Chapter 1

Isolation of Non-parenchymal Cells from the Mouse Liver

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Abstract

Hepatocytes comprise the majority of liver mass and cell number. However, in order to understand liver biology, the non-parenchymal cells (NPCs) must be considered. Herein, a relatively rapid and efficient method for isolating liver NPCs from a mouse is described. Using this method, liver sinusoidal endothelial cells, Kupffer cells, natural killer (NK) and NK-T cells, dendritic cells, CD4+ and CD8+ T cells, and quiescent hepatic stellate cells can be purified. This protocol permits the collection of peripheral blood, intact liver tissue, and hepatocytes, in addition to NPCs. In situ perfusion via the portal vein leads to efficient liver digestion. NPCs are enriched from the resulting single-cell suspension by differential and gradient centrifugation. The NPCs can by analyzed or sorted into highly enriched populations using flow cytometry. The isolated cells are suitable for flow cytometry, protein, and mRNA analyses as well as primary culture.

Key words Liver, Perfusion, Cell isolation, Sinusoidal endothelial cells, Kupffer cells, Hepatic stellate cells

1 Introduction

The principle cell types in a healthy liver are hepatocytes, liver sinusoidal endothelial cells (LSEC), Kupffer cells, and hepatic stellate cells (HSC) [1–3]. Fewer in number are bile duct cells, venous and arterial endothelial cell, hepatic progenitor cells, and dendritic cells. Furthermore, the number and proportion of leukocytes can increase tremendously in an infected or damaged liver [4, 5]. As a result, granulocytes, monocytes, natural killer (NK) and NK-T cells, dendritic cells, CD4+ and CD8+ lymphocytes, and B cells are important determinants of the liver biology. Thus, the dissected dynamics of each cell type can provide powerful information to understand the pathology and immunology of the tissue. This information, in combination with serological, histological and tissue-level observations, allows for a comprehensive assessment of each experimental mouse, thus reducing the number of experimental mice while increasing the likelihood of discovery.

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The purpose of this protocol is to provide a detailed description of materials and methods by which liver cell populations can be isolated from the mouse liver and studied, while also permitting the collection of blood and intact liver tissue. The liver dissociation protocol is derived from the method published by Seglen [6] for isolating rat liver cells. Dr. Seglen provides an extensive description of the theory behind rat liver dissociation that extends to the mouse. We have evolved the method of Seglen to allow rapid, yet effective, isolation of mouse liver cells, permitting the dissociation of up to five livers per hour by two skilled technicians—one conducting perfusions and dissections, the other processing cell suspensions.

The basic protocol relies upon in situ perfusion of the liver via the portal vein. Peripheral blood and cells are flushed from the liver in a Ca^{2+}-free buffer, prior to perfusion with the collagenase digestion solution. Following liver digestion, the liver is removed and mechanically dissociated. Hepatocytes are separated by low-speed centrifugation, and then non-parenchymal cells (NPCs) are enriched by gradient separation. The enriched NPCs allow for relatively efficient cell type-specific analysis and/or further purification by flow cytometry [7]. For purification, magnetic bead-based methods can be applied and in certain circumstances are preferred [8], however, cell sorting allows for multi-way separation from each preparation.

Although liver NPCs are the focus of this protocol, hepatocytes are readily purified and cultured with good success. In addition, it is not yet clear if this protocol is able to isolate the population of sessile Kupffer cells, which are radioresistant and appear somewhat distinct in function from their non-sessile counterparts [2]. This caveat in mind, this protocol establishes a reproducible method to isolate and enable the study of many cell types from the mouse liver. Indeed, a parallel understanding of cell-specific responses associated with tissue immune and pathological responses offers promise of new insights into treatment and prevention of infection and disease.

2 Materials

All solutions and consumables should be purchased as "tissue culture tested" from a trusted commercial source in order to assure minimal contamination with endotoxin and sterility. All surgical instruments should be thoroughly washed, rinsed and autoclaved for sterility, especially if primary culture is the end goal. As with any protocol involving animals, institutional guidelines for handling, anesthesia, and waste disposal should be followed.

2.1 Anesthesia

1. Anesthesia approved for terminal procedures such as Avertin; 1.25 % (w/v) 2,2,2-tribromomethanol, 2.5 % (v/v) 2-methyl-2-butanol, sterile water. Filter-sterilize and then store at 4 °C protected from light (*see* **Note 1**).

2. 28G ½ inch needle, suitable for intraperitoneal injections.
3. 1-cc syringe.

2.2 Perfusion/Liver Dissociation Hardware Components

1. Peristaltic pump; such as Gilson MINIPULS 3 with medium flow-rate pump head.
2. Pump tubing and connectors; such as F1825113 and F1179951.
3. Tubing extension with slip-tip end; such as Hospira 1265528.
4. Catheter; 24G, IV, such as BD 381412 (*see* **Note 2**).
5. Scissors, straight fine-tipped dissection.
6. Forceps, 2 blunt tip.
7. 50-ml conical tubes.
8. 15-ml conical tubes.
9. 5-cm sterile petri dish (optional).
10. 10-cm sterile petri dish.
11. Stainless steel mesh "tea strainer."
12. 10-cc syringe.
13. 100-μm filter.
14. 70-μm filter (optional).
15. Gauze pads, large-size.
16. Surgical tape, such as 3 M Transpore.
17. Disposable absorbent underpads.
18. 37 °C water bath with 50-ml conical rack.

