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Isolation, Characterization, and Purification of Macrophages from Tissues Affected by Obesity-Related Inflammation --Manuscript Draft--

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Abstract:	Obesity promotes a chronic inflammatory state that is largely mediated by tissue-resident macrophages as well as monocyte-derived macrophages. Diet-induced obesity (DIO) is a valuable model in studying the role of macrophage heterogeneity; however, adequate macrophage isolations are difficult to acquire from inflamed tissues. In this protocol, we outline the isolation steps and necessary troubleshooting guidelines derived from our studies for obtaining a suitable population of tissue-resident macrophages from mice following 18 weeks of high-fat (HFD) or high-fat/high-cholesterol (HFHCD) diet intervention. This protocol focuses on three hallmark tissues studied in obesity and atherosclerosis including the liver, white adipose tissues (WAT), and the aorta. We highlight how dualistic usage of flow cytometry can achieve a new dimension of isolation and characterization of tissue-resident macrophages. A fundamental section of this protocol addresses the intricacies underlying tissue specific

	enzymatic digestions and macrophage isolation, and subsequent cell-surface antibody staining for flow cytometric analysis. This protocol addresses existing complexities underlying fluorescent-activated cell sorting (FACS) and presents clarifications to these complexities so as to obtain broad range characterization from adequately sorted cell populations. Alternate enrichment methods are included for sorting cells, such as the dense liver, allowing for flexibility and time management when working with FACS. In brief, this protocol aids the researcher to evaluate macrophage heterogeneity from a multitude of inflamed tissues in a given study and provides insightful troubleshooting tips that have been successful for favorable cellular isolation and characterization of immune cells in DIO mediated inflammation.
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September 04, 2016

Teena Mehta, Science Editor
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Teena Mehta,

We are writing to submit our manuscript entitled, "Isolation, Characterization, and Purification of Tissue Resident Macrophages from Tissues Affected by Obesity-Related Inflammation," for consideration for publication in the Journal of Visualized Experiments (JoVE). This manuscript emerges from our experience in isolating tissue resident macrophages from diet- induced inflamed tissue sources. Primarily this article endeavors to help researchers achieve adequate isolations of macrophages for further characterization in similar models. Through years of consulting with resident experts and following suite with their recommendations and subsequent optimization trials, our research team was able to achieve superb enzymatic isolations and flow cytometry based characterization of tissue resident macrophage populations from the liver, white adipose tissue and aorta. We think researchers would benefit greatly from this format as fatty and inflamed tissues are oftentimes difficult to isolate, digest and process. This manuscript emphasizes troubleshooting options, in which case we have incorporated a comprehensive guide of alternative approaches to help researchers with various options for enriched and adequate isolations.

All authors have approved the written component of this manuscript and it's submission to the Journal of Visualized Experiments and those listed in the author byline have contributed sufficiently to the project. The author contributions are as follows, Joselyn Allen: Substantial contribution to conception and design, data acquisition and interpretation, primary writer and editor of the manuscript; Adwitia Dey: Substantial contribution to drafting and revising the manuscript; Ruth Nissly: Substantial contribution to data acquisition and provided technical expertise; Gayathri Balandaram: Substantial contribution to data acquisition and provided technical expertise; Jeffrey M. Peters: Principal Investigator and provided final approval of the version to be published; Pamela A Hankey-Giblin: Substantial contribution to the revision of the manuscript, provided final approval of the version to be published, primary Principal Investigator and corresponding author.

This manuscript has not been published and is not under consideration for publication elsewhere. If relevant, we briefly discussed these methods in a recently published Journal of Immunology publication (2016). We have no conflicts of interest to disclose, but we do respectfully request that Dr. Peter Murray not be considered as to review our manuscript. If you feel that the manuscript is appropriate for JoVE, we suggest the following reviewers, Ajay Chawla (University of California San Francisco), Philip R. Taylor (Cardiff University School of Medicine), Alan R. Saltiel (University of California San Diego School of Medicine), Tim P. Bushnell (University of Rochester Medical Center), Ernest M. Meyer (University of Pittsburgh Cancer Institute and David Adams (University of Michigan Medical School).

We are grateful to JoVE editors Teena Mehta and Ronald Myers for their guidance and ultimate patience with our submission.

We humbly thank you for taking our manuscript into consideration and review. We sincerely appreciate your time and enthusiastically look forward to your response.

Kind Regards,
Joselyn N. Allen (First Author)
Pamela A Hankey-Giblin (Correspondence Author)

TITLE:

Isolation, Characterization, and Purification of Macrophages from Tissues Affected by Obesity-Related Inflammation

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SHORT ABSTRACT:

This protocol allows a researcher to isolate and characterize tissue-resident macrophages in various hallmark inflamed tissues extracted from diet-induced models of metabolic disorders.

LONG ABSTRACT:

Obesity promotes a chronic inflammatory state that is largely mediated by tissue-resident macrophages as well as monocyte-derived macrophages. Diet-induced obesity (DIO) is a valuable model in studying the role of macrophage heterogeneity; however, adequate macrophage isolations are difficult to acquire from inflamed tissues. In this protocol, we outline the isolation steps and necessary troubleshooting guidelines derived from our studies for obtaining a suitable population of tissue-resident macrophages from mice following 18 weeks of high-fat (HFD) or high-fat/high-cholesterol (HFHCD) diet intervention. This protocol focuses on three hallmark tissues studied in obesity and atherosclerosis including the liver, white adipose tissues (WAT),

and the aorta. We highlight how dualistic usage of flow cytometry can achieve a new dimension of isolation and characterization of tissue-resident macrophages. A fundamental section of this protocol addresses the intricacies underlying tissue-specific enzymatic digestions and macrophage isolation, and subsequent cell-surface antibody staining for flow cytometric analysis. This protocol addresses existing complexities underlying fluorescent-activated cell sorting (FACS) and presents clarifications to these complexities so as to obtain broad range characterization from adequately sorted cell populations. Alternate enrichment methods are included for sorting cells, such as the dense liver, allowing for flexibility and time management when working with FACS. In brief, this protocol aids the researcher to evaluate macrophage heterogeneity from a multitude of inflamed tissues in a given study and provides insightful troubleshooting tips that have been successful for favorable cellular isolation and characterization of immune cells in DIO-mediated inflammation.

INTRODUCTION:

Mouse models have been extensively used to study the dynamics of human diseases. Proper isolation of tissue resident cells from mice in a diseased state can provide a platform for understanding the molecular and cellular contributions to the pathogenesis of a disease¹. One disorder that is of critical importance is obesity. The incidence of obesity continues to rise worldwide in parallel with insulin resistance and type 2 diabetes mellitus, cardiovascular disease, and fatty liver disease^{2,3}. Excessive nutrient consumption is further skewed by decreased physical activity triggering altered signals emanating from adipose tissue, which can alter the cellular milieu of other peripheral tissues such as the aorta and liver⁴. Such disruption of metabolic homeostasis results in a chronic low grade systemic inflammation⁵.

Classical activation of macrophages resident to the aorta and liver as well as their recruitment to white adipose tissue (WAT) has been shown to not only initiate dysregulation of metabolic signals but also sustain inflammation^{6,7}. The phenotypic and functional heterogeneity of macrophages is strongly associated with the pathogenesis of obesity related co-morbidities⁷. The dynamic plasticity in macrophage polarization allows for these cells to exhibit a range of activated phenotypes that coordinate the progression and resolution of inflammation⁸. While classically activated (M1) macrophages are implicated in the propagation of inflammation, alternatively activated (M2) macrophages have been associated with resolution and tissue repair^{9,10}.

As the body undergoes metabolic stress, white adipose tissue abnormally accumulates. The expanded adipose tissue attracts and retains inflammatory cells that profoundly alter normal adipocyte function to promote insulin resistance, hyperglycemia and ultimately type 2 diabetes mellitus, insulin resistance or hyperglycemia^{11,12}. In parallel, white adipose tissue remodels in response to inflammatory signals released by infiltrated classically activated (M1) adipose tissue macrophages (ATMs)^{13,14}. This multi-cellular organ exerts a cascade of signals that derails the normal function of other body organs such as the aorta and liver⁴.

The liver is a metabolic powerhouse that adapts in response to stimuli originating from nearby dysregulated WAT¹⁵. Hepatic macrophages or Kupffer cells, in response to metabolic changes, secrete inflammatory cytokines that transform both parenchymal and non-parenchymal cell

phenotype and promote tissue remodeling. Hepatic lipid accumulation, inflammation, excessive extracellular matrix deposits, necrosis and eventual function loss follows the inflammatory insults contributing to the wide spectrum of liver damage associated with non-alcoholic fatty liver disease^{16–18}.

In parallel to compromised WAT and liver function, large arteries accumulate lipids within the arterial wall as the body undergoes chronic metabolic stress¹⁹. Arterial lipid accumulation triggers the secretion of chemokines by activated endothelial cells and subsequent recruitment of monocytes²⁰. Once recruited, monocytes proliferate, differentiate, ingest lipoproteins and become foam cells. Atherogenesis is initiated and sustained by the pro-inflammatory activity of recruited and tissue resident lipid-laden macrophages. Succumbing to the extracellular and intracellular stress signals relayed in this atherogenic microenvironment, these macrophages then engage in an apoptotic signaling cascade. As these foam cells die, they contribute their lipid filled contents to the necrotic core of the lesion, which then leads to plaque rupture, myocardial infarction, and stroke.

Collectively, the heterogeneity of macrophage phenotypes in part orchestrates the obesity induced by inflammatory changes observed in dysregulated tissues such as WAT, liver and aorta^{8,21}. Characterization of recruited and tissue resident macrophages could provide insight into potential molecular targets that manipulate macrophage phenotype¹. To effectively characterize macrophages from obesity-induced inflamed tissues, a single-cell suspension can be obtained through enzymatic digestion. Such dissociation protocols must be effective in sufficiently degrading connective tissue while minimizing immune cell death and providing optimal cell yield. The enzyme mixture is dependent on the type of tissue and its structural make up. Resilient tissues such as the aorta requires stronger enzymatic activity, as compared to the liver and WAT, to achieve tissue dissociation. From the single cell suspension, tissue resident macrophages can be unambiguously characterized or isolated for further downstream analyses such as transcriptional profiling.

