Here, we provide detailed protocols for the isolation of mouse Kupffer cells – the liver-resident macrophages – for phenotypic (e.g., via flow cytometry, mass cytometry or RNA-sequencing) analyses or for functional experiments involving cell culture. The procedures presented can be adapted for the isolation of other hepatic cell populations.
Protocol
Isolation of mouse Kupffer cells for phenotypic and functional studies

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https://doi.org/10.1016/j.xpro.2021.100831

SUMMARY
Here, we provide detailed protocols for the isolation of mouse Kupffer cells – the liver-resident macrophages – for phenotypic (e.g., via flow cytometry, mass cytometry, or RNA-sequencing) analyses or for functional experiments involving cell culture. The procedures presented can be adapted for the isolation of other hepatic cell populations. For complete details on the use and execution of this protocol, please refer to De Simone et al. (2021).

BEFORE YOU BEGIN
In addition to its metabolic functions, the liver is endowed with unique immunological features (Bénéchet et al., 2019; Ficht and Iannacone, 2020; Iannacone and Guidotti, 2021). It is comprised by parenchymal cells (the hepatocytes) as well as non-parenchymal cells (LNPCs). Among the latter, Kupffer cells (KC) reside within liver sinusoids and represent the most abundant resident macrophage population of the organism. KCs have long been known for their scavenger and phagocytic functions but can also present antigens to CD8+ T cells and promote either tolerance or effector differentiation. Efficient, reproducible methods for the isolation of Kupffer cells suitable for phenotypical and functional analyses are hence of paramount importance in order to study the biology of liver-resident macrophages.

This STAR protocol provides two distinct methods for KC isolation: a quick, scalable procedure for phenotypic analyses (method #1, steps 1 through 26), or a longer, more laborious preparation best suited for in vitro functional studies (method #2, steps 27 through 48).

KCs isolated with either methods are suitable for the desired downstream application, and we provide detailed information and suggestions on how to perform cell culture (steps 49 through 53) as well as flow cytometry analysis and cell sorting (steps 54 through 63).
Prepare the reagents for liver digestion, related to method #1

**Prepare the ex vivo digestion medium**

* Timing: 15 min

1. Prepare a solution of plain RPMI supplemented with 0.2 mg/mL of collagenase, 5 units/mL of Deoxyribonuclease I and 10% FBS.
2. Prewarm the solution at 37°C.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| InVivoMAb anti-mouse CD16/CD32 antibody | Bio X Cell | Bio X Cell Cat# BE0307; RRID: AB_2736987 |
| Brilliant Violet 605(TM) anti-mouse CD31 antibody | BioLegend | BioLegend Cat# 102427; RRID: AB_2563982 |
| Brilliant Violet 650(TM) anti-mouse/human CD11b antibody | BioLegend | BioLegend Cat# 101239; RRID: AB_11125575 |
| FITC anti-Mouse CD45 | BioLegend | BioLegend Cat# 103108; RRID:AB_312973 |
| PE anti-mouse ESAM antibody | BioLegend | BioLegend Cat# 136203, RRID: AB_1953300 |
| PE-CF594 Rat Anti-Mouse Ly-6G | BD Biosciences | BD Biosciences Cat# 562700; RRID: AB_2737730 |
| PE-CF594 Rat Anti-Mouse CD49b | BD Biosciences | BD Biosciences Cat# 562453; RRID: AB_11153857 |
| PE-CF594 Rat Anti-Mouse CD19 | BD Biosciences | BD Biosciences Cat# 562291; RRID: AB_11154223 |
| PE-CF594 anti-Mouse CD3e | BD Biosciences | BD Biosciences Cat# 562286; RRID: AB_11153307 |
| PerCP/Cyanine5.5 anti-mouse I-A/I-E | BioLegend | BioLegend Cat# 107626; RRID: AB_2191071 |
| PE/Cyanine7 anti-mouse Tim-4 | BioLegend | BioLegend Cat# 130010; RRID: AB_2565719 |
| APC anti-mouse CD206 (MMR) antibody | BioLegend | BioLegend Cat# 141708; RRID: AB_10900231 |
| APC/Cyanine7 anti-mouse F4/80 | BioLegend | BioLegend Cat# 123117; RRID: AB_893489 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| DAPI | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# D1306, RRID: AB_2629482 |
| ViaKrome 808 | Beckman | Beckman Cat# C36628 |
| Brilliant stain buffer | BD Biosciences | BD Biosciences Cat# 659611; RRID: AB_2870505 |
| Heparin sodium salt | Sigma Aldrich | Sigma Aldrich Cat# H4784 |
| Liver Perfusion Medium | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# 17701038 |
| Liver Digest Medium | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# 17703034 |
| Hepatocyte Wash Medium | Thermo Fisher Scientific | Thermo Fisher Scientific Cat# 17704024 |
| Deoxyribonuclease I from bovine pancreas | Sigma Aldrich | Sigma Aldrich Cat# D4263 |
| Collagenase from Clostridium histolyticum | Merck Life | Merck Life Cat# C5138 |
| Trypan Blue Solution | Sigma Aldrich | Sigma Aldrich Cat# T8154 |
| Roswell Park Memorial Institute (RPMI) medium 1640 | Gibco | Gibco Cat# 61870-010 |