2.3 Perfusion/Liver Dissociation Solution Components

1. Hank's Balance Salt Solution (HBSS); no Ca^{2+}, no Mg^{2+}, no phenol red.
2. HBSS with phenol red.
3. Phosphate buffered saline (PBS), pH 7.4.
4. Distilled water, TC-grade.
5. PBS, 10×.
6. HEPES; 1 M (Stock).
7. EDTA; 0.5 M (Stock).
8. $CaCl_2$; 0.5 M (Stock).
9. Fetal bovine serum (FBS).
10. Collagenase; *Clostridium histolyticum*, Sigma-Aldrich C5138 (*see* **Note 3**).
11. OptiPrep; 60 % iodixanol solution in water.
12. Tissue fixative; 4 % formaldehyde in PBS.
13. 70 % ethanol.

Table 1
Antibodies for FACS-based purification of some of the major liver NPC and leukocytes

Epitope	Fluorophore	Clone	Dilution
CD8a	Pacific Blue	53-6-7	1:250
CD4	PerCP-Cy5.5	RM4-5	1:250
CD11b	FITC	M1/70	1:200
NK1.1	Per-Cy7	PK136	1:200
Tie2	PE	TEK4	1:250
F4/80	APC	BM8	1:200
GR1	APC-Cy7	RB6-8C5	1:200
N/A	Live/Dead Violet	N/A	1:1000

The antibodies listed here will allow for selection or analysis of some of the most numerous liver NPC as well as some leukocytes

These solutions can be prepared in advance and stored at 4 °C.

1. Perfusion Buffer, 5–10 ml per mouse; HBSS, 5 mM HEPES, 0.5 mM EDTA.
2. Wash Buffer, 50 ml per mouse; PBS, 4 % FBS, 0.5 mM EDTA.
3. PBS Flow Buffer (PFB), 20 ml per mouse; PBS, 1 mM EDTA, 2 % FBS.

These solutions should be prepared on the day of isolation.

1. Collagenase solution, 5–10 ml per mouse; HBSS (w/phenol red), 5 mM HEPES, 0.5 mM $CaCl_2$, 0.5 mg/ml collagenase.
2. 40 % iodixanol in PBS, 2.5 ml per mouse; 1.67 ml OptiPrep + 0.25 ml 10× PBS + 0.58 ml TC-grade water.

2.4 Cell Analysis and Purification Components

1. Flow cytometer; such as BD Biosciences, LSRII or Aria.
2. Flow cytometry tubes (*see* **Note 4**).
3. Antibodies for sorting cell type and/or analysis (Table 1) (*see* **Note 5**).

3 Methods

3.1 Prepare for Perfusion(s)

1. Warm perfusion and collagenase solutions to 37 °C for approximately 15 min prior to beginning the perfusion.
2. Prepare tubing for perfusion (*see* **Note 6**).
3. Prepare perfusion area with absorbent pad, dissection tools, gauze, 10-cm petri dish, tea strainer, and 10-cc syringe (Fig. 1).
4. Fill perfusion line with perfusion solution.

