Journal of Visualized Experiments

Generation of orthotopic pancreatic tumors and ex vivo characterization of tumor-infiltrating T cell cytotoxicity.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60622R1
Full Title:	Generation of orthotopic pancreatic tumors and ex vivo characterization of tumor-infiltrating T cell cytotoxicity.
Section/Category:	JoVE Cancer Research
Keywords:	Immune, digest, tumor, T cell, ex vivo, cytokines, pancreatic, orthotopic, KPC, flow cytometry, intracellular, cytotoxicity
Corresponding Author:	Sarah Spear UNITED KINGDOM
Corresponding Author's Institution:	
Corresponding Author E-Mail:	s.spear@imperial.ac.uk
Order of Authors:	Sarah Spear
	Iain A McNeish
	Melania Capasso
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	London, United Kingdom

TITLE:

Generation of Orthotopic Pancreatic Tumors and Ex vivo Characterization of Tumor-Infiltrating T Cell Cytotoxicity

AUTHORS AND AFFILIATIONS:

Sarah Spear*[1,2], Iain A McNeish[1] and Melania Capasso*[2,3]

1. Division of Cancer, Department of Surgery and Cancer, Imperial College London, London, United Kingdom
2. Centre for Cancer and Inflammation, Barts Cancer Institute, Queen Mary University of London, London, United Kingdom
3. German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany

*Corresponding Authors

Melania Capasso (melania.capasso@dzne.de)

Sarah Spear (s.spear@ic.ac.uk)

Email addresses of co-authors:

Iain A McNeish (i.mcneish@imperial.ac.uk)

KEYWORDS:

Immune, digest, tumor, T cell, ex vivo, cytokines, pancreatic, orthotopic, KPC, flow cytometry, intracellular, cytotoxicity

SUMMARY:

This protocol describes the surgical generation of orthotopic pancreatic tumors and the rapid digestion of freshly isolated murine pancreatic tumors. Following digestion, viable immune cell populations can be used for further downstream analysis, including ex vivo stimulation of T cells for intracellular cytokine detection by flow cytometry.

ABSTRACT:

In vivo models of pancreatic cancer provide invaluable tools for studying disease dynamics, immune infiltration and new therapeutic strategies. The orthotopic murine model can be performed on large cohorts of immunocompetent mice simultaneously, is relatively inexpensive and preserves the cognate tissue microenvironment. The quantification of T cell infiltration and cytotoxic activity within orthotopic tumors provides a useful indicator of an antitumoral response.

This protocol describes the methodology for surgical generation of orthotopic pancreatic tumors by injection of a low number of syngeneic tumor cells resuspended in 5 µL of basement membrane directly into the pancreas. Mice bearing orthotopic tumors take approximately 30 days to reach endpoint, at which point tumors can be harvested and processed for characterization of tumor-infiltrating T cell activity. Rapid enzymatic digestion using collagenase and DNase allows a single-cell suspension to be extracted from tumors. The viability and cell

surface markers of immune cells extracted from the tumor are preserved; therefore, it is appropriate for multiple downstream applications, including flow-assisted cell sorting of immune cells for culture or RNA extraction, flow cytometry analysis of immune cell populations. Here, we describe the ex vivo stimulation of T cell populations for intracellular cytokine quantification (IFNγ and TNFα) and degranulation activity (CD107a) as a measure of overall cytotoxicity. Whole-tumor digests were stimulated with phorbol myristate acetate and ionomycin for 5 h, in the presence of anti-CD107a antibody in order to upregulate cytokine production and degranulation. The addition of brefeldin A and monensin for the final 4 h was performed to block extracellular transport and maximize cytokine detection. Extra- and intra-cellular staining of cells was then performed for flow cytometry analysis, where the proportion of $IFN\gamma^+$, $TNF\alpha^+$ and $CD107a^+$ $CD4^+$ and $CD8^+$ T cells was quantified.

This method provides a starting base to perform comprehensive analysis of the tumor microenvironment.

INTRODUCTION:

This method details, from start-to-finish, the surgical procedure for generating orthotopic pancreatic tumors using a minimal amount of cellular material and the subsequent rapid dissociation of established tumors for comprehensive flow cytometry analysis of immune cell populations, including ex vivo analysis of T cell function.

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive carcinoma with only 8% of patients surviving 5 years[1]. As less than 20% of patients are eligible for surgical resection[2], fresh patient samples are not readily accessible for research and thus in vivo models provide essential tools to investigate this disease. There are multiple murine models of PDAC: orthotopic, subcutaneous, transgenic, intravenous and patient-derived xenograft (PDX), extensively described here[3]. The orthotopic model described here allows the injection of syngeneic PDAC cells into the pancreas of immunocompetent mice. This can be performed in large cohorts of wild-type or mutant mice, and thus provides a cost-effective and consistent model for comparison of therapeutic agents. Importantly, the orthotopic model provides the cognate microenvironment for tumor cell growth and metastasizes in our hands and others[4] to clinically relevant sites (e.g., liver), making it more clinically relevant than the subcutaneous or chemically-induced models. Orthotopic tumors display key features of PDAC, such as a strong desmoplastic reaction with an abundance of fibroblasts and extracellular matrix deposition[5]. Transgenic models of PDAC are the gold-standard of murine model and the most commonly used is the KPC model, which expresses mutant $Kras^{G12D/+}$ and $Trp53^{R172H/+}$ under the pancreas-specific *Pdx-1-Cre* promoter[6]. Additional KPC and other in vivo PDAC models are reviewed here[7]. KPC mice spontaneously develop pancreatic tumors with a disease progression that faithfully replicates features of human PDAC[6]. However, as for all transgenic models, the breeding programme is costly, tumor progression is variable and therefore often requires large cohorts of mice. PDX models use patient-derived tumor cells or pieces which are then grown either orthotopically or more often subcutaneously in immunocompromised mice. Xenograft models provide useful tools for screening therapeutic compounds and account for patient heterogeneity. However, they do not provide a complete immune microenvironment, thus limiting their applications[8,9].

Once established, orthotopic tumors typically take around 1 month or more to grow (depending on the cell line used) and form large tumors that can be readily imaged by ultrasound or MRI to track progression and determine treatment efficacy[4,5,10]. However, once in exponential growth, the last phase of tumor growth can be rapid, so most treatment regimens are commenced relatively early (e.g., 14 days)[11,12]. The immune system plays a critical role in tumor development, including in PDAC, which is characterized by an immunosuppressive tumor infiltrate with relative paucity of T cells and frequent presence of myeloid cells[13]. A high presence of T cells in PDAC confers a better prognosis[14,15]. However, as single agents, immune checkpoint inhibitors that relieve T cell immunosuppression, such as anti-CTLA-4[16] and anti-PD-L1[17], have not shown clinical benefit in PDAC patients, most likely because the overall T cell reactivity is very low. However, agents that prime T cell responses, such as anti-CD40, can overcome anti-PD-L1/CTLA-4 resistance[18,19] and vaccination with GM-CSF-secreting allogeneic PDAC vaccine (GVAX) can increase the immunogenicity of PDAC tumors[20], indicating that enhancing T cell responses forms important therapeutic avenue.

Critical to an antitumoral T cell response is the recognition of tumor-derived antigens via the T-cell receptor (TCR) and the subsequent production of cytotoxic cytokines and granules. While T cell antigen-recognition can be determined by TCR sequencing, this approach is costly and time consuming. However, quantification of tumor-infiltrating T cell subsets provides a good indication of an anti-tumoral response. Further examination of T cell activity ex vivo in terms of degranulation, cytokine production and other cytotoxic factors provides a deeper functional analysis. These assays can be performed on fresh tumor samples and many parameters of T cell function can be measured rapidly by flow cytometry.

$CD8^+$ and $CD4^+$ T cells produce cytokines such IFNγ and TNFα to potentiate an immune response[21]. IFNɣ induces MHCI upregulation on target cells, induces differentiation and recruitment of immune cells and aids cell death. IFNγ production by $CD8^+$ T cells is well-characterized to be part of an antitumoral response and correlates with tumor regression[22,23]. TNFα is another proinflammatory cytokine produced by both $CD8^+$ and $CD4^+$ T cells. It enhances TCR-dependent activation and the proliferation of T cells, aiding the anti-tumoral response. Upon TCR engagement, cytotoxic $CD8^+$ T cells can undergo degranulation, where pre-formed secretory lysosomes containing cytotoxic molecules are released into the immunological synapse to cause target-cell degradation[21]. These molecules include Perforin, a protein that binds to the target cell membrane, forming pores that then disrupt membrane integrity and allow diffusion[21] or endocytosis[24] of other cytotoxic molecules, such as Granzyme B, directly into the cytoplasm of the target cell. Granzyme B is a protease that enacts the degradation of multiple proteins within the target cell, leading to cell death[21]. The release of such molecules requires exocytosis of endosomes to the cell surface, where the endosomal marker CD107a (also known as LAMP-1) is transiently incorporated into the cell membrane[25].

The measurement of cytokine secretion by T cells requires their isolation by either flow-assisted cell sorting or bead-based separation assays, which cannot be readily performed on large number of samples simultaneously. However, measurement of intracellular cytokines does not require

any pre-isolation steps and can be easily be performed on multiple samples at one time, allowing a higher-throughput approach. As cytokines are rapidly secreted by T cells, the intracellular levels can be undetectable and thus the T cell requires stimulation to increase basal cytokine production. To assess antigen-driven cytokine production, the antigen recognized by the TCR must be presented to the T cell by a primed APC in vitro. In cases where the antigen specificity is not known, a broad stimulation approach is required. TCR stimulation can be mimicked using anti-CD3/28 beads that provide both TCR activation and costimulation, which induces cytokine production and proliferation. However, a more cost-effective alternative is the use of PMA and ionomycin, which together broadly activate signaling pathways that lead to the synthesis and release of intracellular cytokines. Specifically, PMA activates protein kinase C (PKC) and ionomycin raises intracellular Ca^{2+} ions, leading to increased cell signaling. In order to preserve intracellular content of cytokines, this stimulation can be effectively combined with protein-transport inhibitors brefeldin A and monensin, which block proteins in the Golgi and thus prevent extracellular release. The use of PMA/ionomycin is a well-established method for stimulating T cells and there is a strong correlation between extracellular-released and intracellular cytokines[26]. Stimulation of T cells with PMA and ionomycin also increases lysosome trafficking to cell membrane and thus CD107a becomes transiently integrated on the cell surface before being recycled into the cell. By including an anti-CD107a antibody during the stimulation, it is possible to use it as a marker of degranulation activity[25].

This method rapidly digests the tumors to provide a single-cell suspension. At this point, individual populations can be directly stained for flow cytometry or purified by downstream methods: flow-assisted cell sorting or magnetic-bead separation. Preparation of a single-cell suspension for flow cytometry analysis allows high-throughput analysis of multiple immune cell populations and their phenotypic markers, providing an accurate quantification of immune cell number and phenotype.