(Continued on next page)
MATERIALS AND EQUIPMENT

**REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER**
--- | --- | ---
Complement inactivated Fetal Bovine Serum (FBS) | Corning | Corning Cat# 35-079-CV
Penicillin-Streptomycin (10,000 U/mL) | Gibco | Gibco Cat# 15140122
Phosphate Buffered Saline (PBS) 1x, pH 7.4 | Gibco | Gibco Cat# 10012023
Phosphate Buffered Saline (PBS) 10x, pH 7.4 | Gibco | Gibco Cat# 70011044
70% Ethanol (v/v) | Sigma Aldrich | Sigma Aldrich Cat# 51976-500ML-F
2,2,2-tribromoethanol | Sigma Aldrich | Sigma Aldrich Cat# T48402
2-methyl-2-butanol | Sigma Aldrich | Sigma Aldrich Cat# 240486
Ammonium chloride (NH4Cl) | Merk | Merk Cat# 254134
Potassium bicarbonate (KHCO3) | Merk | Merk Cat# 237205
Na2EDTA | Merk | Merk Cat# E5134
NaN3 | Merk | Merk Cat# S2002
Percoll | Sigma Aldrich | Sigma Aldrich Cat# P4937

Experimental models: organisms/strains
- Mouse: C57BL/6 Charles River C57BL/6 colony

Software and algorithms
- FlowJo V10 FlowJo https://www.flowjo.com/

Other
- FACS CANTO II BD Bioscience N/A
- CytoFLEX L Beckman Coulter N/A
- FACS Aria Fusion BD Bioscience N/A
- Centrifuge 5810/5810R Eppendorf N/A
- Peristaltic pump PLP 380 Behr Labor Behr labor Cat# B00454739
- Neubauer chamber Blaubrand Blaubrand Cat# 717805
- Student Adson Surgical forceps Fine Science Finescience Cat# 91106-12
- Student fine scissors Fine Science Finescience Cat# 91460-11
- 6-well culture plate flat bottom Corning Corning Cat# 3516
- Petri dishes, polystyrene (60 mm × 15 mm) Sigma Aldrich Sigma Aldrich Cat# PS481
- 10 mL syringe Pic Solution Picosolution Cat# 02076120090300
- Hypodermic needle 18G Pic Solution Picosolution Cat# 02070520300800
- Falcon Cell Strainers 70 µm Falcon Falcon Cat# 352350
- 5 mL polystyrene round bottom FACS tubes Falcon Falcon Cat# 352350
- 15 mL tubes Falcon Falcon Cat# 352096
- 50 mL tubes Falcon Falcon Cat# 352070

**FACS Buffer**

| Reagent | Final concentration | Amount |
|---|---|---|
| FBS | 1% | 5 mL |
| Na2EDTA (0.5 M) | 2 mM | 2 mL |
| NaN3 (10% wt/v) | 0.05% | 2.5 mL |
| PBS | n/a | 490.5 mL |
| Total | n/a | 500 mL |

[Store at 4°C up to one month]

**Alternatives:** 1% FBS can be substituted with 1% BSA.

**Note:** For cell sorting experiments that involve *in vitro* culture, FACS buffer should be prepared under sterile conditions, filtered through 0.2 µm filter and should not contain NaN3.
Note: Adjust pH to 7.2 and filter through 0.2 μm filter. Keep it sterile at 20°C–25°C.