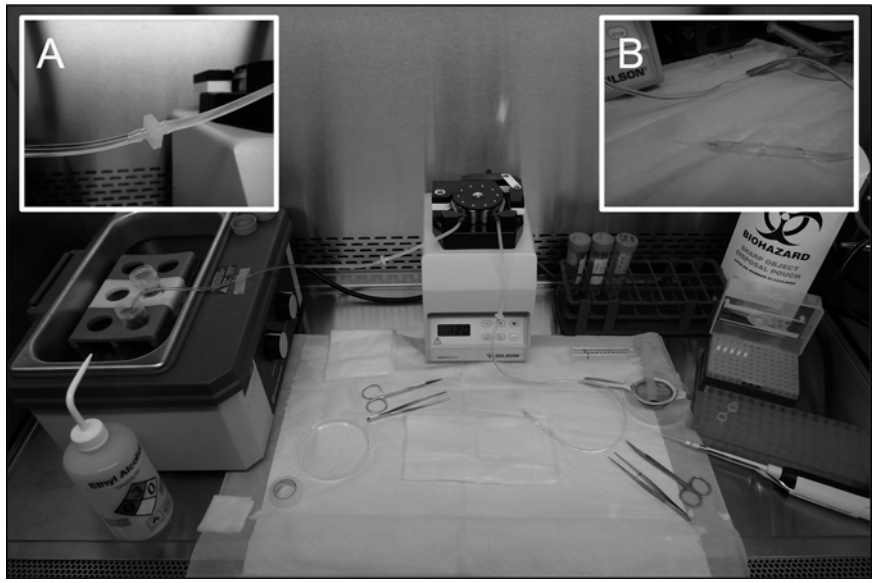

Fig. 1 Suggested workspace set-up. Position the water bath and pump to allow the perfusion tubing to reach the bottom of the 50-ml conical tubes. The water bath should be to the *left*, in order to allow switching of the perfusion line while holding the catheter with the *right hand*. Place absorbent pad on the work surface; this pad will both absorb perfusion solutions and act as the foundation to adhere the mouse. Place large gauze pad in the center of the work area; this small pad will absorb most of the perfusion solutions as well as blood and should be changed after every other if not every mouse. Place tea strainer in a 10-cm petri dish. Place the lid of the dish to the left of the smaller gauze pad. Place one pair of sharp scissors and forceps above the gauze. Place the other scissors and forceps to the right of the gauze. Position the surgical tape, small gauze pads, and 70 % ethanol within easy reach. *Inset* (**a**) illustrates the connection between extension tubing and silicon peristaltic pump tubing. *Inset* (**b**) illustrates the catheter connected to the male end of the extension tubing

3.2 Anesthetize Mouse

1. Inject mouse with appropriate amount of anesthesia.
2. Once adequate level anesthesia is obtained, proceed to Subheading 3.3 (*see* **Note 7**).

3.3 Surgical Preparation

1. Place mouse belly-up on large gauze pad.
2. Secure mouse by footpads using surgical tape in an X orientation (Fig. 2a).
3. Disinfect and wet mouse fur using 70 % ethanol. Wipe off excess.
4. Open skin to expose the peritoneal membrane (Fig. 2b).
5. Open peritoneal membrane (Fig. 2c), gently move intestines and stomach to the right and very gently "stick" the liver to the diaphragm. This should expose the portal vein and descending vena cava (*see* **Note 8**) (Fig. 2d).
6. Use sharp scissors to nick the portal vein; blood will flow (*see* **Note 9**).

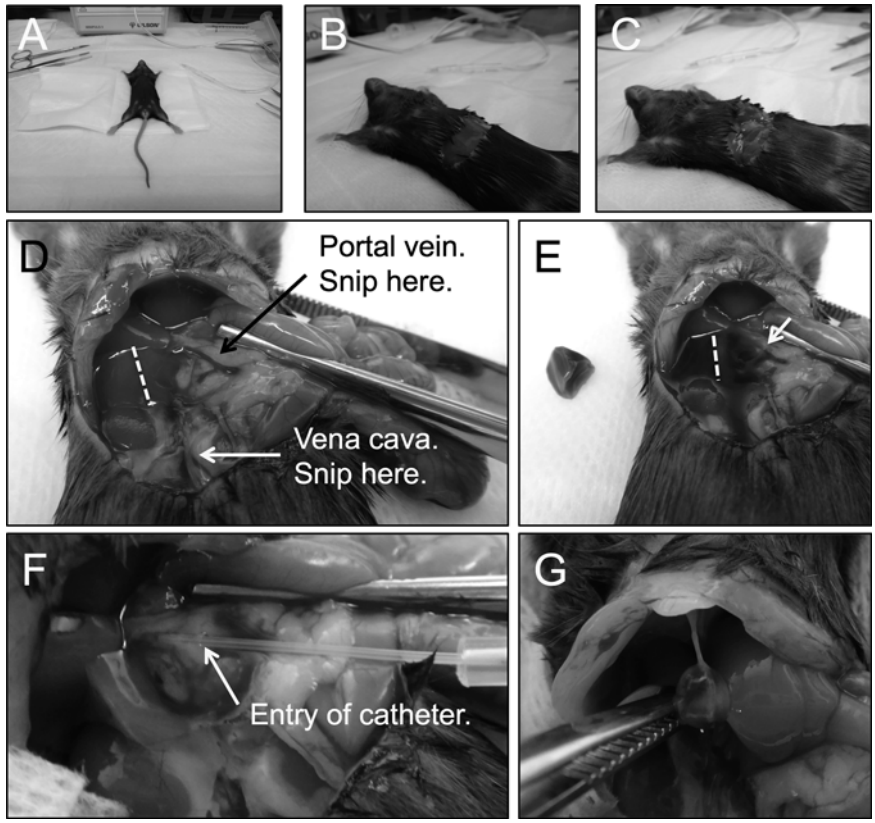

Fig. 2 General perfusion anatomy and procedure. (**a**) Adhere anesthetized mouse overtop of the gauze in an X-configuration. (**b**) Make a crosswise incision through the mouse skin to reveal the peritoneum. (**c**) Being careful to avoid cutting internal organs, make a crosswise incision through the peritoneum. (**d**) Move the gastrointestinal organs to the *left*, revealing the portal vein. Place forceps to hold tissue off of the vein. (**e**) Snip the portal vein (collect blood if desired), then remove a portion of the intact *right* posterior lobe. Catheterize the portal vein, then immediately cut the descending vena cava. (**f**) The liver will blanch once the portal vein is catheterized, and will fully perfuse once the vena cava is cut. Avoid pushing the catheter too far into the vein. The tip of the catheter should be easily observed within the vein. (**g**) Once digested, remove the liver by the falciform ligament, along the top of the medial lobe. The gall bladder is a good landmark for identifying the ligament

3.4 Blood and Tissue Collection (Optional)

1. Collect 0.2–0.5 ml of blood as it pools near the portal vein. Transfer to proper collection tube.
2. Locate and remove ~2/3 of the right posterior liver lobe (Fig. 2d, e). Transfer to 4 % formaldehyde for fixation or further divide for other assessments.

