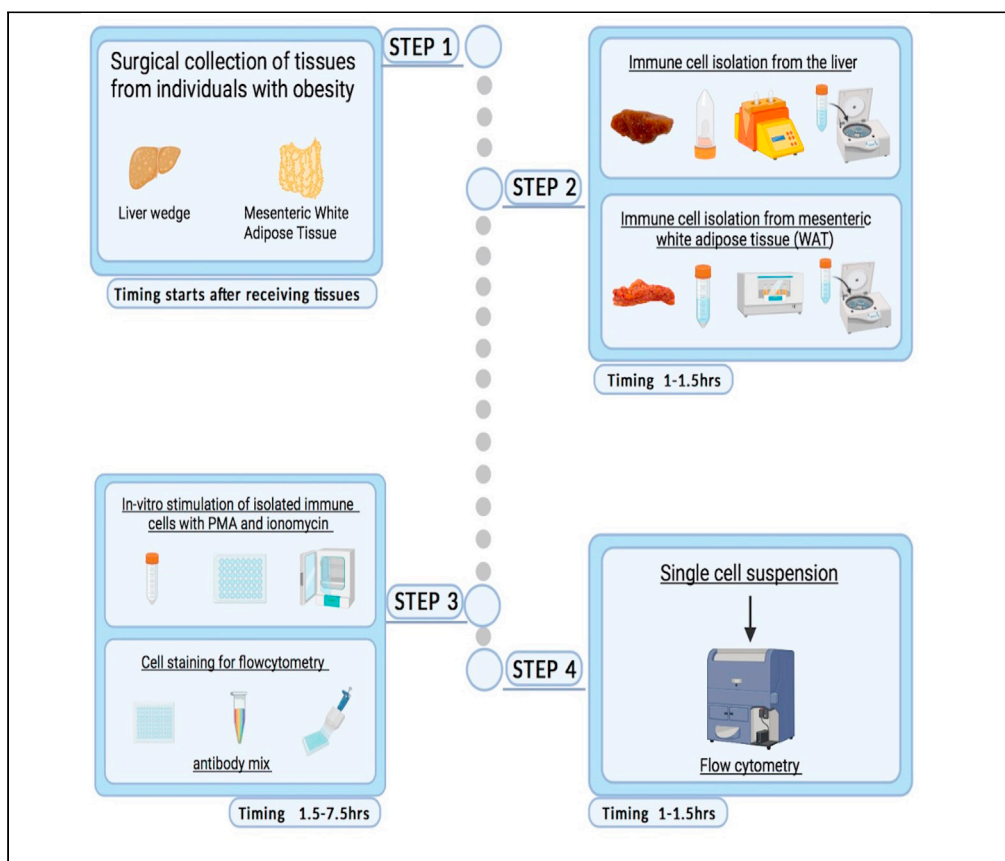


Protocol

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.

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Highlights

Protocol for isolation of highly viable human immune cells from small liver wedges

Protocol for liver wedge processing does not require enzymatic digestion

Detailed steps for isolation of highly viable human immune cells from mesenteric WAT

In vitro activation of primary tissue immune cells and their functional assessment

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Protocol

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

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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 ($22 \pm 2^\circ\text{C}$) prior to use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Zombie UV Dye	Biologend	Cat # 423107
CD45-A700 (clone 2D1)	Biologend	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)	Biologend	Cat # 301047
CD4-BV650 (Clone RPA-T4)	Biologend	Cat # 300535
CD11b-BV421 (Clone ICRF44)	eBioscience	Cat # 301323
CD11c-APC (Clone S-HCL-3)	Biologend	Cat # 371505
CD14- APC Fire750 (Clone 63D3)	Biologend	Cat # 367119
CD15-PE-Cy7 (Clone W6D3)	Biologend	Cat # 323029
HLA-DR-PercPCy5.5 (Clone L243)	Biologend	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
IFN γ -PE-Cy7 (clone 4S.B3)	eBioscience	Cat # 25-7319-41
TNF α AF488 (clone MAb11)	Biologend	Cat # 502915
Immunoglobulin G from human serum	Millipore	Cat # 56834

Biological samples

Human liver wedge	CCHMC Pediatric Diabetes and Obesity center	N/A
<ul style="list-style-type: none"> • Age (years): 17.2 \pm 0.2 • Sex: Females = 41 Males = 19 		
Human mesenteric white adipose tissue (WAT)	CCHMC Pediatric Diabetes and Obesity center	N/A
<ul style="list-style-type: none"> • Age (years): 17.2 \pm 0.2 • Sex: Females = 41 Males = 19 		

