Protocol

Comprehensive analysis of liver macrophage composition by flow cytometry and immunofluorescence in murine NASH

Highlights

Protocol for the isolation and analysis of hepatic macrophages in fatty liver disease

Review of the primary macrophage subsets present in NASH

Outline of macrophage analysis by flow cytometry and tissue imaging

Description of a reproducible and efficient portal vein cannulation technique

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**Protocol**

Comprehensive analysis of liver macrophage composition by flow cytometry and immunofluorescence in murine NASH

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**SUMMARY**

Recently, it has become evident that macrophage diversity increases in the liver during the pathogenesis of non-alcoholic steatohepatitis (NASH). Here, we provide a detailed protocol for the analysis of liver macrophage subsets in mice with non-alcoholic fatty liver disease (NAFLD) and early NASH using flow cytometry and immunofluorescence (IF). These methods can be used to assess the composition and localization of macrophage subsets during NASH.

For complete details on the use and execution of this protocol, please refer to Daemen et al. (2021).

**BEFORE YOU BEGIN**

The protocol below describes the specific steps for isolation of liver macrophages of mice with established NASH. However, we have also used this protocol for isolation and analysis of liver macrophages of healthy, standard diet-fed mice and mice from a variety of genetic backgrounds. As antibodies to CCR2 and CX3CR1 do not work well for flow cytometry following liver digestion the use of Ccr2-GFP or Cx3cr1-GFP reporter mice can be employed to identify Ccr2/Cx3cr1-high liver macrophages. Thus, we will describe here the use of this protocol for both Ccr2/Cx3cr1-GFP and wild-type mice.

**Induction of diet-induced NASH**

© Timing: 0–16 weeks

1. Co-house same sex mice in specific pathogen free animal facilities with regular drinking water. If comparing wild type (WT) to knockout (KO) or transgenic mice it is critical to ensure mixing of genotypes in cages. Littermate controls should also be utilized.

2. Induce NASH in 8-week-old mice by replacing regular chow with a NASH diet, i.e., the fructose, cholesterol, palmitate (FPC) diet or a diet with similar composition (i.e., AMLN), for at least 16 weeks. Provide fresh NASH chow weekly. Although female or male mice can be used the NASH phenotypes are more severe in male mice.

**Note:** Duration of feeding may need to be optimized depending on the experimental question, mouse strain and specific NASH diet.
3. Monitor body weight and record at least every 4 weeks.
4. If needed, perform glucose and insulin tolerance tests 1–2 weeks prior to sacrifice.
5. Sacrifice animals and harvest liver at the end of 16 weeks NASH diet.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rat monoclonal CD11b-APC-Cy7 (clone M1/70) - 1:100 | BioLegend | Cat# 101226 |
| Rat monoclonal CD45-BUV395 (clone 30-F11)-1:100 | BD Biosciences | Cat# 564279 |
| Rat monoclonal F4/80-AF647 (clone BM8)-1:100 | BioLegend | Cat# 123122 |
| Rat monoclonal Ly6C-FITC (clone R86-BC5)-1:100 | BioLegend | Cat# 108406 |
| Rat monoclonal MHCIi-BV605 (clone M5/114.15.2) – 1:300 | BioLegend | Cat# 107639 |
| Rat monoclonal TIM4-BV421 (clone 21H12)- 1:500 | BD Biosciences | Cat# 742773 |
| Rat monoclonal VSG4-PeCy7 (clone NLA14)- 1:200 | Invitrogen-Thermo Fisher | Cat# 25-5752-82 |
| Rat monoclonal CLEC2-PE (clone 17D9) – 1:100 | BioLegend | Cat# 146104 |
| Armenian Hamster monoclonal CD11c-BV711 (clone N418)- 1:100 | BioLegend | Cat# 117349 |
| Chicken polyclonal GFP – unconjugated – 1:500 | Abcam | Cat# ab13970 |
| Rat monoclonal F4/80 - unconjugated (clone BM8) – 1:200 | Invitrogen-Thermo Fisher | Cat# 13-4801-85 |
| Rat monoclonal CD63 - unconjugated (clone NGV-4) – 1:25 | BioLegend | Cat# 143902 |
| Rabbit monoclonal Gpmb - unconjugated (clone EPR18226-147) 1:50 | Abcam | Cat# 188222 |
| Donkey anti-chicken AF488 – 1:500 | Jackson Immunoresearch | Cat# 703-545-155 |
| Goat polyclonal anti-CLEC4F-unconjugated – 1:100 | R&D Systems | Cat# AF2784 |
| Donkey anti-rat AF594 – 1:200 | Invitrogen-Thermo Fisher | Cat# A21209 |
| Donkey anti-rabbit AF647 – 1:500 | Jackson Immunoresearch | Cat# 711-605-152 |
| Goat anti-rabbit AF594 – 1:500 | Jackson Immunoresearch | Cat# 111-585-003 |
| Donkey anti-goat AF647 – 1:500 | Jackson Immunoresearch | Cat# 705-605-003 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Collagenase A      | Sigma  | Cat# C5138 |
| DNase I            | Sigma  | Cat# 10104159001 |
| DMEM               | Sigma  | Cat# D5671 |
| L-glutamine        | Sigma  | Cat# G7513 |
| Penicillin-Streptomycin (P/S) | Gibco | Cat# 15140-122 |
| Fetal Bovine Serum (FBS) | Sigma | Cat# F2442 |
| Sodium Pyruvate    | Corning| Cat# 25-000-Ci |
| Bovine Serum Albumin (BSA) | Lampire Biological Laboratories | Cat # 7500804 |
| EDTA               | Corning| Cat# 46-034-Ci |
| ACK lysis buffer   | Gibco  | Cat# A10492-01 |
| Zombie Aqua        | BioLegend | Cat# 423101 |
| Fc Block           | BD Biosciences | Cat# 553143 |
| 10% Neutral buffered formalin | Sigma | Cat# H5011-1CS |
| Sucrose            | IBI Scientific | Cat# 57-50-1 |
| Tissue-Tek O.C.T.  | Sakura  | Cat# 4583 |