Here a tissue-specific protocol is described that uses collagenase-dependent tissue digestion and polychromatic flow cytometry to effectively isolate and characterize tissue-resident macrophages obtained from traditional diet induced obesity, atherosclerosis, simple steatosis and steatohepatitis mouse models. Simultaneous staining of cell surface markers with antibodies against leukocyte- (CD45 and/or CD11b) and macrophage- (F4/80) specific antigens is often used to identify macrophage populations²². Fluorescence-activated cell sorting (FACS) is a powerful strategy used to sort these identified populations at high purity. The sorted population can then be evaluated for phenotype specific gene profiles using downstream molecular analysis (such as quantitative real time polymerase chain reaction)²³. Although standard flow cytometry and flow cytometry-based cell sorting are powerful tools in distinguishing macrophages within a vastly heterogeneous cell suspension, the former protocols must be optimized to ensure successful output. In this study, protocols that effectively isolate and characterize viable tissue specific macrophages are described; more importantly, this study provides crucial insight into technical issues that often arise, as well as proactive and trouble-shooting strategies to prevent and/or resolve them.

PROTOCOL:

All experimental protocols (Sections 1, 1.2, and 1.3) were approved by the Institutional Animal Care and Use Committee (IACUC) at Pennsylvania State University.

[Please insert Table 1 here]

1) Tissue Isolation and Dissociation

1.1) WAT Isolation and Dissociation

1.1.1) Prepare the tissue-specific buffers as per Table 1, and store them as described.

1.1.2) Prepare the following reagents.

1.1.2.1) Prepare the digestion buffer by thawing the appropriate volume of WAT dissociation buffer and store at 4 °C until use. Immediately before use, warm buffer to 37 °C. Prepare the FACS buffer by preparing 1X phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS).

1.1.3) WAT Isolation

1.1.3.1) Euthanize a mouse in a carbon dioxide (CO₂) filled chamber. Check for effectiveness prior to continuing to the dissection stage.

1.1.3.2) Dip the euthanized mouse briefly in a beaker containing 70% ethanol until thoroughly soaked and coated with ethanol. Place the mouse ventral surface up on the dissection stage and fasten the mouse fore and hind paws to the dissection board using 21-gauge needles.

1.1.3.3) Use medium point tip forceps to grasp the abdominal skin anterior to the urethral opening, then use sharp dissecting scissors to make a nick in the grasped abdominal skin.

1.1.3.4) Insert the lower blade of the scissors into the small incision between the skin and peritoneum and make a lateral incision from abdomen to the rib cage.

1.1.3.5) Gently pull back the skin to expose the intact peritoneal cavity and make a lateral incision through the peritoneum and either fold back the transparent membrane or excise the tissue to expose the abdominal WAT.

1.1.3.6) Collect the perigonadal fat pads as described in Mann *et al.*²⁴

1.1.3.6.1) The perigonadal fat pads in male mice are loosely bound to the epididymis and testes. First locate the testes and then with medium point forceps, grasp hold of the epididymis head and pull gently.

1.1.3.6.2) Using sharp dissecting scissors, excise each epididymal fat pad by cutting along the surface of epididymis (head, body and tail) and the testes.

1.1.3.6.3) With the forceps, gently pull on the fat pad while cutting through the connective tissue directly bound to the epididymis structure.

1.1.3.6.4) Using the forceps, firmly grip the end of fat pad proximal to the epididymis and gently peel the fat pad away from the gonads

1.1.3.6.5) In female mice, the perigonadal fat pads are loosely bond to the uterus body and uterine horn.

1.1.3.6.6) Using medium point forceps, grip the perigonadal fat tissue and gently pull the tissue away from the uterus body and uterine horn.

1.1.3.6.7) Excise each fat pad by cutting along the uterus body and uterine horn using sharp dissecting scissors.

1.1.3.7) Place the fat pads in a petri-dish filled with PBS and keep on ice to keep tissue moist.

1.1.4) Dissociation of WAT into Single Cell Suspensions

1.1.4.1) Remove excess PBS from the petri dish and use a single edge razor blade to mince the abdominal WAT into small pieces.

1.1.4.2) With the sharp edge of the razor blade, gently scrape the minced abdominal WAT into a labeled 15-mL polypropylene collection tube containing 2 mL WAT dissociation buffer.

1.1.4.3) Incubate this tissue-digestion buffer mixture under slow continuous rotation at 37 °C for 45 min.

1.1.4.4) Filter the digested tissue through a 70-µm cell strainer into a 50-mL collection tube while moving the rubber piston of a syringe plunger in a circular motion and continue to sieve the cell suspension through the filter.

1.1.4.5) Pipet 2 mL of Dulbecco's Modified Eagle Medium (DMEM) to a 15-mL collection tube, gently pipet up and down and wash the filter with the single-cell suspension.

1.1.4.6) Wash the filter two additional times with cold DMEM and place the single cell suspension on ice.

1.1.4.7) Centrifuge the cell suspension at 300 × g at 4 °C for 8 min.

1.1.4.8) Use a vacuum aspirator to carefully remove floating adipocytes (first) and remaining supernatant. Make sure not to disturb the pelleted stromal vascular fraction (SVF).

1.1.4.9) With a 1000- μ L pipette, gently re-suspend the pellet in ammonium chloride-potassium (ACK) buffer to lyse erythrocytes according to the manufacturer's instructions.

1.1.4.10) Dilute the above cell suspension with DMEM and centrifuge the cell suspension at 300 g at 4 °C for 8 min.

1.1.4.11) Re-suspend cells in cold PBS containing 2% FBS (FACS Buffer) and count the cells in a Burker chamber/hemocytometer.

1.1.4.12) Transfer either 1×10^6 cells (for basic flow cytometry) or the entire cell suspension (for FACS) to labeled 5-mL round bottom polystyrene tubes. Continue to Section 2 for flow cytometry staining protocol.

1.2) Liver Tissue Isolation and Dissociation

Note: This protocol was adapted from Smedsrød²⁵.

1.2.1) Prepare the tissue-specific buffers as per Table 1 and store them as described.

1.2.2) Prepare the following reagents.

1.2.2.1) Prepare 1X Perfusion Buffer (PB), by diluting 40 mL of PBC into 960 mL ultrapure H₂O. Pre-warm a syringe filled with 50 mL of PB at 37 °C, just before perfusion starts. Eliminate any present air bubbles.

1.2.2.1.1) To eliminate air bubbles, invert the syringe where the leur lock tip is pointing upward. Tap or flick the syringe until air bubbles are to the top of the syringe. Gently push on the plunger to expel the air

1.2.2.2) Prepare the digestion buffer by diluting 0.5 mL of 476 mM CaCl₂ solution to 49.5 mL 1X Perfusion buffer. Add 3600U of Collagenase Type IV to the 4.76 mM CaCl₂ – PB solution. Pre-warm a syringe filled with 50 mL of digestion buffer at 37°C just before perfusion starts. Eliminate any present air bubbles as described in Step 1.2.2.1.1.

1.2.2.3) Prepare the preservation Buffer (PRB) by dissolving 2.5 g bovine serum albumin (BSA) in 250 mL 1X perfusion buffer. Store at 4 °C or keep on ice until use.

1.2.2.4) Prepare FACS Buffer with 1X PBS and 2% FBS.

1.2.3) Liver Tissue Isolation

1.2.3.1) Euthanize a mouse by CO₂ asphyxiation, saturate mouse with 70% ethanol to prevent contamination of intraabdominal organs with fur.

1.2.3.2) Position the mouse ventral side up on a polystyrene foam dissecting board and secure the mouse fore and hind paws to the dissection board using 21-gauge needles.

1.2.3.3) To expose the thoracic wall and peritoneal cavity, grasp the abdominal skin near the urethral opening using a medium point tip forceps. Then use a sharp dissecting scissors to create a small incision to the grasped abdominal skin.

1.2.3.4) Insert the lower blade of the scissors into the small incision between the skin and peritoneum and make a lateral incision from groin to chin.

1.2.3.5) Gently pull back the skin to expose the intact peritoneal cavity and thoracic wall, make a lateral incision through the peritoneum and excise the membrane.

1.2.3.6) Lift the sternum and carefully incise the diaphragm, be sure not to disturb the inferior vena cava (IVC). Move the gastrointestinal organs to the side and locate the subhepatic IVC. Use hemostatic forceps to clamp the suprahepatic IVC to maintain localized perfusion.

1.2.3.7) Attach the syringe filled with pre-warmed PB to the 23-gauge blood collection and infusion set. Gently push on the plunger until the tubing and needle are filled with PB.

1.2.3.8) Insert the 23-gauge needle, parallel to the level of the subhepatic IVC. Secure the needle in place using a 4-cm hemostatic clamp.

Note: Cannulation of the subhepatic IVC is preferred in diet-fed mice in that the IVC can be better visualized and cannulated within the fat-filled cavity.

1.2.3.9) Gently push on the plunger to begin perfusion, color will rapidly flush from the liver (right lobe first) if the needle is appropriately positioned in the vessel. Enlargement of the lobes is also visible if the needle is properly inserted into the subhepatic IVC.

1.2.3.10) Promptly cut the portal vein to allow the PB to flow freely through the liver.

1.2.3.11) Perfuse the liver until blood is no longer visible. On occasion gently massage liver lobes between fore-finger and thumb to facilitate perfusion.

Note: It is important to use non serrated blunt edged tools to handle the liver to prevent tissue damage.