Chemicals, peptides, and recombinant proteins

Fetal Bovine Serum (Seradigm Life Science)	VWR	Cat # 89510
Percoll	Sigma-Aldrich	Cat # P1644
Liberase TM	Roche	Cat # 05401127001
DNase I	Roche	Cat # 10104159001
Bovine Serum Albumin	Sigma-Aldrich	Cat # A9647
DPBS (Calcium and Magnesium Free) 10 \times	Corning	Cat # 20-031- CV
Distilled Water	Gibco	Cat # 15230-147
RPMI 1640 media	Gibco	Cat # 11875-093
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 # 10-548E

Critical commercial assays

BD Cytotfix/Cytoperm Fixation/Permeablization Kit	BD	Cat # 554714
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Other

GentleMACS C tubes	Miltenyi	Cat # 130-104-454
Millepore Stericup receiver bottle, 500 mL	Millipore Sigma	Cat # SC00B05RE
Cell Strainers, 100 μ M, DNase/RNase Free, Non-Pyrogenic, Sterile	VWR	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#130-093-235
Temperature-controlled orbital shaker	New Brunswick Scientific	Classical Series
Temperature-controlled desktop centrifuge	Beckman Coulter	Avanti J-15R Centrifuge

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Scissors and Forceps	Roboz Surgical Instrument Co	Cat# RS-5840SC Cat# RS-8100
Hemocytometer	Hausser Scientific	Bright Line Counting Chamber
Microscope	Nikon	Light Microscope
Flow Cytometer	Beckton Dickinson	LSR Fortessa
Software and algorithms		
FlowJo, Version 10	BD-FlowJo	https://www.flowjo.com/

MATERIALS AND EQUIPMENT

△ **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 × PBS

Reagent	Final concentration	Amount (500mL)
Distilled Water		450 mL
DPBS (Calcium and Magnesium Free) 10×	1×	50 mL

Note: 1 × 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
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



Figure 1. Depiction of liver homogenate from step 6 after centrifugation

An example of a cell pellet after successful homogenization of liver tissue using GentleMACS Tissue Dissociator.

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.

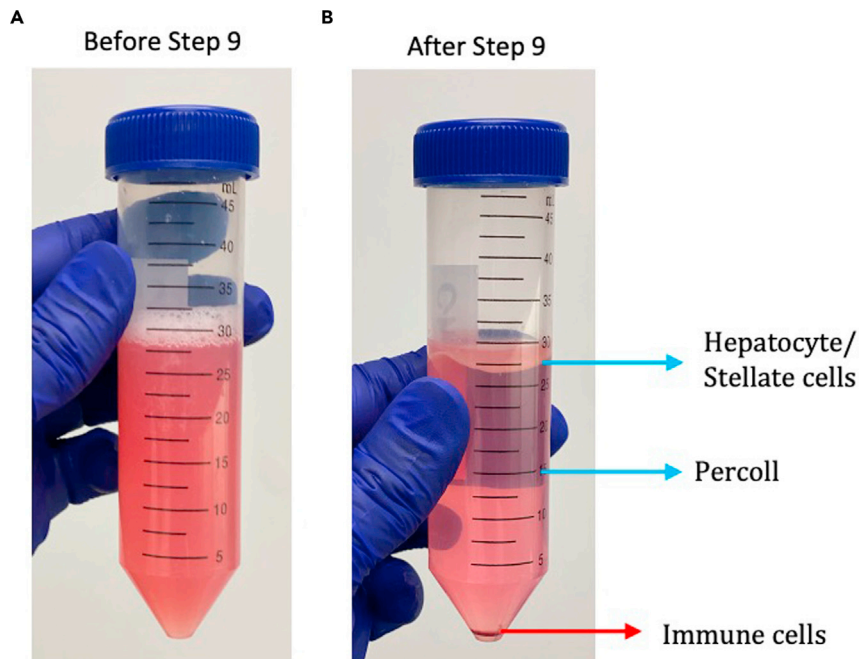


Figure 2. Setup of the Percoll density gradient

(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.

Note: 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 cells 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 brake 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.