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MATERIALS AND EQUIPMENT

Collagenase A stock
Add 13.3 mL DMEM to 1 g collagenase A powder in the supplied vial to achieve concentration of 75 mg/mL. Vortex to mix and incubate at 37°C water bath for 30–40 min to promote dissolution. Store as 1 mL aliquots for up to 12 months at -20°C and avoid repeated freeze/thaw cycles.

DNaseI stock
Add 2 mL DI water to reconstitute 100 mg DNaseI powder in the supplied vial. Transfer solution to new 50 mL conical tube. Rinse the vial with 2 mL of DI water and transfer the rinse to the same conical tube. Add 16 mL DI water to the conical tube to achieve 5 mg/mL stock concentration. Vortex to mix and store as 1 mL aliquots for up to 12 months at -20°C. Avoid repeated freeze/thaw cycles as this will reduce enzyme activity.

Zombie Aqua stock
Prepare according to manufacturer’s instruction. Briefly, reconstitute 1 vial of lyophilized reagent with 100 μL DMSO (included in the kit). Vortex to mix. Store as 10 μL aliquots up to 6 months at -20°C and protect from light.

30% Sucrose solution
Dissolve 150 g of sucrose in 200 mL of autoclaved PBS with a magnetic stir bar. Once dissolved, add more PBS until it reaches the 500 mL line. Aliquot into 50 mL conical tubes for working solutions. Store at 20°C–24°C for up to 6 months.

Complete DMEM

| Reagent                      | Final concentration | Amount   |
|------------------------------|---------------------|----------|
| DMEM                         | n/a                 | 435 mL   |

(Continued on next page)
## Digestion Buffer (for ~1–2 g of liver)

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| DMEM n/a                         |                     | 15 mL  |
| Collagenase A (75 mg/mL)         | 0.75 mg/mL          | 150 μL |
| DNaseI (5 mg/mL)                 | 50 μg/mL            | 150 μL |
| **Total**                        |                     | 15 mL  |

Always prepare fresh digestion buffer on the day of harvest. Digestion buffer can be kept at room temperature during the harvest (1–2 h).

## FACS buffer

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| PBS n/a |                     | 498 mL |
| BSA n/a |                     | 2.5 g  |
| 0.5 M EDTA | 2 mM               | 2 mL   |
| **Total** |                     | 500 mL |

Prepare in a sterile environment and filter through 0.22 μm PES membrane. Store at 4°C for up to 6 months.