1.2.3.12) When 5 mL of PB remains within the syringe, change to the syringe filled with pre-warmed dissociation buffer. Do not disturb the position of the secured needle during this time.

1.2.3.13) Perfuse the liver until fully digested. Gently massage liver lobes to facilitate perfusion.

Note: Separation of Glisson's capsule from parenchyma is observable in a successfully digested liver. To assess this, use the blunt edge of a pair of forceps, to gently press on the left lateral lobe. An impression should appear on the surface and the indentation should fill slowly once the forceps is removed.

1.2.3.14) Remove the needle from the subhepatic IVC. Do not remove the hemostatic forceps clamping the suprahepatic IVC.

1.2.3.15) Excise the whole liver by carefully cutting through connecting ligaments using a short straight blade dissecting scissors and remove the gallbladder.

1.2.3.16) Place the whole liver in a 10-cm petri-dish containing 10 mL ice-cold Preservation Buffer and store at 4 °C until ready to liberate cells.

1.2.4) Liver Cell Dissociation

1.2.4.1) Transfer the liver to a 10-cm dish containing 10 mL ice-cold DMEM.

1.2.4.2) Grip the severed suprahepatic IVC attached to the excised liver using a toothed forceps. Use medium point forceps to release the cells from the Glisson's capsule by applying rapid stroking motion to each lobe.

1.2.4.3) Pass the saturated DMEM through a 70- μ m cell strainer attached to a 50-mL collection tube.

1.2.4.4) Pipet 10 mL ice-cold DMEM to the 10-cm petri dish and repeat steps 1.2.4.1 to 1.2.4.3 until media saturation is no longer observed. Place the cell suspension on ice or at 4 °C.

1.2.4.5) Gently mix the cell suspension by inverting the collection tube and then centrifuge at 54 x g for 2 min at 4 °C.

1.2.4.6) Collect the supernatant in a clean 50-mL collection tube. Then centrifuge the cell suspension at 54 x g for 2 min at 4 °C.

1.2.4.7 Repeat Step 1.2.4.6 two additional times before proceeding to Step 1.2.4.8.

1.2.4.8) Transfer the supernatant to a clean 50 mL-collection tube and centrifuge the cell suspension at 300 x g for 10 min at 4 °C.

1.2.4.9) Re-suspend the non-parenchymal cell pellet in FACS buffer and count them in a Burkert chamber/hemocytometer.

1.2.4.10) Transfer 1×10^6 cells (for basic flow cytometry) or 15×10^6 - 20×10^6 cells (for FACS) to labeled 5-mL round bottom polystyrene tubes. Continue to Section 2 for flow cytometry staining protocol.

1.3) Aorta Isolation and Dissociation

Note: This protocol was adapted from Butcher *et al*²⁶.

1.3.1) Prepare the tissue-specific buffers based upon the recipes provided in Table 1 and store them as described.

1.3.2) Prepare the following reagents.

1.3.2.1) Prepare the digestion buffer by thawing the appropriate volume of WAT dissociation buffer and store at 4 °C until use. Immediately before use, warm buffer to 37 °C.

1.3.2.2) Prepare a 10X heparin solution by dissolving heparin sodium salt in 1X PBS at a concentration of 200 U/mL. Dilute the 10X heparin stock solution with 1X PBS to prepare a 1X Heparin solution. Store the 10X and 1X Heparin solutions at 4 °C until use.

1.3.2.3) Prepare the FACS Buffer with 1X PBS containing 2% FBS.

1.3.3) Aorta Dissection

1.3.3.1) Euthanize a mouse in a carbon dioxide filled chamber and rinse with 70% ethanol.

1.3.3.2) Place the mouse ventral side up on the dissection stage, spread the mouse fore and hind paws and fasten them to the dissection board using 21-gauge needles.

1.3.3.3) Immediately after euthanizing the mouse, perform cardiac puncture. Refer to Steps 1.3.3.4 to 1.3.3.9, if necessary.

1.3.3.4) Locate the xiphoid process at the lower end of the sternum

1.3.3.5) To do so, place a finger midpoint along the width of the animal's neck. Trace along the ventral midline from neck to the caudal end of the sternum until a cartilaginous extension is felt.

1.3.3.6) Use medium point forceps to grasp the cartilaginous extension, and if necessary mark the location of the xiphoid process by removing the patch of hair or skin at the area.

1.3.3.7) Partially insert a 26-gauge needle under the xiphoid process at 20-30° angle.

1.3.3.8) Gently apply negative pressure to the syringe by slowly withdrawing the plunger, then insert the needle further into the cavity until blood flows.

1.3.3.9) If blood flow stops, slowly rotate the needle or move slightly in and out.

1.3.3.10) Use the pair of forceps to grab hold of the abdominal skin anterior to urethral opening, and use a pair of sharp dissecting scissors to cut along the ventral midline through the peritoneum from groin to chin.

1.3.3.11) Pull back the skin to expose abdominal organs and rib cage with fingers or blunt forceps gently move abdominal organs to the side.

1.3.3.12) Use the forceps to grab hold of the xiphoid process, lift the sternum, and incise the diaphragm.

1.3.3.13) Using the dissecting scissors, cut through the lateral ribs on both sides. Gripping the sternum, flip the rib cage upwards and cut through the connecting base. Remove the rib cage exposing the underlying thoracic organs.

1.3.3.14) Perfuse the aorta with a 26-gauge needle attached to a 10 mL-syringe filled with 1X heparin solution by injecting 1-2 mL of solution into the left ventricle (LV) of the heart.

Note: Be sure to perfuse at a slow rate with little pressure to ensure intact atherosclerotic aortic lesions.

1.3.3.15) Excise the thymus, lungs and connective tissue. Refer to Steps 1.3.3.16 and 1.3.3.17, if necessary.

1.3.3.15.1) Anterior to the heart locate the white bi-lobed (or butterfly shaped) organ and firmly grab hold of the thymus with the forceps. Pull both lobes upward away from the cavity and cut at the base with the scissors.

1.3.3.15.2) To remove the lungs, pinch a lung lobe with the forceps, pull the tissue away from the thoracic cavity and cut at the base. Repeat for each remaining lobe.

1.3.3.16) Use a dissection microscope and micro-dissecting tools, and collect the aortic arch (including the innominate artery, left common carotid artery and left subclavian artery), the ascending, descending, thoracic and abdominal aorta.

1.3.3.16.1) Locate the ascending aorta at the left ventricle of the heart.

1.3.3.16.2) With a pair of curved 0.07 x 0.04 mm-tip forceps gently grasp the portion of the ascending aorta emerging from the left ventricle.

Note: Compared to the surrounding yellow-tinged fat tissue, the aorta will be a bright white vessel originating from the LV when sufficiently flushed with the heparin solution.

1.3.3.16.2.1) If the aorta was not properly flushed by cardiac perfusion, flush the aorta again while the aorta remains connected to the cavity. To do so, cut near the heart-aorta junction, remove the heart, and then cut horizontally through the lower-end of the abdominal aorta. Insert a 26-gauge needle in the opening of the ascending aorta and gently flush the aorta with the 1X heparin solution.

1.3.3.16.3) Gently tug on the ascending aorta to better discriminate between the fat tissue and embedded aorta.

1.3.3.16.4) Still gently pulling on the ascending aorta, use the tip of closed spring dissecting scissors to clear the fat that encapsulate the ascending aorta and aortic arch. To do so, stroke the fat tissue with the tip of closed scissor blades to tear away the connecting fat.

Note: Refrain from excising any fat tissue by cutting until the ascending aorta and arch can be clearly visualized. This is to prevent cutting the aorta.

1.3.3.16.5) If the ascending aorta and arch can be clearly delineated from the surround fat tissue, use the spring dissecting scissors to excise excess fat.

1.3.3.16.6) Gently grab hold of the aortic arch with the forceps. Again using the spring scissor's tip, stroke at the fat along the length of the descending, thoracic and abdominal aorta to the caudal end of the abdominal aorta, do so on the right side of the sleeve of fat.

1.3.3.16.7) Continue to grasp along the aorta when tearing away the fat. Be sure to do so gently to prevent damaging the aorta.

1.3.3.16.8) Gently pull the aorta upward toward the microscope so that the fat is now positioned behind the aorta.

1.3.3.16.9) Insert the closed scissor blades between the fat-aorta interface near the anterior end of the descending aorta, sever the fat tissue's attachment to the aorta's surface by bluntly using a sweeping motion.

Note: Thoroughly removing excess fat and connecting tissue while the aorta is still within the cavity is recommended.

1.3.3.16.10) Excise the heart and cut at the caudal end of the abdominal aorta transversely. Remove the whole aorta.

1.3.3.31) Place the aorta in a 35-mm dish and tease away any excess fat on the aorta using two 21-gauge needles. Place the excised aorta in 1.7-mL microcentrifuge tube on ice.

1.3.4) Dissociation of the Aorta into Single Cell Suspensions

1.3.4.1) Pipet 0.2 mL aorta dissociation buffer to the tube containing the aorta. Insert the spring scissor blades toward the tapered end of the tube and rapidly mince the aorta to facilitate enzymatic digestion.

1.3.4.2) Transfer the tissue-solution mixture to a 15-mL collection tube and pipette 1.8 mL aorta dissociation buffer for a total volume of 2 mL.

Note: For easy transfer of the tissue suspension, cut the 1 cm off the tapered end of a standard 1000 μ L pipette tip. Use the shortened tip to transfer the suspension with a micropipette.

1.3.4.3) Incubate the minced aorta in the aorta dissociation buffer for 55 min at 37 °C, shaking at 220 rpm (0.514 x g).

1.3.4.4) Pass the suspension through 50- μ m cell strainer into a 15-mL polypropylene collection tube. Use the rubber piston of a syringe plunger to facilitate filtering.

1.3.4.5) Rinse the 15-mL collection tube with 1 mL FACS buffer, collect the suspension and pass through the cell strainer.

