, provide animals free access to sterile drinking water.

1.2. Donor tumor fragment preparation

1.2.1. Begin by monitoring body weight (BW), via weighing using a balance, and tumor volume (TV), by caliper measurement, of tumor-bearing donor mice.

1.2.2. When TV reaches 500-1200 mm^3, euthanize the animal in a biohazard hood as per protocol.

1.2.3. Sterilize the skin around the tumor using iodophor swabs.

1.2.4. Surgically remove the tumor (details described in section 3: Necropsy and tumor harvest) and place the tumor in a Petri dish containing 20 mL of PBS (pre-chill the media or buffer to 4 °C prior to euthanizing animals).

1.2.5. If there is contaminating blood, transfer the tumor into another Petri dish and wash the tumor with PBS.

1.2.6. Cut the tumor in half, removing any extra skin, vessels, calcification and/or necrosis.

1.2.7. Choose only intact pieces of tumor and place them into a sterile 50 mL centrifuge tube and add 20 mL of PBS then transport the tube to a separate animal room for pharmacology studies.

2. Orthotopic and Subcutaneous (SC) Engraftment

2.1. SC Inoculation

2.1.1. Cut tumors into 2 mm diameter pieces using a scalpel, putting 1 chunk into each trocar, for SC implantation or for later orthotopic implantation (see below).

2.1.2. Anesthetize the recipient animal with 5% isoflurane, which is maintained by a nose cone at 1%. The animal will begin to relax, losing their righting reflex and eventually become immobile.

At this depth of anesthesia they can easily be roused by painful stimuli; allow anesthesia to deepen until such responses to pain are absent.

2.1.3. Once anesthetized, fix the mice on an experiment board in the right lateral position. Sterilize the mouse using iodophor swabs, particularly the areas surrounding the tumors.

2.1.4. With a scalpel, make a 0.5 to 1.0 cm skin incision on the left flank, just cranial to the hip.

2.1.5. Tunnel under the skin towards the forelimb, two to three centimeters, with blunt forceps.

2.1.6. Aseptically transfer one cube of tumor from the medium and place deep inside the subcutaneous tunnel.

2.1.7. Visually confirm that the tumor is deep in the tunnel (and not at the skin incision).

2.1.8. Close the wound with wound clips.

2.1.9. Monitored the animals post inoculation until they regain sufficient consciousness to maintain sternal recumbence. Return animals back to their cage only after their full recovery from anesthesia. Complete the Anesthesia Log, and initiate the Animal Health Chart. Weigh recipient animals daily for three days

2.1.10. For a standard procedure, inoculate 5-10 mice per group for tumor growth monitoring.

2.2. Pancreatic orthotopic implantation

2.2.1. Anesthesia and Analgesia

2.2.1.1. Use a 2 mL ketamine injection and 0.42 mL xylazine injection (20 mg/mL) mixed in 5.91 sterile injection water or saline at a dose volume of 0.06-0.1 mL/20-25 g body weight.

Note: According to animal welfare, analgesia is necessary both pro and post operation. 0.05-0.1 mg buprenorphine /kg, SC. The first dose is pro operation and then dosed 3 times every 4 hours post operation continually.

2.2.2. Surgical operation for orthotopic implantation

2.2.2.1. Anesthetize mice via intramuscular injection (IM) per Step 2.2.1.

2.2.2.2. After the animals are fully anesthetized, fix the mice on an experiment board in the right lateral position.

2.2.2.3. Keep the mice in the right lateral position. Disinfect the skin around the spleen with iodine then de-iodinate with 75% ethyl alcohol.

2.2.2.4. Find the medium point of the spleen and make a 1 cm vertical incision on abdomen to expose the spleen.

2.2.2.5. Draw out a part of pancreas tissue under the spleen gently with flat-tip tweezers, and suture a PDX tumor piece on the pancreas by 9-0 Absorbable surgical suture.

2.2.2.6. Close the abdomen with a 6-0 silk suture by double seam. Achieve homoeostasis by compression.

2.2.2.7. After finishing tumor implantation, if neither bleeding nor tumor tissue leakage occurs, keep the animals in a warm cage.

2.2.2.8. Monitor the animal until it regains sufficient consciousness to maintain sternal recumbency; return the animal to the animal room after full recovery from the anesthesia. Monitor the tumor bearing mice by palpating the abdomen near the spleen and select out the mice bearing orthotopic tumors.

2.3. Tumor-Bearing Mice Health Monitoring

2.3.1. Check the water and food consumption daily.

2.3.2. Examine the mouse appearance for an ungroomed hair coat, lumps, thinness, abnormal breath or ascites.

2.3.3. Palpate the abdomen to check if there are spontaneous tumors on the liver or spleen.

2.3.4. Weigh mice weekly using a balance.

2.3.5. If any of the following clinical signs are observed, the mice are sacrificed for sample collection and necropsy: BW loss >20%; impaired mobility (not able to eat or drink); unable to move normally due to significant ascites and enlarged abdomen; effort in respiration; death.

3. Necropsy and Tumor Harvest

3.1. Inspect visually and by palpation for the presence of palpable tumors before termination.

3.2. For termination, first euthanize mice as per approved protocol, and open the abdomen and visually examine the pancreas for tumors.

3.3. Cut off the tumor by post mortal surgery and add it to cold PBS. Place all animals in a clean, uncharged, translucent euthanasia chamber with a special lid with a port for delivery of the carbon dioxide to be used.

3.4. Discharge gas into the chamber at a flow rate that produces rapid unconsciousness with minimal distress to the animal. The optimum flow rate for CO_2 should be around 2-2.5 liters per minute.

3.5. Visually observe each animal during the euthanasia procedure to assure all animals receive adequate gas concentrations and do not regain consciousness during the euthanize procedure.

3.6. Maintain gas flow for at least 1 min after apparent clinical death to minimize the possibility that an animal may recover (if apneic but not dead).

3.7. Remove adjacent non-tumor tissues. Cleaned tumor can be photographed quickly. Put the tumor tissues into RPMI-1640 and keep on ice before dissociation.

Note: Animal carcasses are bagged and stored in the appropriate freezer await removal.

4. Tumor Tissue Dissociation and Single Cell Preparation

4.1. Reagent preparation

Note: The Tumor Dissociation Kit contains 2 vials of Enzyme D (lyophilized powder), 1 vial of Enzyme R (lyophilized powder), 1 vial of Enzyme A (lyophilized powder) and 1 mL of Buffer A.

4.1.1. Prepare Enzyme D by reconstituting the lyophilized powder in each vial with 3 mL of RPMI-1640. Prepare aliquots of an appropriate volume to avoid repeated freeze-thaw-cycles.

4.1.2. Prepare Enzyme R by reconstituting the lyophilized powder in the vial with 2.7 mL of RPMI 1640. Prepare aliquots of an appropriate volume to avoid repeated freeze-thaw-cycles.