## Blocking buffer for immunofluorescence

| Reagent        | Final concentration | Amount |
|----------------|---------------------|--------|
| PBS n/a        |                     | 50 mL  |
| BSA 1%         |                     | 0.5 g  |
| Triton x100    | 0.3% (v/v)          | 150 μL |
| **Total**      |                     | 50 mL  |

Always prepare freshly on the day of staining for optimal results. Store at 4°C for up to 48 h if needed for next-day secondary antibody staining.

## Zombie Aqua

| Reagent                          | Final concentration | Amount |
|----------------------------------|---------------------|--------|
| Zombie Aqua stock                | 1:250               | 0.4 μL |
| PBS n/a                          |                     | 100 μL |
| **Total**                        |                     | 100 μL |

Prepare fresh for staining. Protect from light and keep on ice.

## Fc Block

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Fc Block | 1:10               | 1 μL   |
| FACS buffer | n/a             | 9 μL   |
| **Total** |                     | 10 μL  |

Prepare fresh for staining. Keep on ice.
**STEP-BY-STEP METHOD DETAILS**

**Assemble perfusion pump and harvest table**

- **Timing:** 10 min

1. Cover the surface of a plastic tray with a sheet of absorbent bench paper and then layer with two paper towels.
2. Assemble tools including: surgical scissor, tweezer, suture, catheter, 2 cotton tips, razor blade, petri dishes, a stack of paper towels, pump, tubing for the pump, a bottle of PBS, a beaker, 70% ethanol, and biohazard bags for waste and carcass disposal.
3. Attach the tubing to the peristaltic pump. Place the inlet end of the tubing into the bottle of PBS and the outlet end in a beaker. Start the pump until the air is cleared from the line and the entire tubing is filled with PBS (Figure 1A).

**Set up reagents for harvest**

- **Timing:** 10–30 min

4. Prepare complete DMEM and FACS buffer (as described in materials and equipment).
5. Prepare and label 3 sets of 50 mL conical tubes for each liver sample.
6. Prepare digestion buffer (as described in materials and equipment) in the first set of 50 conical tubes.
7. If collecting liver samples for IF, prepare 500 μL formalin solution in 1.5 mL Eppendorf tubes.

**Perfusion of mouse liver**

- **Timing:** 15 min/mouse

Proper perfusion of the liver is essential for maximum yield of liver macrophages for downstream analyses including, but not limited to, flow cytometry and immunofluorescence.

8. Euthanize the mouse using carbon dioxide and subsequently perform cervical dislocation.
9. Weigh and record the body weight of the mouse using a balance.
10. Place the mouse in a prone position and sterilize the abdomen with 70% ethanol.
11. Use a tweezer to lift up the skin near the genitals and make an incision with scissors. Pull the skin up to expose the body cavity membrane.
12. Carefully cut open the peritoneal cavity membrane and move the small intestines aside to fully expose the liver.
13. Locate the portal vein underneath the liver. Place a suture underneath the vein and secure a half-knot (Figure 1A).
14. Cannulate the portal vein with the catheter until its tip reaches the branching point of the vein and passes the half-knotted suture. Tighten the knot to secure the catheter and remove the needle. A successful cannulation will result in a flash of blood into the catheter (Figure 1B; Methods video S1).

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**Flow cytometry antibody cocktail**

| Reagent               | Final concentration | Amount   |
|-----------------------|---------------------|----------|
| FACS buffer           | n/a                 | 100 μL   |
| Conjugated antibodies | 1:100–1:500         | 0.2–1 μL |
| Total                 |                     | 100 μL   |

Prepare fresh for staining. Protect from light and keep on ice.
Blood can be collected at this point from portal vein and/or IVC using a 1mL syringe with a 21G 3 1/2 needle. Washing the tube with EDTA can be used to prevent clotting in the tube.

15. Perfuse the portal vein with PBS using the peristaltic pump at a rate of 5 mL/min. After attaching the tubing onto the catheter immediately cut the inferior vena cava (IVC) below the liver to release blood and PBS from the liver. The liver should start to appear pale and beige in color.
CRITICAL: Start the flow of PBS prior to inserting the tubing onto the catheter to prevent the introduction of air bubbles into the liver vasculature.