Finally, the digestion protocol described here prevents cell-surface markers loss and maintains immune cell viability, allowing immune cells to undergo further cell purification steps and culture as required. However, this method has not been tested for deriving epithelial cells from this digestion.

PROTOCOL:

Orthotopic pancreatic tumors were generated as previously described[10] in accordance with the U.K. Home Office Animal and Scientific Procedures Act 1986 and the European Directive 2010/63/EU. All mice were monitored perioperatively for signs of pain or suffering, including but not limited to weight loss (> 15% in 72 h or 20% in any given period), piloerection, narrowing of eyes, raised gait, hunched appearance, as well of signs of wound infection including bleeding, redness and ulceration. Tumor growth was monitored by palpation, and additional clinical signs such as labored breathing, jaundice and cold extremities were also monitored in order to assess if signs of endpoint had been reached. All procedures should be carried out in sterile conditions. All reagents used prior to flow cytometry staining should be prepared in sterile conditions.

1. Preparation of tumor cells for injection

1.1. Take an aliquot of basement membrane from -20 °C and place on ice at 4 °C overnight.

NOTE: Basement membrane concentration may vary from batch to batch; therefore, lot specific basement membrane batches must be tested in vivo to ensure reproducibility. A new batch of basement membrane is thawed on ice at 4 °C overnight then aliquoted in user-defined aliquots, on ice, and then further stored in -20 °C until required. This minimizes the pipetting and freeze-thawing when using basement membrane.

1.1.1. Place sterile PBS at 4 °C overnight to chill.

1.1.2. Place sterile 200 µL and 1000 µL pipette tips at -20 °C overnight to chill.

1.2. Use tumor cells that are mycoplasma free, grown for at least 2- 10 passages post-thawing and in log-phase of growth prior to harvest. This protocol uses the female murine C57BL/6 KPC-derived cell line: TB32048 provided as a generous gift by the lab of David Tuveson.

1.2.1. When tumor cells are required for harvest, remove medium from the flask and wash cells twice in PBS (pre-warmed to 37 °C).

1.2.2. Add 2x trypsin (pre-warmed to 37 °C) to the flask for 10 min at 37 °C (to a T175 flask, add 5 mL).

1.2.3. After 10 min, add an equal volume of complete medium (10% FBS, 1x penicillin, 1x streptomycin in DMEM) to the flask and dissociate cells by gently tapping the flask and resuspending well in medium.

1.2.4. Transfer the cells into a tube and centrifuge for 5 min at 300 x *g* and room temperature (RT).

1.2.5. Remove the supernatant and resuspend cells in complete medium for cell counting.

1.2.6. Centrifuge cells again for 5 min at 300 x *g* and RT, and remove the supernatant.

1.2.7. Resuspend cells in pre-chilled PBS to achieve a concentration of $1x10^6$ cells/mL.

NOTE: This stock concentration is prepared to achieve a final injection concentration of 1,000 cells in 5 µL. We found injection of a lower number of cells in a low injection volume minimized cell leakage and therefore increased reproducibility. However, tumor growth may be cell-line dependent; therefore, users should optimize each cell line.

1.3. Alongside this, place a pre-aliquoted basement membrane aliquot, on ice, into the hood.

1.3.1. The ratio of the final solution of basement membrane, PBS and tumor cells in PBS prepared for injection is 5:3:2. Therefore to a 500 µL aliquot of basement membrane add 300 µL of pre-chilled PBS using a pre-chilled 1000 µL pipette tip.

1.3.2. Add the PBS directly to the basement membrane aliquot to minimize pipetting.

1.3.3. Keep the p1000 set to 300 µL and resuspend the PBS and basement membrane, making sure to keep the tube on ice to preserve the basement membrane in liquid state.

1.3.4. When finished ejecting all the basement membrane from the p1000 tip, leave the tip in the tube to allow any basement membrane/PBS to come down the pipette tip.

1.3.5. After 5-10 min, eject more basement membrane from the p1000 tip back into the tube and leave the tube to sit on ice.

1.4. Take 200 µL of resuspended tumor cells in PBS and add directly to the basement membrane using a pre-chilled 200 µL pipette tip.

1.4.1. Take a fresh pre-chilled p1000 pipette tip, set the pipette at 300 µL and resuspend 30-40 times. A larger pipette tip, set on a low volume, is preferable as basement membrane can travel up the tip and touch the pipette tip filter during resuspension.

1.4.2. The tumor cells are ready for injection. Keep tumor cell/basement membrane on ice during surgery.

2. Orthotopic injection of tumor cells

2.1. Acclimate the mice in the animal facility for 7 days.

2.1.1. Around 2 h prior to surgery, shave the left-hand side of abdomen and back, then administer pre-operative analgesic subcutaneously under the scruff of the neck (Buprenorphine at 50-100 µg/kg).

2.1.2. Prepare surgical field, with a heat mat to lay mouse on and drapes for surrounding equipment and over mouse. Sterilize all surgical tools; prepare enough sets of tools for each mouse.

2.1.3. Place the mouse in a 5% isoflurane with O_2 chamber until unconscious.

2.1.4. Transfer the mouse, lying on its back, onto a heat mat and maintain anesthesia using a mask, usually at a lower 2-3% isoflurane.

2.1.5. Confirm deep anesthesia; as identified by loss of the pedal-withdrawal reflex when the

hind paw is pinched and monitor breathing rate remains constant.

2.1.6. Cover the body in drape, with only the shaved portion exposed. Ensure the mouse is securely in the anesthesia mask.

2.1.7. Using a sterile cotton bud, add iodine solution in a circular motion over the shaved area: starting from the center and circling out to the edge. Repeat process again with fresh cotton bud and iodine.

2.2. Use scalpel to make a 1 cm incision directly above the pancreas/spleen location (upper-left quadrant). Sterile scissors may also be used to make the incision, if preferred.

2.2.1. Pull the skin apart using forceps. With new forceps, locate the peritoneal wall and use scissors to make another 1 cm incision through the peritoneal wall.

2.2.2. Extract the pancreas, which may come with the spleen, from the body using the second pair of forceps.

2.2.3. Gently invert the vial of tumor cells/basement membrane several times to mix.

2.2.4. Prepare the glass syringe with 5 µL containing 1,000 tumor cells in basement membrane and place on the heat mat for a few seconds to allow it to warm.

NOTE: The brief warming of the syringe will allow the basement membrane to start solidifying, in order to make it easier to inject without leaking. However, this must be kept brief, if left too long the basement membrane will solidify completely and will not be injected. The use of a glass syringe allows a low volume to be precisely injected.

2.2.5. Hold the pancreas at the tail to extend it and insert the needle directly into the center of the pancreas, parallel to the pancreas itself with an effort to avoid visible blood vessels.

NOTE: The center of the pancreas has a large area and it is easiest to inject. However, the head or tail of the pancreas may also be specifically injected if preferred.

2.2.6. Slowly inject 5 µL of basement membrane into the pancreas and hold the needle steady in the pancreas for at least 30 s after injection to allow the basement membrane to solidify and prevent leaking. The basement membrane should be visible as a small clear bubble will have formed; however, it may not be visible.

NOTE: Larger volumes of tumor cells/basement membrane can be injected; however, the exact volume must be tested to ensure leakage does not occur.

2.2.7. Remove the needle from the pancreas and wait to confirm no bleeding has occurred. Gently insert the pancreas back into the abdominal cavity, taking care not to touch the basement

membrane bubble.

2.2.8. Pull the peritoneal wall together and perform a single suture, or two interrupted sutures if needed.

2.2.9. Pull the two sides of the skin incision together and perform multiple interrupted sutures as needed or insert two surgical clips.

2.3. Administer another subcutaneous injection of buprenorphine into the scruff.

2.3.1. Transfer the mouse into a heated 37 °C cage for at least 30 min post-surgery to maintain body temperature before transferring back into a fresh cage.

2.3.2. Prepare a mash diet available in the cage, to ensure rehydration and body weight.

2.3.3. Re-administer post-operative analgesia as recommended and watch closely for signs of wound opening, pain or infection and weight loss. If using surgical clips, these can be removed 7-10 days later using a clip remover.

2.3.4. After around 14 days the scar tissue will have healed sufficiently to begin palpating the abdomen. Monitor tumor size closely via palpation until mice reach endpoint.

2.3.5. At endpoint the mouse is culled via cervical dislocation followed by decapitation. The skin and peritoneal cavity are opened using scissors and the pancreas tumor excised using forceps to hold the tumor, and scissors to remove surrounding tissue.

3. Digestion of pancreatic tumors

3.1. Place the dissected pancreatic tumor, metastatic site tumors, or healthy pancreatic tissue in ice-cold PBS, and store on ice.

3.1.1. Use forceps to transfer the tumor onto a Petri dish.

3.1.2. Add 5.0 mL of digestion medium (2 mg/mL Collagenase, 0.025 mg/mL DNase RPMI) into a 50 mL tube; store on ice to prevent enzyme activity commencing.

NOTE: This protocol uses Collagenase Type V, which has an activity of ≥1 units/mg FALGPA and > 125 collagen digestion units (CDU)/mg solid. Collagenase and DNase aliquots can be stored at -20 °C and thawed on ice before use. When both are completely solubilized in sterile RPMI, they can be passed through a 0.2 µm filter to remove contaminants. Collagenase must be completely solubilized before filtering to avoid loss of material.

3.1.3. Take a small aliquot of this solution to cover the tumor on the Petri dish.

3.1.4. Use sterile scalpel and forceps to cut the tumor into small pieces, roughly less than 3 mm in length.

3.1.5. Scrape the tumor pieces into the tube and gently invert the tube until all the pieces are submerged in digestion media. Store on ice if other tumor samples need to be prepared in a batch.

3.1.6. Transfer onto a shaking device for 20 min at 37 °C. Make sure all pieces of tumor are submerged and not stuck to the edge of the tube. If shaking is not possible, then vortex the sample every 5 min to aid digestion.

4. Preparation of single-cell suspension from digested tumor

4.1. Immediately after the digestion step, place the tube on ice to slow enzyme activity.

4.1.1. Add EDTA to achieve a final concentration of 20 mM and briefly vortex sample to mix. This will further slow enzyme activity.

4.1.2. Open the tube and rinse any tumor digest off the lid of the tube with fresh RPMI medium.

4.1.3. Prepare a 70 µm strainer (the µm size of the strainer can be altered as desired) on a 50 mL open tube, on ice.

4.1.4. Pre-wet the filter with medium.

4.1.5. Resuspend the digested cells and wash the sides of the tube using a 25 mL stripette, or larger. The wider opening of the stripette is important to allow the thick digest to pass easily.

4.1.6. Transfer all of the digest, using the 25 mL stripette, onto the strainer.

4.1.7. Mash the tumor on top of the filter using a 1 mL syringe plunger. Mash only directly up and down to minimize shear stress to cells.

4.1.8. Continuously wash cells through the strainer with RPMI. Make sure to wash with enough force to push cells through.

4.1.9. If there is still material to mash, but the RPMI stops flushing through, the strainer will be saturated. Therefore, transfer the sample into a new filter and continue.