4.1.3. Prepare Enzyme A by reconstituting the lyophilized powder in the vial with 1 mL of Buffer A supplied with the kit. Do not vortex. Prepare aliquots of an appropriate volume to avoid repeated free-thaw-cycles.

4.1.4. Prepare the Enzyme mix by adding 2.35 mL of RPMI-1640, 100 µL of Enzyme D, 50 µL of Enzyme R and 12.5 µL of Enzyme A into each gentleMACS C-tube.

4.2. Tumor Dissociation

4.2.1. For each tumor prepare one C-tube with tumor digestion mix, please refer to section 4.1 for digestion buffer dilution and preparation.

4.2.2. Use 3 mL digestion buffer for tumor fragments <0.8 g, and ensure the tumor is fully digested.

4.2.3. Label with the study code, tumor, mouse ID, treatment group, and tumor weight.

4.2.4. Collect the tumor from the mouse, wash the tumor in cold PBS and clean out the tissue attached on the tumor (such as blood vessels, fat, fascia, *etc*.).

4.2.5. Put the tumor in digestion media in one well of a sterile 6 well plate.

4.2.6. Hold the tumor in place with sterile tweezers/forceps and slice with a scalpel. Slice the tumor well enough to break into smaller pieces (~1 mm^3).

4.2.7. Place the tumor pieces back into the C-tube and use the remaining digestion buffer to wash the plate and then transfer the fluid into the C-tube that is placed on ice until digestion.

4.2.8. Switch on the dissociator with heaters.

4.2.9. Place tumor dissociation C-tubes upside down into sleeves of the vacant positions and adjust the status of tube positions from Free to Selected. Choose a dissociation program (37_c_m_TDK_1), followed by the selection of the required folder, where the list of Programs is displayed.

4.2.10. After termination of the program, take C-tubes off the dissociator and spin briefly (300 x g, 4 °C) to pellet the sample.

4.2.11. Re-suspend samples and put into a cell strainer above a 50 mL tube. Wash the cells through the cell strainer with 10 mL of wash buffer to provide a single-cell suspension.

4.2.12. Centrifuge the tubes at 300 x g for 5 min, discard the supernatant and re-suspend the cells with 5 mL of wash buffer, count viable cells using trypan blue and/or by cell counter and adjust the cell concentraton to 1x10^6 cells per tube or per sample. Include the correct isotype control antibodies to ensure staining is specific

5. Immune Panel Design and Flow Data Acquisition

5.1. Panel design

5.1.1. Please see **Table 1**.

5.2. Immunostaining

5.2.1. Fc-Block sample cells: re-suspend the cells in 200 µL of staining buffer with 1 µg/mL Fc-Block, followed by incubation on ice or 4 °C refrigeration for 15 min in the dark.

5.2.2. Stain cells using the desired antibody/fluorescence panels (*e.g*. T-cell panel, macrophage panel, *etc*.): Add the antibody mixture diluted in Fc blocking buffer to each sample, stain for at

least 30 min on ice in the dark.

5.2.3. Add 1 mL of ice cold PBS to each tube and re-suspend the cells gently, followed by centrifugation at 300 x g for 5 min. Discard the resulting supernatant.

5.2.3.1. Repeat to wash the cells twice.

5.2.4. Stain for intracellular markers if needed, following steps 6-10, otherwise jump to step 10.

5.2.5. Re-suspend the cell pellet by pulse vortex and add 200 µL of prepared Fixation/Permeabilization working solution for each sample. Pulse vortex again, and then incubate at 4 °C overnight (preferred) or 30 min at room temperature in the dark.

5.2.6. Spin down the cells and remove the supernatant.

5.2.7. Wash twice by adding 1 mL of 1x Permeabilization Buffer (made from 10x Permeabilization Buffer, diluted with distilled H_2O) followed by centrifugation and decanting of supernatant.

5.2.8. Add intracellular marker antibody in 1x Permeabilization Buffer and incubate at room temperature for 30 min in the dark.

5.2.9. Wash cells twice with 1 mL of 1x Permeabilization Buffer. Centrifuge and decant supernatant.

5.2.10. Resuspend cells in 150 µL of Staining Buffer and analyze on a cytometer. Due to the fixation and permeabilization procedure, the FSC (forward-light scatter)/SSC (sidelight scatter) distribution of the cell population will be different to live cells. Therefore, the gate and voltages will need to be modified.

5.3. FMO Controls (Fluorescence Minus One Control)

Note: Multi-color flow analysis is particularly important for the analysis of tumor-infiltrating immune cells. Therefore, there is a need to find a way to identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel. FMO (Fluorescence Minus One) Control is an important approach for this purpose.

5.3.1. To this end, include additional mice in each group for FMO controls (at least 2 per Rx) and processed individually for each tissue. After dissociation, pool tissues. For example, in a study with 4 Rx groups, 8 additional tumors should be processed individually and then pooled into one sample for FMOs.

5.4. Flow Instrument Setup

5.4.1. Make compensation beads while the machine is warming up (at least 20 min).

5.4.2. Use CS&T beads to check performance.

5.4.3. Voltage and compensation settings: use UltraComp beads, vortex the Comp beads thoroughly before use.

5.4.4. Label a separate 12 x 75 mm sample tube for each fluorochrome-conjugated antibody.

5.4.5. Add 100 µL of staining buffer to each tube. Add 1 full drop (approximately 60 µL) of the beads to each tube.

5.4.6. Add antibodies and perform the staining procedure exactly as the sample process stated in section 4.

5.4.7. Add 0.5 mL of staining buffer each, to completely re-suspend bead pellets via vortex.

5.4.8. Set flow cytometer PMT voltage per target tissue for the given experiment.

5.4.9. Run through flow cytometer for data acquisition, by gating on the singlet bead population per FSC and SSC readings.

5.4.10. Set Flow rate around 200-300 events per second.

5.4.11. Set appropriate compensation for a given fluorescein [FITC]-conjugated antibody, use an FL1 *vs.* FL2 dot plot.

5.4.12. Place a quadrant gate so that negative beads are within lower left quadrant and the positive beads are in the upper or lower right quadrant. Adjust the compensation values until the median fluorescence intensity (MFI) of each population (as shown in the quadrant stats window) is approximately equal (*i.e.* for FL2-%FL1, the FL2 MFI of both beads should be similar).

5.4.13. Repeat steps 5.4.11 and 5.4.12 for all tubes.

5.4.14. Proceed to acquiring the actual stained samples. Run the compensation wizard and save the settings with the format “date experiment your initials”.

6. Flow Data Analysis and Presentation

6.1. Analyze data by Flowjo and/or Kaluza.

REPRESENTATIVE RESULTS:

Orthotopic implantation of PDAC resulted in rapid tumor growth similar to that seen for SC implantation. After the donor tumor fragments were implanted into recipient mice, both subcutaneously and orthotopically according to the protocols described in Steps 2.1 and 2.2, the

implanted KPC homograft tumors demonstrated similar rapid growth as shown in **Figure 1A**. KPC homograft tumors harvested at different time points are shown in **Figure 1B** and representative H&E images are shown **Figure 1C**. Our data demonstrated similar growth of SC and orthotopic implants.