3.5 In Situ Liver Dissociation

1. Turn on pump to flow of ~2 ml/min.
2. Drip perfusion buffer onto the cut portal vein.
3. Use gauze sponge to draw perfusion solution to the left.
4. Identify the opening in the vein (*see* **Note 10**).

5. Gently catheterize the vein; the liver should blanch (*see* **Note 11**) (Fig. 2f).
6. Cut the descending vena cava; blood and buffer should visibly flow from the vena cava.
7. Relax your hand (*see* **Note 12**).
8. Perfuse liver with 5–10 ml of perfusion buffer. Most perfusion tubing setups hold about 5 ml of solution, thus once the descending vena cava is cut, proceed to **step 9**.
9. Stop pump.
10. Switch line to collagenase, using the left hand.
11. Resume pump flow (*see* **Note 13**).
12. Swell the liver using forceps to occlude buffer flow from the vena cava, every 45–60 s for 5–10 s. If part of the right posterior lobe was removed, use the forceps to occlude flow into this lobe (*see* **Note 14**).
13. Perfuse liver with 5–10 ml of collagenase buffer. After 3–4 min, the liver should soften and the left lobe will begin to fall over the portal vein. When this happens, use forceps to lift up the lobe to periodically check that the catheter is properly positioned. After 5 min, the internal structure of the liver cracks. This indicates a good digestion, and is most evident in the right anterior lobe.
14. Stop the pump.
15. Remove catheter from vein.
16. Reverse pump to return unused collagenase solution to the 50-ml conical tube.
17. Switch line back to perfusion solution and refill the line in preparation for the next mouse.

3.6 Single Cell Suspension

1. Using wide-tipped forceps, grasp the liver just to the left of the gall bladder along the falciform ligament (Fig. 2g).
2. Use scissors to separate the liver from the diaphragm and all other points of connection. Care should be taken to avoid cutting the gastrointestinal tract.
3. Transfer the digested liver into the tea strainer within a 10-cm petri dish.
4. Remove the gall bladder (*see* **Note 15**).
5. Add 30 ml of cold wash buffer to the dish.
6. Use the rubber plunger of 10-cc syringe to gently massage the liver through the tea strainer, shake the strainer to disperse the cells. The liver should easily disperse with only the capsule and ligament remaining in the strainer.

7. Use 10-cc syringe (or 10-ml pipet) to gently disperse any clumps.
8. Filter (100 μm) the cell suspension into a 50-ml conical tube.
9. Store on ice or at 4 °C for no longer than 15 min before proceeding to Subheading 3.8.

3.7 Isolate Splenocytes (Optional, See Note 16)

1. Locate and remove spleen.
2. Place spleen into 5-ml petri dish filled with 10 ml of PFB.
3. Place the spleen on the rough surface of a glass slide.
4. Use the rough surface of a second glass slide to dissociate the spleen by gentle pressure applied in a circular motion. Continue this gentle mashing until the tissue is clearly dispersed.
5. Scrape the cells into the buffer using the edge of the slide.
6. Disperse the cells by pipetting.
7. Filter (70 μm) into 50-ml conical tube.
8. Store on ice until the NPC isolation reaches Subheading 3.10, **step** 7, then process as NPC.

3.8 Crude Liver Cell Fractionation

1. Centrifuge the cell suspension at 50 × *g* for 3 min at room temperature. At this speed and duration, hepatocytes and debris will pellet while most NPCs will remain in suspension.
2. Transfer the supernatant, which contains the hepatocyte-depleted NPCs, to a new 50-ml conical tube.

3.9 Hepatocyte Enrichment (Optional)

1. Wash the hepatocyte pellet in 40 ml of wash buffer.
2. Pellet at 50 × *g* for 3 min.
3. Resuspend in 10 ml of media.
4. The resulting hepatocytes can be further enriched by magnetic bead depletion of contaminating cells and/or plated on collagen-coated tissue culture dishes. For the mouse, anti-CD45 and anti-CD146 microbeads will deplete most immune cells and endothelial cells, respectively.

3.10 Non-parenchymal Cell Enrichment

1. Pellet the NPC suspension at 500 × *g* for 5–7 min at 4 °C.
2. Gently resuspend in 2.5 ml of PFB.
3. Mix cell suspension with 2.5 ml of 30–40 % iodixanol solution in 15-ml conical. A final concentration of 20 % iodixanol will enrich for most if not all intact NPCs.
4. Gently overlay with 2 ml of PFB.
5. Centrifuge at 1500 × *g* for 25 min at room temperature. If available, turn the brake OFF on the centrifuge to minimize disturbance to the cell interface.

6. During the centrifugation add 10 ml of cold PFB to a 15 ml conical tube.
7. After centrifugation a well-defined interface of cells should be visible. Carefully transfer this cell layer from the iodixanol gradient to the 10 ml of PFB in order to wash away excess iodixanol.
8. Centrifuge at 500 × *g* for 5 min at 4 °C.
9. Resuspend the enriched NPC pellet in 0.5 ml of cold PFB or appropriate buffer for desired applications.