| ACK Buffer | Final concentration | Amount |
|------------|---------------------|--------|
| NH₄Cl      | 0.15 M              | 8.02 g |
| KHCO₃      | 10 mM               | 1 g    |
| Na₂EDTA (0.5M) | 0.1 mM       | 200 µL |
| double-distilled H₂O (ddH₂O) | n/a | 1 L   |
| Total      | n/a                 | 1 L    |

Note: Dissolve 2.5 g of tribromoethanol into 5 mL of 2-methyl-2-butanol by warming to 55°C with gentle swirling. Add slowly (dropwise) the 5 mL of dissolved tribromoethanol into 200 mL of sterile ddH₂O. Filter through 0.2 μm filter and store at 4°C for up to 4 months.

| Avertin | Final concentration | Amount |
|---------|---------------------|--------|
| 2,2,2-tribromoethanol | 45 mM | 2.5 g |
| 2-methyl-2-butanol     | 2.5%  | 5 mL  |
| ddH₂O                  | n/a   | 195 mL|
| Total                  | n/a   | 200 mL|

Note: Mix 7.2 mL of Percoll (100%) with 0.8 mL of PBS (10X) to achieve a iso-osmolar Percoll solution. Then, mix this solution with 12 mL of plain RPMI to achieve a final Percoll density of 36%.

| Percoll 36% | Final concentration | Amount |
|-------------|---------------------|--------|
| Percoll (100%) | 36%  | 7.2 mL |
| PBS 10x     | 1 x                 | 800 µL |
| RPMI        | n/a                 | 12 mL  |
| Total       | n/a                 | 20 mL  |

Note: Mix 7.2 mL of Percoll (100%) with 0.8 mL of PBS (10x) to achieve a iso-osmolar Percoll solution. Then, mix this solution with 12 mL of plain RPMI to achieve a final Percoll density of 36%.

**STEP-BY-STEP METHOD DETAILS**

**Harvesting the liver for ex vivo LNPCs isolation (method #1)**

© Timing: 10 min

1. Euthanize the mouse according to the authorized ethical guidelines.
2. Sanitize the mouse abdomen surface with 70% ethanol (Figure 1A).
3. Cut through the peritoneum (Figure 1B), by first performing a vertical cut from the lower end of the abdomen to the ribcage, and then performing horizontal cuts at the mid-section of the abdomen to both sides until the organs of the mouse are exposed. Pay attention not to damage any organs and gently expose the liver.
4. Push the intestine right-hand sideward by using the blunt end of a forceps to get access to the liver (Figure 1C).
5. Load a 10 mL syringe with 10 mL of PBS and a 27G needle.
6. Carefully insert the needle in the inferior vena cava and perfuse 2 mL until the organ starts to swell (Figure 1D).
7. Cut the portal vein with scissors, paying attention not to damage the liver (Figure 1E).
8. Complete the perfusion until 10 mL of PBS have been injected in about 2 min (Figures 1F and 1G).
9. Remove the gallbladder (Figure 1H) and cut the liver ligaments connecting the liver to surrounding tissues (Figures 1I and 1J).

10. Collect the liver in ice-cold plain RPMI until next step.

**Note:** The following protocol has been adapted from (Blériot et al., 2020) and optimized for the digestion of half a liver. However, the volume of the digestion medium can be adjusted according to the amount of liver to be processed. If KC isolation is performed to be followed by a cell culture experiment, both mouse surgery and organ processing should be performed under sterile conditions (e.g., under a cell culture hood).

### Processing the liver for *ex vivo* LNPCs isolation

**Timing:** 60 min

11. Transfer the liver to a 60 mm Petri dish and use blunt-end scissors to mince it in 1–2 mm³ small pieces.

12. Transfer the liver pieces in a 50 mL Falcon tube containing 10 mL of pre-warmed *ex vivo* digestion medium (see above).

13. Incubate at 37°C for 30 min.

14. Vortex the sample every 10 min at 2000 rpm.

15. After incubation, gently homogenize the digested liver pieces by forcing them 7–8 times into a 10 mL syringe loaded with a 18G needle.

