A protocol for isolation of primary human immune cells from the liver and mesenteric white adipose tissue biopsies

Isolation of viable immune cells from human tissues is critical for the characterization of cellular and molecular processes underlying disease pathogenesis. Here, we describe protocols for the isolation of highly viable immune cells from liver wedges and mesenteric white adipose tissue resections from obese persons. Notably, characterization of the isolated single-immune cell suspensions, via utility of basic immunological interrogations and genetic approaches, promises to generate an improved understanding of altered immunological pathways in obese individuals with or without metabolic diseases.

Moreno-Fernandez et al., STAR Protocols 2, 100937 December 17, 2021 © 2021 The Authors.
https://doi.org/10.1016/j.xpro.2021.100937
Protocol

A protocol for isolation of primary human immune cells from the liver and mesenteric white adipose tissue biopsies

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https://doi.org/10.1016/j.xpro.2021.100937

SUMMARY

Isolation of viable immune cells from human tissues is critical for the characterization of cellular and molecular processes underlying disease pathogenesis. Here, we describe protocols for the isolation of highly viable immune cells from liver wedges and mesenteric white adipose tissue resections from obese persons. Notably, characterization of the isolated single-immune cell suspensions, via utility of basic immunological interrogations and genetic approaches, promises to generate an improved understanding of altered immunological pathways in obese individuals with or without metabolic diseases. For complete details on the use and execution of this protocol, please refer to Moreno-Fernandez et al. (2021).

BEFORE YOU BEGIN

© Timing: 9–10 h

Decontaminate working space (biosafety cabinet- with UV light) and equipment with 70% ethanol to maintain a sterile space and prevent contamination.

Note: The protocol below describes the specific steps for isolation of immune cells from human liver and WAT explant. However, this protocol has similarly been used for successful isolation of mouse and macaque liver and WAT immune cells. Prior to proceeding with this protocol, it is necessary to obtain approval by the institutional review board for collection of human material.

⚠ CRITICAL: Allow RPMI 1640, DMEM and Percoll to reach room temperature (22 ± 2 °C) prior to use.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Zombie UV Dye       | Biolegend | Cat # 423107 |
| CD45-A700 (clone 2D1) | Biolegend | Cat # 368513 |
| CD3-APC-eF780 (Clone OKT3) | eBioscience | Cat # 47-0037-41 |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| CD8-BV510 (Clone RPA-T8) | Biolegend | Cat # 301047 |
| CD4-BV650 (Clone RPA-T4) | Biolegend | Cat # 300535 |
| CD11b-BV421 (Clone ICRF44) | eBioscience | Cat # 301323 |
| CD11c-APC (Clone S-HCL-3) | Biolegend | Cat # 371505 |
| CD14-APC Fire750 (Clone 63D3) | Biolegend | Cat # 367119 |
| CD15-PE-Cy7 (Clone W6D3) | Biolegend | Cat # 323029 |
| HLA-DR-PercPCy5.5 (Clone L243) | Biolegend | Cat # 307629 |
| CD123-PE (clone 9F5) | BD Bioscience | Cat # 340545 |
| CD3-BUV395 (Clone SK7) | BD Bioscience | Cat # 564000 |
| CD16-BUV395 (Clone 3G8) | BD Bioscience | Cat # 563784 |
| CD19-BUV395 (Clone SJ25C1) | BD Bioscience | Cat # 563551 |
| IFNγ-PE-Cy7 (clone 4S.B3) | eBioscience | Cat # 25-7319-41 |
| TNFα AF488 (clone MAb11) | Biolegend | Cat # 502915 |
| Immunoglobulin G from human serum | Millipore | Cat # 56834 |

**Biological samples**

| Human liver wedge | Biological samples | N/A |
|-------------------|-------------------|-----|
| Age (years): 17.2 ± 0.2 | CCHMC Pediatric Diabetes and Obesity center | N/A |
| Sex: Females = 41 | | |
| Males = 19 | | |

| Human mesenteric white adipose tissue (WAT) | Biological samples | N/A |
|---------------------------------------------|-------------------|-----|
| Age (years): 17.2 ± 0.2 | CCHMC Pediatric Diabetes and Obesity center | N/A |
| Sex: Females = 41 | | |
| Males = 19 | | |