3.11 Staining NPCs for Flow Cytometry

1. Prepare the necessary number of flow cytometry tubes.
2. Add anti-CD16/anti-CD36 (Fc receptor blocking) antibody to each sample to a final concentration of 1:250 (*see* **Note 17**).
3. Incubate for 5 min at room temperature.
4. Add antibody cocktail (*see* Table 1).
5. Vortex briefly and gently.
6. Incubate for 20 min at 4 °C.
7. Wash the cells by adding 1 ml of PFB to each sample.
8. Centrifuge at 500 × *g* for 5 min at 4 °C.
9. Aspirate supernatant.
10. Resuspend cell pellet in 0.5 ml of PFB.
11. In order to minimize clogs during cell sorting, filter the cell suspension.

3.12 Identifying and Sorting Liver NPC by Flow Cytometry

Liver NPCs have yet to become absolute in their defining characteristics. However, many distinct cell populations can be sorted from a mouse liver. Those identified here represent a cross-section of major cell types, including endothelial cells, macrophage, quiescent hepatic stellate cells, lymphocytes, and natural killer cells. If a population of cells appears diffuse in characteristics, separation by an additional dimension may reveal multiple cell populations. The successful isolation of pure and viable cells is as much art as science and will be aided by the direction and advice of a skilled flow cytometrist with an appreciation for the complexity of sorting from dissociated tissue. The gating strategy depicted in Fig. 3 is one approach to sorting liver NPCs.

3.13 Quality Control Analysis of Enriched Liver Cell Populations

Quality control analysis of enriched and sorted liver cell populations can be conducted by in vitro culture of the cells to confirm morphology and/or function [7]. In addition, enriched cells can be analyzed for expression of genes known to be relatively specific to cell types. The basic protocol and representative results are presented below.

