TITLE PAGE

Title : **An optimized method for mouse liver sinusoidal endothelial cell isolation**

Keywords : LSEC, mice, purification, CD11b, stabilin-2

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<u>List of abbreviations :</u>	Ac-LDL	Acetylated low-density lipoprotein
	FACS	Fluorescence-activated cell sorting
	FCS	Forward-scattered light
	FT	Flow-through
	HDMS	Hexamethyldisilazane
	LSEC	Liver sinusoidal endothelial cell
	MACS	Magnetic-activated cell sorting
	P	Pellet
	SN	Supernatant
	SSC	Side-scattered light

Abstract

The objective of the present study was to develop an accurate and reproducible method for liver sinusoidal endothelial cell (LSEC) isolation in mice. Non-parenchymal cells were isolated using a modified two-step collagenase digestion combined with Optiprep density gradient centrifugation. LSEC were further purified using two prevalent methods, short-term selective adherence and CD146+ magnetic-activated cell sorting (MACS), and compared in terms of cell yield, viability and purity to our purification technique using CD11b cell depletion combined with long-term selective adherence. LSEC purification using our technique allowed to obtain 7.07 ± 3.80 million LSEC per liver, while CD146+ MACS and short-term selective adherence yielded 2.94 ± 1.28 and 0.99 ± 0.66 million LSEC, respectively. Purity of the final cell preparation reached $95.10 \pm 2.58\%$ when using our method. In contrast, CD146+ MACS and short-term selective adherence gave purities of $86.75 \pm 3.26\%$ and $47.95 \pm 9.82\%$, respectively. Similarly, contamination by non-LSEC was the lowest when purification was performed using our technique, with a proportion of contaminating macrophages of only $1.87 \pm 0.77\%$. Further, isolated cells analysed by scanning electron microscopy presented typical LSEC fenestrations organized in sieve plates, demonstrating that the technique allowed to isolate *bona fide* LSEC. In conclusion, we described a reliable and reproducible technique for the isolation of high yields of pure LSEC in mice. This protocol provides an efficient method to prepare LSEC for studying their biological functions.

1 Introduction

2

3 Liver capillaries are lined by specialized endothelial cells unique by their morphology [1-4] and their
4 functions [2-6], the liver sinusoidal endothelial cells (LSEC). Located at the strategic interface between
5 blood and parenchymal cells, LSEC have been shown to play crucial roles in liver immunity [7-9], acute and
6 chronic liver injuries [10, 11] and liver regeneration [12, 13]. While parenchymal cells, i.e. hepatocytes,
7 accounts for 77.8% of liver cells in rats, non-parenchymal cells, i.e. LSEC, resident macrophages (Kupffer
8 cells), fat-storing cells and pit cells, accounts for 2.8%, 2.1%, 1.4% and less of liver cells, respectively [14].
9 Proportions of non-parenchymal cells in mice were reported to be similar, with LSEC representing 50% of
10 non-parenchymal cells, and resident macrophages and stellate cells 23% and 14%, respectively [15].

11 In order to study the mechanisms by which LSEC interact with blood and liver cells, reliable methods for
12 the isolation of LSEC from mouse liver have been developed during the last decades. Recent protocols have
13 relied on liver tissue dispersion using liver physiological perfusion with a calcium-free solution followed by
14 a calcium-supplemented solution containing collagenase, a technique originally described by Howard *et al.*
15 and adapted by Berry and Friend [16, 17]. Further steps include the discard of parenchymal cells and the
16 isolation of non-parenchymal cells, using differential centrifugation combined with density gradient
17 centrifugation, and finally, the purification of LSEC from the non-parenchymal cell fraction [18]. This last
18 step was found to be crucial for LSEC isolation procedures, as resident macrophages cannot be efficiently
19 discriminated from LSEC based on their respective densities. Of note, the presence of contaminating
20 macrophages in the final preparation could severely impair the reliability of studies using primary cells.

21 Recent technologies, such as magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting
22 (FACS), have emerged as methods allowing to purify LSEC based on targeting specific surface antigens.
23 MACS for CD146+ [19-23], CD31+ [24], CD105+ [25, 26], CD45- [27] cells and FACS for CD146+ [28] or
24 CD45- CD146+ [29] cells were reported for LSEC isolation in mice.

However, purification techniques based on LSEC phenotype have encountered limitations due to the controversies regarding the expression of specific markers on LSEC [3, 4]. The use of surface antigens targeting subpopulations of LSEC or endothelial cells not restricted to liver sinusoids may lead to inappropriate magnetic sorting and purity assessment [18]. For instance, cells obtained using the widespread CD31+ MACS technique were proven to be devoid of fenestrations organized in sieve plates [30], a characteristic morphological feature of LSEC. Moreover, CD31 expression was shown to be restricted to the central vein of liver lobules [4, 31] and to depend on the LSEC micro-environment [32].

The objective of the present study was to develop an accurate and easily reproducible method for pure LSEC isolation in mice. First, we improved the method for liver cell dispersion, to obtain an executable and straight-forward technique for research laboratories. Second, considering the difficulties in identifying a specific phenotypic marker for mouse LSEC, we exploited the capacity of LSEC to adhere to culture plates to purify LSEC from non-adherent lymphoid cells. Therefore, following liver tissue dispersion and non-parenchymal cell isolation, we depleted the cell preparation from macrophages using MACS targeting CD11b cells and proceeded to a long-term selective adherence of the cell fraction devoid of macrophages to remove non-adherent cells and retain LSEC.