16. Filter the homogenized liver solution through a 70 μm cell strainer in a new 50 mL Falcon tube and wash the filter with 10 mL of plain RPMI.
17. Centrifuge at 50 rcf for 3 min.
18. Recover the aqueous phase and transfer it in a new 50 mL Falcon tube. Discard the pellet containing dead hepatocytes and debris.
19. Centrifuge at 400 rcf for 5 min.
20. After this centrifugation step the pellet contains target LNPCs. Discard the top aqueous phase.
21. Lyse red blood cells resuspending the cell pellet with 2 mL of ACK and incubate for 30 s at 20°C–25°C.
22. Add 20 mL of plain RPMI to restore osmolarity and centrifuge at 400 rcf for 5 min.
23. Resuspend the cell sediment in 10 mL of ice-cold plain RPMI. From now on all the steps are done at 4°C.
24. Filter cell suspension through a 70 μm cell strainer in a new 50 mL Falcon tube.
25. Mix an aliquot of the cell suspension cell suspension 1:2 in trypan blue and determine cell count of viable cells with a Neubauer chamber.
26. Proceed with the desired downstream application (steps 49 through 53 for cell culture or steps 54 through 63 for flow cytometry and cell sorting).

**CRITICAL:** cell sediment in step 20 is loose, pay attention if a vacuum aspiration system is used to discard the supernatant.

**Optional:** at steps 20–21 there might be clogs in the cell pellet, cut the tip of a 1 mL pipette to facilitate the resuspension process.

**In situ liver digestion for the isolation of hepatocytes and LNPCs (method #2)**

*Timing: 90 min*

The following step-by-step section describe an alternative, longer but more gentle protocol for the isolation of LNPCs. This method also allows the simultaneous recovery of hepatocytes and replaces steps 1 through 28.

27. Prewarm the Liver perfusion medium and liver digestion medium in the water bath at 37°C (see Figure 2 for pump configuration).
28. Prime and wash the pump tubes with prewarmed HBSS. Pump flux during the procedure should be set at 5 mL/min.
29. Inject the mouse intravenously with 200 μL of heparin solution (100 Units in PBS) to avoid coagulation issues that can potentially interfere with the perfusion and with the digestion.
30. Anesthetize the mouse with Avertin (600 μL for a mouse of 25 grams) at 37°C intraperitoneally.
31. Spread the mouse with 70% EtOH and cut through the peritoneum, by first performing a vertical cut from the lower end of the abdomen to the ribcage, and then performing horizontal cuts at the mid-section of the abdomen to both sides und the organs of the mouse are exposed.
32. Gently push the intestine right-hand sideward by using the blunt end of a forceps to get access to the liver.
33. Canulate the inferior vena Cava (25G needle).

**Optional:** Place an open silk suture around inferior vena Cava to help the canula to be in place.

34. Start the perfusion with Liver Perfusion Medium for 3 min after severing the portal vein. This medium is meant to clean the liver from blood and to start the loosening of cell-to-cell contacts.
35. Perfuse with warm Liver digestion medium for 12 min and pay attention that no air bubbles should be introduced during the entire process. After 8 min, start checking the liver consistency with a wet cotton applicator. Digested liver loses elasticity and assumes a reticulated appearance.
36. Once the liver is completely digested, remove the gallbladder, and cut the liver ligaments connecting the liver to the surrounding tissue.
37. Transfer liver into a 60 mm Petri dish with Hepatocyte Wash Medium and carefully decapsulate
the organ using surgical forceps (for more information see the online Methods video S1).

38. Gently shake the liver while holding it with a tweezer. This procedure allows digested cells (Hepatocytes
and LNPCs) to be released in the Hepatocyte wash medium (online Methods video S1).

39. Transfer the obtained cell suspension to a 50 mL falcon tube and reach 50 mL of volume with the
Hepatocyte Wash Medium.

40. Centrifuge at 20 rcf for 3 min.

Note that at the end of each 20 rcf centrifugation the pellet is enriched by Hepatocytes, while soluble
fraction by LNPCs.

41. At this step the pellet is composed mainly by Hepatocytes (HC) and the soluble fraction by LNPCs.

42. Repeat steps 40 to 41.

43. Collect separately HC and LNPC and resuspend each fraction in separate 15 mL tubes contain-
ing 10 mL of 36% Percoll solution and gently mix.

44. Centrifuge 2000 rpm for 20 min without brake.

Note: the purpose of the Percoll separation is to get rid of cellular and tissue debris to obtain a
clearer cell preparation.

45. At the end of the centrifugation, discard the Percoll soluble fraction (containing cellular debris)
and resuspend each cell pellet in the appropriate medium (LNPC fraction should be suspended
in RPMI, hepatocytes in hepatocyte medium).

46. Filter LNPCs fraction through a 40 μm cell strainer.

Optional: lyse red blood cells with 2 mL of ACK for 30 seconds at 25°C, centrifuge and resus-
pend the cell pellet in 10 mL of RPMI.