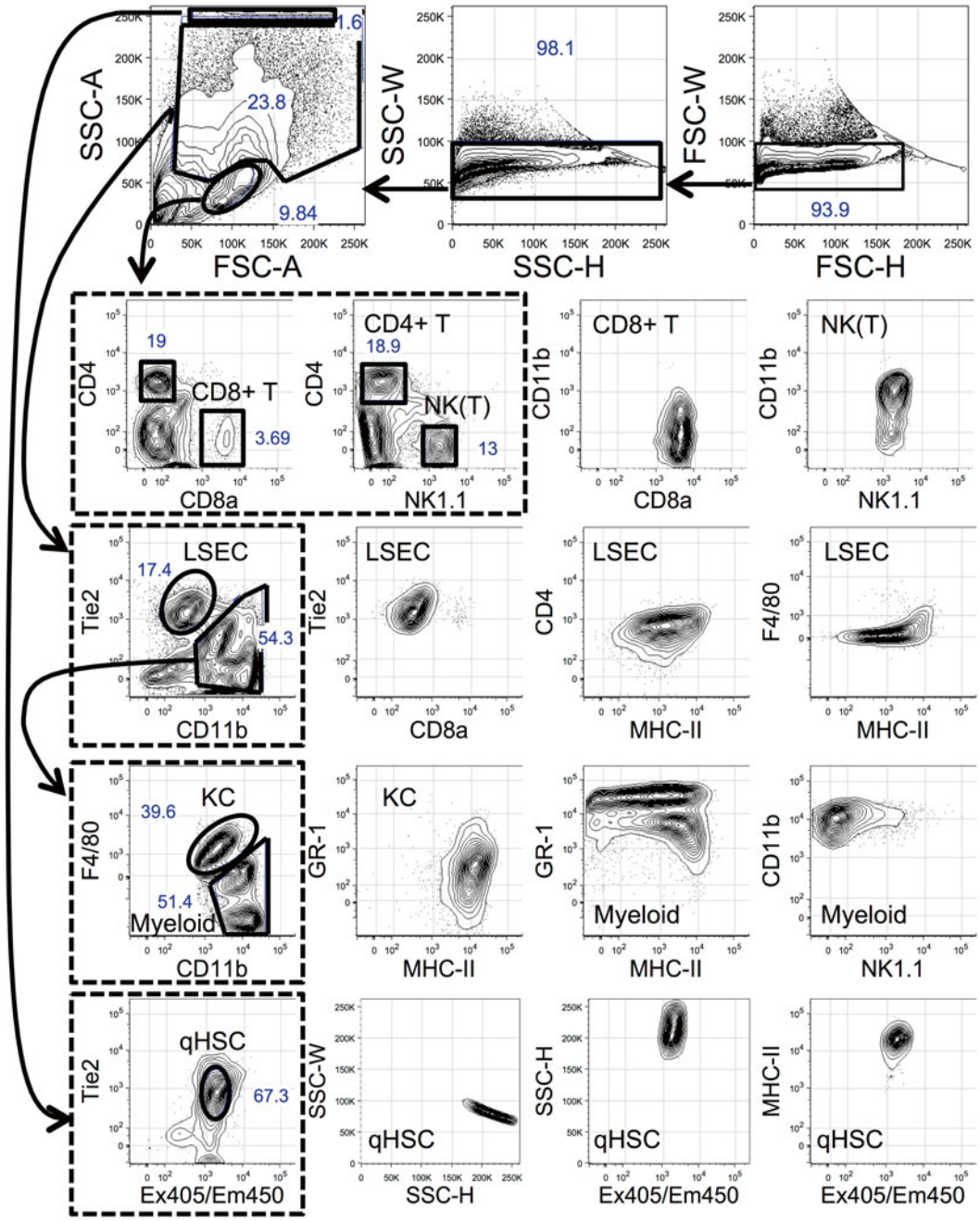

Fig. 3 NPC sort strategy. Representative NPC sorting strategy from a C57BL/6J mouse 68 h following injection of 50,000 *Plasmodium yoelii* sporozoites. Labeled gates are sorted populations. Exclude doublets by FSC-H vs. FSC-W and SSC-H vs. SSC-W, but if quiescent hepatic stellate (qHSC) are desired, be sure to include the SSC-H events. From a standard FSC-A vs. SSC-A scatter plot, separate lymphocyte-sized cells from cells with high granularity (SSC) and larger size (FSC). Hepatic stellate cells contain highly refractive retinol droplets and are autofluorescent when excited with 405 nm and emitting at 450 nm. Lymphocytes can be separated into many populations. Here, CD8+ T cells are collected against CD8a vs. CD4. CD4+ T cells and NK(T) cells are collected against NK1.1 vs. CD4. A significant population of NK-T cells are CD4+ in the mouse. The best identifier of NK-T cells is CD1d (stained by tetramer, not conducted here). NK(T) cells induce CD11b expression when activated. From the larger cells, LSEC, Kupffer cells (KC), and infiltrating myeloid cells (including monocytes and granulocytes) can be collected. LSEC are selected against CD11b vs. Tie2. From the CD11b$^{int/hi}$ Tie2$^{int/lo}$

The described liver cell isolation method was used to purify liver LSECs, Kupffer cells (KCs), qHSCs, and hepatocytes from five 9-week-old C57BL/6 J male mice purchased from The Jackson Laboratory (Bar Harbor, ME). Briefly, hepatocytes were processed through Subheading 3.9 and enriched using anti-CD45 and anti-CD146 microbeads. Liver NPCs were processed through Subheading 3.10 and then stained for cell sorting on a BD Aria III, as touched upon in Subheading 3.12. The antibodies used to discriminate cell populations during sorting were as follows: Live/Dead Violet (Pacific Blue), CD11b (BV605), IA/IE (FITC), Tie2 (PE), Ly6C (PerCP-Cy5.5), F4/80 (APC), and Ly6G (APC-Cy7). There were minimal differences in the concentration of antibodies used in sorting (*see* **Note 18**) and while the gating strategy was similar to that shown in Fig. 3, it was not identical (*see* **Note 19**).

Post-sort analyses of sorted LSECs, KCs, and qHSCs show average cell purities of 93.02 %, 93.82 %, and 87.02 % respectively (averaged value of $n = 5$). To further assess the purity of these cell populations, RNA was isolated using TRIzol (Invitrogen) and then cDNA was synthesized (QIAGEN QuantiTect) and quantified using microfluidic PCR (Fluidigm Corp, South San Francisco CA, USA) with cell-type-specific TaqMan® assays (Invitrogen) (Fig. 4). The qRT-PCR analysis shows that isolated hepatocytes, LSECs, KCs, and qHSCs are enriched for their cell-type-specific genes. Genes commonly associated with each cell type—*Alb* for hepatocytes, *Tek* (Tie2) for LSECs, *Emr1* (F4/80) for KCs, and *Pdgfrb* for qHSCs—are enriched in the expected populations (*see* **Note 20**).

4 Notes

1. Avertin becomes toxic when exposed to light. Although concentrated stock solutions can be prepared, preparation of smaller volumes of working solution minimizes the likelihood of accumulating toxic by-products.
2. Some researchers use the needle to catheterize, others simply use a 24G needle. We prefer to use the Vialon™ catheter alone and reuse it on multiple mice.

Fig. 3 (continued) cells, KC and general myeloid cells can be distinguished by CD11b vs. F4/80 staining. Here we see that KC are MHC class-II high and GR-1 (Ly6G/Ly6C) intermediate. The myeloid infiltrate contains GR1^{hi} and GR1i^{int} population with varying degree of MHC-II staining. Lastly, qHSC show very high SSC and autofluorescence (Ex 405 nm/Em 450 nm) and often show autofluorescence in many channels. Since many of these characteristics are that of dead cells or debris, the best validation of sorted qHSC is direct observation under a light microscope. In all cases, heterogeneity may exist in these populations, and further selection or validation of purity may be needed