1.3.4.6) Wash the 50- μ m cell strainer two additional times with 1 mL FACS buffer.

1.3.4.7) Pellet the cells by centrifugation at 300 x g for 5 min at 4 °C. Re-suspend cells in cold FACS Buffer. Count the cells in a Burker chamber/hemocytometer.

1.3.4.8) Transfer either 1×10^6 (for basic flow cytometry) cells or the entire cell suspension (for FACS) to a labeled 5-mL round bottom polystyrene tubes. Continue to Section 2 for flow cytometry staining protocol.

2. Flow cytometry and FACS staining

[Please insert Table 2 here]

2.1) Collect and prepare the following items: FACS Buffer 5-mL round bottom polystyrene tubes, anti-mouse CD16/32 Fc receptor blocking antibody, fluorophore conjugated or unconjugated primary antibodies, fluorophore-conjugated secondary antibodies (if necessary), 1X phosphate-buffered saline (PBS).

Note: For staining large amounts of cells, antibody concentration is most critical rather than cell number. For example, if staining 10×10^6 cells the staining buffer volume and antibody concentration should be the same as if staining 1.0×10^6 cells in 100 μ L buffer volume. For staining 1.0×10^8 cells, increasing the antibody amount 5-fold is recommended.

2.2) Add additional ice-cold FACS Buffer to each FACS tube if necessary, pellet cells by centrifugation at 751 x g for 5 min (4 °C). Aspirate the supernatant following centrifugation.

2.3) Add anti-mouse CD16/CD32 Fc receptor blocking antibody to all samples according to the manufacturer's instructions. Incubate for 15 min at 4 °C or on ice.

2.4) Add fluorophore-conjugated primary antibody cocktail directly to the Fc receptor blocking antibody-containing cell suspension and incubate samples at 4 °C for 30 min. Protect samples from light to minimize bleaching of fluorophore-conjugated antibodies.

2.4.1) In the event unconjugated primary antibodies were used, follow these steps subsequent to completing Step 2.4.

2.4.2) Wash primary antibody stained samples by centrifugation at 751 x g for 5 min (4 °C).

2.4.3) Remove supernatant by decanting and re-suspend pellet in 100 µL FACS buffer.

2.4.4) Add conjugated secondary antibody to all necessary samples and incubate for 30 min at 4 °C. Proceed to Step 2.5.

2.5) Following antibody incubation, add 2 mL of ice cold FACS buffer then pellet cells at 751 x g for 5 minutes (4 °C). Decant supernatant and be sure not to disturb pellet.

2.6) Mix cells gently and then re-suspend with 200-400 µL of FACS buffer, then keep this in the dark at 4 °C until standard flow cytometry analysis or cell sorting.

2.7) For short term fixation of stained cells, proceed to Steps 2.8 to 2.10. Use fixation for standard flow cytometry only.

2.8) Immediately following Step 2.5, re-suspend cells in .5 mL 2-4% paraformaldehyde (PFA) for 15-30 minutes in 4°C.

Note: Caution: PFA is a known irritant with carcinogenic and toxic effects. When using PFA, handle with extreme care. Be sure to use the appropriate personal protective equipment and exhaust ventilation.

2.9) Following fixation, wash cells by adding 2 mL FACS buffer to all samples and centrifuge at 751 x g in 4 °C for 5 min. Remove supernatant following centrifugation.

2.10) Resuspend fixed cells in 200 µL of ice cold FACS buffer and store at 4 °C. Store fix cells up to 1 week at 4 °C, ideally perform flow cytometry acquisition within 48 hours to minimize autofluorescence.

2.11) Acquire flow cytometry data according to the flow cytometer manufacturer's instructions or perform fluorescent activated cell sorting as described by Basu *et al.*²³

REPRESENTATIVE RESULTS:

When using apolipoprotein E deficient (ApoE KO) C57BL/6 (BL6) mice maintained on a high fat high cholesterol diet (HCHFD or HCD) for 18 weeks, $1 \times 10^4 - 2 \times 10^4$ CD45⁺F4/80⁺ aortic macrophages can be isolated when two samples are pooled. Livers dissected from HFHCD-fed ApoE KO mice, produced greater than 5×10^5 sorted Kupffer cells (which depends on available sorting time). When using high fat diet (HFD) fed wild type (WT) C57BL/6 mice, 5×10^5 to 1×10^6 resident adipose tissue macrophages (ATMs) can be sorted from the stromal vascular fraction (SVF). The mentioned total number of macrophages that can be sorted from a given tissue was adequate enough for performing gene expression analysis using quantitative real time polymerase chain reaction (qPCR). For tissues where fewer numbers of macrophage populations are recovered, such as the aorta, it is recommended to use co-precipitants (for example glycogen) and overnight precipitation when isolating RNA is needed for these analyses.

Here, the effects of diet-induced metabolic disorders on macrophage phenotype using basic flow cytometry, fluorescence-activated cell sorting (FACS), and downstream post sort analyses are shown. These findings corroborate previously published observations that mice fed a HFD or HFHCD exhibit increased infiltration of classically activated (M1) macrophages in affected tissues such as the aorta (Figure 1B). Taking advantage of flow cytometry, FACS, and gene expression profiling (via quantitative polymerase chain reaction (qPCR), the predominating phenotype for Ron receptor tyrosine kinase-expressing CD45⁺ F4/80⁺ macrophages derived from tissues isolated from diet-fed mice was observed. Ron receptor-expressing aortic macrophages which demonstrated an anti-inflammatory phenotype were decreased in HFHCD-fed mice (Figure 1C and D). Sorted Ron receptor-expressing macrophages derived from digested aortas demonstrated increased arginase 1 (Arg1) gene expression, which is a well-established M2 macrophage marker (Figure 1E). Pro-inflammatory macrophages which were characterized as CD45⁺ F4/80⁺ CD11c⁺ were increased in aortas isolated from HFHCD-fed mice (Figure 1B and D). Characterizing liver-resident macrophages further elucidated the prevailing phenotype of Ron receptor-expressing subpopulations (Figure 2A). CD11c⁺ pro-inflammatory macrophage populations demonstrated a decrease expression of genes that are strongly associated with an anti-inflammatory (M2) phenotype such as Arg1 and Ron (Figure 2B). Similar trends were observed in macrophage populations isolated from digested white adipose tissue (Figure 3). Macrophage population with a pro-inflammatory signature showed decreased surface expression of the Ron receptor (Figure 3). Combining the approaches, basic flow cytometry and FACS, resulted in conclusive data that further validates the Ron receptor as a regulator of alternative (M2) activation in macrophages.³² Such bias toward an anti-inflammatory (M2) phenotype has been shown to play a protective role in the development and progression of obesity, atherosclerosis and steatohepatitis.³¹

Figure 1. Characterization of macrophages isolated from dissociated aorta removed from ApoE KO mice maintained on a HCD for 18 weeks. A) Cells were first gated on CD45⁺ leukocytes, excluding cellular debris. B) CD45 staining in conjunction with F4/80 is used to delineate double

positive populations presumed to be macrophages. Additional gating was used to demonstrate the percentage of CD11c⁺CD45⁺F4/80⁺ (M1) and C) Ron⁺CD45⁺F4/80⁺ (potential M2) macrophages in aortas derived from mice fed on either a normal chow or high cholesterol diet for 18 weeks. D) Increased percentage of CD11c⁺CD45⁺F4/80⁺ (M1) macrophages, as well as a decreased percentage of Ron⁺CD45⁺F4/80⁺ (potential M2) macrophages were observed in aortas derived from mice fed HCD compared to mice fed a normal chow diet. E) Gene expression analysis of sorted Ron⁺CD45⁺F4/80⁺ macrophages demonstrated increased expression of arginase I (a well-known murine M2 marker). Values were obtained using Student's t-test analyses performed using statistical analysis software and represented as mean \pm SEM. P < 0.05 was considered statistically significant. (*P<0.05, ***P<.001) Figure has been modified from Yu *et al.* (2016).³¹

Figure 2. Gene transcript profiling of Kupffer cell populations sorted from digested livers dissected from ApoE KO mice maintained on a HCD for 18 weeks. A) General gating scheme for characterizing and sorting Kupffer cell populations. B) Gene expression analysis of sorted Ron expressing (Ron⁺) and non-expressing (Ron⁻) CD45⁺F4/80⁺ macrophages by quantitative RT PCR. C) Transcript expression analysis of sorted CD45⁺F4/80⁺CD11c⁻ and CD45⁺F4/80⁺CD11c⁺ macrophage populations. Student's t-test analyses were performed using statistical software and values were represented as mean \pm SEM. P < 0.05 was considered statistically significant. (*P<0.05, ***P<.001) Figure has been modified from Yu *et al.* (2016).³¹

Figure 3. Characterization of ATMs isolated from white adipose tissue dissected from WT BL6 mice fed a HFD for 18 weeks. A) General gating scheme for characterizing and sorting adipose tissue derived macrophage populations B) Percentage of Ron⁺ cells within CD45⁺F4/80⁺CD11c⁻ and CD45⁺F4/80⁺CD11c⁺ macrophage populations sorted from WAT. Figure has been modified from Yu *et al.* (2016).³¹

Table 1. Tissue specific perfusion buffer recipes.

Table 2. List of fluorophore tagged antibodies specific for discriminating tissue resident macrophages.

Table 3: Troubleshooting unsuccessful tissue dissociation.