Viable tumor cells and cells in the TME, including tumor-infiltrating immune cells, originating from either orthotopic or SC implantation, can be efficiently recovered. Tumors were harvested and digested to prepare single cell suspensions for subsequent FACS analysis using a commercial dissociator (**Figure 2A**) according to the protocol described in Step 4. We usually obtained reasonably high viable cell yields from the tumor samples of both types of implantation (~80% viability based on Trypan Blue); the representative FACS plot shows viable cells from tumor, separated from the dead cells/cell debris (**Figure 2B**).

Tumor-infiltrating immune cells of different subsets have been identified in both orthotopic and SC implanted tumors, while their profiles have differences. The single cell suspension prepared from tumor tissues by using the method described in step 4.2 were subjected to FACS analysis after staining with a 16 color panel of markers shown in **Table 1**, which covers different lineages of important immune cells as well as tumor cells. The gating strategy or immune lineage hierarchy along with representative flow plots are shown in **Figure 3A**. CD45, a marker for all mature immune cells, was used for distinguishing tumor cells and tumor-infiltrating immune cells. All immune lineages were subsequently analyzed from $CD45^+$ populations as displayed by the panel (**Table 1**).

Beside markers, cell size is also used to differentiate different subpopulations (**Figure 3B left**) to quantify cell subsets, including T, B lymphocytes, macrophages and MDSCs, etc. Tumor infiltrating immune profile comparison of SC *vs.* orthotopic homografts of pancreatic cancers. The major enumerated cell populations of several key subsets of tumor infiltrating immune cells are shown **Figure 3C**. The data clearly shows that the tumor has significantly increased immune cell infiltration compared with the pancreas of healthy mice. In addition, different percentages of subset of tumor-infiltrating immune cells were found in orthotopic *vs*. SC homografts, *e.g.* significantly more B-cells in orthotopic than in SC.

FIGURE AND TABLE LEGENDS:

Table 1: 16-color flow panels designed for analysis of tumor-infiltrating mouse immune cells.

Figure 1. Both orthotopic and SC (subQ) implanted PDAC homograft tumors in C57BL/6 mice demonstrated similar growth with or without gemcitabine treatment. Panel A growth curve: SC-left, and orthotopic-right. Blue: Vehicle; Gold: Gemcitabine (initiated on Day 10 post inoculation when average SC tumor volume reached 200 mm^3). Panel B: tumor tissues at different time points. Panel C: Representative H&E staining of both types of homografts (40X10).

Figure 2. Tumor tissue dissociation to prepare viable single cell suspension. Panel A: the use of the disassociator; Panel B: the viable cells, separated from dead cells, as shown by flow analysis.

Figure 3. Multi-color flow analysis of tumor-infiltrating immune cells of both orthotopic and SC PDAC homografts. The raw data from each tumor sample were acquired from the flow cytometry instrument, followed by analysis using Flowjo FACS analysis software. Panel A. The standard gating strategy for the analysis, is displayed as an example; Panel B. Representative flow gating and analysis data using the standard gating strategy; Panel C. Representative tumor-infiltrating immune cellsare displayed to include B-cells, Treg and macrophages.

DISCUSSION:

Although studies using SC tumor are more readily conducted, orthotopically implanted tumor models can potentially be more relevant for preclinical pharmacology studies (particularly I/O investigations) to provide enhanced translatability. This report aims at helping the interested readers/audience to be able to directly visualize the technical procedures that can be used in their respective research. Our protocols demonstrate that orthotopic implantation of PDAC can result in efficient tumor growth, similar to SC implantation. Our observations also seem to suggest the presence of different immune profiles of TMEs from the different implantations. The major challenges to adapt orthotopic, as compared to SC implantations, are that complex skills are required, the process is time consuming, the surgical procedures for implantation are labor intensive and also the difficulty in monitoring orthotopic tumor growth in life.

There are four critical steps to ensure that orthotopic pancreatic tumor experiments are successfully performed: 1) the surgical procedure of implantation; 2) the careful and timely monitoring of tumor development; 3) the importance of performing pre-experiment tests first to familiarize with the procedure and assess the take rate and tumor growth rate; 4) using single cell suspensions of dissociated tumors as an alternative engraftment method. This report procedure is helpful to readers for performing research using this specific homograft, as well as other pancreatic orthotopic models, and even other orthotopic models involving abdomen-opening surgery.

Flow cytometry or FACS is currently the most important tool to perform immunoprofiling. Immunophenotyping of tumors by FACS significantly differs from that of cells from different organs, such as peripheral blood, spleen, lymph node and bone marrow in the following ways. Generally, there is a very small percent of immune cells present in tumors (small sample size). The extreme heterogeneity of tumors and the small number of immune cells present make recovering viable rare immune cells technically challenging, requiring custom-developed tumor tissue dissociation involving machines. Both previous points make simultaneous multi-parameter measurement using multi-color flow cytometry essential. Multi-color flow requires complex marker panel design, compensation, and gating strategies, due to fluorescence spectral overlap. This report also attempted to demonstrate to the interested readers/audience the process of tumor immune profiling via defined tumor tissue dissociation and multi-color flow cytometry analysis.

Three critical steps could be particularly important to yield productive TIL analysis: first, a high yield of viable cells recovered from dissociated tumors using customized tumor dissociation

procedures; second, the optimized design of large multi-color staining panels based on available reagents; third, an optimized gating strategy in the analysis. The authors would like to emphasize the training and experience of the operators of both data acquisition and analysis are essential for the successful flow cytometry analysis of TIL.

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DISCLOSURES:

All authors are current full-time employees of Crown Bioscience, Inc.