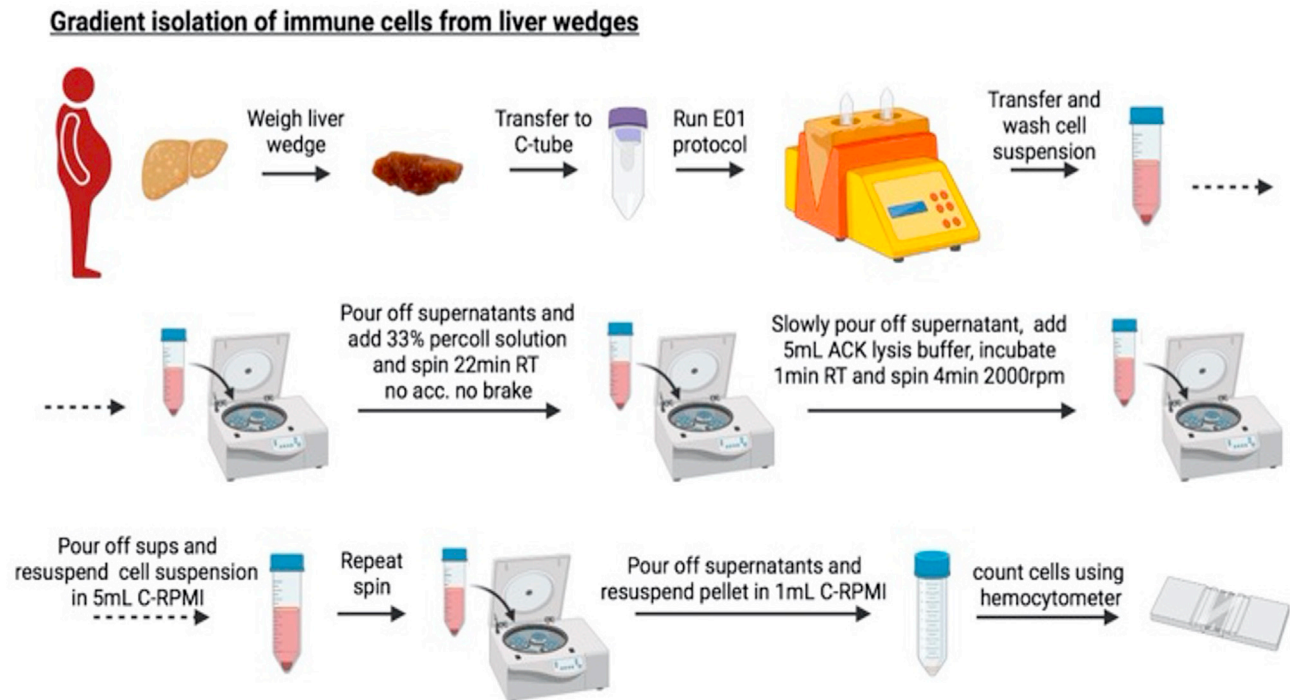


Figure 3. Immune cell isolation from liver wedge

Detailed schematics illustrating isolation of liver immune cells by Percoll gradient.

- Resuspend the isolated primary immune cell pellet in 5 mL ACK (RBC lysis buffer) and incubate for 1 min at room temperature.
- Spin at $800 \times g$ for 4 min at 4°C .
- Gently decant supernatant and resuspend pellet in 5 mL of C-RPMI.
- Spin at $800 \times g$ for 4 min at 4°C .
- Gently decant supernatant and resuspend pellet in 1 mL of C-RPMI.
- Count cells using a hemocytometer (mix $10 \mu\text{L}$ of sample with Trypan blue at 1:10 ratio ($10 \mu\text{L}$ cell sample + $90 \mu\text{L}$ Trypan blue), load $10 \mu\text{L}$ of this dilution into the hemocytometer and count cells. A liver wedge of $\sim 500 \text{ mg}$ will yield between $0.5\text{--}2 \times 10^6$ cells following this protocol.
- 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.