DISCUSSION:

Diet-induced metabolic disorder models that mimic co-morbidities such as atherosclerosis, simple steatosis, steatohepatitis and type 2 diabetes are extensively used to better understand the underlying molecular mechanisms of disease progression. Collagenase dependent digestion is often used to dissociate tissues to liberate cells from the extracellular matrix (ECM)^{16,27}. Enzymes such as collagenase disrupt collagen which provides structural support for neighboring cells. The tissues' structural composition dictates the tissue matrix stiffness (resistance to deformation) and which crude collagenase product is most efficient in ensuring successful ECM disruption²⁸. WAT, which is composed of "soft matrix" is often digested with Collagenase Type II to liberate adipocytes and resident immune cells while simultaneously maintaining the integrity of cell surface insulin receptors²⁹. The microarchitecture of the fibril components of the aorta

contributes to the “stiff matrix”, for which effective aortic digestion with collagenase type XI (which has the highest collagenase activity) is used in combination with additional enzymes. Unlike the aorta, the liver digestion uses a collagenase with a weaker enzymatic activity, Collagenase Type IV, to disrupt the matrix and liberate viable parenchymal and non-parenchymal cells³⁰. Chronically inflamed tissues derived from diet-fed wild type or apolipoprotein E deficient (ApoE KO) mice on a C57BL6 background often undergo remodeling that can prevent proper enzymatic digestion. This section will discuss strategies to minimize and/or eliminate the possibility of improper tissue dissociation and low cell recovery.

A common feature of tissues derived from diet-fed mouse models that mimic obesity, atherosclerosis, and/or non-alcoholic fatty liver disease, is abnormal tissue remodeling. In white adipose tissue, the aorta, and liver, ECM composition is altered, often resulting in excessive deposition of fibrillar components such as collagens. Wild type C57BL6 mice maintained on a high fat diet for eighteen weeks, experience tissue-specific abnormalities such as expanded white adipose tissue and enlarged fatty liver (simple steatosis). Often times these metabolic features are not troublesome obstacles to overcome during the tissue dissociation process. On the other hand, in other diet induced models that mirror more exacerbated phenotypes, the extensive remodeling of the tissue can pose a problem during tissue digestion. HFHCD fed ApoE KO mice are often used to model atherosclerosis and steatohepatitis. A common feature associated with advanced steatohepatitis is the excessive deposition of ECM in the liver (or fibrosis). Fibrotic liver has been shown to be quite problematic during enzymatic tissue dissociation and often produces low cell yield³¹. To improve cell yield, it is critical that the digestion protocols be followed without deviation. Although normal livers can be minced and submerged in digestion buffer to achieve dissociation, perfusion with digestion buffer maximizes contact between the extracellular matrix of the liver and the collagenase solution; and therefore this approach is highly recommended. Additionally, 20-30 mL of digestion buffer is often an adequate volume for successful dissociation of normal livers; however, in regards to mouse models that develop enlarged and/or fibrotic livers (1.5 - 2.0 g), perfusion with 50 mL of digestion buffer is preferred to ensure proper digestion while minimizing cell death. For livers with weights that exceed 2.0 g, increasing the volume of digestion buffer is recommended to guarantee successful digestion.

[Please Insert Table 3 here]

Flow cytometry based cell sorting or FACS is a powerful technique for isolating cell populations where high purity is a necessity. When purifying cells by cell sorting, achieving high cell yield but also a high purity sort requires using the proper sorting strategies. In this section, methods for improving multi-fluorophore flow cytometry-based cell sorting of tissue resident macrophage derived from diet-fed mice are described. For distinct tissue resident macrophage isolation, surface marker selection is a critical step. Macrophages derived from WAT, aorta, and liver are often distinguished using a CD45⁺, CD11b⁺, F4/80⁺ gating strategy. Additional flow cytometric panels can be used to identify tissue resident macrophages. These panels include antibodies which probe for the surface expression of macrophage specific glycoproteins (CD64, CD68, and CD14), major histocompatibility complexes (MHCII) and apoptotic cell tyrosine kinase receptors (MerTK)^{32,33}. Specific macrophage phenotype can then be delineated by probing for the selective

surface expression of M2 (CD163, CD209, and CD206) or M1 markers (CD38, CD40, CD80, and CD86)^{34,35}. Auto-fluorescence generated by lipid-laden macrophages can present some issues when gating populations. Using antibodies tagged with fluorochromes that are excited by the yellow-green laser (such as PE, PE/Cy5, PE/Cy7) or red laser (such as APC, APC Cy7) results in emitted fluorescence that is significantly brighter than the auto-fluorescence and thus can improve results³⁶. When selecting fluorophore conjugates with such high staining index and potential emission spectra overlap, inclusion of appropriate controls is critical. When identifying gating boundaries, inclusion of single stain (SS) controls and isotype controls allow for the delineation of positive/negative populations and the measurement of non-specific background signal caused by primary antibodies, respectively³⁷. For circumstances where cells are scarce, we recommend using compensation particles for SS and isotype controls. Compensation particles typically emit brighter signals than biological controls and also have less variance in background fluorescence. Additionally, fluorescence minus one (FMO) controls are ideal for delineating gating boundaries. By including FMO controls, the maximum fluorescence expected for a staining subset is revealed in a given channel when the fluorochrome-tagged antibody specific for that particular fluorescence channel is excluded³⁸. In our experience, in addition to single stained compensation particle controls, an unstained biological comparison control should be included for setting more precise positive/negative boundaries.

Excluding cellular debris and cell aggregates in the gating strategy is also an additional approach used to minimize auto-fluorescence. To distinguish cellular debris from the viable cell population, using forward scatter (FSC) and side scatter (SSC) is the most common gating strategy. For isolated cells that will be used for post sort analyses and require higher viability for DNA/RNA extractions, it is recommended that viability stains not be used with any fixation and/or permeabilization procedures. Formaldehyde fixing of cells can compromise nucleic acid integrity due to nucleic acid-protein crosslinking and thus limit isolation efficiency, detection, and accurate quantification. Cell aggregates as mentioned before cannot only contribute to emitted auto-fluorescent signals, but also result in “coincidence aborts”. Such action occurs to maintain high purity in a sort but if too frequent, it reduces the sorted cell yield. Sorting buffer (FACS buffer) supplemented with DNase and MgCl₂ during cell staining can minimize cell aggregation. It is recommended to not use EDTA in combination with DNase as it inhibits the enzyme’s activity. Filtering the single cell suspension through a 70-µm cell strainer prior to sorting can also liberate cells from aggregates. It is imperative to note that to minimize aggregation, cell suspensions should be at a concentration of 5 million cells per mL in a minimum volume of 0.4 mL. Cells isolated from lipid laden tissues tend to be more prone to aggregation and this can result in coincidence aborts and low sort yield. It is recommended that samples are further diluted if aggregation persists. Sorted macrophages can be collected in polypropylene round-bottom collection tubes containing fetal bovine rich medium to maximize cell recovery. An initial high FBS concentration ensures cell recovery since the concentration eventually becomes diluted with each sorted droplet. For collecting cells that will be used for DNA or RNA analysis, cells can be sorted directly into the appropriate extraction reagent (*e.g.* TriZol) to prevent RNase contamination. When sorting high volumes, sorting cells first into culture media supplemented with lower concentrations of FBS, is recommended. Immediately following sorting, cells should then be pelleted and lysed for DNA/RNA extraction. The isolated genetic material can then be

profiled for altered gene expression. Pro-inflammatory genes including $\text{TNF}\alpha$, $\text{IL1-}\beta$, IL-6 , IL-12 , IL-23 , $\text{IFN}\gamma$, Nos2 , and MCP1 (CCL2) are often upregulated in macrophages exhibiting a classically activated (M1) phenotype³⁹. On the other hand, alternatively activated (M2) phenotype in macrophages is often marked by induction of genes encoding Chi3l3 (Ym1), Fizz1 , Arginase 1 , CD206 , CD163 , CD209 , IL-10 , and $\text{TGF}\beta$ ^{35,40}. Recently, CD38 , Fpr2 , and Gpr18 have been validated as M1-specific genes and c-Myc and Egr2 as M2-specific genes³⁴.

Although multi-color flow cytometry-based cell sorting is a valuable tool for isolating macrophages at high purity, this approach can be costly. The powerful advantages of FACS mediated sorting are dependent on operations personnel that can maneuver a cell sorter in addition to the high cost of cell sorter maintenance reagents. Alternative approaches can be used in substitute of costly flow cytometry based cell sorting. They include magnetic activated cell sorting (MACS) or density gradient centrifugation. The first alternative cell sorting method mentioned encompasses magnetic and/or microbead column isolation kits to separate cells of interest from blood or solid tissues⁴¹. The second cell sorting approach separates a heterogeneous cell suspension based on density and force of centrifugation. Unfortunately, density mediated centrifugation is not practical for isolating macrophages from aorta- or WAT-derived single cell suspensions. Oftentimes, the product obtained from differential centrifugation is contaminated, and of low yield. Consequently, smaller tissues (such as the aorta or WAT) that result in few cells initially following enzymatic digestion are not ideal candidates for differential centrifugation. On the other hand, cell suspensions derived from dissociated livers can produce an adequate number of sorted macrophages that can be used in post sort experiments and analyses such as culture stimulations, qPCR, or western blotting analysis. These alternative approaches can also be used to enrich populations prior to FACS, allowing for cleaner sorts. Of note, tissue resident macrophages make up a small percentage of the entire cell population in WAT, liver and the aorta. Enrichment of cells prior to FACS sorting has been an approach used when isolating populations of cell that are less frequent. One issue that is common in isolating small populations is that large cell numbers must be processed to obtain enough cells for subsequent analysis. Enrichment or pre-sorting can be used to resolve such an issue. This method aids in obtaining a more concise population of cells through positive and negative selection but it also allows conservation of time as FACS sorting can be an enduring process for dense tissue sources such as the liver.