NOTE: Eventually only extracellular matrix components will remain in the filter, all single cells should have passed through.

4.2. Centrifuge the tube for 5 min at 300 x *g* and 4 °C.

4.2.1. Carefully resuspend the cell pellet in complete RPMI and pass directly through another filter to remove any extracellular matrix or large cell clumps that cannot be adequately resuspended.

4.2.2. At this point, if no stimulation is required, immediately stain the isolated cells for flow cytometry analysis by skipping to Step 6.1. Alternatively, resuspend them in freezing medium (10% DMSO in FBS) and store at -80 °C followed by long-term storage in liquid nitrogen.

NOTE: The freezing step can allow purification of immune cells at a later date; however, the quantification of immune cell subsets may require optimization to confirm that cell numbers and phenotype is not affected by the freeze/thaw process. The ex vivo T cell stimulation is best performed on fresh tumor samples. At this point the sample can be further purified by bead-based dead-cell removal or immune cell enrichment assays if required.

5. Preparing cells for ex vivo stimulation

5.1. Count the cells to achieve a concentration of 2 x 10^6/ 100 µL in complete medium (RPMI 10% FBS, 1x penicillin and 1x streptomycin).

NOTE: The high number of total cells plated ensures that there will be adequate T cells within this sample to analyze. However, the number can be scaled up or down depending on sample availability and the rare nature of T-cell subsets of interest.

5.1.1. Plate 100 µL of cells in a U-bottomed 96-well plate.

5.1.2. Add 100 µL of complete medium containing a 2x preparation of PMA/ ionomycin (to achieve a final concentration 0.081 µM and 1.34 µM, respectively, as recommended by the manufacturer).

NOTE: If measuring degranulation/exocytosis, also include here a fluorescently conjugated anti-mouse CD107a in the media. A control sample that does not contain CD107a must also be performed.

5.1.3. Place in 37 °C incubator with 5% CO_2 for 1 h.

5.1.4. Add 20 µL of a 10x preparation of brefeldin A and monensin (to achieve a final concentration 1.06 µM and 2.0 µM, respectively (as recommended by the manufacturer) in complete media.

NOTE: Brefeldin A and monensin are protein transport inhibitors and thus block extracellular release of cytokines, etc. permitting their detection by flow cytometry. If measuring cytokine release into the supernatant by ELISA or similar methods – then this step can be skipped.

5.1.5. Place the plate in a 37 °C incubator with 5% CO_2 for further 4 h.

6. Extracellular and intracellular staining for flow cytometry

6.1. Remove the plate and resuspend each well to transfer all material to a V-bottomed plate, placed on ice.

NOTE: Epithelial cells, macrophages and other adherent cells may not be fully retrieved by resuspending. However as the downstream analysis is only on T cells, this is not a problem.

6.1.1 Centrifuge the plate for 5 min at 300 x *g* and 4 °C (use these conditions for subsequent steps unless stated).

6.1.2. Remove the supernatant by flicking the plate upside down in one sharp movement.

6.2. Resuspend in 50 µL of a fixable viability dye, prepared in ice-cold PBS. When resuspending, set the pipette to a lower volume to avoid making bubbles.

6.2.1. Incubate for 20 min at 4 °C, in the dark.

6.2.2. Wash step: Add 100 µL of FACS buffer, centrifuge and remove the supernatant.

6.3. Resuspend each well with 50 µL of anti-CD16/CD32 (2.5 µg/mL) in FACS buffer (0.5% BSA, 2.0 mM EDTA in PBS) to block non-specific binding of detection antibodies to Fc receptors.

6.3.1. Incubate for 15 min at 4 °C, in the dark.

6.4. Add directly to each well a 2x mastermix of fluorochrome-conjugated anti-mouse CD45, CD3, CD4 and CD8 (further extracellular markers can be added as desired) in FACS buffer.

6.4.1. Incubate for 30 min at 4 °C, in the dark.

6.4.2. Wash step: Add 100 µL of FACS buffer, centrifuge and remove the supernatant.

6.5. Add 100 µL of 1x Intracellular (IC) fixation buffer and incubate for 30 min at RT, in the dark.

6.5.1. Prepare the centrifuge at RT.

6.6. Add 100 µL of FACS buffer, centrifuge for 5 min at 300 x *g* and RT and remove the supernatant. Repeat with 1x permeabilization buffer and centrifuge for 5 min at 300 x *g*; then remove the supernatant.

6.7. Add 50 µL of 1x mastermix of fluorochrome-conjugated anti-mouse IFNγ, TNFα, and other intracellular markers prepared in 1x permeabilization buffer.

6.7.1. Incubate for 1 h at RT, in the dark.

6.7.2. Add 100 µL of permeabilization buffer to wash. Then centrifuge for 5 min at 300 x *g* and RT and remove the supernatant.

6.7.3. Add 100 µL of FACS buffer to wash, centrifuge for 5 min at 300 x *g* and RT and remove the supernatant.

6.8. After this final centrifugation, resuspend cells in a volume compatible for the flow cytometer. It may vary depending on the size of FACS tubes.

6.8.1. Transfer this volume into appropriate FACS tubes for acquisition.

6.8.2. Cover from light and store in the fridge and acquire the samples within 24 h.

REPRESENTATIVE RESULTS:

After injecting 1000 TB32048 cells into the pancreas, orthotopic tumors take approximately 30 days to develop (**Figure 1A,B**). Basement membrane leakage during surgery can cause large tumors to form directly on the peritoneal wall, which are prominently visible through the skin (**Figure 1C**). We would remove these mice from the study. However, with good surgical skills the incidence of leakage is minimized. Orthotopic tumors harvested at endpoint can grow to a substantial size in C57BL/6 wild-type mice (**Figure 1D**). Harvested orthotopic tumors require digestion in collagenase/ DNase for 20 min in order to achieve a single-cell suspension (**Figure 2**). At this point, tumor-derived cells can be plated in a U-bottomed plate at 2 x 10^6 cells/well. The number of cells plated can be altered depending on the prevalence of T cells within the sample; the cell number can be lowered if T cells are at a high density. Control spleen or lymph node samples can also be plated at this point for stimulation. Each well is stimulated with PMA and ionomycin for 5 h and after 1 h incubation, brefeldin A and monensin are added in order to block extracellular release of cytokines (**Figure 2**). After the incubation, samples are stained for extracellular epitopes and intracellular cytokines for analysis by flow cytometry (**Figure 2**).

Samples of spleen and tumors from mice bearing orthotopic tumors were analyzed by flow cytometry. The gating strategy used in flow cytometry analysis for the spleen and orthotopic tumors excludes debris using FSC-A, SSC-A, doublets by FSC-A/FSC-H and SSC-A/SSC-W, then dead or apoptotic cells as positive for fixable viability dye (**Figure 3A**). Immune cells are then gated on as $CD45^+$, and T cells further gated on as $CD3^+$ from which $CD4^+$ and $CD8^+$ subsets are defined (**Figure 3A**). A fluorescence minus one (FMO) is performed to determine background fluorescence for gating and a brefeldin A/monensin only control is performed to determine basal production of cytokines (**Figure 3B-D**).

For IFNγ, incubation with brefeldin A/ monensin resulted in no increase in IFNγ over FMO control in both spleen and tumor samples. However, the addition of PMA and ionomycin increased the% of intracellular IFNɣ detectable in both splenic and tumor-derived $CD4^+$ and $CD8^+$ T cells.

Splenic $CD4^+$ and $CD8^+$ T cells, used as a positive control, have a relatively higher IFNγ production than tumor-infiltrating T cell subsets, with an average of 6.60 ± 1.5% and 12.97 ± 3.4% compared to 4.81 ± 1.0% and 4.13 ± 1.3%, indicating immunosuppression occurs within the tumor (**Figure 4A, Figure 5A**). Using the same strategy for TNFα, we visualized that a high percentage of splenic $CD4^+$ T cells are positive for intracellular TNFα (65.93 ± 2.3%), compared to tumor-infiltrating $CD4^+$ T cells produce (22.45 ±5.4%). Splenic and tumor-infiltrating $CD8^+$ T cells produce similar levels of TNFα (25.15 ± 3.7% and 19.91 ± 5.1%, respectively) (**Figure 3B, Figure 4B**).

Finally, CD107a is an endosomal marker that is expressed transiently on the cell surface during the exocytosis of cytotoxic granules and cytokines, as such, it is used as a surrogate marker for cytotoxicity. The benefit of staining for CD107a during the stimulation is that all transiently cell-surface expressed CD107a will be captured by the fluorescent-antibody. The basal levels of CD107a are shown in brefeldin A/monensin only treated cells. For splenic $CD8^+$ T cells, stimulation with PMA/ ionomycin increases the level of CD107a detected, with the strongest upregulation in $CD8^+$ cells which were 23.95 ± 3.5% $CD107a^+$, compared to 5.8 ± 1.9% in tumor-infiltrating $CD8^+$ cells, indicating splenic CD8+ had a greater rate of degranulation. On the other hand, splenic and tumor-infiltrating $CD4^+$ expressed comparable levels of CD107a 9.37 ± 1.5% and 11.50 ± 1.8%.

Overall these results highlight that orthotopic tumors can be generated from the injection of a very low number (1,000) of tumor cells into the pancreas. These tumors can be rapidly digested for the isolation of T cells for ex vivo stimulation. Detection of intracellular cytokines is possible and highlights the basal level of immunosuppression of infiltrating T cells, compared to T cells in secondary lymphoid organs.

FIGURE AND TABLE LEGENDS:

Figure 1: Generation of orthotopic pancreatic tumors.
(**A**) Schedule of in vivo experiments. (**B**) The macroscopic appearance of orthotopic tumors within the abdominal cavity (left) and after excision (right) where the tumor shown has been cut in half. (**C**) Evidence of basement membrane leakage during surgery can cause tumors to develop which are visible through the skin (upper photo) and form on the peritoneal wall (lower photo). (**D**) Orthotopic pancreatic tumor weights harvested from mice which had reached endpoint (n=22). Each data point represents an individual mouse, bar graph shows mean ± SEM. The data in this figure has been modified from previously published work[10].

Figure 2: Schematic of processing orthotopic tumors for ex vivo T cell stimulation.
After harvesting, pancreatic tumors are rapidly digested in Collagenase (2 mg/mL) and DNase (0.025 mg/mL) for 20 min at 37 °C. Following this, cells are resuspended at 2 x 10^6/mL in complete RPMI media and plated in a U-bottomed plate. A stimulation cocktail of PMA and ionomycin is added for 5 h, at which point the anti-mouse CD107a antibody can also be added to the culture. After 1 h incubation the intracellular transport blockers, brefeldin A and monensin, are added. After ex vivo stimulation the cells are transferred into a v-bottomed plate for staining with the fixable viability dye (in PBS) for 20 min 4 °C. Cells are washed in FACS buffer and incubated in anti-CD16/32 (FcR block) for 15 min (in FACS buffer) and then incubated with extracellular

fluorescent-conjugated antibodies for a further 30 min (in FACS buffer). Cells are washed again in FACS buffer and resuspend in intracellular fixation buffer for 20 min. After this, cells are washed once in FACS buffer and once in 1x permeabilization buffer. Cells are resuspended for 1 h at RT in intracellular fluorescent-conjugated antibodies for 1 h (in 1x permeabilization buffer). Cells are washed once in 1x permeabilization buffer and once in FACS buffer before resuspending in FACS buffer for acquisition on the flow cytometer with 24 h.