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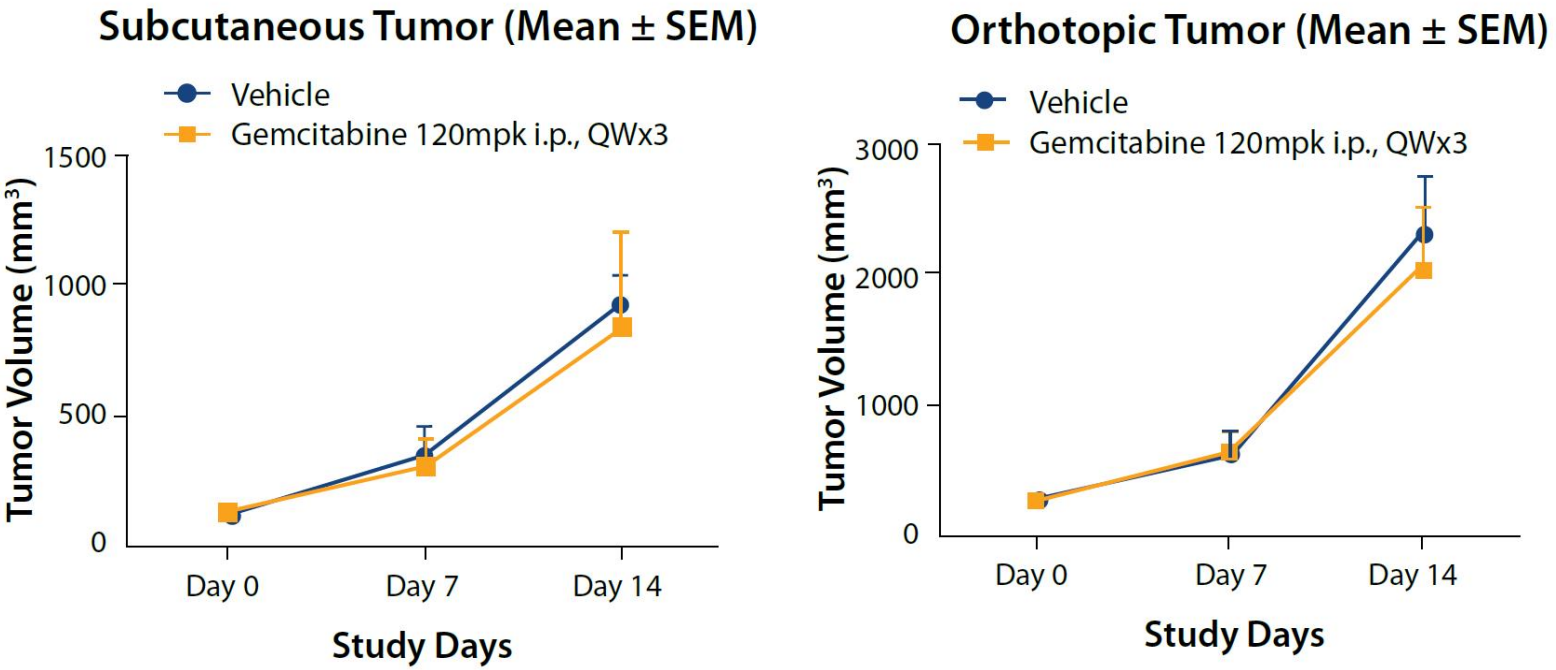
Figure 1

A.



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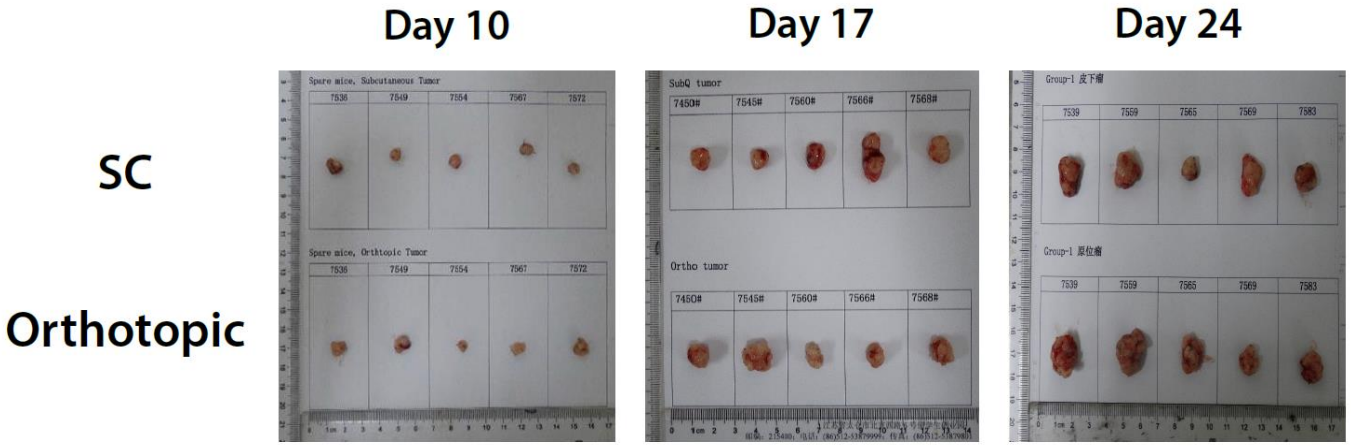

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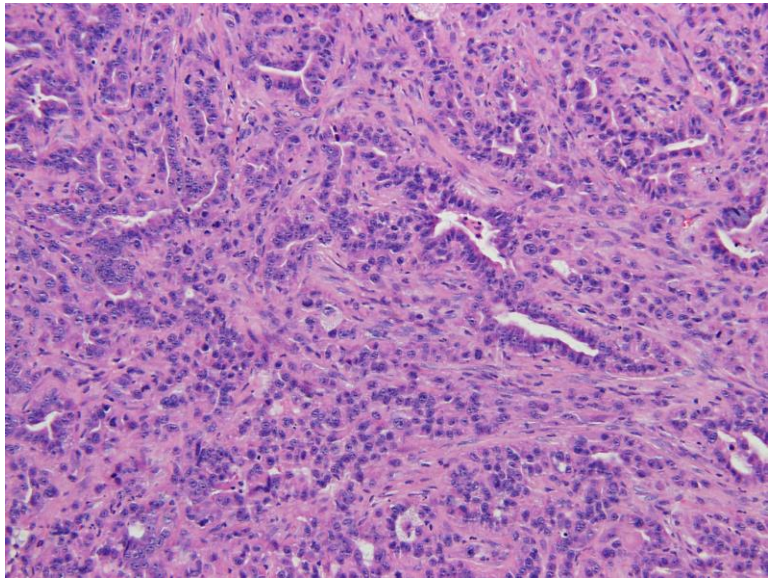

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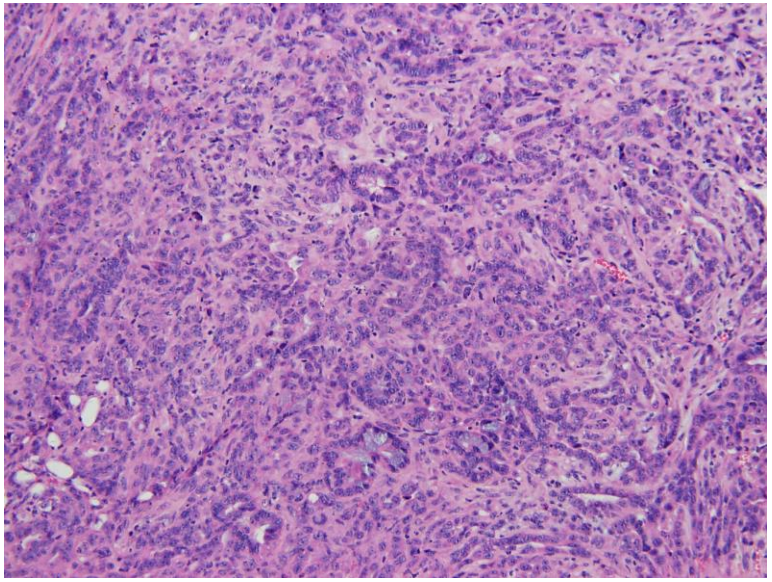