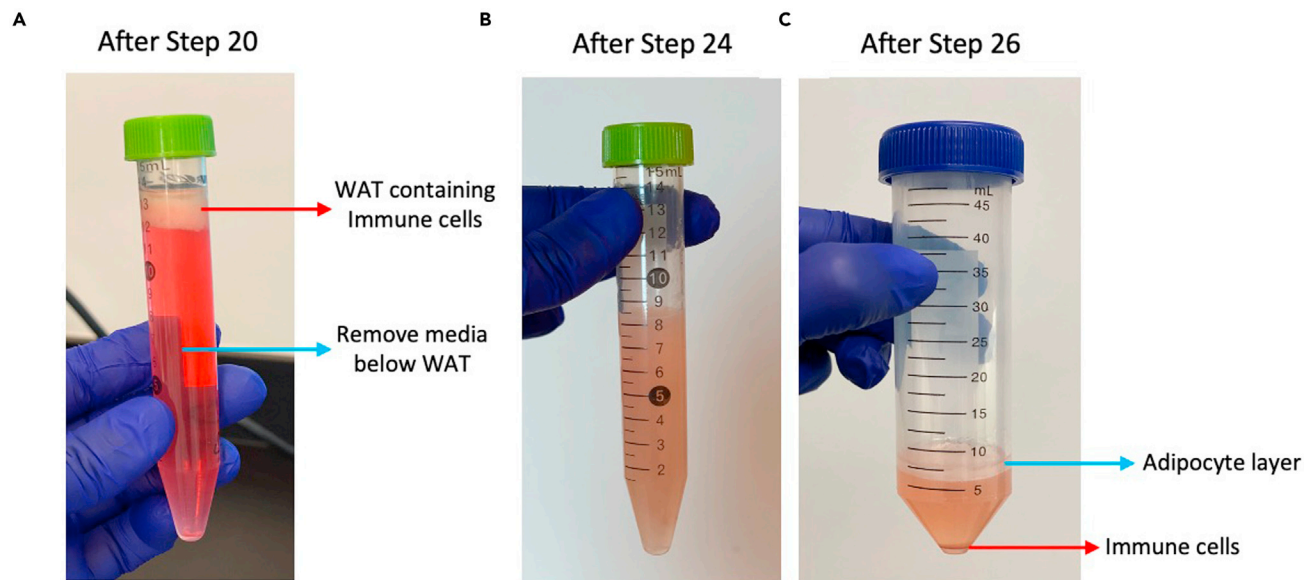


Figure 4. Setup of the immune cell isolation from mesenteric WAT

(A) An example of the different layers after mincing (after step 20).

(B) An example of the WAT homogenates after enzymatic digestion (after step 24).

(C) Depiction of the various layers post enzymatic digestion and after centrifugation (after step 26).

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 $1000 \times g$ 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.

△ 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 $100 \mu\text{M}$ cell strainers into a 50 mL conical tube.
26. Centrifuge at $800 \times g$ for 4 min at 4°C . [Figure 4C](#).

△ 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 $800 \times g$ for 5 min at 4°C .