47. Mix an aliquot of each cell suspension 1:2 in trypan blue and determine cell count of viable cells
with a Neubauer chamber.

48. Proceed with the desired downstream application (step from 49 to 53 for cell culture or step
from 54 to 66 for flow cytometry and cell sorting).

△ CRITICAL: make sure that the tube carrying the digestion medium to the liver is at 37°C
when reaches the vena cava. If the solution is at lower temperature, enzymatic activity
might be suboptimal.

Note: Hepatocytes are extremely fragile cells and should be maintained at 37°C during the
entire procedure to maximize cell vitality.
Isolating KCs by cell adhesion

© Timing: 3 h

49. Prepare the culture medium supplementing plain RPMI with 10% FBS and 100 U/mL Penicillin/Streptomycin.
50. Resuspend LNPCs to a density of $1 \times 10^7$/mL in culture medium.
51. Plate $1 \times 10^7$ LNPCs per well in a 6-well culture plate.
52. Incubate for 2 h at 37°C with 5% CO₂.
53. Remove cell debris and non-adherent cells by gently washing adherent cells with cold PBS.

Note: more than 95% of adhering cells are Kupffer cells (Li et al., 2014). To maintain Kupffer cell differentiation, add 50 ng/mL of recombinant M-CSF (R&D system cat #416-ML) in the culture medium.

Staining KCs from isolated LNPCs for FACS sorting

© Timing: 2 h

54. Transfer LNPCs in FACS tubes at $5 \times 10^7$/mL.

Note that in order to sort $10^6$ KCs, a good starting point is $2 \times 10^7$ of total LNPCs.

55. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
56. Incubate with 200 μL of anti-CD16/CD32 (5 μg/mL in FACS buffer) for 15 min at 4°C.
57. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
58. Incubate with 300 μL of antibody mix for 40 min at 4°C.
59. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
60. Incubate with 200 μL of DAPI (5 μg/mL in FACS buffer) for 5 min at 4°C.
61. Wash with 1–2 mL of FACS buffer and spin down for 5 min at 400 rcf.
62. Resuspend in FACS buffer at $5 \times 10^6$ cells per mL.
63. Sort target cells at 4°C using a 100 μm nozzle.

△ CRITICAL: sheath pressure should not exceed 20 psi to preserve cell viability.

Recommended: To enhance viability of sorted cells, collection FACS tubes pre-coated with 5% BSA or 5% FBS for 14–16 h at 4°C to neutralize the electrostatic charges of polystyrene tubes should be used. Otherwise, polypropylene tubes can be used since have neutral charge.

Note: If cells are sorted for functional downstream applications (e.g., cell culture), do not supplement FACS buffer with NaN₃. To further improve cell viability, collection media should contain at least 10% FBS.

| Antibody mix | Fluorophore | Clone | Final concentration (μg/mL) |
|--------------|-------------|-------|----------------------------|
| CD31         | BV605       | 390   | 5                          |
| CD11b        | BV650       | M1/70 | 1                          |
| CD45         | FITC        | 30F11 | 2                          |
| I-A/I-E (MHCII) | PerCP-Cy5.5 | M5/114.15.2 | 1 |
| ESAM         | PE          | 1GB/EASAM | 1 |

(Continued on next page)
Optional: brilliant stain buffer can be used in place of FACS buffer to minimize Brilliant Violet dyes staining artifacts.

Alternatives: fixable Live/Dead (L/D) dyes can be used to gate live cells instead of DAPI. In this case, at the end of LNPC isolation procedure, cells must be washed in plain PBS and Live/Dead staining should be performed at 20°C–25°C for 20 minutes.

Note: sessile LNPCs (e.g., KCs and Liver Sinusoidal Endothelial cells - LSECs), once extracted from the liver during the digestion process, are slightly auto fluorescent and have more proteins on their membrane surface. For this reason, since fixable dyes stain amine reactive groups, the use of Live/Dead might be suboptimal in separating dead cells. DAPI – being impermeable to the membrane of live cells – is more appropriate when possible (see Figure 3).

EXPECTED OUTCOMES

Usually, this protocol allows to recover 2–3*10^7 of total LNPCs and about 1.5–2*10^6 KCs from one healthy liver of a 7–9 week-old mouse. Considering the LNPC fraction, we do not find major differences in terms of absolute numbers when comparing the ex vivo and in situ digestion protocols.