Recent advances in inflammation biology highlight the importance of phenotypic and functional characterization of macrophage heterogeneity to further the understanding of the complex role of these immune cells in regulating chronic inflammation. In brief, this comprehensive protocol provides a multi-dimensional approach to characterizing tissue resident macrophages from three hallmark tissues studied in established diet induced obesity and inflammation models. More importantly this protocol takes into account the difficulty and the measures necessary to isolate clean single cell suspensions from dysregulated inflamed tissues such as WAT, aortic plaques and fatty livers. The protocol allows the researcher to apply flow cytometry and FACS sorting tools in an innovative dimension to characterize tissue resident macrophages, key regulators of inflammation in obesity. In-depth characterization of macrophage population dynamics can provide insight into monocyte trafficking in inflamed tissues and allow for continued mechanistic

evaluations through a multitude of experimental approaches on sorted macrophages. Further characterization of macrophage populations can provide a pivotal insight into the biological underpinnings that regulate macrophage heterogeneity in health and disease.

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DISCLOSURES:

Authors have no conflict of interests to disclose.

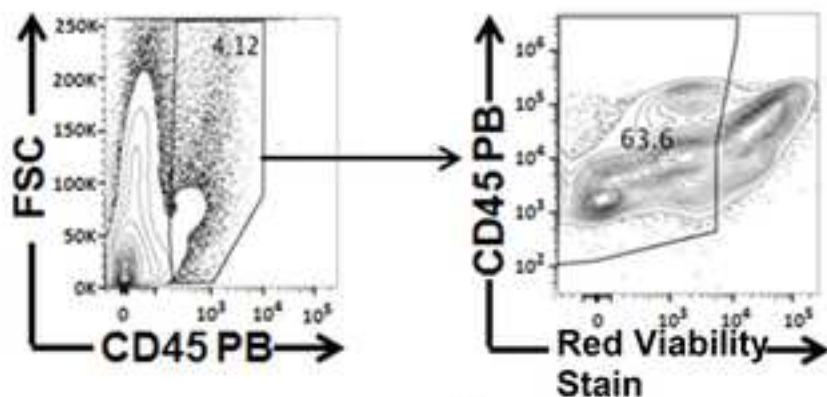
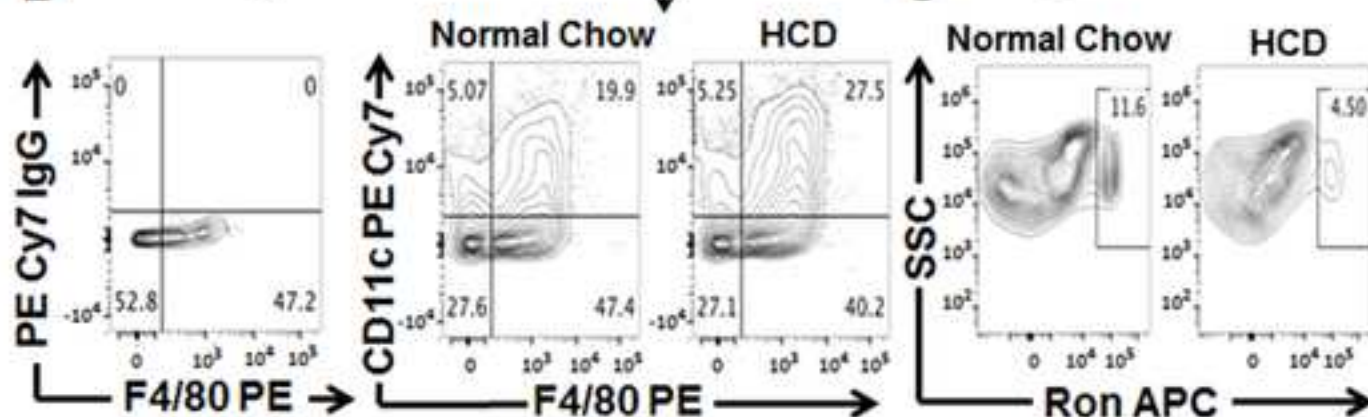
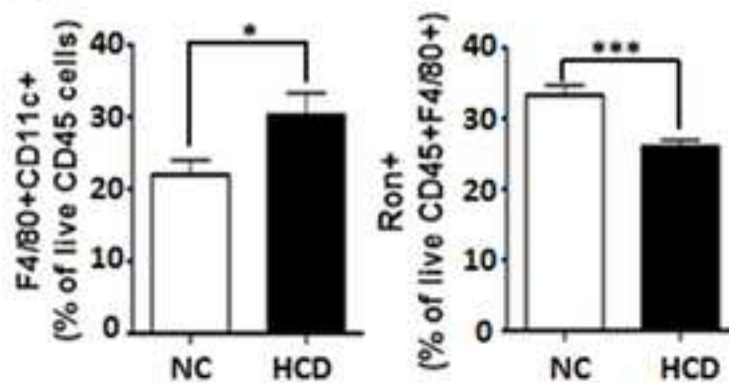
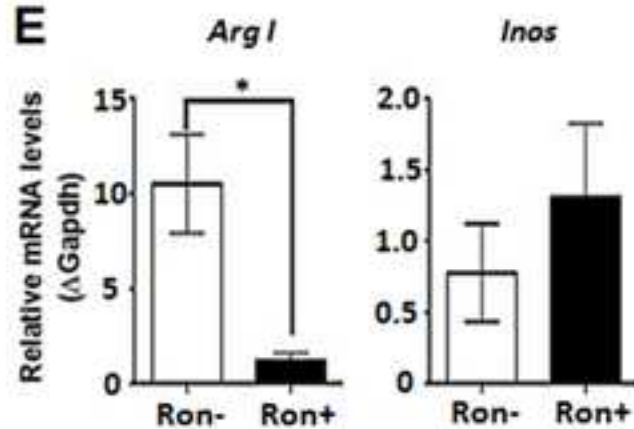
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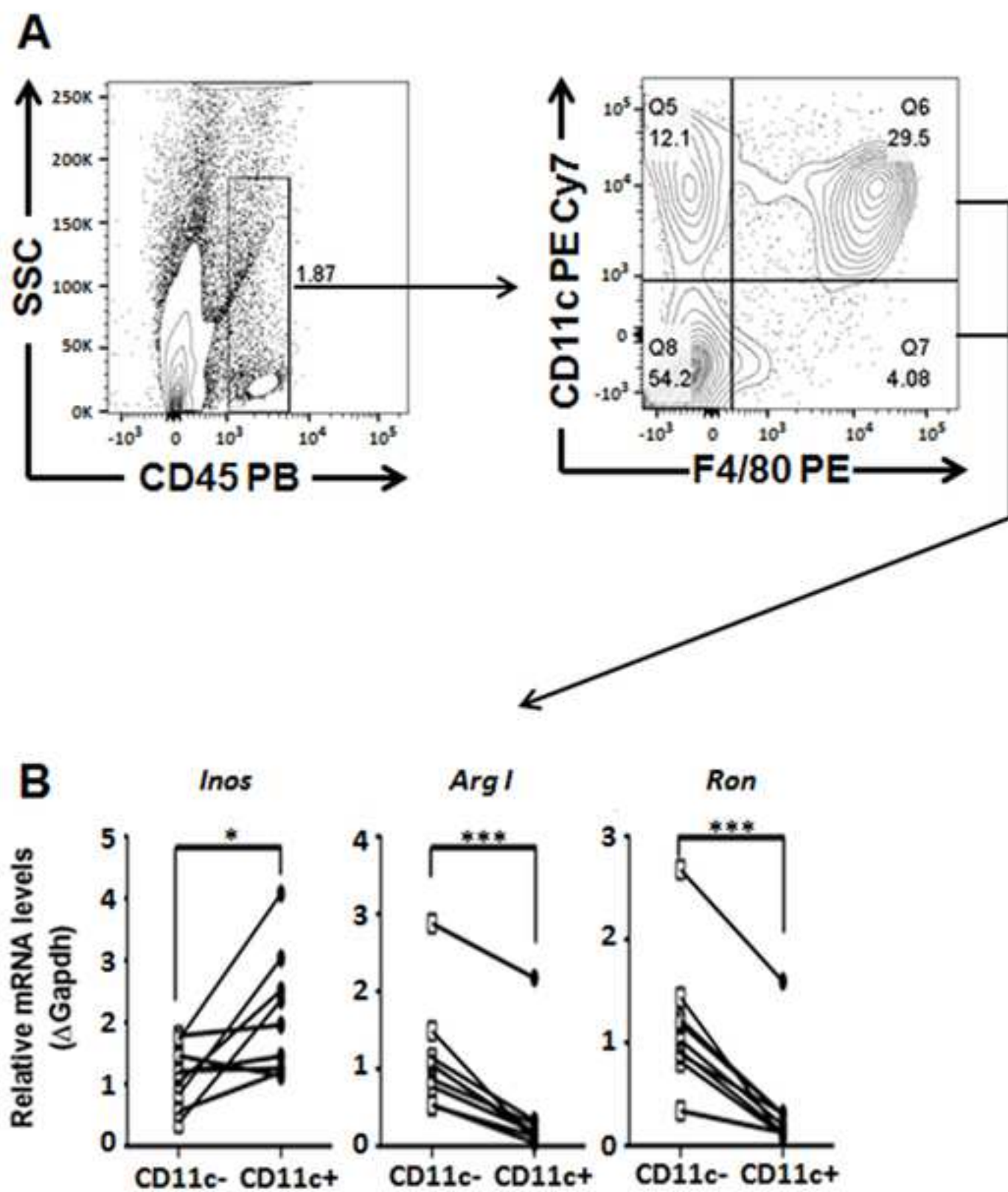
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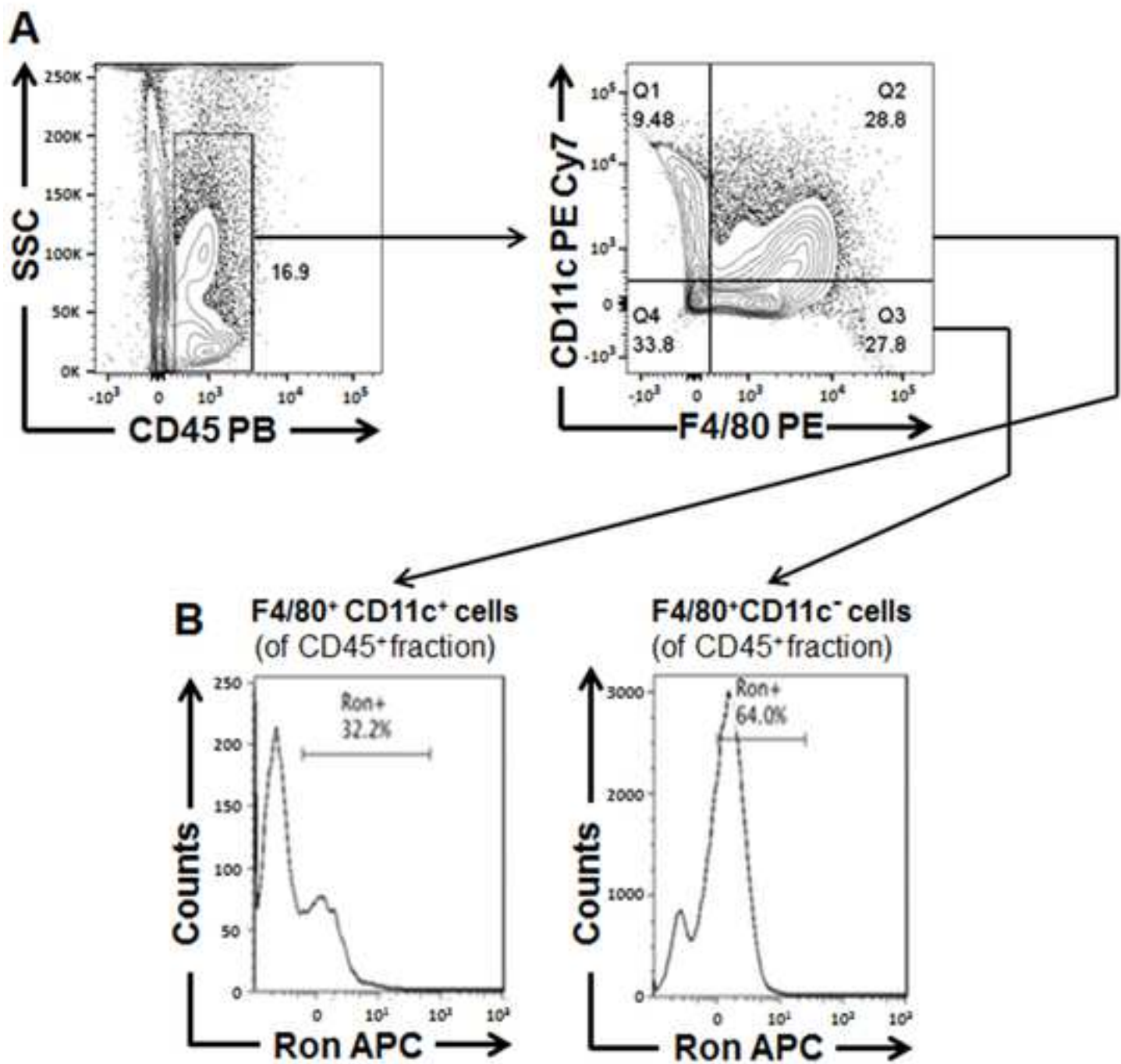
(Figure 1)