Enzymatic digestion and isolation of immune cells from mesenteric WAT

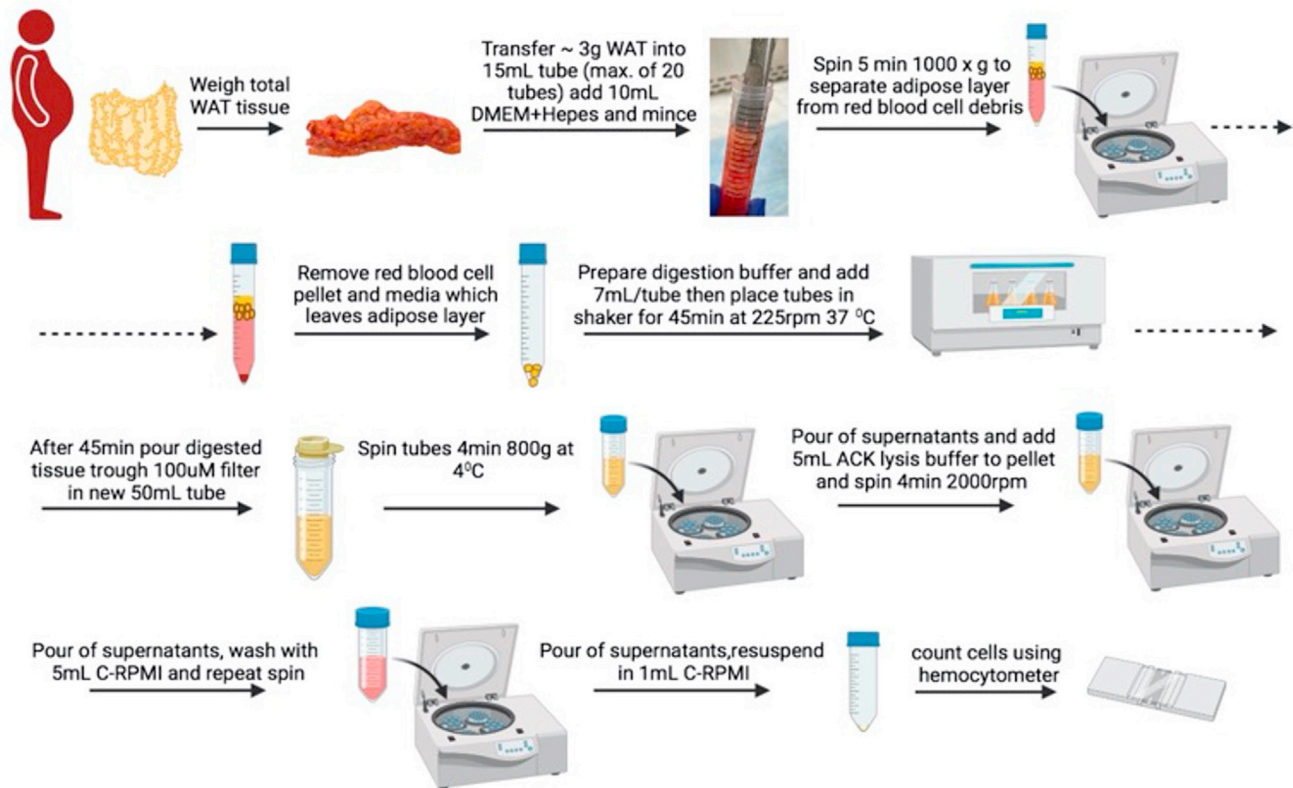


Figure 5. Immune cell isolation from mesenteric WAT

Detailed scheme illustrating isolation of WAT immune cells by enzyme digestion isolation protocol.

29. Decant supernatant and resuspend pellet in 1 mL C-RPMI.
30. Count cells using a hemocytometer (mix 10 μ L of sample with Trypan blue at 1:10 ratio (10 μ L cell sample + 90 μ L Trypan blue), load 10 μ L of this dilution into the hemocytometer and count cells. From white adipose tissue (60 grams) the provided protocol should yield $\sim 20 \times 10^6$ 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.

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

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

Antibody	Dilution	Amount per sample
FACS buffer		50 μ L
Zombie UV	1:250	0.2 μ L
CD45 AF700	1:50	0.1 μ g
CD3 APCeF780	1:50	0.025 μ g
CD8 BV510	1:50	0.1 μ g
CD4 BV650	1:10	0.35 μ g
CD11b-BV421	1:15	0.3 μ g
CD11c-APC	1:50	0.1 μ g
CD15-PE-Cy7	1:15	0.6 μ g
HLA-DR-PercPCy5.5	1:15	0.6 μ g
CD123-PE	1:10	0.0625 μ g

34. Place the 0.5×10^6 cells into a 96-well round bottom plate (non-tissue culture treated).

35. Spin plate at $800 \times g$ 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 μ L of FACS buffer.

38. Spin $800 \times g$ 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 μ L of FACS buffer and 2 μ L 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 μ L of cold FACs Buffer.