LIMITATIONS

The ex vivo digestion combined with the mechanical dissociation of the liver has been designed to obtain reproducible whole LNPC preparations in a reasonable amount of time. This protocol is easily scalable, allowing the isolation of LNPC from several liver samples at the same time. The viability of recovered cells is greater than 94%, which makes the single cell suspensions obtained with this procedure suitable for functional and phenotypical analysis by multicolor flow cytometry, cell sorting and RNAseq (see Figure 4 for a suggested flow cytometry data analysis).

Although this protocol gives highly reproducible preparations, it is worth noting that the execution of critical steps (e.g., the homogenization step with the 18G syringe) should be performed consistently to minimize inter-user variability.

However, because of the mechanical force being used, the quick LNPC preparation does not permit the isolation of hepatocytes and enables only the partial recovery of other non-parenchymal cells like Hepatic Stellate Cells (HSC). For this purpose, the in situ intravenous injection of the enzymatic medium has the main advantage to completely digest the liver tissue without the need for mechanical disruption (Guidotti et al., 2015). This passage is essential to preserve hepatocytes integrity since those cells are extremely fragile and are lost during the quick ex vivo procedure.

In situ digestion might be preferred if hepatocytes or stellate cells must be isolated contextually and/or if a gentler preparation is demanded by the experimental design. Here, the main limitation is represented by the need of dedicated equipment (e.g., the peristaltic pump) and by the laboriousness of the in situ digestion itself, which makes this protocol not suitable for the processing of a large quantity of samples.
TROUBLESHOOTING

Problem 1
Liver perfusion during the ex vivo protocol at step 6 does not efficiently remove blood from the liver.

Potential solution
Failure in performing an effective liver perfusion leads to an irreversible contamination of LNPC preparation with non-resident, blood-borne cells. Therefore, this step is critical since it can be a potential source of sample-to-sample variability in term of LNPC composition and preparation.

To reach 100% success in the liver perfusion, insert the 27G needle of a PBS-charged 10 mL syringe in the inferior vena cava paying particular attention to be inside the vasculature. Start injecting 1–2 mL of PBS into the vena cava without cutting the portal vein at this step. Only once the liver vasculature becomes engorged, cut the portal vein with surgical scissors. This will make the hepatic perfusion more effective and the liver will clear immediately turning into a light brown color. At this point, complete the perfusion with the remaining PBS.

For more details, please refer to Figure 1.

Problem 2
Liver in situ digestion is suboptimal (step 36).

Potential solution
Enzymes need a controlled, stable temperature of about 37.5°C to perform efficiently. For this reason, it is mandatory that the output pipe carrying the digestion mix reaches the vena cava at the correct temperature (see Figure 2). To avoid unwarranted cooling of the digestion mix during its way from the thermostatic bath to the vena cava, one strategy might be to rise the temperature of the water bath up to 39°C–40°C. However, consider that higher temperatures can damage the enzymatic activity.

If necessary, shortening the output pipe tube might also prevent excessive dissipation of the heat. Alternatively, place the output pipe in the water bath again in order to re-warm the buffer (see Figure 2, label #7).

Checking the temperature of the digestion mix at the end of the tube with a digital thermometer is also recommended.

Problem 3
After the ex vivo digestion, at step 15, liver pieces are still too big and cannot pass through the 18G needle for the homogenization step.

Figure 3. Different strategies to gate live cells
Representative dot plots of LNPC preparation in which live cells have been gated with DAPI staining (A) or with fixable Live/Dead staining (B). In separate samples, LNPCs have been mixed in 1:1 ratio with LNPCs placed at 70°C for 5 min to show actual dead cell population.
Potential solution
Make sure to cut the liver pieces as small as possible before starting the enzymatic digestion. At the end of the mechanical disruption the liver should have a pulp consistency.

Alternatively, if liver pieces are still too big, use a serological 10 mL pipette and flush up and down for about 10 times before homogenizing with the syringe.

Please note that the homogenization through the 18G needle is another critical step and should be done consistently (e.g., same number of homogenizations for each sample) in order to minimize sample-to-sample variations.

Problem 4
The recovery of LNPC is low (step 25).

Potential solution
If cell recovery is below $2 \times 10^7$ LNPC per liver, make sure that the enzymatic digestion has been performed for the correct amount of time (e.g., 30 min) at the correct temperature (e.g., 37°C). Alternatively, the homogenization step might not have been performed properly. In the latter case, make sure to move the plunger up and down for at least 7–10 times. At the end of this step no more liver pieces should be evident in the cell suspension. Moreover, after the filtration of the homogenized liver solution, limited debris should be present on the surface of the cell strainer.