**Chemicals, peptides, and recombinant proteins**

| Fetal Bovine Serum (Seradigm Life Science) | Sigma-Aldrich | Cat # 05401127001 |
| Percoll | | |
| Liberase TM | Roche | Cat # 10104159001 |
| DNase I | Roche | Cat # 10875-093 |
| Bovine Serum Albumin | Sigma-Aldrich | Cat # A9642 |
| DPBS (Calcium and Magnesium Free) 10× | Corning | Cat # 20-031-CV |
| Distilled Water | Gibco | Cat # 15230-147 |
| RPMI 1640 media | Gibco | Cat # 89510 |
| DMEM (Dulbecco’s Modification of Eagle’s Medium) | Gibco | Cat # 12430062 |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich | Cat # P1585 |
| Ionomycin (Calbiochem) | EMD Millipore | Cat# 407952 |
| Brefeldin A | GoldBio | Cat # B-930 |
| Penicillin and streptomycin | Thermo Fisher Scientific | Cat # 15240062 |
| Glutamax | Gibco | Cat # 35050061 |
| Trypan Blue, 0.4% Solution in PBS | MP Biomedicals | Cat # 091691049 |
| ACK red blood Lysing Buffer | Lonza | Cat # 10-54BE |

**Critical commercial assays**

| BD Cytofix/Cytoperm Fixation/Permeabilization Kit | BD | Cat # 554174 |

**Other**

| GentleMACS C tubes | Miltenyi | Cat # 130-104-454 |
| Millepore Stericup receiver bottle, 500 mL | Millipore Sigma | Cat # SC00805RE |
| Cell Strainers, 100 uM, DNase/RNase Free, Non-Pyrogenic, Sterile | WWR | Cat # 10199-658 |
| 15 mL Centrifuge tubes | BioExpress | Cat # C-3394-1 |
| Falcon 50 mL Conical Centrifuge Tubes | Fisher Scientific | Cat # 14-959-49A |
| 96 well plates U bottom non tissue culture treated | Falcon | Cat # 351177 |
| GentleMACS Dissociator | Miltenyi Biotec | Cat # 130-09-235 |
| Temperature-controlled orbital shaker | New Brunswick Scientific | Classical Series |
| Temperature-controlled desktop centrifuge | Beckman Coulter | Avanti J-15R Centrifuge |

(Continued on next page)
CRITICAL: Many chemicals used in this protocol are hazardous and/or toxic. Institutional safety guidelines should be followed for safe use and disposal.

Note: To avoid unwanted baseline stimulation of the isolated immune cells, all reagents are tested either in lab (using Lonza, Kinetic Chromogenic LAL) Assay for endotoxin detection (Catalog #: 50–650U) or by the manufacturer providing reagents for endotoxin levels. A maximum level of 0.05 endotoxin units (EU) is acceptable for all reagents used.

Media and buffers

Note: Protocols are tissue specific, proceed carefully with cell isolations.

The following media and buffers are required:

Media:

- RPMI 1640
- DMEM, high glucose, L-glutamine and HEPES

Buffers:

- 1 x PBS

| Reagent                          | Final concentration | Amount (500mL) |
|----------------------------------|---------------------|---------------|
| Distilled Water                  |                     |               |
| DPBS (Calcium and Magnesium Free) | 1 x                 | 50 mL         |

Note: 1 x PBS can be prepared ahead of time and stored at room temperature for several weeks prior to use.

- Complete RPMI 1640 (C-RPMI)

| Reagent             | Final concentration | Amount (500mL) |
|---------------------|---------------------|---------------|
| RPMI 1640           |                     | 440 mL        |
| Fetal bovine serum  | 10%                 | 50 mL         |
| Glutamax            | 1%                  | 5 mL          |
| Penicillin/Streptomycin | 1%       | 5 mL          |
**Note:** C-RPMI can be prepared ahead of time and stored at 4°C for several weeks prior to use.