16. Increase the flow rate to 10 mL/min. Apply gentle pressure on the IVC periodically and massage the liver with cotton tipped wood applicator to promote clearance of sinusoidal blood. Perfuse for a minimum of 3 min, or until the liver is completely pale (Figure 1A). The total perfusion volume of PBS should be about 30 mL.

17. Remove catheter, suture, and gallbladder from the liver. Resect the liver from the body cavity (Figure 1A).

18. Weigh and record total liver weight.

19. Resect specific lobes of the liver for downstream analyses: for example, left medial and lateral lobe for flow cytometry and right lateral lobe for immunofluorescence. For flow cytometry between 0.5-1 gm of tissue is recommended. (Further processing for immunofluorescence: see section Immunofluorescence of liver tissue)

Optional: Depending on experimental question, the remaining liver tissue can be processed for other analyses including, but not limited to, histology, RNA, protein, or triglyceride quantification. For histology, submerge tissue into 500–750 mL formalin and incubate at 4°C for 24 h. Transfer tissue into 70% ethanol for subsequent paraffin embedding. For RNA, protein and triglyceride quantification, flash freeze tissue in liquid nitrogen and transfer to −80°C.

Note: It is a lab-dependent choice to decide which lobe to use for specific analysis, but it is encouraged to maintain consistency across different experimental groups and different experiments.

Enzymatic digestion and dissociation of liver tissue into single-cell suspension

© Timing: 1.5 h

This step describes the process of collagenase A digestion and differential centrifugation of liver tissue to prepare single-cell suspension for flow cytometry (Figure 1B).

Note: The cell suspension obtained after this step can also be used for alternative purposes, for example flow sorting, single-RNA sequencing, and/or ex vivo function assays.

20. Weigh and record the liver tissue dedicated for flow cytometry.

21. Place the liver in a petri dish and mince finely with a razor blade.

22. Transfer the minced liver into a prepared digestion buffer and keep on ice until the end of the tissue harvest.

23. At the end of the harvest, warm up all samples in a 37°C water bath for 5 min.

24. Place the liver mixture on a shaker at 37°C with rotation for 30 min to digest. Vortex mixture after 15 min.

25. Pre-wet 70 µm cell strainers placed onto new 50 mL falcon tubes with 5 mL cold complete DMEM.

26. Transfer the digested liver mixture onto the cell strainers to allow pass-through of cells into the new 50 mL tubes.

27. To inactivate enzymatic activity, add 15 mL cold complete DMEM to the original tubes and carefully pour the rinse onto the cell strainers, while mashing any undigested liver pieces using the end of a syringe plunger.

28. Centrifuge the strained cell suspension at 50 × g for 3 mins at 4°C to initially separate hepatocytes (pellet) and non-parenchymal cells (NPCs) (supernatant).

29. Carefully pour the supernatant containing NPCs into a new 50 mL falcon tube. Discard pellet containing hepatocytes. Of note, ~10% of KCs will be lost in the hepatocyte pellet.

Note: Hepatocyte pellet may be loose, use caution while pouring or use a pipette.
Optional: Hepatocytes in the pellets can also be processed separately if of interest.

30. Centrifuge the NPC suspension at 163\,g for 7 min at 4°C to pellet the NPCs. The supernatant can be discarded.
31. Resuspend the NPC pellet with 1 mL ACK lysis buffer and incubate for 5 min, at 20°C to lyse red blood cells. Add 10 mL PBS to wash.
32. Centrifuge cell suspension at 163\,g for 7 min at 4°C to re-pellet the NPCs. At this point the pellet should be pale and free of red blood cells.
33. Resuspend the cells in 1 mL PBS and transfer to 1.5 mL Eppendorf tubes. Keep on ice.

Pause point: Samples can be kept on ice for 1–2 h until ready to proceed to the next step. If pause, resuspend cells in 1 mL FACS buffer instead of PBS.

Staining for flow cytometry

© Timing: 2 h

This step details the method of staining liver NPCs for identifying macrophage populations (Kupffer cells (KCs), monocyte-KCs, hepatic lipid-associated macrophages, and monocytes) by flow cytometry. Key markers include CD45, CD11b, F4/80, TIM4, VSIG4, CLEC2, Ly6C and MHCII. For this protocol, the antibody panel in Table 1 will be used as an example.

Optional: Depending on experimental question, additional markers may be added, for example CD11c for dendritic cells, Ly6G for neutrophils, and/or Siglec F for eosinophils.