Figure 3: Flow cytometry analysis of ex vivo stimulated spleen- and tumor-derived T cells.
(**A**) Flow cytometry gating strategy used for spleen (positive control) and orthotopic tumors samples. Cells are discriminated from debris using FSC-A/SSC-A and single cells are further isolated using FSC-A/FSC-H and SSC-A/SSC-W. Dead or apoptotic cells are excluded using the fixable viability dye -FVD506 and immune cells are gated on by $CD45^+$. Following this $CD3^+$ T cells and $CD4^+$ and $CD8^+$ subsets are defined. Data was acquired on a BD Fortessa. (**B**) The gating strategy used to quantify $IFN\gamma^+$ $CD4^+$ and $CD8^+$ T cells. A fluorescent minus one (FMO) control is used on fully stimulated samples (PMA/ionomycin/brefeldin A/monensin) to determine the background fluorescence. A brefeldin A/monensin only control (B+M only) is used to determine the basal cytokine production. The fully stimulated sample is then used to calculate the% $IFN\gamma^+$ T cells. (**C**) The gating strategy used to quantify $TNF\alpha^+$ $CD4^+$ and $CD8^+$ T cells. An FMO control is used on fully stimulated samples (PMA/ionomycin/brefeldin A/monensin) to determine the background fluorescence. A brefeldin A/monensin only control (B+M only) is used to determine the basal cytokine production. The fully stimulated sample is then used to calculate the% $TNF\alpha^+$ T cells. (**D**) The gating strategy used to define $CD107a^+$ $CD4^+$ and $CD8^+$ T cells. An FMO control is used on fully stimulated samples (PMA/ionomycin/brefeldin A/monensin) to determine the background fluorescence. A brefeldin A/monensin only control (B+M only) is used to determine the basal degranulation. The fully stimulated sample is then used to calculate the% $CD107a^+$ T cells.

All flow cytometry data was analyzed on FlowJo Version 10.6.1. The data in this figure has been modified from previously published work[10].

Figure 4: Quantification of ex vivo spleen- and tumor-derived T cell activity.
The proportion of $CD4^+$ and $CD8^+$ T cells positive for (**A**) $IFN\gamma^+$ (**B**) $TNF\alpha^+$ and (**C**) $CD107a^+$ was quantified in the spleen (n=4) and tumor (n=7) of orthotopic-tumor bearing mice. Each data point represents an individual mouse and error bars display mean ± SEM. Statistical significance was tested using an unpaired t-test where * = $p<0.05$, ** = $p<0.01$, ***= $p<0.001$ and ****=$p<0.0001$.

All data was analyzed using Prism 8. The data in this figure has been modified from previously published work[10].

DISCUSSION:
In vivo models of pancreatic cancer provide invaluable tools to understand disease progression and assess new therapeutics targets[3]. The orthotopic model in particular is a cost-effective and reproducible model that can be applied in large cohorts of mice simultaneously[4,27]. The orthotopic model also provides the cognate microenvironment and intact immune system for

tumor growth, making it more appropriate than the subcutaneous and PDX-models. However, we have found that some elements of immune infiltration can differ between the orthotopic model and KPC mice, the gold-standard murine model[10]. One reason for this could be the accelerated tumor growth seen in the orthotopic model. Further differences in the density of immune cell subsets have been described between the orthotopic and subcutaneous models[3,28]. Therefore, although the transgenic KPC model is more costly and variable[6], key findings should be verified in a small cohort of KPC mice where possible.

The preparation of tumor cells for the orthotopic surgery is a critical step in the protocol. Cells should always be in the log phase of growth and mycoplasma- and infection-free. Orthotopic surgery should be postponed if there are any concerns over tumor cell growth. The use of basement membrane improves tumor incidence rate over injecting cells without it[29] and reduces cell leakage and thus peritoneal spread[27]. However, once suspended in basement membrane, tumor cells should be rapidly injected (within 2 hours) to avoid any cell loss. The number of tumor cells required to generate tumors is likely to be cell-line dependent, and a range of cell numbers should be tested (e.g., from 100 – 100,000) which may also determine the time to reach endpoint. It is likely there will be a margin of error when preparing 1,000 cells per mouse for injection; therefore, if multiple days of surgery are required, treatment of groups should be spread equally across days to control for batch effects. Most surgical steps can be modified depending on preferences; however, care must be taken not to disturb the basement membrane when replacing the pancreas in the abdominal cavity or closing the peritoneal wall. Basement membrane leakage can cause tumor cell growth on the peritoneal wall, which form rapidly and can result in having to sacrifice the animal earlier.

Ideally, pancreatic tumors should be rapidly digested post-harvest and prepared for ex vivo stimulation immediately. However, this might not be possible if there is a large batch of tumors to harvest, in this case tumors should be kept on ice and digested in batches. The type, concentration and length of exposure to digestive enzymes have all been shown to affect a large number of surface molecules on immune cells[30–32]. The digestion time is also deliberately short to limit cell death[33]. Digested cells can be frozen down in freezing medium for long-term storage; however, some cell loss will occur when thawing. The digestion process can be less than optimal if the tumor pieces are not sufficiently diced before collagenase incubation and this will be evident as hard tumor pieces will remain in the filter after digestion. The collagenase concentration can be lowered if working with healthy pancreas or early-stage tumors; reports on extracting healthy pancreatic ductal cells use significantly lower concentrations[34]. A high degree of epithelial cell death is to be expected during the digestion; however, immune cells should tolerate the process well. Alternative methods exist to isolate viable epithelial cells for organoid growth[35] or to preserve tissue architecture[36].

Modifications to the stimulation protocol can be made easily, depending on the desired read-out and immune cell analyzed (e.g., macrophages or B cells). The use of pan-stimulation reagents PMA/ionomycin does not discriminate for TCR-antigen specificity, making it useful when the antigen is not known. However, the production of IFNɣ is closely associated with TCR engagement[37] and both IFNɣ and TNFα production are critical in PDAC antitumoral responses[38].

PMA/ionomycin stimulation reflects the maximal capacity of T cells to produce cytokines, which might or might not be produced by the T cells within the tumor microenvironment. Endogenous production can be measured without the need for stimulation; however, levels may be far lower or undetectable. There are alternative methods to stimulate T cells: anti-CD3/28-coated beads, which also do not require antigen or indeed other immune cell populations. The benefit of this method is allowing quantification of cytokine production by specific T cell subsets without the need for separation methods. Other markers of cytotoxicity (granzyme B and Perforin A), activity (IL-2) or immunosuppression (IL-10) can also be added[21]. However, high-quality flow cytometry antibodies are not available to detect all cytokines and factors of interest. Therefore, if there are other applications such as ELISA required the stimulation can be performed without the inclusion of brefeldin A/monensin, allowing cytokine release into the supernatant. However, of note, this will permit total cell cytokine release and it will not be possible to determine which cell populations contributed.

IFNγ production is a dominant feature of an antitumoral T cell response, often used as a substitute for TCR-antigen recognition[37,38]. Other in vivo methods that more accurately define antigen-specific responses utilize tumor cells expressing a known antigen, such as Ovalbumin or SV40. The universal antigen can then be used ex vivo to test T cell recognition or in combination with a TCR-restricted host mouse. Alternatively, where the antigen is unknown, quantification of T cell clonal expansion can be performed by bulk-TCR sequencing, or more recently single-cell TCR sequencing[39,40]. To fully understand the state of the intratumoral T cell response, markers indicative of exhaustion or inhibitory receptors should also be measured including: CTLA-4, PD-1, LAG-3, TIM3, 2B4. As well as markers of effector T cells ($CD44^{hi}$, $CD62^{lo}$) and proliferative activity, $Ki67^{+}$ or CSFE dilution[41–44]. Overall, the orthotopic model provides a useful platform to rapidly test therapeutic strategies, in particular that may modulate the antitumoral T-cell response, that can be then validated on a smaller cohort of transgenic, KPC, mice.

ACKNOWLEDGMENTS:

We would like to thank the Animal Technician Service and Dr. Alzbeta Talarovicova (Barts Cancer Institute, Queen Mary University of London, London, United Kingdom) for their assistance during the orthotopic surgery. We would also like to thank Dr. Jennifer Morton (Beatson Institute for Cancer Research, Glasgow, United Kingdom) for her guidance in surgical technique, Dr. Cristina Ghirelli (Barts Cancer Institute, Queen Mary University of London, London, United Kingdom) for her advice on tumor digestion and Dr. Fabienne McClanahan (Barts Cancer Institute, Queen Mary University of London, London, United Kingdom) for her advice regarding ex vivo T cell stimulation protocols. We also would like to thank the Medical Research Council (MRC), Pancreatic Cancer Research Fund (PCFR) and Ovarian Cancer Action who funded this research.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Siegel, R. L., Miller, K. D., Jemal, A. Cancer statistics, 2018. *CA: A Cancer Journal for Clinicians*. **68**(1), 7–30 (2018).