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

45. Gently aspirate media.

46. Add 100 μ L of BD Cytfix Buffer to each well then resuspend cell pellet by pipetting up and down.

Liver immune cell gating strategy

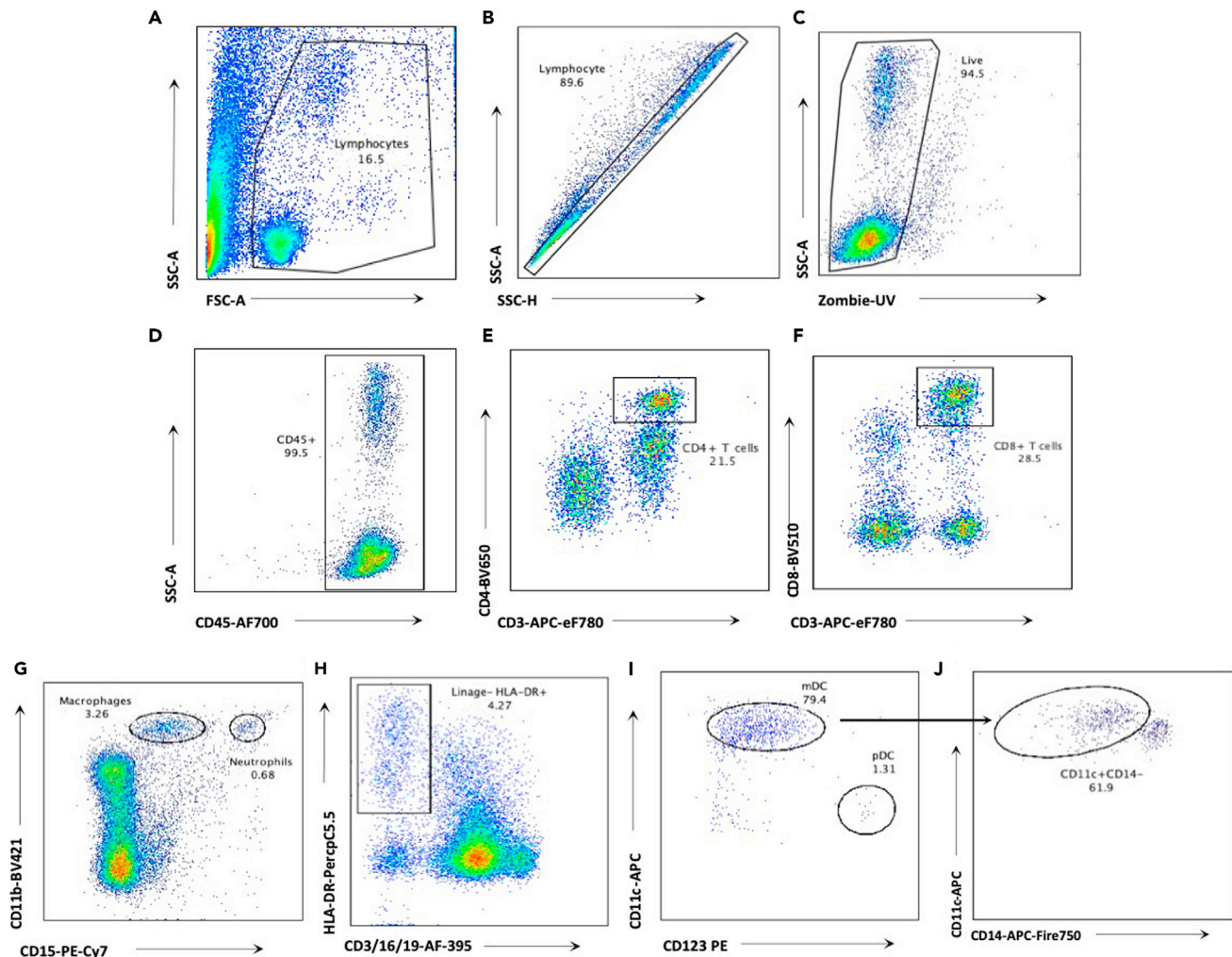


Figure 6. Gating strategy for FACS analyses of Liver immune cells

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.

(G) from the second staining panel we gated forward and side scatter gating on lymphocytes to exclude cellular debris then in singlets to exclude doublets followed by live cell and CD45⁺ cells positive gating. (G) CD11b and CD15 positive gating for macrophages/monocytes CD11b⁺CD15^{low} and neutrophils CD11b^{high}CD15^{high}.

(H) HLA-DR high and CD3/CD16/CD19 negative gating for dendritic cells.

(I and J) (I) CD11c vs CD123 for plasmacytoid DC (CD11c⁺CD123⁺) and myeloid DC (CD11c⁺CD123⁻) and (J) CD11c vs CD14 for monocyte exclusion.