Note: This staining protocol utilizes Zombie Aqua to assess live/dead status of cells prior to staining for surface markers, hence requiring cell suspension to be free of BSA in step 36 Alternatively, DAPI can also be used to identify live cells. For DAPI staining, resuspend stained-cells in 1× DAPI solution in FACS buffer (according to manufacturer instruction) prior to analyzing samples on the cytometer.

34. Pellet the cells using a bench-top centrifuge at 650 × g for 3.5 min at 4°C.
35. If cells are in buffers containing BSA, wash off and exchange with PBS.
36. Resuspend pellet with 100 μL Zombie Aqua solution at 1:250 dilution in PBS for live/dead stain. Incubate on ice and in the dark for 15 min.
37. Wash the cells by adding 700 μL of FACS buffer to cell suspension. Pellet the cells at 650 × g for 3.5 min at 4°C. Aspirate the supernatant.
38. Resuspend the pellet with 10 μL Fc block solution. Incubate on ice and in the dark for 5 min.

### Table 1. Example staining panel for analysis by BD X20 flow cytometer

| Reagent             | Final concentration |
|---------------------|---------------------|
| CD45 - BUV395       | 1:100               |
| CD11b - APC-Cy7     | 1:100               |
| F4/80 - AF647       | 1:100               |
| CLEC2 - PE          | 1:100               |
| TIM4 - BV421        | 1:500               |
| VSIG4 - PE-Cy7      | 1:200               |
| Ly6C - FITC         | 1:100               |
| MHC II - BV605      | 1:300               |
| CD11c - BV711       | 1:100               |
| Zombie Aqua         | 1:250               |

For exact antibodies, see key resources table.
39. Add 90 μL of antibody cocktail to the cell suspension. Resuspend and incubate on ice and in the dark for at least 45 min (Table 1).

40. Wash the cells by adding 700 μL FACS buffer to the cell suspension. Pellet the cells at 650 × g for 3.5 min at 4°C. Aspirate the supernatant.

41. Resuspend the cells in 300 μL FACS buffer.

42. Filter the cell suspension through a 70-μm cell strainer into 5 mL polystyrene round-bottom flow tube and keep on ice in the dark. The samples are now ready to be analyzed on a flow cytometer.

**Immunofluorescence of liver tissue**

© Timing: 1–2 days

This step will explain immunofluorescence staining of macrophage markers in liver tissue. This can be used for analysis of identified NASH-associated macrophage markers, i.e., TREM2, Gpnlmb and CD63, that have been proven difficult to analyze via flow cytometry (Daemen et al., 2021; Remmerie et al., 2020). Additional information that can be obtained is localization of macrophage subsets in the tissue as well as their proximity to other parenchymal and non-parenchymal cells.

43. Following perfusion, harvest liver lobe and place the tissue into a tube containing formalin. It is critical to ensure the tissue is submerged in formalin, which typically requires a volume of 500–700 μL of fixative. We typically use the right medial liver lobe for IF. Keep on ice until all tissue is harvested.

△ CRITICAL: To maintain tissue architecture of the liver, immediate incubation in fixative following tissue isolation is essential.

44. Incubate at 4°C for 24 h.

45. Transfer liver lobe to 750 μL 30% sucrose in PBS at 4°C for 24 h. A minimum incubation of 24 h is required, but incubation may be extended up to 72 h.

46. Submerge and freeze liver lobe in OCT in mold and store at −80°C. Ideally, the liver will be sectioned in the horizontal plane and therefore the smooth dome shaped portion of the liver lobe should be placed face down in the OCT media.

47. Cut 8 μM tissue sections using a cryostat on glass slides. Tissue sections can be stored at −80°C for several months.

48. Prepare 50 mL blocking buffer fresh on the day of staining.

49. Retrieve frozen slides from −80°C and let them air dry for 15–20 min at 20°C.

△ CRITICAL: Prevent drying of the tissue sections at any step during the rest of the staining protocol.

50. Incubate slides in PBS for 5 min in glass chamber, followed by 45 min in blocking buffer.

51. Prepare primary antibody, 50 μL per section, in blocking buffer.

52. Carefully extract slides from glass chamber and dry slides around the tissue sections with Kim-wipe. Encircle sections with using a hydrophobic pen and add 50 μL primary antibody solution on each section.