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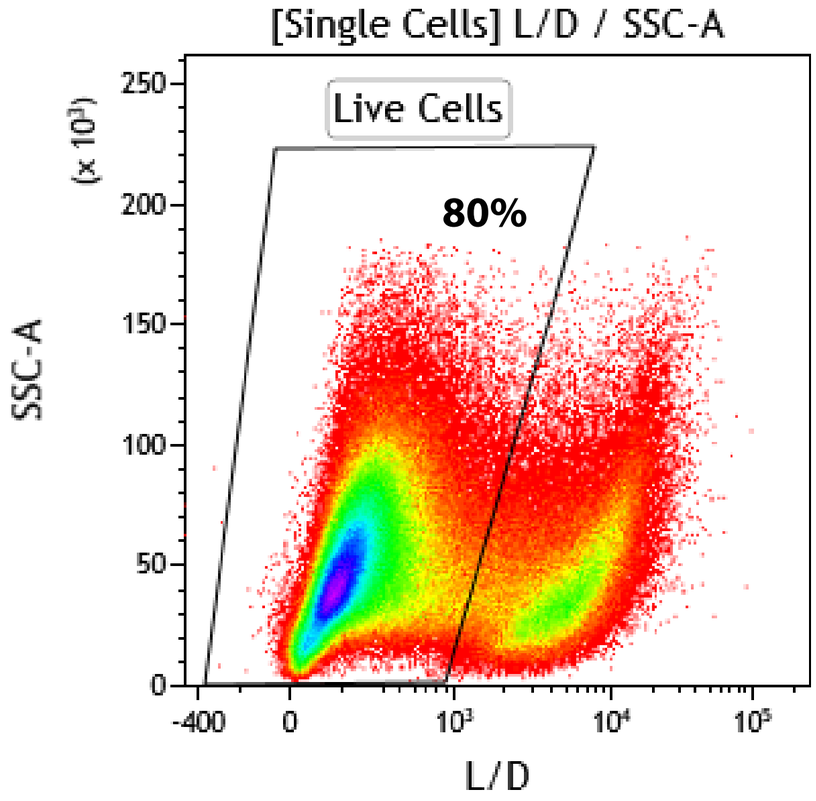

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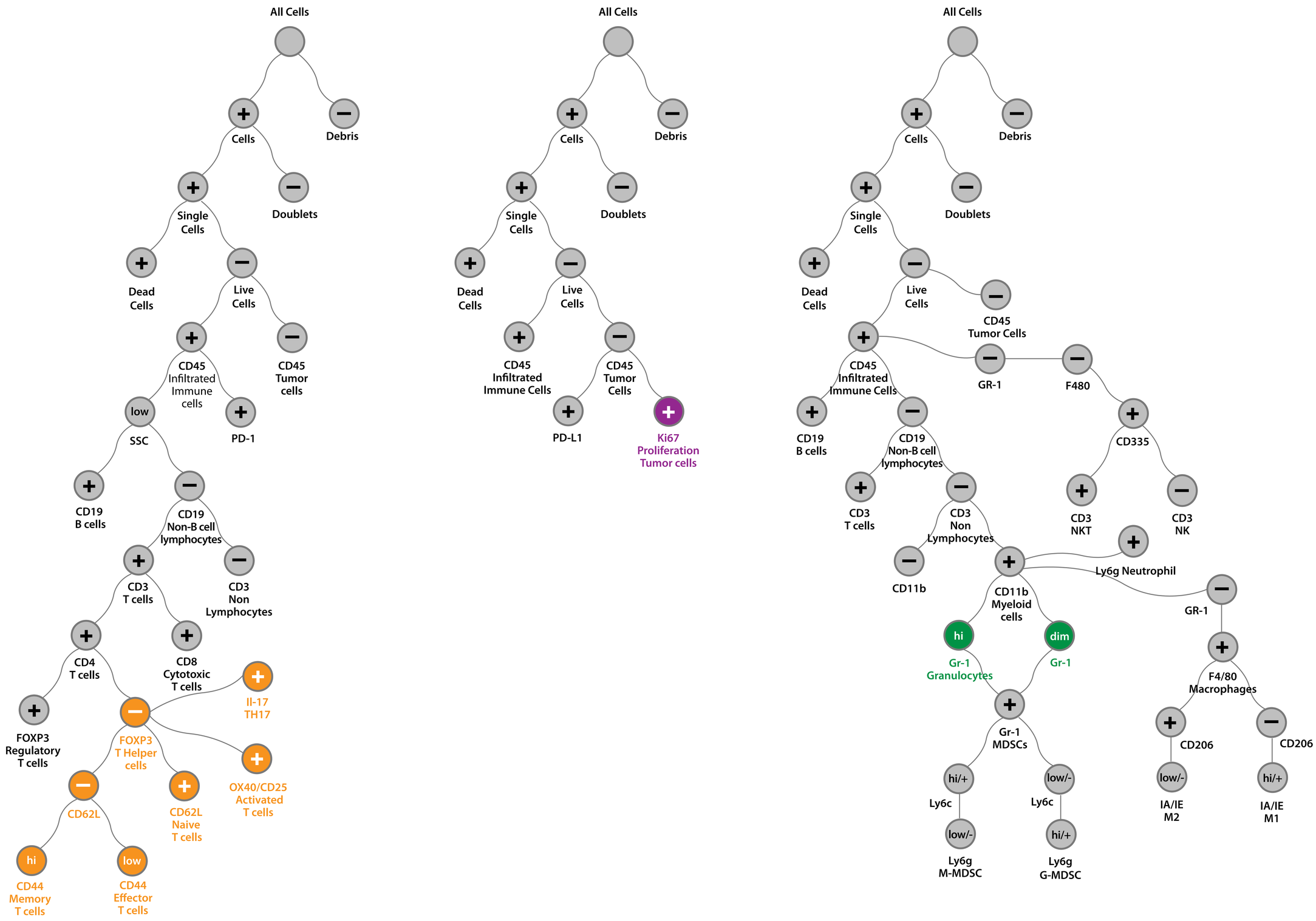