- **33% Percoll solution** (for gradient density isolation of immune cells from the liver, 30mL per sample)

| Reagent             | Final concentration | Amount  |
|---------------------|---------------------|---------|
| RPMI 1640           | 67%                 | 20.1 mL |
| Percoll             | 33%                 | 9.9 mL  |

**Note:** Percoll solution has to be made fresh on day of isolation and kept at room temperature prior to use.

- **WAT Digestion Buffer** (for gradient isolation of immune cells from the WAT, 7 mL per sample)

| Reagent                        | Final concentration | Amount                        |
|--------------------------------|---------------------|-------------------------------|
| DMEM, high glucose, L-glutamine and HEPES | 7 mL per sample      |                               |
| Liberase TM                    | 0.03 mg/mL (equals 0.156 U/mL) | 21 µL (from 10 mg/mL pre prepared stock) per sample (equals 1.09 U/sample) |
| DNase I                        | 50 U/mL             | see lot # for unit concentration |
| Bovine Serum Albumin           | 2%                  | 140 mg per sample             |

**Note:** Make a new WAT digestion buffer the day of isolation just prior to start of protocol and keep at room temperature until use.

- **FACS Buffer**

| Reagent            | Final concentration | Amount  |
|--------------------|---------------------|---------|
| PBS                |                     | 49 mL   |
| Fetal Bovine Serum | 2%                  | 1 mL    |

**Note:** keep FACS buffer at 4°C degrees and use within two weeks.

**Equipment**
The following equipment was used for the described protocols. Please check the protocols for specific instructions.

- Laminar flow biosafety cabinet
- GentleMACS® Dissociator (Miltenyi Biotec, Cat#130-093-235)
- Temperature-controlled orbital shaker adjusted to 37°C
- Temperature-controlled desktop centrifuge
- Sterile scissors, forceps
- Hemocytometer
- Light Microscope
- Flow Cytometer
- FlowJo
**STEP-BY-STEP METHOD DETAILS**

**Immune cell isolation from liver**

© Timing: 1 h

In this section we describe how to isolate primary immune cells from liver wedges of obese adolescents. We recommend the initiation of primary immune cell isolation process within 1 h after surgical tissue collection. Following tissue biopsy, the liver wedge is placed in a sterile gauze socked in sterile saline solution and promptly transported to the processing lab. For schematic overview see Figure 1.

**Note:** Keep the liver tissue at room temperature

1. Add 10 mL of C-RPMI (room temperature) into a GentleMACS C tube.
2. Place the liver wedge (~500 mg) into a GentleMACS C tube containing the C-RPMI.
3. Use GentleMACS Tissue Dissociator (program E.01, pre-programmed by manufacturer) to homogenize the liver tissue.
If the liver tissue did not homogenize completely, repeat the same dissociation program (E.01) 2–3 additional times. We do not recommend using other methods of homogenization since most mechanical dissociation techniques are either too gentle or too harsh to obtain robust yields of viable tissue immune cells.

4. Transfer homogenized liver tissue sample into a sterile 50 mL conical tube.
5. Wash GentleMACS C tube with an additional 10 mL of C-RPMI and transfer into the same 50 mL conical tube from step 4. Spin the homogenate at $800 \times g$ for 10 min at 4°C (Figure 1).

Note: While samples are spinning, prepare 33% Percoll in RPMI 1640 at room temperature.

6. Decant supernatant (from step 6) gently.
7. Add 30 mL of 33% Percoll solution directly to tube containing the cell pellet then briefly shake tube by hand to mix cell pellet with Percoll solution prior to centrifugation.

Note: This protocol does not involve layering of immune cells onto Percoll gradient.