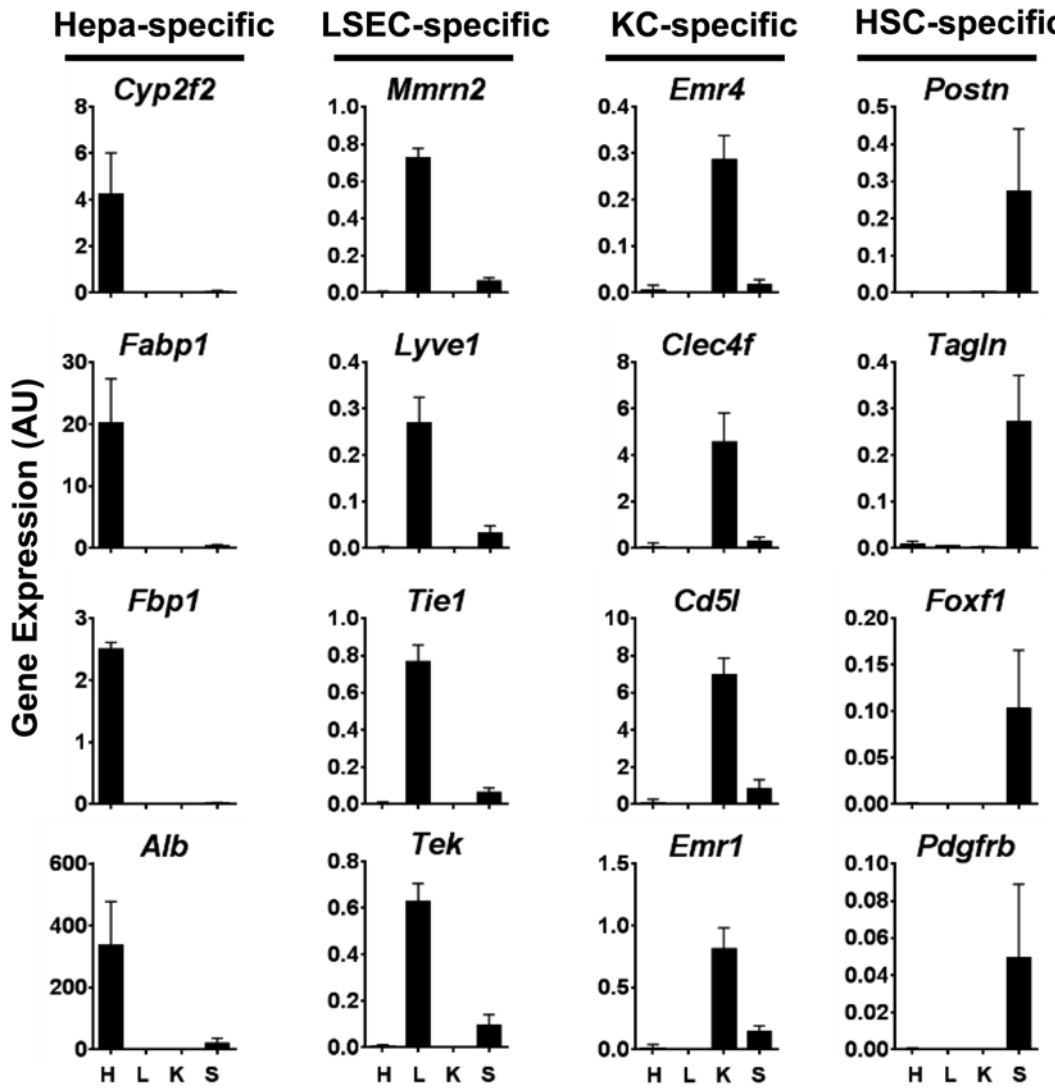

Fig. 4 Quality control by qRT-PCR of hepatocytes and sorted liver NPCs. The relative expression of genes in enriched liver cell types illustrates the efficacy of the method. Gene expression is normalized to the average of three house-keeping genes: *Gapdh*, *Actb*, and *Hprt*. Each bar is the mean (+SD) of n = 5 mice. H = Hepatocyte, L = LSEC, K = Kupffer cell, and S = Hepatic stellate cell. Graphed using Prism6 (GraphPad Software, San Diego CA, USA)

3. Collagenase is available in many fractions and sources. We have found that collagenase from *C. histolyticum*, Type IV, from Sigma-Aldrich dissociates the liver efficiently and maintains expected cell function.
4. Standard polystyrene tubes are suitable for most applications. However, prior to sorting samples, it is important to filter (40 μm) each sample in order to reduce the likelihood of clumps and clogs. Use sterile tubes when necessary.