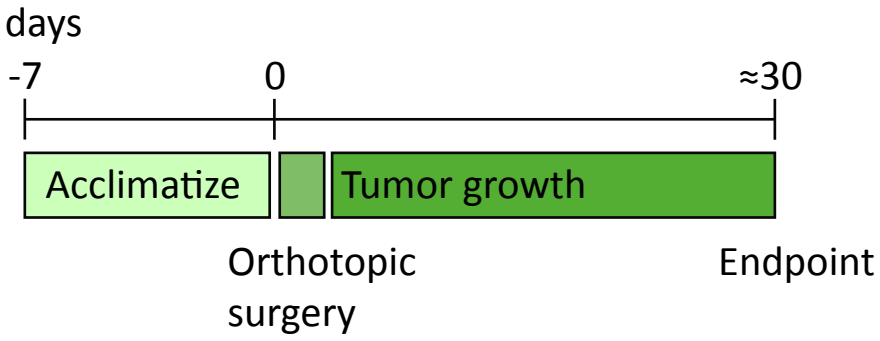
2. Conroy, T. et al. Current standards and new innovative approaches for treatment of pancreatic cancer. *European Journal of Cancer*. **57**, 10–22 (2016).
3. Lee, J. W., Komar, C. A., Bengsch, F., Graham, K., Beatty, G. L. Genetically engineered mouse models of pancreatic cancer: The KPC model (LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre), its variants, and their application in immuno-oncology drug discovery. *Current Protocols in Pharmacology*. **2016**, 14.39.1-14.39.20 (2016).
4. Tseng, W. W. et al. Development of an Orthotopic Model of Invasive Pancreatic Cancer in an Immunocompetent Murine Host. *Clinical Cancer Research*. **16** (14), 3684–3695 (2010).
5. Majumder, K.et al. A Novel Immunocompetent Mouse Model of Pancreatic Cancer with Robust Stroma: a Valuable Tool for Preclinical Evaluation of New Therapies. *Journal of Gastrointestinal Surgery*. **20** (1), 53–65 (2016).
6. Hingorani, S. R. et al. Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. **7** (5), 469–483 (2005).
7. Herreros-Villanueva, M., Hijona, E., Cosme, A., Bujanda, L. Mouse models of pancreatic cancer. *World Journal of Gastroenterology*. **18** (12)1286–94 (2012).
8. Witkiewicz, A. K. et al. Integrated Patient-Derived Models Delineate Individualized Therapeutic Vulnerabilities of Pancreatic Cancer. *Cell Reports* (2016).
9. Nicolle, R. et al. Pancreatic Adenocarcinoma Therapeutic Targets Revealed by Tumor-Stroma Cross-Talk Analyses in Patient-Derived Xenografts. *Cell Reports* . (2017).
10. Spear, S. et al. Discrepancies in the Tumor Microenvironment of Spontaneous and Orthotopic Murine Models of Pancreatic Cancer Uncover a New Immunostimulatory Phenotype for B Cells. *Frontiers in Immunology*. **10**, 542 (2019).
11. Zhu, Y. et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Research* (2014).
12. Lee, J. J., Huang, J., England, C. G., McNally, L. R., Frieboes, H. B. Predictive Modeling of In vivo Response to Gemcitabine in Pancreatic Cancer. *PLoS Computational Biology* (2013).
13. Clark, C. E. et al. Dynamics of the Immune Reaction to Pancreatic Cancer from Inception to Invasion. *Cancer Research*. **67** (19), 9518–9527 (2007).
14. Fukunaga, A. et al. CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. *Pancreas*. **28** (1), e26-31 (2004).
15. Tewari, N. et al. The presence of tumor-associated lymphocytes confers a good prognosis in pancreatic ductal adenocarcinoma: an immunohistochemical study of tissue microarrays. *BMC Cancer*. **13** (1), 436 (2013).
16. Royal, R. E. et al. Phase 2 Trial of Single Agent Ipilimumab (Anti-CTLA-4) for Locally Advanced or Metastatic Pancreatic Adenocarcinoma. *Journal of Immunotherapy*. **33** (8), 828–833 (2010).
17. Brahmer, J. R. et al. Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. *New England Journal of Medicine*. **366** (26), 2455–2465 (2012).
18. Winograd, R. et al. Induction of T-cell Immunity Overcomes Complete Resistance to PD-1 and CTLA-4 Blockade and Improves Survival in Pancreatic Carcinoma. *Cancer Immunology Research*. **3** (4), 399–411 (2015).

19. Beatty, G. L. et al. CD40 Agonists Alter Tumor Stroma and Show Efficacy Against Pancreatic Carcinoma in Mice and Humans. *Science*. **331** (6024), 1612–1616 (2011).
20. Lutz, E. R. et al. Immunotherapy converts nonimmunogenic pancreatic tumors into immunogenic foci of immune regulation. *Cancer Immunology Research*. **2** (7), 616–31 (2014).
21. Barry, M., Bleackley, R. C. Cytotoxic T lymphocytes: all roads lead to death. *Nature Reviews. Immunology*. **2** (6), 401–9 (2002).
22. Mojic, M., Takeda, K., Hayakawa, Y. The dark side of IFN-γ: Its role in promoting cancer immunoevasion. *International Journal of Molecular Sciences* (2018).
23. Castro, F., Cardoso, A. P., Gonçalves, R. M., Serre, K., Oliveira, M. J. Interferon-gamma at the crossroads of tumor immune surveillance or evasion. *Frontiers in Immunology* (2018).
24. Thiery, J. et al. Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells. *Nature Immunology* (2011).
25. Betts, M. R. et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *Journal of Immunological Methods*. **281** (1–2), 65–78 (2003).
26. Schuerwegh, A. J., De Clerck, L. S., Bridts, C. H., Stevens, W. J. Comparison of intracellular cytokine production with extracellular cytokine levels using two flow cytometric techniques. *Cytometry*. **55B** (1), 52–58 (2003).
27. Partecke, L. I. et al. A syngeneic orthotopic murine model of pancreatic adenocarcinoma in the C57/BL6 mouse using the panc02 and 6606PDA cell lines. *European Surgical Research* (2011).
28. An, X. et al. Immunophenotyping of Orthotopic Homograft (Syngeneic) of Murine Primary KPC Pancreatic Ductal Adenocarcinoma by Flow Cytometry. *Journal of Visualized Experiments*. (140) (2018).
29. Jiang, Y. J. et al. Establishment of an orthotopic pancreatic cancer mouse model: Cells suspended and injected in Matrigel. *World Journal of Gastroenterology* (2014).
30. Autengruber, A., Gereke, M., Hansen, G., Hennig, C., Bruder, D. Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. *European Journal of Microbiology & Immunology*. **2** (2), 112–20 (2012).
31. Trapecar, M. et al. An Optimized and Validated Method for Isolation and Characterization of Lymphocytes from HIV+ Human Gut Biopsies. *AIDS Research and Human Retroviruses* (2017).
32. Liu, Q. et al. Effects of enzymatic digestion, cell culture and preservation conditions on surface CD62L expression of primary murine CD3+CD4+T cells. *Biomedical Research (India)* . **29** (10), 2153–2159 (2018).
33. Seaman, S. A., Tannan, S. C., Cao, Y., Peirce, S. M., Lin, K. Y. Differential effects of processing time and duration of collagenase digestion on human and murine fat grafts. *Plastic and Reconstructive Surgery*. (2015).
34. Huch, M. et al. Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *EMBO Journal* (2013).
35. Boj, S. F. et al. Organoid models of human and mouse ductal pancreatic cancer. *Cell* (2015).
36. Misra, S. et al. Ex vivo organotypic culture system of precision-cut slices of human pancreatic ductal adenocarcinoma. *Scientific Reports* (2019).
37. Moran, A. E., Polesso, F., Weinberg, A. D. Immunotherapy Expands and Maintains the

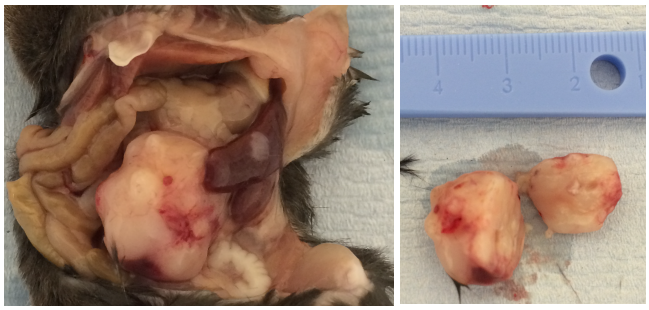
Function of High-Affinity Tumor-Infiltrating CD8 T Cells In Situ. *The Journal of Immunology* (2016).
38. Stromnes, I. M. et al. T Cells Engineered against a Native Antigen Can Surmount Immunologic and Physical Barriers to Treat Pancreatic Ductal Adenocarcinoma. *Cancer Cell* (2015).
39. Singh, M. et al. High-throughput targeted long-read single cell sequencing reveals the clonal and transcriptional landscape of lymphocytes. *Nature Communications*. **10** (1), 3120 (2019).
40. Jiang, N., Schonnesen, A. A., Ma, K.-Y. Opinion Ushering in Integrated T Cell Repertoire Profiling in Cancer. *Trends in Cancer*. **5**, 85–94 (2019).
41. Schietinger, A. et al. Tumor-Specific T Cell Dysfunction Is a Dynamic Antigen-Driven Differentiation Program Initiated Early during Tumorigenesis. *Immunity*. **45** (2), 389–401 (2016).
42. Raghav, S. K. et al. Exhaustion of tumor-specific CD8+ T cells in metastases from melanoma patients. *Journal of Clinical Investigation* (2011).
43. Gros, A. et al. PD-1 identifies the patient-specific in filtrating human tumors. *The Journal of Clinical Investigation* (2014).
44. Miller, B. C. et al. Subsets of exhausted CD8 + T cells differentially mediate tumor control and respond to checkpoint blockade. *Nature Immunology* (2019).