8. Spin at $1000 \times g$ for 20 min at room temperature.

Note: Ensure that centrifuge is set to acceleration 1 and brake 0. Activating the break on the centrifuge may disturb the separation gradient and in turn may lower the cell number yield.

9. Gently and carefully decant the supernatant following centrifugation to limit disruption of immune cells pellet collected at the bottom of the 50 mL conical tube Figure 2.

△ CRITICAL: Cell pellet does not stick to tube bottom of the conical tube. Thus, extra caution should be employed to gently and carefully decant the supernatant to avoid the loss of isolated liver tissue primary immune cells.

Figure 2. Setup of the Percoll density gradient
(A) An example of a prepared gradient prior to centrifugation.
(B) Depiction of the resulting gradient layers after centrifugation. The desired immune cells pellet should be collected by discarding top fractions.
10. Resuspend the isolated primary immune cell pellet in 5 mL ACK (RBC lysis buffer) and incubate for 1 min at room temperature.

11. Spin at 800 × g for 4 min at 4°C.

12. Gently decant supernatant and resuspend pellet in 5 mL of C-RPMI.

13. Spin at 800 × g for 4 min at 4°C.

14. Gently decant supernatant and resuspend pellet in 1 mL of C-RPMI.

15. Count cells using a hemocytometer (mix 10 μL of sample with Trypan blue at 1:10 ratio (10 μL cell sample + 90 μL Trypan blue), load 10 μL of this dilution into the hemocytometer and count cells.

A liver wedge of ~500 mg will yield between 0.5–2 × 10^6 cells following this protocol.

16. Proceed to downstream application. See Figure 3 for step-by-step guide.

### Immune cell isolation from mesenteric white adipose tissue (WAT)

© Timing: 1.5 h

Note: Prepare Millipore Stericup receiver bottle with 300 ml DMEM, high glucose, L-glutamine and HEPES for tissue collection.

In this section we describe how to isolate immune cells from the mesenteric WAT. We recommend the initiation of primary immune cell isolation process within 1 h after surgical tissue collection. Following surgical collection, the WAT is placed in a sterile Millipore Stericup receiver bottle, containing 300 mL of DMEM, high glucose, L-glutamine and HEPES and promptly transported to the processing lab. For schematic overview see Figure 5.

17. Weigh entire mesenteric WAT biopsy.

Note: Be sure to keep sample sterile in hood.
18. Place approximately 3 grams of WAT into a 15 mL conical tube containing 10 mL of DMEM, high glucose, L-glutamine and HEPES. Prepare 10–20 tubes total (depending on amount of sample received).
19. Thoroughly mince tissue into small pieces using scissors.
20. Spin at 1000 \( g \) for 5 min at 4°C. Figure 4A

**Note:** in the meantime, prepare WAT digestion buffer. Prepare fresh WAT digestion buffer every time the protocol is done. Do not make stocks, enzymes can undergo degradation.

\( \Delta \) **CRITICAL:** After spinning, the mesenteric WAT layer will remain at the top of the tube while unwanted red blood cells and cellular debris will form a pellet at the bottom of the tube.

21. Remove the media and pellet from the bottom of each tube by inserting a 5 mL pipet through WAT layer. Keep the top WAT layer in the tube (Figure 4A).
22. Add 7 mL of WAT digestion buffer to the tube containing the remaining WAT layer.
23. Place on a shaking incubator at 37°C for 45 min at 225 rpm.
24. After 45 min of digestion vigorously shake tubes with hand. Figure 4B.
25. Filter the digested mesenteric WAT through 100 \( \mu \)M cell strainers into a 50 mL conical tube.
26. Centrifuge at 800 \( g \) for 4 min at 4°C. Figure 4C.

\( \Delta \) **CRITICAL:** The immune cells will be pelleted at the bottom whereas the mature adipocytes and lipid layer will be floating. Please make sure to remove as much as possible of the mature adipocytes and lipid layer to prevent downstream interference of these cells and lipids in flow cytometry analysis.