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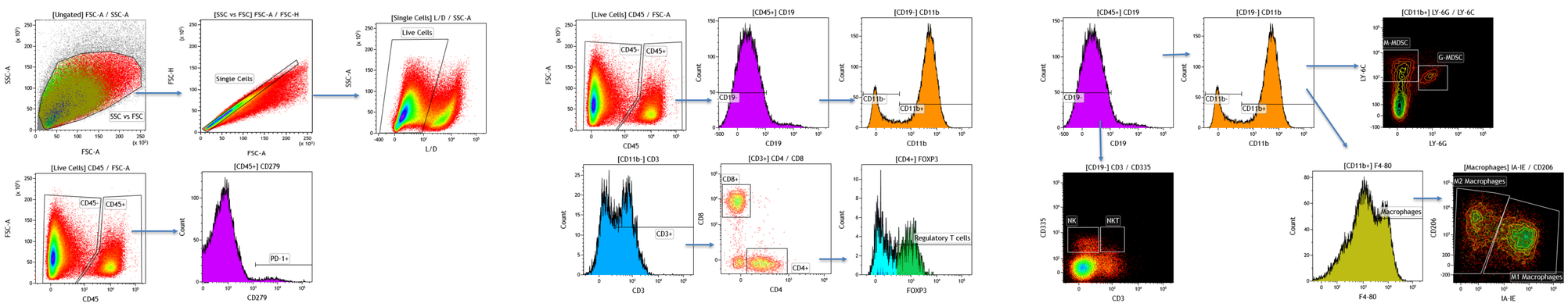

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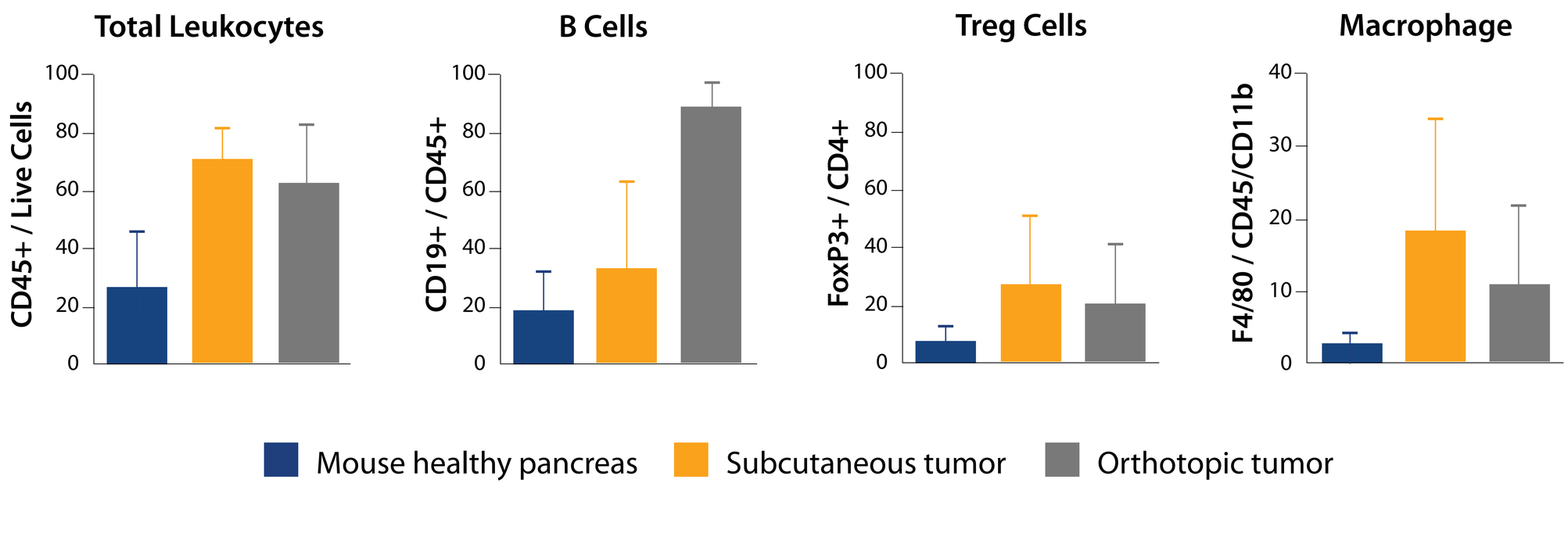

Table1

Click here to download Table Table 1 Murine FACS Panel for TIL Analysis.xlsx

Marker	**Immune Cell Population**
CD45	Total leukocytes
CD3	Total T cells
CD4	CD4+ T helper cells
CD8	CD8+ cytotoxic T cells
CD44/CD62L*	Naïve, memory and effector T cells
CD69/CD44/OX40/CD25 *etc* *	Activation markers
CD4+CD25+FoxP3+	Regulatory T cells
CD11b+Ly6c/Ly6g	G-MDSC and M-MDSC
CD11b+ F4/80	Macrophages
IA/IE/CD206	M1 and M2 macrophages
CD3-CD335+	NK cells
CD3+CD335+	NKT cells
CD19	B cells
TNF-a/IFN-r/IL-7/IL-3 *etc* *	Cytokines
PD-1/PD-L1/CTLA-4/TIM-3 *etc* *	Checkpoint inhibitors
Granzym B *etc* *	Commonly requested markers
KI67/Brd U/PNCA *etc*	Proliferation
Live/dead (fixable)	Live/dead

*Note: Further markers can be added as needed

Materials Table

Click here to download Materials Table Copy of JoVE_Materials-aa.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anesthesia machine	\	SAS3119	\
Trocar 20	\	\	2mm
Petri dish	\	\	20mm
100x antibiotic and antimycotic	\	\	\
Iodophor swabs	\	\	Daily pharmacy purchase
Alcohol swabs	\	\	Daily pharmacy purchase
Liquid nitrogen	Air chemical	\	\
Biosafety hood	AIRTECH	BSC-1300IIA2	\
FACS machine LSRFortessa X-20	BD	LSR Fortessa	\
antibodies	BD	\	\
Trevigen MD or BD Matrigel Basement Membrane Matrix High concentration	BD	354248	\
FACS buffers	BD	554656	Mincing buffer
Brilliant Staining Buffer	BD	563794	\
Mouse BD Fc Block	BD	553142	\
cell filters	BD-Falcon	352350	70μm
routine blood tube	BD-Vacutainer	365974	2mL
Kaluza	Beckman		vs 1.5
6-well plates	Corning	3516	\
Foxp3 Fix/Perm kit	ebioscience	00-5523-00	\
UltraComp eBeads	ebioscience	01-2222-42	\
Centrifuge	eppendorf	5810R,5920R	\
FlowJo software	FlowJo LLC	\	vs 10.0
PBS	Hyclone	SH30256.01	50mL
RPMI 1640	Hyclone	SH30809.01	\
Disposable, sterile scalpels	Jin zhong	J12100	11#
knife handle	Jin zhong	J11010	\
eye scissors and tweezers	Jin zhong	Y00030	Eye scissors 10cm
	Jin zhong	JD1060	Eye tweezers 10cm with teeth