Figure 1

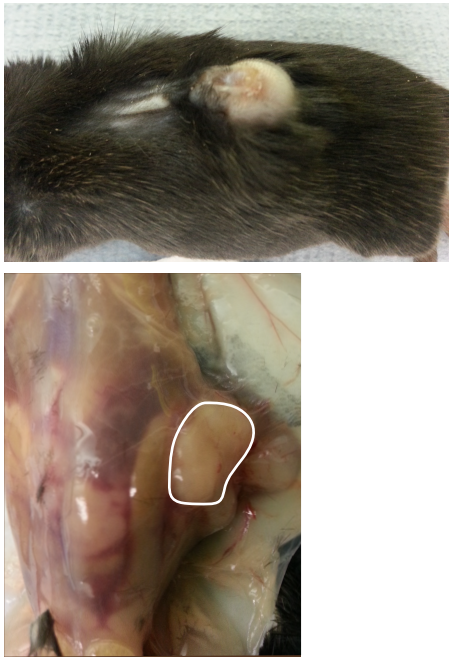
A



B Typical orthotopic pancreatic tumor



C Evidence of Matrigel Leakage



D Expected tumor weights

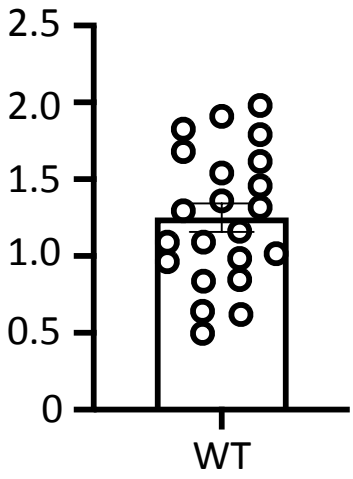


Figure 2 revised

Click here to access/download;Figure;Figure 2_revised.ai

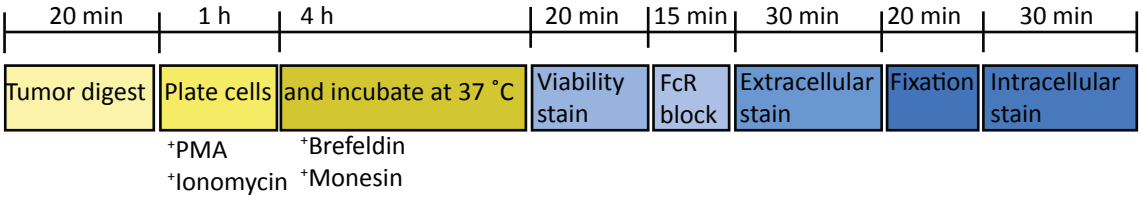
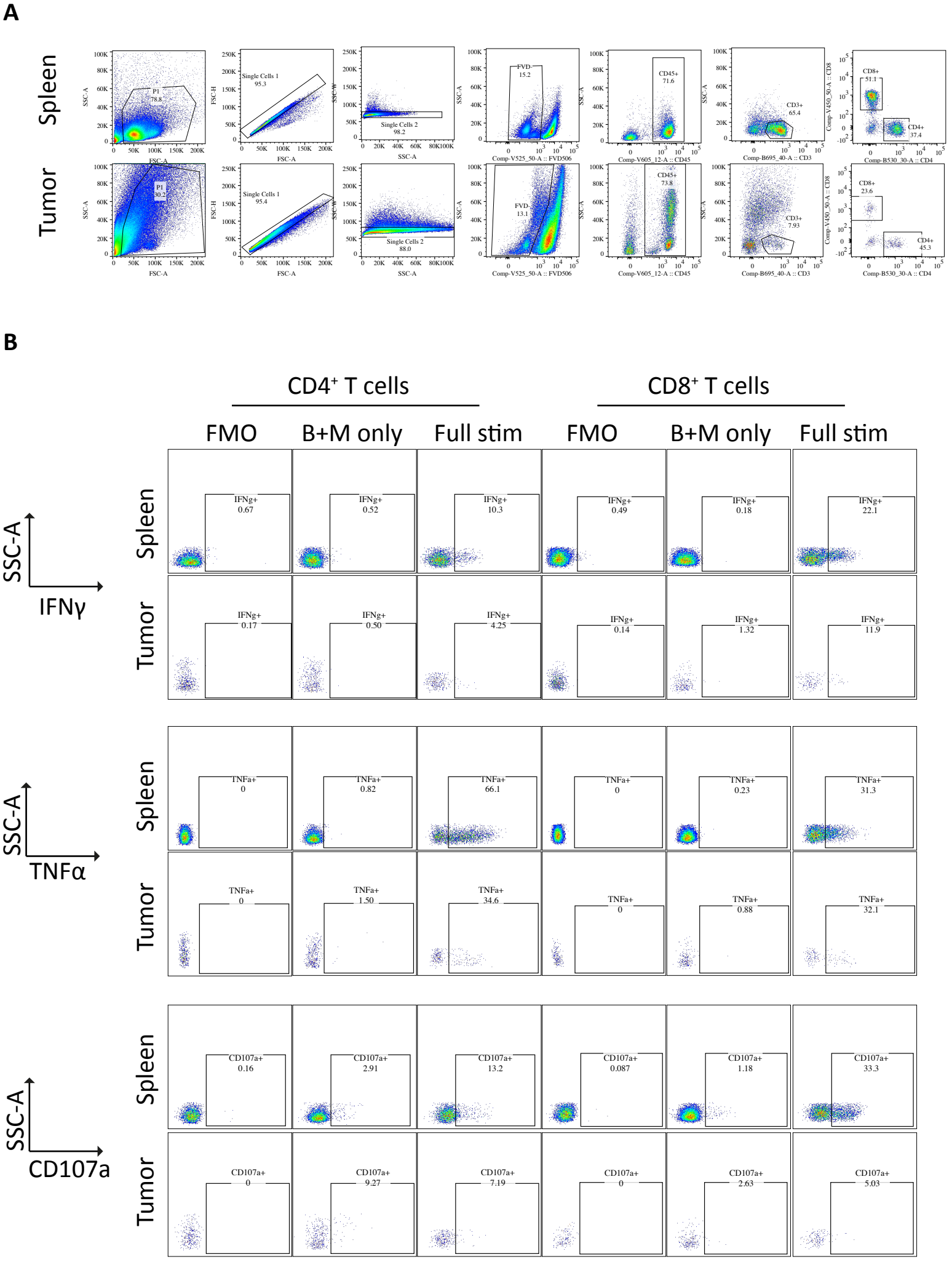
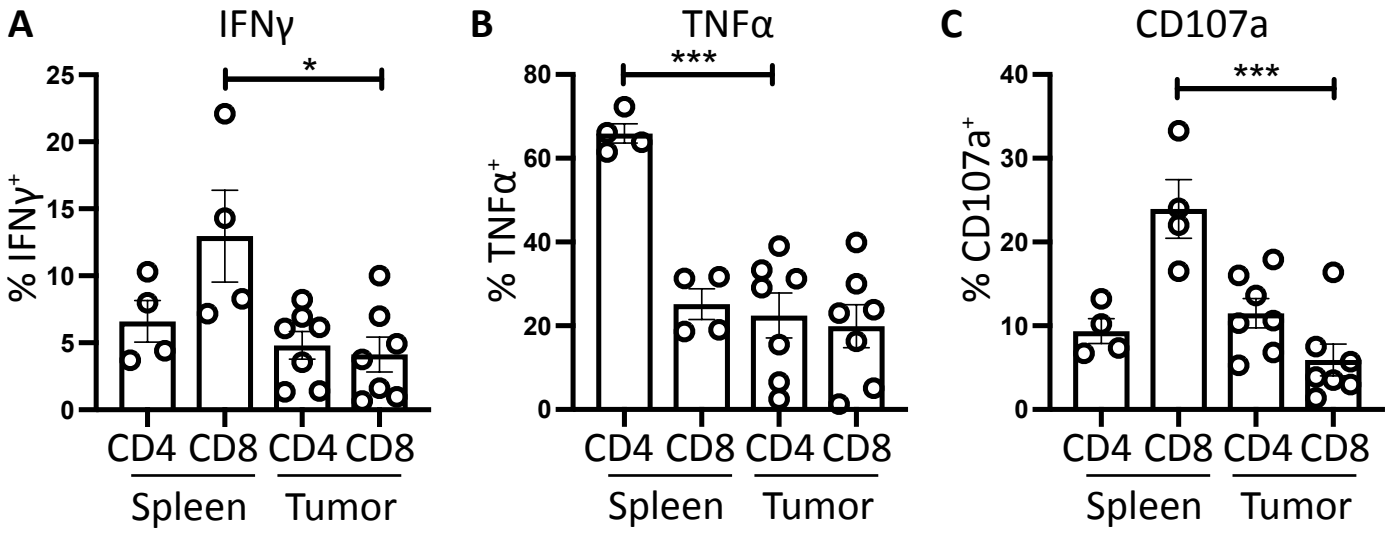


Figure 3 revised

Click here to access/download;Figure;Figure 3_revised.ai





Name of Material/ Equipment	Company	Catalog Number
6/0 gauge coated vicryl absorbable sutures	Ethicon	W9500T
70 µm pore-size cell strainer	Fisher Scientific	11597522
9 mm Clay Adams clips	VetTech Solutions	IN015A
anti-CD107a PE (clone 1D4B)	Biolegend	121612
anti-CD16/CD32	BD Biosciences	553142
anti-CD3 PerCP eFluor710 (clone 17A2)	Biolegend	46-0032
anti-CD4 FITC (clone GK1.5)	eBioscience	11-0041
anti-CD45 Brilliant Violet 605 (clone 30-F11)	Biolegend	103140
anti-CD8 Brilliant Violet 421 (clone 53-6.7)	Biolegend	100738
anti-IFN-gamma PE/Cy7 (clone XMG1.2)	Biolegend	505826
anti-TNF-alpha Alexa Fluor 647 (clone MP6-X)	Biolegend	506314
BD Matrigel Basement Membrane Matrix High Concentration	BD Biosciences	354248
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A4503
Cell Stimulation Cocktail (500x) (phorbol 12-myristate 13-acetate (PMA) and ionomycin)	eBioscience	00-4970-03
Clay Adams Autoclip Applier	VetTech Solutions	IN015B
Clay Adams Autoclip remover	VetTech Solutions	IN015B
Collagenase Type V from *Clostridium histolyticum*	Sigma-Aldrich	C9263
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	D2650-100mL
DMEM High glucose (4.5 g/L) with L-Glutamine	PAA	E15-810
DNase (Deoxyribonuclease I from bovine pancreas Type II-S) stock 10 mg/mL in 0.15 M N	Sigma-Aldrich	D4513
Fixable Viability Dye 506 (FVD506)	eBioscience	65-0866
Foetal calf-serum (FCS)	GE Healthcare	A15-104
Hamilton syringe 700 series, 25 µL volume, 22s gauge needle bevel tip	Fisher Scientific	10100332
Intracellular Fixation buffer and Intracellular Permeabilisation Buffer	eBioscience	88-8824-00
Penicillin/streptomycin	PAA	15140122
Protein transport inhibitor cocktail (500x) (brefeldin A and monesin)	eBioscience	00-4980-03

RPMI-1640 (containing 0.3 g/L Glutamine)	Sigma-Aldrich	R8758
Surgical Scalpel Blade No.10	Swann-Morton	0501
Trypsin-EDTA Solution 10x	Sigma-Aldrich	594-18C
U-bottomed 96 microwell plate	VWR	734-2080
Universal Cotton Tipped Applicators - 6 inch x 100	Medisave	UN982
V-bottomed 96 microwell plate	VWR	735-0184

Comments/Description

1:100 to culture media

Use at final dilution 1:200
Use at final dilution 1:50
Use at final dilution 1:100
Use at final dilution 1:200
Use at final dilution 1:100
Use at final dilution 1:50
Use at final dilution 1:50

Aliquot on ice and store in -20 °C

1x Final concentration PMA 0.081 µM, ionomycin 1.34 µM

2 mg/mL in media

Final concentration in digestion media 0.025 mg/mL
Use at 1:200 in PBS
10% in RPMI

Dilute permeabilisation buffer to 1x in H_2O
100 units/mL Penicillin, 100 µg/mL Streptomycin
1x Final concentration Brefeldin A 10.6 µM, monensin 2 µM

Trypsin (0.1%) EDTA (0.4%) final concentration

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript for any spelling or grammar issues.

2. Please print and sign the attached UK Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

We have signed this document and uploaded it to the account.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Matrigel, Falcon

We have substituted “Matrigel” for “basement membrane” and “falcon” for “tube”. No other commercial products were referenced in the text to our knowledge.

Protocol:
1. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:
1. 1.2: Where do these cells come from?

We have added “provided as a generous gift by the lab of David Tuveson.”

2. 3.1: How is the pancreatic tumor dissected?

We have added the following dissection step to our protocol, so it precedes the section being filmed.

2.3.7 At endpoint the mouse is culled via cervical dislocation followed by decapitation. The skin and peritoneal cavity are opened using scissors and the pancreas tumor excised using forceps to hold the tumor, and scissors to remove surrounding tissue.

Figures:
1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

Data used in this JoVE manuscript also appears in our previous publication, in Frontiers in Immunology. Frontiers in Immunology allows reprints and the editorial policy has been uploaded into our Editorial Manager account.

A link to Frontiers in Immmunology copyright policy is also provided here:
https://www.frontiersin.org/legal/copyright-statement

2. Please remove 'Figure 1' etc. from the figures themselves.

The titles have been removed from the figures.