53. Keep slides in a container on wet paper towels 16–24 h at 4°C.

54. Incubate slides in PBS for 5 min in glass chamber to wash and perform this step three times.

55. Carefully extract slides from glass chamber and dry slides around the tissue sections with Kim-wipe. Re-apply hydrophobic barrier with pen if necessary.

56. Prepare secondary antibody solution and add 50 μL per section. Incubate for 1 h at room temperature in the dark.

57. Incubate slides in PBS for 5 min in glass chamber to wash, 2 times.

58. Prepare 50 mL Hoechst dye (1:25000) and add solution to slides in glass chamber. The nuclear dye should be prepared fresh for each experiment.
59. Retrieve slides from glass chamber, dry carefully, and apply a small drop of prolong gold anti-fade reagent. Place cover slip on top and let slides dry for minimum of 1 h in dark.

60. Imaging can be done with light or confocal microscopy. For optimal result, image within one week of staining.

EXPECTED OUTCOMES

The estimated yield per gram of liver tissue of CD45-positive cells and total F4/80\textsuperscript{hi} macrophages can vary from 0.5–1.5 and 0.1–0.4 million cells, respectively. Exact cell number is dependent on many factors including perfusion quality and variation in digestion efficiency. The gating strategy described below may be used to identify the liver macrophage subsets previously described in NASH, but alternative gating strategies have been utilized by other labs (Remmerie et al., 2020; Tran et al., 2020). When using this protocol, it is expected that ~ 90% of the CD45\textsuperscript{pos} cells will be viable by live-dead staining. Although this can be somewhat variable, if the viability is below 70% this may indicate a problem with the isolation protocol.

All liver macrophages are identified as F4/80\textsuperscript{hi}, CD11b\textsuperscript{int} (Figure 2A) and consist of the sinusoidal macrophages as well as a small number of liver capsular cells. The sinusoidal macrophages are composed of embryonically derived resident Kupffer cells (KCs), which can be identified by high expression of TIM4 (Figure 2B) and recruited monocyte-derived macrophages (MdMs) which are almost exclusively TIM4\textsuperscript{lo}, although a subset of mo-KCs can begin to express TIM4 after several weeks in the liver (Figure 2B)(Scott et al., 2016). Typically, we have found the percentage of TIM4\textsuperscript{lo} MdMs correlates with NASH severity. MdMs can be further subdivided into the monocyte-derived Kupffer cells (mo-KCs) which are TIM4\textsuperscript{lo}, VSIG4\textsuperscript{hi} (or CLEC4F\textsuperscript{hi}), and lipid-associated macrophages (LAMs), which are TIM4\textsuperscript{lo}, VSIG4\textsuperscript{lo} (Figure 2C)(Daemen et al., 2021). LAMs are likely the equivalent of the previously described NASH-associated macrophages (NAMs) as these populations both exhibit high expression of the markers TREM2, CD9, CD63 and Gpnmb (Xiong et al., 2019). The use of Ccr2-GFP or Cx3cr1-GFP reporter mice allows for further identification of Ccr2\textsuperscript{hi}/Cx3cr1\textsuperscript{hi} LAMs (C-LAMs) (Figure 2C)(Daemen et al., 2021). Several recent papers have also demonstrated CLEC2 as a marker of sinusoidal liver macrophages (Seidman et al., 2020). CLEC2 is highly expressed by most liver macrophages except for a small subpopulation of MdMs, which are high in Ccr2 and Cx3cr1. These cells are found in the C-LAM gate by flow cytometry (Figure 2D). This CLEC2\textsuperscript{lo} population may include liver capsular cells and/or an early precursor of LAMs, but additional studies will be necessary to delineate the trajectory of these macrophages.

Liver Ly6C\textsuperscript{hi} monocytes can be found within the CD11b\textsuperscript{hi}, F4/80\textsuperscript{int} population and can be identified by gating for Ly6C\textsuperscript{hi}, MHCh\textsuperscript{lo}, and SSC\textsuperscript{lo} (Figure 2E). Alternatively, antibodies to Ly6G and SiglecF can be used here to gate on neutrophils and eosinophils. When using Ccr2-GFP or Cx3cr1-GFP reporter mice, classical monocytes can also be identified as Ly6C\textsuperscript{hi}, Ccr2\textsuperscript{hi}/Cx3cr1\textsuperscript{hi} (Figure 2E).