47. Incubate plate for 30 min at 4°C.

48. Spin at 800 × 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.

WAT immune cell gating strategy

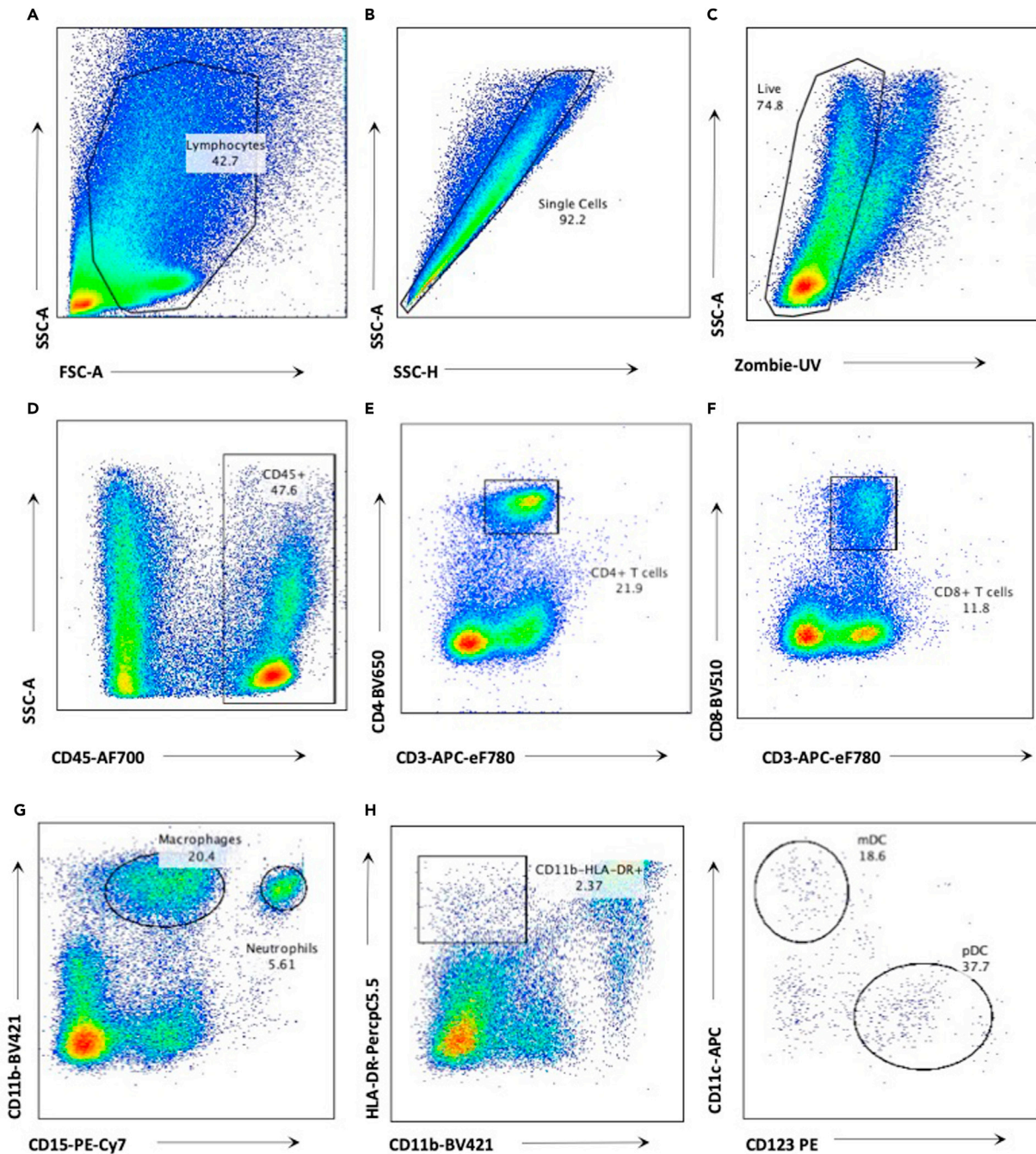


Figure 7. Gating strategy for FACS analyses of mesenteric WAT immune cells

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.

Figure 7. Continued

(G) from the second staining panel we gated forward and side scatter gating on lymphocytes to exclude cellular debris then in singlets to exclude doublets followed by live cell and CD45⁺ cells positive gating. (G) CD11b and CD15 positive gating for macrophages/monocytes CD11b⁺CD15^{low} and neutrophils CD11b^{high}CD15^{high}.

(H) Left panel, HLA-DR high and CD11b negative gating for dendritic cells and right panel, CD11c vs CD123 for plasmacytoid DC (CD11c⁺CD123⁺) and myeloid DC (CD11c⁺CD123⁻).

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 0.5×10^6 cells in 48-well plates in 500 μ L 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 μ L (from 40 ug/mL pre prepared stock)
Ionomycin	1 μ g/mL	1 μ L (from 1 mg/mL pre prepared stock)
Brefeldin A	10 μ g/mL	1 μ L (from 10 mg/mL pre prepared stock)

54. Add 25 μ L 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 μ L
Zombie UV	1:250	0.2 μ L

(Continued on next page)

Continued

Antibody	Dilution	Amount per sample
CD45 AF700	1:50	0.1 µg
CD3 APCe780	1:50	0.025 µg
CD8 BV510	1:50	0.1 µg
CD4 BV650	1:10	0.35 µg

57. After incubation transfer the 0.5×10^6 cells unstimulated cells and the 0.5×10^6 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 Cytfix 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× BD Perm Wash by diluting 10× BD Perm Wash to 1× in distilled water.