27. Decant supernatant and floating adipocytes and resuspend the cell pellet in 3 mL ACK lysis buffer, incubate for 1 min at room temperature.
28. Centrifuge at 800 \( g \) for 5 min at 4°C.
29. Decant supernatant and resuspend pellet in 1 mL C-RPMI.
30. Count cells using a hemocytometer (mix 10 μL of sample with Trypan blue at 1:10 ratio (10 μL cell sample + 90 μL Trypan blue), load 10 μL of this dilution into the hemocytometer and count cells. From white adipose tissue (60 grams) the provided protocol should yield ~20 x 10^6 cells.
31. Proceed to downstream application. See Figure 5 for step-by-step guide.

**Immunostaining of immune cells from liver and mesenteric WAT using flow cytometry**

**Timing:** 1.5 h

⚠ CRITICAL: We recommend using the manufacturer suggested volume per antibody concentration. However, we perform titration of each antibody prior to use for optimal standardization of flow cytometry staining in our lab.

**Note:** Prepare all Flow reagents in the hood with the light OFF. Prepare all required controls for flow cytometry analysis including compensation controls (unstained cell control, viability control (Zombie) and single-color controls) for proper set up of the cytometer.

32. Prepare Master Mix for surface markers for liver Macrophages, neutrophils, and DC.
33. Prepare Master Mix for surface markers for WAT T cells Macrophages, neutrophils, and DC

| Antibody                  | Dilution | Amount per sample |
|---------------------------|----------|-------------------|
| FACS buffer               |          | 50 μL             |
| Zombie UV                 | 1:250    | 0.2 μL            |
| CD45 AF700                | 1:50     | 0.1 μg            |
| CD11b-BV421               | 1:15     | 0.3 μg            |
| CD11c-APC                 | 1:50     | 0.1 μg            |
| CD14-APC Fire750          | 1:50     | 0.1 μg            |
| CD15-PE-Cy7               | 1:15     | 0.6 μg            |
| HLA-DR-PercPCy5.5         | 1:15     | 0.6 μg            |
| CD123-PE                  | 1:10     | 0.0625 μg         |
| CD3-BUV395                | 1:50     | 0.2 μg            |
| CD16-BUV395               | 1:50     | 0.2 μg            |
| CD19-BUV395               | 1:50     | 0.2 μg            |

34. Place the 0.5 × 10^6 cells into a 96-well round bottom plate (non-tissue culture treated).
35. Spin plate at 800 × g for 4 min at 4°C.
36. Following spin completion, gently aspirate media from the side of each well. Avoid touching the pellet.
37. Add 200 μL of FACS buffer.
38. Spin 800 × g for 4 min at 4°C.
39. Following spin gently aspirate media from the side of each well. Avoid touching the pellet.
40. Add 25 μL of FACS buffer and 2 μL of human IgG (1 mg/mL) and incubate for 10 min at 4°C

**Alternatives:** Fc receptors can be blocked by incubating cells with 10% normal human serum or Fc Block (BD-Bioscience Cat# 564219).

41. Add antibody master mix prepared in the steps 32 and 33 above. Pipet up and down to mix.
42. Incubate plate for 30 min at 4°C.

⚠️ CRITICAL: Ensure to protect plate from light.

43. Following incubation, add 150 μL of cold FACs Buffer.
44. Spin at 800 × g for 4 min at 4°C.
45. Gently aspirate media.
46. Add 100 μL of BD Cytofix Buffer to each well then resuspend cell pellet by pipetting up and down.
47. Incubate plate for 30 min at 4°C.
48. Spin at 800 x g for 4 min at 4°C.
49. Re-suspend the cell pellet in 200 μL of cold FACS buffer and transfer to flow tube for acquisition.
   Store at 4°C.

Note: Cells can be run in the flow cytometer the following day.
Figure 7. Gating strategy for FACS analyses of mesenteric WAT immune cells
A representative of 60 individual human samples.
(A) Forward and side scatter gating on lymphocytes to exclude cellular debris.
(B) Singlets gating to exclude doublets.
(C) Live cell positive gate.
(D) CD45^+ cells positive gating.
(E) CD3^+ and CD4^+ gating for CD4^+ T cells
(F) CD3^+ and CD8^+ positive gating for CD8^+ T cells.
Acquire the data using available Flow Cytometer.