Importantly, in the flow cytometric analysis, LAMs are currently identified by the absence of KC surface markers (TIM4, VSIG4), because flow cytometry of known LAM markers, i.e., TREM2, Gpnmb, and CD63, after tissue digestion has been challenging. Therefore, the presence and number of these cells also be assessed by immunofluorescence and confocal imaging. With this approach, co-staining of CD63 or Gpnmb with CLEC4F and Ccr2-GFP allows for simultaneous visualization and separation of LAMs, KCs/mo-KCs and C-LAMs, respectively (Figures 2F–2G). TIM4 staining can also be used to aid in the identification of Em-KCs vs. mo-KCs.

QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify the flow cytometry absolute cell number should be corrected for the amount of liver tissue initially digested. Given the day-to-day variation in efficiency of cell isolation, same day comparison is recommended when analyzing parallel experimental groups. Quantification of immunofluorescence can be done using software such as ImageJ and can be expressed as number of cells per field of view. Quantification of total fluorescent area could also be performed, but importantly this does not correct for changes in macrophage size which may vary with disease state.
Figure 2. Example flow plots and immunofluorescence images to identify macrophage sub-populations in the NASH liver

(A) After gating for CD45-positive, live and singlet cells, liver macrophages can be identified as F4/80$^{\text{hi}}$, CD11b$^{\text{int}}$ cells (orange circle).

(B) In a standard diet fed mouse almost all liver macrophages are resident TIM4$^{\text{hi}}$ Kupffer cells (KCs) (green box). In contrast, the NASH liver also contains significant numbers of recruited TIM4$^{\text{lo}}$ monocyte-derived macrophages (MdMs) (red box).

(C) TIM4$^{\text{lo}}$ macrophages can be further subdivided into VSIG4$^{\text{hi}}$ monocyte-KCs (Mo-KCs) and VSIG4$^{\text{lo}}$ lipid-associated macrophages (LAMs). Using Ccr2$^{-}$ or Cx3cr1-GFP reporter mice also facilitates the identification of Ccr2/Cx3cr1-GFP-positive LAMs (C-LAMs).
Figure 2. Continued

(D) Although most liver macrophages are CLEC2hi, there is a CLEC2lo subpopulation that can be identified within the TIM4hi, VSIg4lo MdMs. These CLEC2lo macrophages express high levels of Ccr2/Cx3cr1.

(E) Monocytes can be identified by subsequent gating for CD11bhi, MHCIi, Ly6Chi, and low side scatter area (SSC-A). They can also be identified as Ccr2/Cx3cr1hi cells within the CD11bhi, MHCIi, Ly6Chi gate when using reporter mice.

(F and G) With immunofluorescence imaging LAMs can be identified using antibodies against Gpmb or CD63 (red) and monocytes/C-LAMs by Ccr2-GFP (green). CLEC4F staining (white) allows for the identification of KCs as well as mo-KCs. A magnification image is shown to illustrate identification of macrophage subsets in the liver tissue. Nuclei are presented in blue. Scale bar is 50 μM.

LIMITATIONS

The described protocol can be used for both standard diet healthy livers and NASH livers. However, when using healthy livers NPC pellet is larger when compared to the same tissue volume of a NASH liver. This can result in slow and difficult flow cytometry as the number of macrophages per given volume is reduced. When regularly using standard diet controls, the use of a percoll gradient may be preferred for better purification of macrophages, although this dramatically lowers the yield (Lynch et al., 2018). Fibrosis of the liver can also result in more difficult perfusion, digestion, and extraction of liver macrophages. Consequently, this may result in lower macrophage yield and/or favor extraction of specific macrophage populations, thereby giving distorted results on liver macrophage composition. Confirmation of flow cytometry results with immunofluorescence is therefore recommended. As an alternative direct perfusion of the liver with collagenase improves liver digestion and macrophage yield, however, this excludes the use of any tissue for immunofluorescence, histology, RNA, and protein analysis.

TROUBLESHOOTING

Problem 1
Unsuccessful cannulation of portal vein (step 14)

Potential solution
Cannulation of the IVC for perfusion is a possible alternative, though this often results in lower quality perfusion and subsequently lower cell yield. It is advised to practice portal vein cannulation until achieving reliable perfusion quality. Please see video file of portal vein cannulation (Methods video S1).