3. Figure 2: Please use 'h' and 'min' for units of time.

This has been corrected in the figures, legends and text.

References:
1. Currently parentheses around issue numbers are inverted; please correct.

This has been corrected.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have added missing materials to the Table of Materials.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is a very useful protocol, not duplicative of any currently on JOVE. My lab performs these kinds of experiments in a very similar fashion, so I think this is a good example of standard protocol for orthotopic pancreatic tumor injections and analysis of immune infiltrates. The introduction and discussion are well written.

We thank the reviewer for their kind feedback and are glad this protocol will prove useful.

Major Concerns:
none

Minor Concerns:
Line 39: Delete cell number (1000 cells) from this line as it is confusing to the flow of the sentence. The number of cells injected is dependent on the cell line used and the rate of growth desired. Discussion section currently highlights 1000 cells as a critical number - please indicate that this is cell line and user dependent, so first time users may want to try several cell numbers ranging 1000-100,000.

We have deleted the cell number from line 39. This is an important point we had overlooked, we have now edited the discussion accordingly to highlight that cell-line/user dependent optimisation is important, on lines 636-638.

Lines 200-214: If the Matrigel is already aliquoted, why do you need to transfer it with pipette tips? We have pre-aliquoted Matrigel frozen in Eppendorf tubes and add the cell suspension directly to the Matrigel to minimize pipetting.

We also pre-aliquot Matrigel in Eppendorf tubes to minimize pipetting and agree this is an important step that we have not explained sufficiently. To make this clearer we have added a sentence at line 183, line 217 and 223 to highlight this.

Note on Matrigel: The ratio of Matrigel required for solidification varies from batch to batch. Please add a note that Matrigel is not a defined substance and that each lot must be individually tested. People understand this for fetal calf serum, but I think it is less well known for Matrigel. Maybe make a section just about Matrigel and move the instructions on proper pipetting to this section as well.

This is a useful point and we have added text at line 180 to highlight that Matrigel batches can vary and must be tested.

Line 249: You use a scalpel, not scissors, to cut open the skin?

Yes, we use disposable scalpels as they guarantee blade sharpness and it means we can replace the scalpel for each mouse, ensuring sterility. We have not experienced any problems with this method, however scissors would certainly provide an alternative so we have added them as such on line 274.

Line 259: Why are you warming the syringe? Is the intention to solidify the Matrigel prior to transfer? We transfer 20uL of Matrigel/cell solution using insulin gauge needles. Syringes are kept on ice and minimally handled at room temperature.

We very briefly place it on the heat mat to start the process of solidification, so that when injected it becomes solid as quickly as possible and thus minimises leakage. We have added some sentences to explain this clearer and highlight the fact that it is a very brief warming at line 287.

Line 293: Best to specify an endpoint or add humane monitoring criteria here.

We agree, this is a useful point which we have missed. As humane endpoints and monitoring criteria apply to the entire *in vivo* experiment, we have chosen to now add this information at the start of the protocol, line 168.

Line 381: Cell-cell contact is not required for PMA/ION stimulation. Please delete this note.

Thank-you for highlighting this mistake, we have deleted this sentence from line 423.

Line 497: Cell surface expression of CD107a means that the cell degranulated and is used as a surrogate for cytotoxicity. It's a bit semantic, but please do not directly call CD107a expression cytotoxicity, especially in the case of CD4 T cells.

We agree that the sentence was not accurate and amended the text at line 543:

Finally, CD107a is an endosomal marker that is expressed transiently on the cell surface during the exocytosis of cytotoxic granules and cytokines, *as such, it is used as a surrogate marker for cytotoxicity.*

And line 548: *splenic CD8+ had a greater rate of degranulation*

Note on PMA/ION: Best to explain that you are measuring the maximum capacity for cytokine production that those cells could be doing. It does not mean that the CD4 and CD8 T cells in the tumors are actually making those cytokines. You could perform the same protocol without PMA/ION and hope to capture endogenously stimulated cytokine production, but this of course is much lower. Lots of people use PMA/ION so I think it is fine to present this protocol, so long as this caveat is explained. Line 609 seems a good place to insert this comment.

Thank-you for this useful comment, we have added a comment about this caveat at line 667.

PMA/ionomycin stimulation reflects the maximal capacity of T cells to produce cytokines, which might or might not be produced by the T cells within the tumor microenvironment. Endogenous production can be measured without the need for stimulation; however, levels may be far lower or undetectable.

Line 627: "Alternatively, where the antigen is unknown, quantification of antigen-specific T cell responses can be performed by bulk-TCR sequencing, or more recently single-cell TCR sequencing". Change to "Alternatively, where the antigen is unknown, quantification of T cell clonal expansion can be performed by bulk-TCR sequencing, or more recently

single-cell TCR sequencing". Determining antigen specificity from TCR sequences is still a difficult endeavor.

We have changed this sentence as suggested at line 685.

Please cite PMID: 27248578, which is the authoritative reference comparing spontaneous KPC tumors to orthotopic and SQ versions.

We thank the reviewer for this useful suggestion and have added this reference in the Introduction (line 68) and in the discussion (line 627).

Reviewer #2:
Manuscript Summary:
The manuscript describes methods to generate pancreatic orthotopic tumors in immunocompetent mice, their isolation, the generation of cell suspensions and the flow-cytometry analyses of T cell present within the tumor mass.

Major Concerns:
There are no major concern regarding the techniques described and approach taken.

We thank the reviewer for this feedback.

Minor Concerns:
The authors should consider the following issues (no particular order):
1- text: replace "derivatives" with "in vivo PDAC models" p3 L79

We have added the sentence at line 78: *Additional KPC and other in vivo PDAC models are reviewed here[7].*

2- Define/list the volume (final of solution prepared for injection (500ul Matrigel + 300 ul PBS + 200 ul Cells)

We hope we understood this comment correctly and have hopefully clarified the sentence on line 218 to read:

1.3.1. The ratio of the final solution of basement membrane, PBS and tumor cells in PBS prepared for injection is 5:3:2. Therefore to a 500 µl aliquot of basement membrane add 300 µl of pre-chilled PBS using a pre-chilled 1000 µl pipette tip.

3- Confirm the volume used (5ul) and add the use of Hamilton syringe in the text. Provide an alternate option of using larger volume up to 20-40 ul and the potential benefit in limiting technical challenge associated with low injected volume (dead space, precision...)

We have added "5 µl" into line 298 to clarify this is the volume injected, and also added to that we used a glass syringe on line 284. In line with JoVE's requirements, we have limited the use of commercial language in the text, however the Hamilton syringe is detailed in the

methods section. The Hamilton syringe does allow low volumes, in this case up to 25 µl to be precisely measured with minimal dead volume. We cannot ascertain if injecting larger volumes would produce the same effect, however we have added a sentence that suggest users can of course optimise this, added at line 303.

4- Discuss the site of the injection in the pancreas

We have included an additional sentence to line 295 to highlight our choice of injection site.

5- Address the collagenase quality, and activity as it will affects the quality and speed of the digestion

We do not have specific information regarding the collagenase quality but we do have information regarding the collagenase activity which we have added to the text at line 347, which will allow users to compare it to other collagenases.

6- Nylon filter can be replaced with 70um strainer (Falcon)

We agree this suggestion is clearer and have changed the text to state *70 µm strainer*. However, we have now omitted the use of Falcon from the main text to comply with JoVE's requirements on commercial language.

7- Reference/justify the final concentration for PMA, Oimycin, Brefeldin, Monensin

These concentrations are recommended by the manufacturer and we have added a sentence to explain this at line 426 and line 435.

8- The authors may want to discuss the adaptation of this protocol to adherent immune cells (e.g., macrophages)

We have not performed *ex vivo* stimulation of adherent macrophages from orthotopic tumours, however we have modified a sentence in the discussion to expand on the idea that this protocol can be adapted for different applications on line 663.

Reviewer #3:
Journal of Visualized Experiments
Generation of orthotopic pancreatic tumors and ex vivocharacterization of tumorinfiltrating T cell cytotoxicity.

Sarah Spear*1,2, Iain A McNeish1 and Melania Capasso1,3
1 Division of Cancer, Department of Surgery and Cancer, Imperial College London, London, United Kingdom.
2 Centre for Cancer and Inflammation, Barts Cancer Institute, Queen Mary University of

London, London, United Kingdom.
3 German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany.

General comments

*** The authors describe the methodology for orthotopic implantation of a KPC derived cell line into the pancreas and the ex vivo disaggregation of the tumours to stimulate T cells ex vivo to profile cytokine production by flow analysis. The methods for pancreatic orthotopic model development (KPC) have been previously published, inclusive of this journal (An, X., Ouyang, X., Zhang, H., Li, T., Huang, Y.y., Li, Z., Zhou, D., Li, Q.X. Immunophenotyping of Orthotopic Homograft (Syngeneic) of Murine Primary KPC Pancreatic Ductal Adenocarcinoma by Flow Cytometry. J. Vis. Exp. (140), e57460, doi:10.3791/57460 (2018)).**

Author should cite it and comment on difference and justifies scientific values of this new paper. Furthermore, it would be more desirable to provide comparison with the convention subcutaneous model of the same tumor biologically.

We agree with the reviewer, multiple publications describing the *in vivo* orthotopic model have been published, many of which we reference in our text. We thank the reviewer for highlighting this additional publication, which we have now added to the discussion (line 627). The above-mentioned article describes the orthotopic and subcutaneous generation of tumours using implantation of KPC-derived tumour explants in the pancreas or under the skin respectively. In this publication we describe the injection of a KPC-derived cell line, resuspended in Matrigel injected directly into the pancreas, thus the methodology for tumour generation is different. Furthermore, while the above publication describes the digestion of orthotopic tumours for flow cytometry, we expand on this protocol and detail specifically the *ex vivo* stimulation of T cells, isolated from orthotopic tumours, in order to characterise their cytokine production.

Since we have not used a subcutaneous model in our immunological studies, we feel we would not be qualified to describe experiments comparing the two models. We have now highlighted two references (ref 3 and ref 28) which have compared the orthotopic and subcutaneous models within the text at line 68 and 627.

*** Many tumor dissociation methods have been described. It would be meaningful to compare with, or also at least comment on its differences (pro- and con) from the other methods published by others, to claim optimisation for immune cells.**

We agree many dissociation methods have been described and in our discussion we highlight other publications that use alternative methods for digestion when the focus in on extracting healthy pancreatic ducts (ref 34), epithelial cells (ref 35) or preserving structure (ref 36). We also reference other publications that have described the harmful effects of other digestive enzymes (e.g. dispase) on immune cell surface markers, explaining our choice of collagenase as well as publications that suggest a limited digestion time can limit cell death (lines 649-660).

*** The authors should clearly specify whether rapidly test novel therapeutics and impact on cytokine production as measured by ex vivo assay described is novel, not published before, or provide citation of other's similar work. if indeed novel, the focus of paper should be emphasized and therefore the title and abstract should reflect that .**

We have previously used this protocol to analyse *ex vivo* stimulated T cells in another publication, which we reference (ref 10) and many additional publications exist that utilise the orthotopic model. As we understand in order to publish in JoVE the methodology is not required to be novel, and the emphasis is on providing a detailed methodology that can be filmed to allow other scientists to learn from it.