Analyze FCS files using desired flow cytometry analysis software. We routinely use FlowJo for flow cytometry data analysis (Figures 6 and 7).

50. Acquire the data using available Flow Cytometer.

51. Analyze FCS files using desired flow cytometry analysis software. We routinely use FlowJo for flow cytometry data analysis (Figures 6 and 7).

**In-vitro stimulation of liver isolated immune cells with PMA and ionomycin for intracellular cytokines detection in T cells**

- **Timing**: 4 h

52. Plate 0.5 x 10^6 cells in 48-well plates in 500 μL of C-RPMI one well for unstimulated and one well for PMA/Ionomycin (PMA/Iono) stimulation.

  **Note:** Only plate the cells that are going to be used for the intracellular cytokine staining

53. Prepare the following stimulation solution:

| Reagent       | Final concentration | Amount per mL of culture |
|---------------|----------------------|--------------------------|
| Complete RPMI |                      | 46.75 mL                 |
| PMA           | 50 ng/mL             | 1.25 μL (from 40 μg/mL pre prepared stock) |
| Ionomycin     | 1 μg/mL              | 1 μL (from 1 mg/mL pre prepared stock) |
| Brefeldin A   | 10 μg/mL             | 1 μL (from 10 mg/mL pre prepared stock) |

54. Add 25 μL of the solution prepared in step 53 above to the stimulated well.

55. Incubate at 37°C in 5% CO2 for 4 h.

**Extra- and intracellular immunostaining of in-vitro stimulated immune cells from liver using flow cytometry**

- **Timing**: 3.5 h

  **Critical:** We recommend using the manufacturer suggested volume per antibody concentration. However, we perform titration of each antibody prior to use for optimal standardization of flow cytometry staining in our lab.

56. 30 min prior to the end of the incubation period with PMA/Ionomycin, prepare flow antibody master mix for extracellular antigens as follow:

  **Note:** Prepare all Flow reagents in the hood with the light OFF. Prepare all required controls for flow cytometry analysis including compensation controls (unstained cell control, viability control (Zombie) and single-color controls) for proper set up of the cytometer.

| Master Mix for surface markers for liver T cells | Antibody | Dilution | Amount per sample |
|------------------------------------------------|----------|----------|-------------------|
| FACS buffer                                    |          |          | 50 μL             |
| Zombie UV                                      | 1:250    |          | 0.2 μL            |

(Continued on next page)
57. After incubation transfer the $0.5 \times 10^6$ cells unstimulated cells and the $0.5 \times 10^6$ stimulated cells into a 96-well round bottom plate (non-tissue culture treated).

58. Spin plate at 800 $\times$ g for 4 min at 4°C.

59. Following spin completion, gently aspirate media from the side of each well. Avoid touching the pellet.

60. Add 200 µL of FACS buffer.

61. Spin 800 $\times$ g for 4 min at 4°C.

62. Following spin gently aspirate media from the side of each well. Avoid touching the pellet.

63. Add 25 µL of FACS buffer and 2 µL of human IgG and incubate for 10 min at 4°C

**Alternatives:** Fc receptors can be blocked by incubating cells with 10% normal human serum or Fc Block (BD-Bioscience Cat# 564219).

64. Add antibody master mix prepared in the step 56 above. Pipet up and down to mix.

65. Incubate plate for 30 min at 4°C.

△ **CRITICAL:** Ensure to protect plate from light.