Problem 2
Low yield of liver macrophages (step 42)

Potential solution
A low yield can result from low perfusion quality or incomplete digestion of the liver. Ensure the liver is properly perfused until a pale and beige appearance. In addition, ensure that the digestion buffer with collagenase is prepared fresh and the samples are heated to 37°C before digestion on the shaker.

If a higher yield of macrophages is required, for example for flow sorting and/or plating of the macrophages, direct perfusion of the liver with collagenase coupled with digestion on the shaker can be considered. However, as described before this excluded use of liver tissue for other analyses.

Problem 3
Kupffer cell autofluorescence (step 42)

Potential solution
KCs are extremely vacuolar macrophages and as such have significant autofluorescence in the green channel. Therefore, it is critical to include appropriate isotype control antibodies for the green channel to ensure that the positive signal is real. Similarly, when using GFP-reporter mice it is important to include some NTG littermate controls to confirm the findings, see figure 4 of the Daemen et al. manuscript (Daemen et al., 2021).
Problem 4
Low Kupffer cell viability (step 42)

Potential solution
As mentioned above, it is expected that CD45 positive cells will have a viability of ~90% using this protocol and gating strategy. If the viability is below 70% this may indicate a problem with the isolation protocol. The most likely steps that could lower cell viability are the collagenase digestion at 37 deg and/or the red cell lysis step. If either of these steps is allowed to continue beyond the recommended time, then immune cell viability can be compromised. It is also important to keep samples on ice with FACS buffer, rather than PBS to maximize cell viability.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joel D. Schilling (schillij@wustl.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate a new dataset or code for analysis.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100511.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.D., M.M.C., and J.D.S.; methodology, S.D., M.M.C., and J.D.S.; investigation, S.D., M.M.C., and J.D.S.; writing-original draft, S.D., M.M.C., and J.D.S.; writing-review & editing, S.D., M.M.C., and J.D.S.; and funding Acquisition, J.D.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Daemen, S., Gainullina, A., Kalugotla, G., He, L., Chan, M.M., Beals, J.W., Liss, K.H., Klein, S., Feldstein, A.E., Finck, B.N., et al. (2021). Dynamic shifts in the composition of resident and recruited macrophages influence tissue remodeling in NASH. Cell Rep. 34, 108626.

Lynch, R.W., Hawley, C.A., Pellicoro, A., Bain, C.C., Iredale, J.P., and Jenkins, S.J. (2018). An efficient method to isolate Kupffer cells eliminating endothelial cell contamination and selective bias. J. Leukoc. Biol. 104, 579–586.

Remmerie, A., Martens, L., Thone, T., Castoldi, A., Seurinck, R., Pavie, B., Roels, J., Vanneste, B., De Prijck, S., Vanhockerhout, M., et al. (2020). Osteopontin expression identifies a subset of recruited macrophages distinct from kupffer cells in the fatty liver. Immunity 53, 641–657.e14.

Scott, C.L., Zheng, F., De Baetselier, P., Martens, L., Saëys, Y., De Prijck, S., Lippens, S., Abels, C., Schoonooghe, S., Raes, G., et al. (2016). Bone marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. Nat. Commun. 7, 10321.

Seidman, J.S., Troutman, T.D., Sakai, M., Gola, A., Spann, N.J., Bennett, H., Bruni, C.M., Ouyang, Z., Li, R.Z., Sun, X., et al. (2020). Niche-specific reprogramming of epigenetic landscapes drives myeloid cell diversity in nonalcoholic steatohepatitis. Immunity 52, 1057–1074.e7.

Tran, S., Baba, I., Poupel, L., Dussaud, S., Moreau, M., Gelineau, A., Marcelin, G., Mageau-Davy, E., Ouhachi, M., Lesnik, P., et al. (2020). Impaired kupffer cell self-renewal alters the liver response to lipid overload during non-alcoholic steatohepatitis. Immunity 53, 627–640.e5.

Xiong, X., Kuan, H., Ansari, S., Liu, T., Gong, J., Wang, S., Zhao, XY, Ji, Y., Li, C., Guo, L., et al. (2019). Landscape of intercellular crosstalk in healthy and NASH liver revealed by single-cell secretome gene analysis. Mol. Cell 75, 644–660.e5.