Problem 5
The vitality of LNPC is below 90% (step 25).

Potential solution
Possible cell-damaging steps are represented by the liver homogenization and by the red blood cell lysis. Avoid excessive (e.g., more than 15) homogenizations through the 18G needle and be gentle in pushing/pulling the plunger in order to avoid incorporating air bubbles.

Otherwise, do not exceed 60 s incubation with the ACK solution.
RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact.

Materials availability
This study did not generate new unique reagents.

Data and code availability
This protocol did not generate data sets.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100831.

ACKNOWLEDGMENTS
We thank all the members of the Iannacone laboratory for helpful discussions. Flow cytometry was carried out at FRACTAL, a flow cytometry resource and advanced cytometry technical applications laboratory established by the San Raffaele Scientific Institute. M.I. is supported by the European Research Council (ERC) Consolidator Grant 725038, ERC Proof of Concept Grant 957502, Italian Association for Cancer Research (AIRC) Grants 19891 and 22737, Italian Ministry of Health (MoH) Grant RF-2018-12365801, Lombardy Foundation for Biomedical Research (FRRB) Grant 2015-0010, the European Molecular Biology Organization Young Investigator Program, and a Funded Research Agreement from Gilead Sciences. F.A. is the recipient of a Fondazione Umberto Veronesi postdoctoral fellowship. X.F. is the recipient of the iCARE fellowship 23906 from AIRC. M.K. is supported by the Italian Ministry of Education, University and Research grant PRIN-2017ZXT5WR. F.G. is supported by the Singapore Immunology Network (SIN) core funding, the Singapore National Research Foundation Senior Investigatorship (NRFI) NRF2016NRF-NRF1001-02 and the European Molecular Biology Organization Young Investigator Program.

AUTHOR CONTRIBUTIONS
All authors contributed to setting up or optimizing the protocols and in writing the manuscript.

DECLARATION OF INTERESTS
M.I. participates in advisory boards/consultancies for Gilead Sciences, Roche, Third Rock Ventures, Amgen, Allovir. M.I. is an inventor on patents filed, owned, and managed by San Raffaele Scientific Institute, Vita-Salute San Raffaele University and Telethon Foundation on technology related to work discussed in this manuscript (WO2020/016434, WO2020/016427, WO2020/030781, WO2020/234483, EU patent applications n. 19211249.8 and n 20156716.1, and UK patent application n. 1907493.9). F.G. is a member of the Immunity advisory board.

REFERENCES
Bénéchet, A.P., De Simone, G., Di Lucia, P., Cilenti, F., Barbiera, G., Bert, N.L., Fumagalli, V., Lusito, E., Moalli, F., Bianchessi, V., et al. (2019). Dynamics and genomic landscape of CD8+ T cells undergoing hepatic priming. Nature 574, 200–205.

Blieriot, C., Li, S., Kairi, M.F.B.M., Newell, E., and Ginhoux, F. (2020). Kupffer cells, methods and protocols. Methods Mol. Biol. 2164, 87–99.

Ficht, X., and Iannacone, M. (2020). Immune surveillance of the liver by T cells. Sci. Immunol. 5, eaaba2351.

Guidotti, L.G., Inverso, D., Sironi, L., Di Lucia, P., Fioravanti, J., Ganzzer, L., Fiocchi, A., Vacca, M., Aiolfi, R., Sammicheli, S., et al. (2015). Immunosurveillance of the liver by intravascular effector CD8+ T cells. Cell 161, 486–500.

Iannacone, M., and Guidotti, L.G. (2021). Immunobiology and pathogenesis of hepatitis B virus infection. Nat. Rev. Immunol. 1–14.

Li, P., Li, J., Li, M., Gong, J., and He, K. (2014). An efficient method to isolate and culture mouse Kupffer cells. Immunol. Lett. 158, 52–56.

De Simone, G., Andreata, F., Blieriot, C., Fumagalli, V., Laura, C., Garcia-Manteiga, J.M., Di Lucia, P., Gelotto, S., Ficht, X., De Ponti, F.F., et al. (2021). Identification of a Kupffer cell subset capable of reverting the T cell dysfunction induced by hepatocellular priming. Immunity 54, 1–12.