We agree, the concluding sentence of the discussion did not reflect the methodology described and we have modified it to the following (line 690): *Overall, the orthotopic model provides a useful platform to rapidly test therapeutic strategies, in particular that may modulate the antitumoral T-cell response, that can be then validated on a smaller cohort of transgenic, KPC, mice.*

Specific comments

*** Methodology for implantation of cells into the pancreas to generate orthotopic models described in sections 1-2 of the protocol has previously been published in detail - although these refer to xenograft models the cell harvesting and laparotomy procedure is similar.**
¬ Moreno, J.A., Sanchez, A., Hoffman, R.M., Nur, S., Lambros, M.P. Fluorescent Orthotopic Mouse Model of Pancreatic Cancer. J. Vis. Exp. (115), e54337, doi:10.3791/54337 (2016).
¬ Chai, M.G., Kim-Fuchs, C., Angst, E., Sloan, E.K. Bioluminescent Orthotopic Model of Pancreatic Cancer Progression. J. Vis. Exp. (76), e50395, doi:10.3791/50395 (2013).

We agree with the reviewer, the orthotopic model is extensively used and many publications describe the methodology. The references above provide useful methodology for generating orthotopic tumours, however both protocols inject a larger number and volume of cells; Chai *et al.* inject 0.4 x10^6 in 20 µl and Moreno *et al.* inject 3 x10^6 cell in 50 µl.

In this protocol we suggest a refinement by injecting a lower number of cells in only 5 µl Matrigel to prevent cell leakage. However, the main benefit of detailing the surgical procedure is to provide the background methodology performed prior to isolating the syngeneic tumours for *ex vivo* T cell analysis. Indeed, as highlighted, it is the tumour digestion and T cell stimulation which we have suggested would form the video component of this JoVE publication.

*** Spear et al here describe the establishment of a syngeneic model (homograft) using a cell line derived from KPC model - Protocol step 1.2 needs to be corrected to C57BL/6 not C56BL/6.**

We thank the reviewer for finding this mistake and we have now corrected it accordingly.

*** The laparotomy surgical procedure needs some additional information about how the authors confirm the mouse is fully anaesthetised, what needle gauge is used, humane endpoints for this specific model or clinical symptoms other than palpation, as well as what metastatic site incidence is expected using this method described.**

We agree, information regarding the anaesthesia has now been added on line 261.

We have also included an expanded paragraph detailing the humane endpoint used in this study at the start of the protocol, line 168.

The needle gauge is provided in the methods table.

*** Imaging is a refinement for orthotopic modelling especially when cell lines can be easily engineered as in Moreno et al or Chai et al with bioluminescence or fluorescence enabling optical imaging - imaging may not be available for different reason therefore more details on humane endpoints.**

As above, now added on line 168.

*** Digestion of pancreatic tumors is described in section 3 and single cell suspension in section 4 by the authors however method for pancreatic tumour disaggregation has been previously published.**
*** An, X., Ouyang, X., Zhang, H., Li, T., Huang, Y.y., Li, Z., Zhou, D., Li, Q.X. Immunophenotyping of Orthotopic Homograft (Syngeneic) of Murine Primary KPC Pancreatic Ductal Adenocarcinoma by Flow Cytometry. J. Vis. Exp. (140), e57460, doi:10.3791/57460 (2018).**

We agree, similar publications exist for the digestion of pancreatic tumours, however we have included it in this protocol to help users understand how we digested the tumour prior to *ex vivo* T cell stimulation.

*** Preparing cells for ex vivo stimulation in section 5 for flow analysis using Brefeldin A and Monensin is described but the advantage of flow over ELISA methodology is not explained which would assist researchers in selecting whether this step is needed.**

We highlighted in the discussion that quantification of cytokines using intracellular flow cytometry allows you to determine exactly which cell type was responsible for the cytokine production, whereas ELISA methodology provides you with the total cytokine production in a tumour, lines 675-679.

Reviewer #4:
Manuscript Summary:
The manuscript by Spear et al. describes a protocol for orthotopic injection of syngeneic

pancreatic cancer cells and subsequent analysis of intratumoral T cell activity. The protocol is reasonably clear, but some of the experimental details and discussion could be improved.

We thank the reviewer for their feedback, and we have addressed the points raised.

Major Concerns:

1. One major concern is the number of cells used for injection is quite low, and so is the amount of Matrigel/PBS solution that is being injected. Potential for missing injection or under/over estimating tumor cell number that was injected into each mouse is quite large. The authors need to discuss this pitfall, especially when it comes to being able to determine tumor size differences between experimental conditions. Another potential caveat is that for the number of cells injected, these tumors seem to grow extremely quickly, and are likely very aggressive. While the authors mention cell type dependency, they need to be more descriptive as to how this quick tumor growth, which is distinct from other published models injecting tens of thousands of cells, might result in potentially different tumor microenvironment composition.

We suggest that the low injection volume reduces the likelihood that Matrigel will leak and thus provides a more reliable methodology for orthotopic injection. However, as the cell number is low we do acknowledge that there is a margin of error, and that possible batch effects may occur. For this reason, we suggest always performing experimental controls with each batch (lines 639-641). Regarding the fast nature of orthotopic tumour growth, we do find other publications report a similar time frame, however there are undoubtably impacts on the tumour microenvironment. We hope the modified text (from line 623) in the discussion better highlights this caveat. We have also added additional text at line 636 to highlight how the growth and time to endpoint of orthotopic tumours will most likely be dependent on cell line used and number of cells injected.

2. Authors should comment on their decision to forgo Ficoll or similar bulk immune cell isolation method during tumor processing. Similarly, there is no use of dead cell removal kit, which would allow for immune cells to avoid being incubated with a lot of inflammatory cell death debri during cytokine detection assays. Please discuss.

We agree, immune cell isolation is a refinement of this protocol and certainly dead-cell removal would be advantageous. In this protocol our priority was to allow analysis of large numbers of tumour samples harvested simultaneously in a reasonable time frame, with minimal manipulation. Additional steps of purification would be challenging to perform with large cohorts of samples. However, as these suggestions may be useful for other users of the protocol we have included them from line 411: *At this point the sample can be further purified by bead-based dead-cell removal or immune cell enrichment assays if required.*

3. Figure 4: one should not compare cytokine production between CD4 and CD8 T cells, it is like comparing apples and oranges. Subsets can be compared to each other, but not otherwise.

Thank-you for this feedback, we have amended the comparisons accordingly in the results section and deleted the statistical significance comparisons for CD4 to CD8 in the figures. As we now only compare CD4/CD4 and CD8/CD8 we have also changed the statistic test to the more appropriate unpaired t-test.

Minor Concerns:

1. The authors cite general metastatic dissemination potential of orthotopically injected pancreatic cell lines, but it is not clear from Figures that the cell line being used is actually metastatic. Please clarify.

We agree, we were not clear on this point. The cell line we use is metastatic and we have observed metastasis on the liver, mesentery and diaphragm. However, as we have not recorded the exact incidence for all our mice we are not able to put an exact number on this. We have amended the text to clarify our findings at line 73.

Importantly, the orthotopic model provides the cognate microenvironment for tumor cell growth and metastasizes in our hands and others[4] to clinically relevant sites e.g. liver, making it more clinically relevant than the subcutaneous or chemically-induced models.

2**. Please provide information on sutures that are used for the body wall and skin, including if they are absorbable or not.**

The details regarding the sutures are provided in the methods table and we have added that the are absorbable for clarification.

3. What do the authors suggest in case of Matrigel leakage and body wall tumor formation - are these tumors normally included in studies?

This is a useful point and we were not clear about this. We suggest this protocol minimises the likelihood of Matrigel leakage and the image in Figure 1 is shown only to demonstrate to a new user the result of leakage. We would suggest these mice should be eliminated from the study, although surgical skill should reduce the incidence. To make this clear we added a sentence to the results section at line 506.

Frontiers Copyright Statement

All content included on Frontiers websites (including Loop), such as text, graphics, logos, button icons, images, video/audio clips, downloads, data compilations and software, is the property of Frontiers if created by Frontiers, or of the person or entity who or which owned it prior to submission to Frontiers. If not owned by Frontiers, it is licensed to Frontiers Media SA (Frontiers) or its licensees and/or subcontractors.

The copyright in the text of individual articles (including research articles, opinion articles, book reviews, conference proceedings and abstracts) is not the property of Frontiers, and its ownership is not affected by its submission to or publication by Frontiers. Frontiers benefits from a general licence over all content submitted to it, and both Frontiers and its users benefit from a **Creative Commons CC-BY licence** over all content, as specified below.

Images and graphics not forming part of user-contributed materials are the property of or are licensed to Frontiers may not be downloaded or copied without Frontiers' explicit and specific permission or in accordance with any specific copyright notice attached to that material.

The combination of all content on Frontiers websites, as well as the design and the look and feel of the Frontiers websites, and the copyright and all other rights in such content and combination, are the sole property of Frontiers.

As an author or contributor you grant permission to others to reproduce your articles, **including any graphics and third-party materials supplied by you**, in accordance with the **Frontiers Terms and Conditions**. The licence granted to third parties over all contents of each article, **including third-party elements**, is a Creative Commons Attribution ("CC BY") licence. The current version is **CC-BY, version 4.0**, and the licence will automatically be updated as and when updated by the Creative Commons organisation.

You may include a requirement to reproduce copyright notices in materials contributed by you, but you may not restrict the right to reproduce the entire article, including third-party graphics. This means that you must obtain any necessary third-party consents and permissions to reproduce third-party materials in your articles submitted to Frontiers.

E-books are subject to the same licensing conditions as the articles within them.

Articles published prior to the effective date of this notice: Please note that reproduction of third-party graphics and other third-party materials contained in articles published prior to the effective date of this notice may be subject to third-party notices prohibiting their reproduction without permission. You must comply with those notices.

Articles published prior to July 2012: The licence granted for these articles may be different and you should check the pdf version of any article to establish what licence was granted. If an article, dating from before July 2012, carries only a non-commercial

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article:	Generation of orthotopic pancreatic tumors and ex vivo characterization of tumor-infiltrating T cell cytotoxicity
Author(s):	Sarah Spear, Iain McNeish and Melania Capasso

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode ; "**CRC NonCommercial License**" means the Creative Commons Attribution-NonCommercial 3.0 Agreement (also known as CC-BY-NC), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License. If the "Standard Access" box

has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC NonCommercial License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties**. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

discretion andwithout giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contaminationdue to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Sarah Spear
Department:	Surgery and Cancer
Institution:	Imperial College London, London, United Kingdom
Title:	Generation of orthotopic pancreatic tumors and ex vivo characterization of tumor-infiltrating T cell cytotoxicity
Signature:	
Date:	23/08/2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140