Portable liquid nitrogen tank	Jinfeng	YDS-175-216	\
Electronic balance	Metter Toledo	AL204	0-100g
Miltenyi C-tubes	Miltenyi	130-096-334	\
Miltenyi Gentle MACS with heater blocks	Miltenyi	120-018-306	\
Tumor Dissociation Kit	Miltenyi	130-096-730	\
Cell counter	Nexcelom	Cellometer	Cellometer Auto T4
cryopreservation tube	Nunc	375418	1.8ml
Cultrex High Protein Concentration (HC20+) BME	PathClear	3442-005-01	\
syringes	Shanghai MIWA medical industry	\	1-5mL
Studylog software	Studylog	\	software
Studylog-Balance and supporting USB	OHAUS	SE601F	Balance and supporting USB
Studylog-Data line of vernier calipers	Sylvac	926.6721	Data line of vernier calipers
Caliper	Sylvac	910.1502.10	Sylvac S-Cal pro
Sterilized centrifuge tubes	Thermo	339653	50mL
	Thermo	339651	15mL
Ice bucket	Thermo	KLCS-288	4℃
Ice bucket	Thermo	PLF-276	—20℃
Ice bucket	Thermo	DW-862626	—80℃
RNAlater	Thermo	am7021	\

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Author(s): Xiaoyu An, Xuesong Ouyang, Hui Zhang, Tingting Li, Qixiang Li

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December 7, 2017

Alisha DSouza, Ph.D.
Review Editor
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Dear Dr. Dsouza,

Thank you very much for reviewing our manuscript entitled "Immunophenotyping of orthotopic homografts (syngeneic) of murine primary KPC pancreatic ductal adenocarcinoma by flow cytometry" by An *et al*, and the suggested revisions. I sincerely appreciate the time and the thorough review by the three reviewers. We are in complete agreement with reviewers' critiques. We have now completed the revision of the manuscript per editor/reviewers' suggestions and are submitting the final version for publication. We have also addressed the reviewers' comments item by item, as shown in **Red bold font** as below. In addition, we have carefully edited the text and hope that it is now acceptable for publication.

Please note that I have attached a clean version along with the one with tracked change for reference. I have also attached original Figure 1C Tiff file photo in case the problem with resolution.

Please do not hesitate to contact me if you have any question regarding this submission. Thank you again, and I look forward to hearing from you.

Sincerely,

Sincerely,

Henry Q.X. Li, Ph.D.
Vice President, Translational Oncology, Crown Bioscience, Inc.
#6 W. Beiijng Rd., Taicang, Jiangsu, China
Visiting Professor, Peking University, the State Key Laboratory, China
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Dear Dr. Li,

Your manuscript, JoVE57460 Immunophenotyping of orthotopic homografts (syngeneic) of murine primary KPC pancreatic ductal adenocarcinoma by flow cytometry, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits. After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **Dec 19, 2017**.

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the mentioned sections as per the suggestion. Meanwhile, we will also revise our other manuscript (chapter) for the same rationale, so both final versions will be sufficiently different.

3. Figure 1: Please provide scale bars for panel C. **We added "40x10" in the figure legend.**

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7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. **Revised per suggestions.**

8. What type of mouse is used? **We added C57BL/6 mice in the text and Figure 1 legend.**

9. How is the tumor surgically removed? We need more details on the surgical procedure.
We now added the details per suggestions.

10. Please mention how proper anesthetization is confirmed.
We now added the confirmation per suggestions.

11. 4.1.4: Inoculate the tumor how?
Now, we added the description.

12. Please specify all surgical tools used and when.
Now, we added more descriptions per suggestion.

13. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency. **Added.**

14. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered. **Added.**

15. What is the gentleMACS program? Please use generic terms. **Added description.**

16. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. **Done**

17. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. **Done.**

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol **(described)**

b) Any modifications and troubleshooting of the technique **(done)**

c) Any limitations of the technique **(added)**

d) The significance with respect to existing methods **(yes)**

e) Any future applications of the technique **(done)**

Reviewers' comments:

Reviewer #1: Manuscript Summary: The manuscript by An, et al. describes the method of immunophenotyping of a orthotopic pancreatic adenocarcinoma. The paper appears to have all the necessary details to successfully reproduce the experiment

Major Concerns:

Minor Concerns: Since identifying and quantifying the various immune cells is a key part of the manuscript, more details in the Figure legend and the Results section for Figure 3 would greatly enhance the manuscript. **Added more description per suggestions.**

Reviewer #2: Manuscript Summary:

The manuscript describes the method for setting up a mouse model for orthotopic homograft of pancreatic tumor and flow cytometry-based phenotyping of immune cells in that tumor. The authors have extensive previous experience with patient tumor-derived xenograft mouse models. Now they are applying their expertise to the orthotopic homograft tumor mouse model. Although immunotherapy made an important breakthrough in treating cancer patients, majority of patients are still not getting clinical benefits. There is an increasing awareness that the more "physiological" pre-clinical tumor models will provide new insights into the improvement of cancer immunotherapy. Therefore, the aim and scope of this paper is timely. The manuscript is

well written and is easy to follow. The level of technical details provided in this manuscript with video demonstration is appropriate and will help others implement this model.

Major Concerns:

The quality of results section needs to be improved.

1) Fig. 1A showed vehicle vs Gemcitabine. Gemcitabine was not effective. Is there any reason to believe showing gemcitabine data is helpful to understand the model? **We agree with the reviewer that the Gemcitabine treatment may not necessarily be needed to demonstrate the procedure of this model. It is just that this model happened to be insensitive to gemcitabine as the SOC, which was observed in our early model validation experiment. In the video portion of manuscript, we will not include the treatment portion.**

2) The H & E staining for SubQ Tumor in Fig. 1C needs to be replaced with the one with higher resolution. Scale needs to be provided as well.
Replaced as suggested.

3) Fig. 2 is not necessary.
Figure 2 intends to show that tissue dissociator plays a critical role in successful TIL analysis.

4) It is unclear why PD-1+ cells were excluded from T cell analysis in the Lymphocytes panel in Fig.3 A. **We revised. There is no PD-1 involved in this study.**

5) For tumor cells in Fig. 3A, are PD-L1+ cells and Ki-67+ cells mutually exclusive?
We revised. PD-L1 and KI-67 were not involved in this study.

6) For Lymphoid gating in Fig. 3B, CD11b staining for CD19- population was mistakenly shown.
This is for extra parent gates for further T cell analysis. We gated $CD19^-$ cells from $CD45^+$ (total leukocytes) which identifies all non-B cells (myeloid cells plus T cells), followed by gating for $CD11b^-$ cells to remove $CD11b^+$ myeloid cells from $CD19^-$ cells, in another word, we tried to remove as much as non-T cells. In the end, we gated the $CD3^+$ T cells from the remaining cell population.

7) For Fig. 3C, authors should show data in counts in addition to %. They should also show statistics. The graph should also show individual data points or mention the number of mice analyzed. It is unclear how many times experiments were repeated.
Done. We have edited the plots to bar graph plus dot plots to represent each individual animal.