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

Antibody	Dilution	Amount per sample
1× Perm Wash		50 µL
IFN γ PE-Cy7	1:16	0.075 µg
TNF α 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× BD Perm Wash.

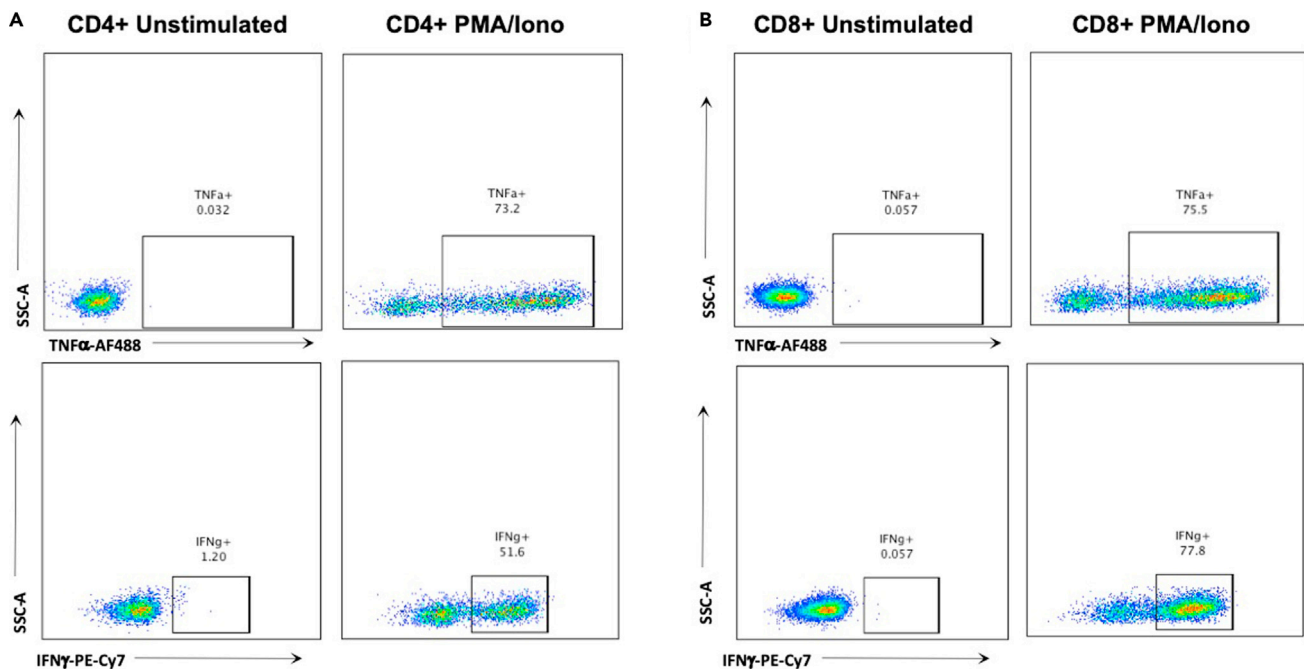


Figure 8. Gating strategy for FACS analyses of CD4⁺ and CD8⁺ T cytokine production after *in vitro* stimulation with PMA and Ionomycin

A representative of 60 individual human samples. Gating strategy for intracellular detection of TNF α and IFN γ from liver T cells.

(A) Intracellular detection of TNF α and IFN γ in CD3⁺CD4⁺ T cells (gated from Figure 6E) with and without stimulation with PMA/Ionomycin.

(B) Intracellular detection of TNF α and IFN γ 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.

77. Spin at 800 \times g for 4 min at 4°C.
78. Gently aspirate supernatant from well. Do not touch the cell pellet.
79. Add 220 μ L of cold FACS Buffer.
80. Spin at 800 \times 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 μ L 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 (\sim 500 mg) the provided protocol should yield between $0.5\text{--}2 \times 10^6$ cells, while from mesenteric WAT (\sim 60 grams) the provided protocol should yield $\sim 20 \times 10^6$ cells. Some variability in attainment of highly-viable cell numbers may occur 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

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 steel 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 μ M 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].

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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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