66. Following incubation, add 150 µL of cold FACs Buffer.

67. Spin at 800 $\times$ g for 4 min at 4°C.

68. Gently aspirate media.

69. Add 100 µL of BD Cytofix Buffer to each well then resuspend cell pellet by pipetting up and down.

70. Incubate plate for 30 min at 4°C.

71. Spin at 800 $\times$ g for 4 min at 4°C.

**Break point: after fixation** Cells can be stored to continue the intracellular staining at a later time up to 24 h. Resuspend cells in FACS Buffer for storing cells at 4°C for

**Note:** If decide to continue with staining procedure, while plates are spinning prepare the $1 \times$ BD Perm Wash by diluting $10 \times$ BD Perm Wash to $1 \times$ in distilled water.

72. Prepare intracellular stain for the liver T cells well as follows:

| Antibody          | Dilution | Amount per sample |
|-------------------|----------|-------------------|
| 1× Perm Wash      | 1×       | 50 µL             |
| IFNγ PE-Cy7       | 1:16     | 0.075 µg          |
| TNFα AF488        | 1:16     | 0.15 µg           |

73. Gently aspirate supernatant from well. Do not touch the cell pellet.

74. Add antibody master mix prepared in step 72 above. Pipet up and down to mix.

75. Incubate plate for 1 h at 4°C.

76. Following incubation, add 200 µL of cold $1 \times$ BD Perm Wash.
77. Spin at 800 g for 4 min at 4°C.
78. Gently aspirate supernatant from well. Do not touch the cell pellet.
79. Add 220 μL of cold FACs Buffer.
80. Spin at 800 g for 4 min at 4°C.
81. Gently aspirate supernatant from well. Do not touch the cell pellet.
82. Re-suspend the cell pellet in 200 μL of cold FACs buffer and transfer to flow tube for acquisition.
Store at 4°C.

Note: Cell can be run in the flow cytometer the following day.

83. Acquire the data using available Flow Cytometer.
84. Analyze FCS files using desired flow cytometry analysis software. We routinely use FlowJo for flow cytometry data analysis, Figures 6 and 8.

EXPECTED OUTCOMES
Described protocols for both liver wedge and mesenteric WAT primary immune cell isolation provide a consistent and reproducible method for attainment of highly-viable tissue immune cells. These protocols are based on gradient isolation or enzymatic digestion followed by flowcytometric analysis of immune cells. Of note, from a liver wedge (~500 mg) the provided protocol should yield between 0.5–2 × 10^6 cells, while from mesenteric WAT (~60 grams) the provided protocol should yield ~20 × 10^6 cells.
Some variability in attainment of highly-viable cell numbers may occurs given the biological differences between individuals, their metabolic state, and degree of disease severity at time of isolation.

LIMITATIONS
We acknowledge that limitations to the protocols above exist. For example, our described protocol has been tested in combination with the use of GentleMACS Tissue Dissociator. Although other protocols

Figure 8. Gating strategy for FACS analyses of CD4+ and CD8+ T cytokine production after in vitro stimulation with PMA and Ionomycin
(A) Intracellular detection of TNFα and IFNγ in CD3+CD4+ T cells (gated from Figure 6E) with and without stimulation with PMA/Ionomycin.
(B) Intracellular detection of TNFα and IFNγ in CD3+CD8+ T cells (gated from Figure 6F) with and without stimulation with PMA/Ionomycin. Left panels unstimulated control. Right panels PMA/Ionomycin stimulated cells.
that use enzymatic digestion for isolation of single immune cell suspensions from tissues exist and may be applicable for the overall scientific question at hand, such protocols have not been tested in our hands. One line of caution is that the use of mechanical mincing or tissue homogenization (use of steal blades) may be too gentle or too harsh respectively to obtain a high yield of viable single immune cell suspensions. In addition, we acknowledge that the addition of enzymatic digestion may increase cell yields (e.g., DC) from the liver wedges, however such approach was not tested in our hands. Further, liver wedge and mesenteric WAT were not perfused. Thus, a possibility of partial contamination from immune cells originated from the circulation may occur, while the presence of excess red blood cells during the isolation process may present challenges during the in vitro stimulation portion of the experimental approach. Similarly, adding a Percoll gradient step to our mesenteric WAT prep may further improve immune cell purity. However, to limit the potential impact on extra processing time on cell viability, given the high numbers of immune cells in mesenteric WAT, we opted to exclude the Percoll gradient step from our protocol. Of note, the autofluorescence of cells derived from liver and mesenteric WAT tissues may present challenges in the discrimination of various immune cell populations during FACS analysis. Thus, appropriate controls must be included to ensure rigor in gating analyses. Lastly, although large body of literature has demonstrated effective use of FBS in in vitro culturing of human immune cells, human AB serum may represent a more natural reagent for long term cultures of human immune cells. Of note, our protocol only requires short term in vitro culturing of human immune cells (4 h)—something that has been previously reported to be effective method in functional characterization of human immune cells isolated from livers of individuals with NAFLD (Moreno-Fernandez et al., 2021). Together, these data demonstrate that using our described protocol allows for efficient and effective attainment and functional analyses of a human immune cells.