A**B****D****E**

(Figure 2)



(Figure 3)



Tissue	Dissociation Buffers Preparation	Final Volume	Storage
White Adipose Tissue (WAT)	Dissociation Buffer: 2.5% HEPES, 10 mg/mL Bovine serum albumin (BSA), 3 mg/mL (0.3%) Collagenase Type II in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose without L-glutamine and sodium pyruvate	500 mL	minus 80°C (10 mL aliquots)
Liver	25X Perfusion Buffer Concentrate (PBC): 3.55 M NaCl, 168 mM KCl, 240 mM HEPES, 150 mM NaOH in distilled deionized H ₂ O	500 mL	minus 20°C (40 mL aliquots)
	Preservation Buffer (PRB): 1% BSA in 1X Perfusion Buffer	1L	4°C
	Dissociation Buffer: 1X Perfusion Buffer supplemented with 4.76 mM CaCl ₂ and 72 U/mL Collagenase Type IV	50 mL (per mouse)	Prepare immediately prior to use
Aorta	Dissociation Buffer: 125 U/mL Collagenase Type XI, 60 U/mL Hyaluronidase type I, 60 U/mL DNase I, 450 U/mL Collagenase Type I, 20 mM HEPES in 1X Phosphate Buffered Saline (PBS)	500 mL	minus 80°C (10 mL aliquots)

Fluorophore	R-Phycoerythrin (PE)	PE/Cyanine (Cy) 7	PE/Cyanaine (Cy) 5
Laser (nm)	Blue (488 nm) / Yellow (561-570 nm) - Green (532 nm)	Blue (488 nm) / Yellow (561-570 nm) - Green (532 nm)	Blue (488 nm) / Yellow (561-570 nm) - Green (532 nm)
Excitation _{MAX} (nm)	496	496	496
Emission _{MAX} (nm)	578	785	667
Phenotypic Marker	F4/80	CD11c	CD11b
	EMR1, Ly71	αX integrin, integrin αX chain, CR4, p150, ITGAX	αM integrin, Mac-1, Mo1, CR3, Ly-40, C3biR, ITGAM
Targeted Cell Type	Tissue Resident Macrophages	Classicaly Activated (M1) Macrophages	Monocytes / macrophages
	Subset of Dendrttic cells	Dendritic cells, NK cells, Activated T cells, and a subset of intestinal intraepithelial lymphocytes (IEL)	Granulocytes, dendritic cells, NK cells, and subsets of T and B cells
Dilution Factor	1:50	1:100	1:100
Isotype Controls	PE Rat IgG2a	PE/Cy7 Armenian Hamster IgG	PE/Cy5 Rat IgG2b

Pacific Blue (PB)
Violet (405 nm)
401
455
CD45
T200, Ly-5, LCA
Leukocytes (Macrophages / monocytes, lymphocytes, and granulocytes)
1:100
Pacific Blue Rat IgG2c

Tissue	Problem	Possible Reason	Solution
White Adipse Tissue (WAT)	Poor cell dissociation	Poor Digestion	Make sure digestion buffer is at 37°C
	Cell Death	Excessive collagenase digestion	Reduce time of digestion Reduce volume of digestion buffer
Aorta	Poor cell dissociation	Poor Digestion	Make sure digestion buffer is at 37°C
		Aorta pieces in digestion buffer were too large	Make sure aorta is cut into approximately 1 mm pieces
		Aorta pieces were not shaking in dissociation buffer during incubation	Be sure aorta pieces are shaking in the digestion solution
	Cell Death	Excessive collagenase digestion	Reduce time of digestion Reduce volume of digestion buffer
Liver	Poor cell dissociation	Poor Digestion	Make sure digestion buffer is at 37°C
			Increase volume of digestion buffer
		Improper cannulation of IVC (swelling of tissue surrounding IVC occurs)	Be sure needle is properly inserted into the IVC
		Ruptured IVC	Properly secure needle in IVC prior to perfusion Reduce perfusion speed
		Ruptured tissue	Reduce perfusion speed
	Cell Death	Improper release of cells from the Glisson's capsule	Be sure to dissociate cells from capsule using previously discussed stroking method
		Excessive collagenase digestion	Reduce time of digestion

			Reduce volume of digestion buffer
--	--	--	-----------------------------------

Name of Material/ Equipment	Company	Catalog Number
26G x 5/8 in Needles	BD	305115
23G x 0.75 in needle x 12 in. tubing Blood Collection Set	BD	367297
21G 1 1/2 in. Needles	BD	305156
1mL syringe with rubber stops	BD	309659
10mL Syringes	BD	309604
1mL Syringe	BD	309659
F4/80 PE	Biolegend	123110
CD11c PE/Cy7	Biolegend	117318
CD11b PE/Cy5	eBioscience	15-0112-81
Anti-mouse CD16/32 Fc Block	Biolegend	101320
CD45 Pacific Blue	Biolegend	103126
PE Rat IgG2a	Biolegend	400508
PE/Cy7 Armenian Hamster IgG	Biolegend	400922
PE/Cy5 Rat IgG2b	Biolegend	400610
Pacific Blue Rat IgG2c	Biolegend	400717
Dulbecco's Modified Eagle Medium (DMEM)	Cellgro	15-017-CV
1X Phosphate Buffered Saline (PBS)	Cellgro	21-031-CV
70 micron cell strainers	Corning, Inc.	352350
1.7 mL microcentrifuge tube	Denville	C2170
Paraformaldehyde Aqueous Solution -16X	Electron Microscopy Sciences	CAS #30525-89-4
Micro Dissecting Scissors, 3.5 inch, Straight, 23mm, Sharp	Stoelting	52132-10P
Micro Forceps, 4in, full curve, 0.8mm	Stoelting	52102-37P
Spring dissection scissors – 3 mm Cutting edge	Fine Science Tool	15000-10
Curved 0.07 x 0.04 mm Tip Forceps	Fine Science Tool	11297-10
Hemostatic Forceps (Curved)	Fine Science Tool	13021-12
Heparin Sodium Salt	Fischer Scientific	9041-08-1
35mm Cell Culture/Petri Dishes	Fischer Scientific	12-565-90
Polystyrene Petri Dishes (10 cm) w/lid	Fischer Scientific	08-757-100D
15mL Conical Centrifuge Tubes (Polypropylene)	Fischer Scientific	14-959-53A
50mL Conical Centrifuge Tubes (Polypropylene)	Fischer Scientific	14-432-22
5mL Round-Bottom Polystyrene Tubes	Fischer Scientific	14-959-5
Fetal Bovine Serum	Gemini Bio-Products	100-106
Ethanol (Stock Ethyl Alcohol Denatured, Anyhydrous)	Millipore	EX0285-1
Bovine Serum Albumin	Rockland	BSA-50
HEPES	Sigma-Aldrich	H3375
Collagenase Type II	Sigma-Aldrich	C6885
Collagenase Type XI	Sigma-Aldrich	C7657
Hyaluronidase Type I	Sigma-Aldrich	H3506
DNAse	Sigma-Aldrich	DN25