Minor Concerns:

1) Authors should clearly convey the benefit of the orthotopic homograft (e.g., allow to synchronize timing of tumor growth) in Introduction or Discussion. **Added now in the Introduction.**

2) Line 125: Current most up-to-date FlowJo version is v10.4. Is there any reason to use v10.0?
We have yet to upgrade the software. We do not believe our current version (10.0) will not affect the analysis for this study.

3) Line 145-146: It is unclear if you mean any of three is OK. **Now removed.**

4) Line 163-167: numbers are not consecutive. They should be #27-31 rather than #17-21. **Now removed.**

5) Line 166: Supposed to be "Sterile PBS"? **now removed.**

6) Line 172: Please specify "the local IACUC". Is this study approved by the Crown Bioscience Inc. IACUC? **Now specified.**

7) Line 195-197: How did you measure the tumor volume? **Using caliper, now added the description.**

8) Line 255-257: How frequently did you observe this to occur? **We believe that this is more model specific. For this particular model, we do not have sufficient data to know the frequency.**

9) Line 289: section 6.1.1 instead of 5.1.1. **done**

10) Material table was truncated. It also misses information for company and catalog number. The table needs to be complete.
Corrected.

Reviewer #3: Manuscript Summary:

Using the immunophenotyping of murine orthotopic PDAC homograft, authors aims at profiling the tumor immuno-microenvironment. This is a well-organized paper with protocol in details. This protocol would be applicable for likely a majority of tumor models used. My opinion is " accepted with minor revision".

Major Concerns:

Digestion with enzymes can be performed. This will allow for a more thorough single cell

suspension. However, it should be noted that such digestion protocols can affect surface antigen expression, so caution should be taken in interpreting these results. This can be verified using enzyme treated or un-treated samples.

Minor Concerns:

1. Page 7, line 199, after" containing 20 mL PBS", "Chill media or buffer to 4 °C prior to euthanization" is recommended; **now revised per suggestions.**

2. Page 7, change the sequence of step 3 and 5 on "3.2. Donor tumor fragment preparation"; **Changed now.**

3. Page 7, add "Only intact pieces were chosen" to step 6; **Revised per suggestion.**

4. Page 7, line 203, what is "transport medium"? Please give the detail. **Changed to PBS.**

5. Page 8, line 233, the sentence is recommended to use "Once anesthetized, the mice were fixed on an experiment board in the right lateral position." **Revised per suggestions.**

6. Page 8, line 240, please add "homoeostasis was achieved by compression" to the end of item e; **revised.**

7. Page 8, line 242, please add "If neither bleeding nor tumor tissue leakage occurred" to the ahead of "After finishing tumor implantation"; **revised per suggestions.**

8. Page 9, line 299, PBS should be cold; **revised.**

9. Page 10, line 314, add "4 °C" after "300 x g"; **done.**

10. Page 10, line 320, replace " count the cell number" with "count viable cells using trypan blue" ; **revised.**

11. Page 10, line 320, add "Include the correct isotype control antibodies to ensure staining is specific." to the end of item 2; **Revised.**

12. Figure 1, C, left photo (H&E) is unclear, ple
Now changed

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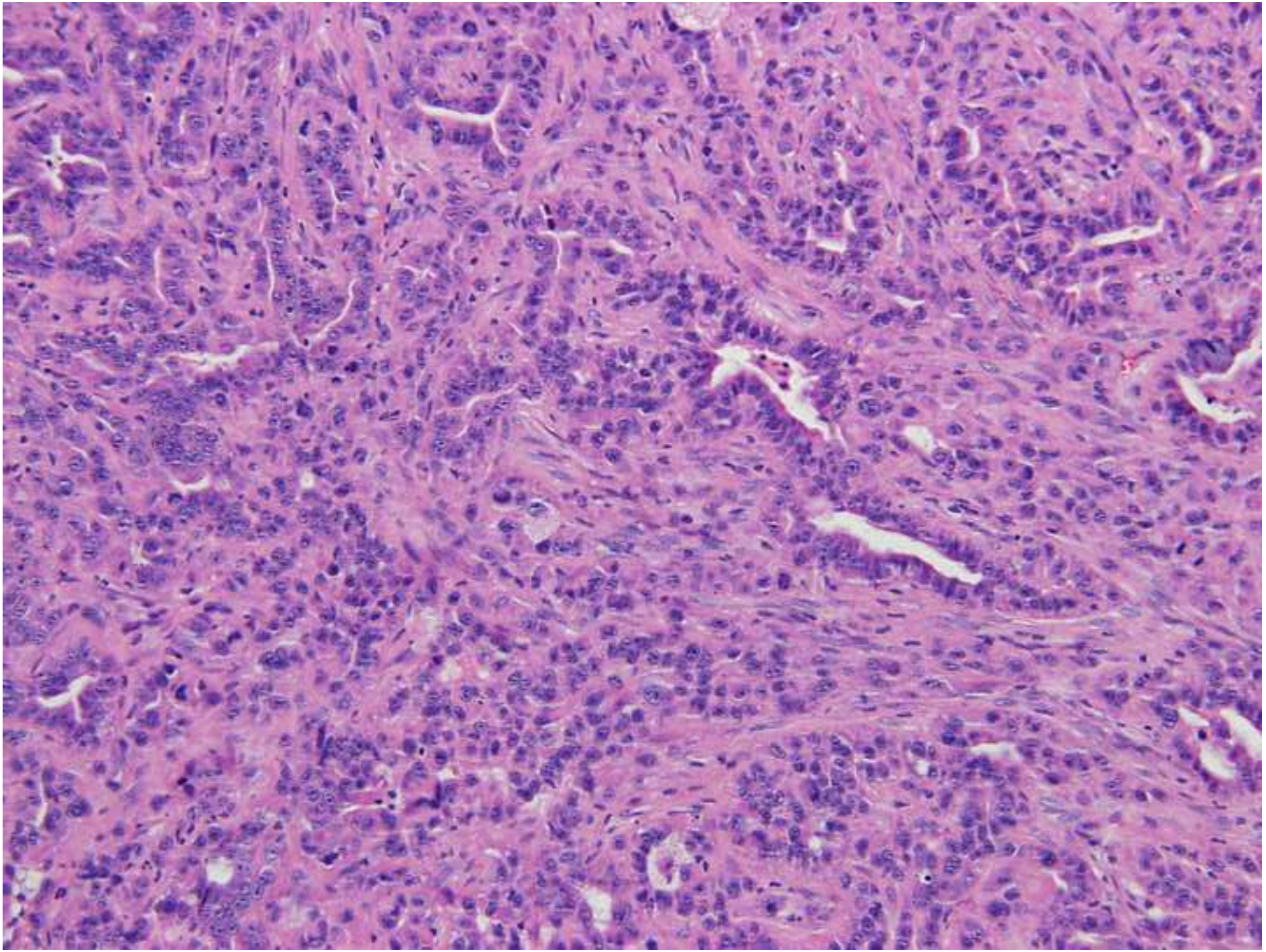

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