5. The choice of antibodies and fluorophores is highly dependent upon the cell(s) of interest. Those listed in Table 1 allow for separation of relatively pure populations of LSEC, Kupffer cells, CD8+ T cells, CD4+ T cell, infiltrating myeloid cells, and quiescent HSC. During inflammation or pathology, the morphology and cell surface molecules of most cells change, resulting in heterogeneity. Thus, additional antibodies may be necessary to achieve homogenous cell populations.
6. Prepare a perfusion tubing set using two IV extensions, two adaptors, and one length of silicon peristaltic pump tubing. Cut and discard the male end from one extension set and the female from the other; remove the slide clamps from both and discard. Connect the cut ends of the extension tubes to the pump tubing using the connectors (Fig. 1a). Connect the catheter to the male end (Fig. 1b). Perfusion tubing may be reused for many months with proper cleaning and storage. Flush the tubing with 5 ml of 70 % ethanol followed by 15 ml of sterile distilled water. Allow the tubing to run dry then store in a plastic zip-lock bag, protected from light. Tubing will become brittle with prolonged exposure to ethanol. Upon reuse, flush the tubing with ethanol then distilled water, run dry, then fill with perfusion buffer.
7. Toe-pinch reflex is a standard method for assessing depth of anesthesia in mice. The mouse should not flinch. If flinching occurs, allow more time or administer additional Avertin.
8. Exercise care when moving the liver in order to avoid hemorrhage. Once the portal vein is exposed, place forceps in order to hold back other tissue. Excess fat (or pancreas) may partially hide the portal vein. This is more likely in older (>8 months of age) male mice.
9. It will be more difficult to catheterize the vein if it is cut clean through. A “nick” in the vein will allow an entry point for a catheter without a needle, while preserving structural support.
10. Blood will generally flow from the right—the opening towards the intestines. As perfusion buffer washes the blood away, the nick in the portal vein should be clear. Gently catheterize the vein towards the liver.
11. Do not cut the descending vena cava until the portal vein is catheterized. The liver should blanch as soon as the vein is catheterized, if it doesn’t the vein is not catheterized, so try again. The most likely “miss” occurs in the smooth muscle layer surrounding the vein. This layer will puff up. If this occurs, remove the catheter and try for the vein opening again. The catheter is visible within the vein.
12. It is important to maintain a steady hand or else the catheter will slip, tear or puncture (if pushed too far toward the liver)

the vein. Mindful relaxation of the right hand will minimize shaking and fatigue, which is especially important for multiple mouse experiments. If with time and practice, shaking persists and is the cause of failed perfusions, consider the method of Seglen and secure the catheter with a noose. Alternatively, with practice, the catheter can be fully released from grip, once the mouse has expired.

13. When switching the line from perfusion buffer to collagenase, air bubbles are occasionally introduced. A small bubble (<3 mm) in the line is not an issue. Larger bubbles may occlude the perfusion of regions of the liver, but not always. If a very large bubble (>2 cm) is seen in the line, it is best to remove the catheter just before the bubble reaches the catheter, run the bubble out, then catheterize the vein again. With practice, this is easily done. When in doubt, let the bubble run its course.
14. The liver should swell. The left lobe, in particular, should clearly swell and fall over the portal vein. If the liver does not swell, inspect the catheter in the portal vein. Diseased livers (i.e., fibrotic) do not digest or swell very well.
15. The gall bladder contains bile acid salts, digestive enzymes, and fat-soluble compounds destine for excretion in the feces. Ideally, the gall bladder is removed intact. However, we have not observed a difference in NPC phenotype due to gall bladder rupture. If working with an assistant, pass the digested liver to the assistant to complete the processing, then continue with Subheading 3.7 or begin another mouse.
16. Although it is optional, removal and isolation of cells from the spleen is useful for preparing compensations for flow cytometry and providing additional (and validating) immunological information. The cell surface staining of lymphocytes isolated from the liver often shows a bias towards mixed activation and polarization states. By contrasting the immunophenotype of cells in the liver to those of the spleen, one is better able to assure: (1) an intact and "proper" immune system, (2) proper staining protocol, and (3) proper gating strategy in flow cytometry.
17. A concentration of 1:50 Fc block is generally adequate to identify populations of liver NPCs with proper compensation. Increasing the concentration of Fc block will reduce the amount of nonspecific antibody binding, and has the potential to further resolve cell populations. LSEC and KC are abundant liver NPC and show affinity for most flow cytometry antibodies.
18. Anti-CD16/anti-CD36 (Fc receptor blocking) was used at a final concentration of 1:50 to increase the resolution between cell populations. The Live/Dead Violet stain was used at a final concentration of 1:1000. All other antibodies were used at a final concentration of 1:200.

19. Cells were sorted in the manner illustrated in Fig. 3, with modification. In brief, following size gating on the FSC-A vs. SSC-A plot, Live/Dead was used to exclude dead cells. Cells staining $CD11b^{int/hi}Tie2^{int/lo}$ CD11b vs. Tie2 plot were further gated on CD11b vs. Ly6G to gate out the $Ly6G^{hi}$ neutrophils then selecting Kupffer cells by CD11b vs. F4/80. Of note, since the size gate from the FSC-A vs. SSC-A plot is mostly sufficient to separate qHSC from LSEC and KC, this panel uses the Pacific Blue channel to sort qHSC as the highly autofluorescent population and to define dead cells as the Pacific Blue positive population. The qHSC are generally more Pacific Blue "positive" than dead cells.
20. The low-level expression of LSEC- and KC-specific genes in the qHSC population is likely due to a small contaminating fraction of dead LSECs and KCs, and resulting from the use of the Pacific Blue channel for both qHSC autofluorescence and the Live/Dead-violet stain. We suspect this issue to be solved if a different channel for Live/Dead discrimination is used. Autofluorescence from debris, however, renders many other channels frustratingly nonspecific for absolute exclusion of dead cells. As a result, exclusion of dead cells should be considered on a case-by-case basis.

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