Collagenase Type I	Sigma-Aldrich	C0130
NaOH	Sigma Aldrich	1310-73-2
CaCl ₂	Sigma Aldrich	449709-10G
500mL beaker	Sigma Aldrich	02-540M
4 cm Hemostatic clamp	Stoelting	52120-40
Toothed forceps	Stoelting	52104-33P
50 micron Disposable filters	Systemex	04-0042-2317
Collagenase Type IV	ThermoFischer Scientific	17104019
Ammonium Chloride Potassium (ACK)	ThermoFischer Scientific	A1049201
Razors (0.22 mm (0.009"))	VWR International	55411-050
Texas Red Live/Dead stain		

[illegible]

Red viability stain (in Figure 1A)



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 Author(s): tissues affected by obesity-related inflammation. Joselyn Allen, Adwitia Dey, Ruth
Nissly Gayathri Balandaram, Jeff Peters and Pamela Hankey-Giblin

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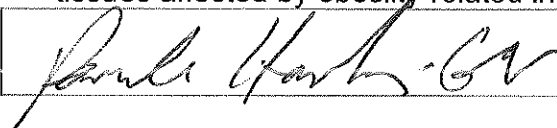
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Institution: The Pennsylvania State University
Article Title: Isolation, Characterization, and Purification of Tissue Resident Macrophages from tissues affected by obesity-related inflammation
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AUTHOR RESPONSES:

We would like to thank the editor and reviewers for their insightful comments. We will address each of these comments point by point below. Thank you again for your time and consideration.

Editor:

1) Please copyedit the manuscript for numerous grammatical errors. In particular, the protocol is full of comma splices. Please use conjugates like “and” appropriately. See 1.2.3.15 as an example, but please correct this throughout.

The proper conjugates are now present throughout the manuscript. The manuscript has been edited for further grammatical errors.

2) “Forceps” is plural. See 1.2.3.13 note, 1.2.4.2, etc. This also applies to scissors, which should not be preceded by “a” – see 1.2.3.15.

The “a” preceding forceps was deleted.

3) 2.5 – “pellet cells 751 x g”

The sentence was edited and now reads “pellet cells at 751 x g”.

4) 2.7 – Please use imperative tense.

The sentence was re-written using imperative tense.

5) Discussion – “that can hamper with”

The following sentence was corrected and “prevent” is used instead of hamper.

6) Please ensure that the references appear in the following format: [Last name, F.I., Last Name, F.I., Article Title. Source. Volume (Issue), First Page – Last Page, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al.

a. Please abbreviate all journal titles.

b. Please include volume, issue numbers, and DOIs for all references.

The listed references now meet the proper format.

7) Please include y axis tick labels on Figure 3 A (center, right).

This issue have been addressed and corrected.

8) Please use larger fonts for Figure 1, Figure 3, Figure 4, x and y axis tick labels.

A larger font size was used for all axes tick labels.

9) Please add more details to steps 1.2.2.1 and 1.2.2.2 describing how to eliminate air bubbles.

The method used to eliminate air bubbles was described.

10) Please specify the instrument used to make the incision in steps 1.2.3.3, 1.3.3.13.

The instrument used in these steps was specified.

11) Please correct step numbers referenced in 1.2.4.4. (18 and 19), and 2.4.4. (Step 4.4)

The referenced steps (mentioned above) were corrected.

12) Please describe centrifuge speeds as "x g" instead of the machine-dependent "rpm", in step 1.3.4.3.

All centrifuge speeds are now described as x g.

13. Formatting:

-1.3.3.16 and 1.3.3.17 should be substeps of 1.3.3.15.

-1.3.3.19-1.3.3.30 should be substeps of 1.3.3.18.

-2.4.4 – There is no step 4.4. Please correct the step number.

-2.5 – Paraformaldehyde is toxic. Please provide a caution statement as a note.

-First sentence of results section – Please fix the text formatting.

All the above formatting errors mentioned have been corrected.

14) Length exceeds 2.75 pg of highlighted material and should be reduced accordingly (1 pg minimum). We cannot film the procedures for all three tissues so it is recommended to only focus on one.

The highlighted material has been reduced to 2pgs and focuses solely on the liver dissociation protocol.

15) Visualization: Protocol is discontinuous. For instance, the aorta dissection should not be highlighted as insufficient detail will be filmed. Please choose which sections are most important to be visualized and highlight a continuous section of protocol. Only steps that are not essential to the section being filmed should be unhighlighted. We will not be able to produce a video from isolated steps in the sections.

Highlighted material now focuses on the liver dissociation protocol (the most challenging tissue) and provides a continuous script.

16) Additional detail is required:

-1.1.3.1, 1.3.3.2 – Is the mouse placed ventral side up or down? One assumes down, but the subsequent incisions made suggest that it is in fact up.

-1.2.3.2 – What orientation is the mouse placed in?

Additional details regarding the orientation have been provided.

17) Results:

-Please discuss the results shown in Figure 3 and Figure 4 in the Results section. What is the interpretation of the data?

Each figure and its sub content have been thoroughly discussed within the result section.

-Please define all statistical tests and p values and also define the error bars (SD, SEM, etc.) in the figure legends of Figures 2 & 3.

All statistical tests, p values and error bars are now properly defined.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

(A) Title: The title is to the point, describes the intent of the work and offers a practical example of the work.

The reviewer expressed no concerns, section remains unchanged.

(B) Abstract:

1. Short abstract could mention isolation of resident macrophages, not just characterization

The short abstract was re-written to address the reviewer's feedback.

2. Long abstract should still be to the point; the authors spend 4 sentences with background about obesity before mentioning that this publication is essentially about the techniques of isolating and characterizing macrophages. The abstract, at times, is the only thing that many readers will read. It needs to grab the attention of the reader and convey the purpose of the article.

The long abstract was re-written to address the reviewer's feedback.

(C) Introduction:

The introduction provides sufficient background to preface the purpose of the study. The authors include a motivation for the work.

The reviewer expressed no concerns, section remains unchanged.

(D) Results: This submission does not present novel results. The strength is the detailed protocol and as a way of demonstrating the quality of isolated macrophages, the authors cite the results of their previous publication. The figures included with this submission are DIRECT copies of the figures from the previous publication.

1. According to the Peer Review Specialist that was consulted, this is permissible, providing that, "they gained permission from the original journal. "

The permission for re-print was granted by the Journal of Immunology.

2. This reviewer recommends that the flow cytometry data figures (histograms) have better labels, including the specific fluorochromes used for the staining and detection. As a methods paper, details to this degree would be extremely useful in reproducing the protocol and results. Just because JI let you publish poorly labeled figures, the authors should be held to a higher standard for such a well written method based paper such as this.

The labels for all flow cytometry plots have been improved and included as required.

(E) Methods:

The methods described are well written, have adequate detail and are well organized.

The reviewer appreciates the attention to detail and enjoyed working through the protocol.

The reviewer expressed no concerns, section remains unchanged.

(F) Discussion: The Discussion is a very useful component of the overall submission. It includes not only discussion of results, but in depth troubleshooting and practical tip and advice. This discussion is very well done and useful.

The reviewer expressed no concerns, section remains unchanged.

1. Although the authors mention the use of viability dyes to enrich viable collections, there are no examples of this technique in the figures.

A plot demonstrating the use of the viability dye Texas Red live/dead stain has been included (Figure 1).

2. The authors mention that sorting can be used to collect cells for DNA and RNA analysis. The authors also mention the collection of cells in fetal bovine serum to enhance recovery (the reviewer assumes that this would be to collect live functional cells, not cells for DNA/RNA analysis). However, serum contains RNAses that would harm RNA downstream analysis. The authors should specify the collection medium for the specific downstream application

The reviewer makes an important point and consequently this was addressed. The collection medium needed for specific downstream application that minimizes RNA degradation is now explained.

References: Appropriate references cited.

The reviewer expressed no concerns, section remains unchanged.

Reviewer #2:

Manuscript Summary:

The manuscript by Allen et al. gives an excellent overview about current methods for cell isolation of adipose tissue, liver and the aorta. The step wise procedure is clear and the materials are listed properly.

(1) Especially, for researchers that are new in the field, this manuscript could be very useful. However, more established M2 markers, such as CD206 and CD301 should be discussed.

This is an excellent point and we have addressed more established M2 and M1 macrophage markers for flow cytometry and QRT PCR applications.

(2) Further, background staining of CD11c (clone N418) is usually very high, compared to the isotype control. Therefore, comparison of blanks, CD11c isotype and CD11c staining in lean and obese mice should be shown and the gating schemes should be recommended.

Although this is an excellent observation, the purpose of this publication is to focus on the isolation and characterization of macrophage populations from different tissues inflamed during diet induced metabolic disorders and consequently we wish to not focus on the disadvantages of the CD11c (clone N418) antibody. The high background of the CD11c antibody (clone N418) can be corrected for by using the proper controls to identify accurate gating boundaries. The use of proper controls (including SS, isotype controls and FMO) to define reliable gating boundaries is stressed in the manuscript. Additionally alternative M1 phenotype antibodies are available and can be used in substitute. Unfortunately, isotype controls could not be included for each experimental group (normal chow, HFD, HFHCD). This is because doing so will require additional time to obtain the data files from the other co-first author of the 2016 JI publication and this individual is no longer at our institution. Again, the aim of this publication is not to focus on the disadvantage of mouse CD11c antibodies since the selection for other M1 specific antibodies have grown over the years.



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Supplemental File (as requested by JoVE)

Allen 197 256-1.pdf