TROUBLESHOOTING

Problem 1

Low cell yield: A low number of immune cells may occur when: (a) time between surgery and isolation is extended; (b) time of gradient isolation or enzymatic digestion is prolonged; (c) calculations required for the preparation of Percoll buffer or digestion buffer are incorrect; and (d) prolonged exposure to ACK lysis buffer at room temperature (step 10 and 27).

Potential solution

To avoid excessive loss of immune cells during various steps of the protocol a timely and precise isolation protocol should be used. Further, one can prepare standard calculations for amounts of buffers and enzymes to be used to limit the mathematical mistakes at time of performing the protocol.

Problem 2

Low cell viability: Lower cell viability can occur when cells are exposed to a prolonged period of time to the collagenase digestion and/or the red cell lysis step (step 10, 23 and 27).

Potential solution

Try to keep the digestion and lysis steps to the recommended time keep samples, keep samples in media or PBS containing FBS to maximize cell viability.

Problem 3

Clumping of cells: Clumping of cells can occur during isolation process due to dead cells and cell debris. We do not recommend using EDTA to avoid clumping because EDTA is an ion chelator that interferes with the in-vitro stimulation using PMA/Ionomycin (step 54).

Potential solution

Please be gentle during isolation process, keep the different steps to the recommended time to avoid excessive cell death which contributes to clumping. Additionally, after isolation from the tissue, cells suspension can be filter in 100 μM filters to remove clumps.
Problem 4
Autofluorescence: This is attributed to the presence in the cell suspension prep of some non-immune cells such as epithelial cells and the higher frequency of macrophages and myeloid cells in this tissue (step 84).

Potential solution
Macrophages and myeloid cells have significant autofluorescence in the green and UV channels. Therefore, it is critical to include appropriate control (unstained cells, isotype and FMO) to ensure that the positive signal is real. In addition, we recommend to use antibodies for such populations to be label with fluorochromes for that do not emit in the green or UV spectrum.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Senad Divanovic, senad.divanovic@cchmc.org.

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate/analyze [datasets/code].

ACKNOWLEDGMENTS
This work was supported in part by NIH R01DK099222, Department of Defense (DoD) W81XWH2010392, American Diabetes Association (ADA) 1-18-IBS-100, CCHMC Pediatric Diabetes and Obesity Center, and CCRF Endowed Scholar Award (to S.D.); R01DK099222-02S1 (associated with S.D., and M.E.M.-F.); American Heart Association (AHA) 17POST33650045 and ADA 1-19-PMF-019 (to M.E.M.-F.); and PHS Grant P30 DK078392 Pathology of the Digestive Disease Research Core Center at CCHMC (associated with S.D.).

AUTHOR CONTRIBUTIONS
M.E.M.-F., M.S.M.A.D., and S.D. participated in the conception and design of the study and wrote the manuscript. M.E.M.-F. and M.S.M.A.D. participated in analysis and interpretation of data. All authors have reviewed the manuscript and approve the final version.

DECLARATION OF INTERESTS
S.D. is a consultant for Janssen Research & Development.

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