We compared the outcomes (cell yield, purity and viability) of this refined purification technique with the two most prevalent methods for LSEC purification in mice: CD146+ MACS [19-23] and short-term selective adherence [33]. To reach high accuracy, purity was estimated using both a new highly specific phenotypic marker for mouse LSEC, stabilin-2 [34], and the conventional functional acetylated low-density lipoprotein (Ac-LDL) uptake assay, in combination with a counter-stain for contaminating macrophages [18]. Finally, the phenotype of LSEC obtained with MACS-based CD11b cell depletion combined with long-term selective adherence were confirmed by revealing the presence of LSEC-specific fenestrations organized in sieve plates using scanning electron microscopy.

1 **Materials and methods**

3 **Animals**

4 C57BL/6 male mice aged 10-16 weeks were obtained from Janvier Labs (Le Genest-Saint-Isle, France). All
5 procedures were performed in accordance with protocols approved by the animal ethics committee of the
6 Geneva Veterinarian Office and the University of Geneva (Geneva, Switzerland). All mice were maintained
7 under standard conditions at the animal facility of the University of Geneva. Water and standard chew
8 were provided *ad libitum*.

10 **Materials**

11 Twenty-two-gauge intravenous catheters were purchased from Hospira Inc. (Lake Forest, USA).
12 Heidelberg perfusion lines were obtained from B. Braun (Melsungen, Germany). 70µm cell strainers were
13 from Beckton Dickinson Biosciences (Bedford, USA). CD11b and CD146 microbeads for mice, LS columns,
14 QuadroMACS separator and MACS MultiStand platform were purchased from Miltenyi Biotec (Bergisch
15 Gladbach, Germany). Falcon polystyrene adherent Petri dishes, 15 and 50ml polypropylene conical tubes,
16 24 and 48-well polystyrene tissue culture plates were obtained from Beckton Dickinson Labware (Franklin
17 Lakes, USA). References for materials are listed in S1 Table.

19 **Reagents**

20 Calcium-free Dulbecco's phosphate buffered saline, calcium-free Hank's balanced salt solution, Dulbecco's
21 modified Eagle medium with Glutamax supplement, Iscove's modified Dulbecco's medium with Glutamax
22 supplement, HEPES buffer solution, foetal bovine serum, penicillin-streptomycin and 0.25% trypsin-EDTA
23 were purchased from Life Technologies (Grand Island, USA). Heparin was from Drossapharm (Arlesheim,
24 Switzerland). 40% glucose was from Grosse Apotheke Dr. G. Bichsel AG (Interlaken, Switzerland).
25 Collagenase type 4 (320 units/mg) was provided by Worthington Biochemical Corporation (Lakewood,

USA). DNase type 1 (2000 units/mg) was purchased from Roche Diagnostics (Risch, Switzerland). OptiPrep density gradient medium was obtained from Axis-Shield (Oslo, Norway). EGTA, EDTA, tricine and trypan blue were purchased from Sigma-Aldrich (Buchs, Switzerland). Endothelial cell medium for animals was obtained from Cell Biologics (Chicago, USA). Glutaraldehyde, sodium cacodylate buffer, osmium tetroxide, sucrose, hexamethyldisilazane (HDMS) and tannic acid were purchased from Electron Microscopy Sciences (Hatfield, USA). All other reagents were of analytical grade. References for reagents are listed in S1 Table.

Antibodies and acetylated low-density lipoprotein

Purified anti-mouse CD16/32 (clone 93), allophycocyanin-conjugated anti-mouse F4/80 (clone BM8), isotype controls allophycocyanin-conjugated rat IgG2ak (clone RTK2758) and Alexa Fluor 488-conjugated rat IgG2ak (clone RTK2758) were purchased from Biolegend (San Diego, USA). Alexa Fluor 488-conjugated anti-mouse stabilin-2 (clone #34-2) was from MBL International (Woburn, USA). Isotype control Alexa Fluor 488-conjugated rat IgG2ak (clone eBR2a) was purchased from eBioscience (San Diego, USA). Alexa Fluor 488-conjugated acetylated low-density lipoprotein from human plasma was obtained from Molecular Probes (Eugene, USA). Alexa Fluor 488-conjugated chicken anti-rat IgG (H+L) antibody was purchased from Life Technologies (Grand Island, USA).

Isolation procedures

Mouse liver perfusion and digestion

All procedures were optimized for isolation of LSEC from one mouse. All solutions were constituted at the day of the isolation procedure and warmed at 37°C in a water bath. Table 1 summarizes the media and reagents used for their composition. S1 Fig shows a schematic representation of the liver perfusion system used for the procedure. Mice were anesthetized with isoflurane. Abdominal midline and subcostal bilateral incisions were performed, quickly followed by bilateral para-sternal incisions to expose the heart. The animals were carefully exsanguinated by cardiac puncture (Fig 1B, step 1). The supra-hepatic portion of the

1 inferior *vena cava* was cannulised using a 22-gauge catheter passing through the right atrium and perfused
2 with pre-warmed wash solution at 5ml/min using a peristaltic pump (Ismatec, Zürich, Switzerland). A few
3 seconds later, when the liver became swollen and discoloured, the portal vein was quickly sectioned to
4 allow the effluent perfusion to escape (Fig 1B, step 2). Washing perfusion was maintained for 5 minutes
5 and then the tap was switched for 5-7 minutes perfusion with the digestion solution under a warming
6 infrared light (Fig 1B, step 3). Liver perfusion was stopped after checking liver cell dispersion by a gentle
7 pressure on the liver capsule that had to result in subcapsular tissue disruption. The liver was then
8 carefully removed from the abdominal cavity by sectioning the supra-hepatic portion of the inferior *vena*
9 *cava* and liver ligaments, (Fig 1B, step 4) and placed on a Petri dish. After removal of the gallbladder, the
10 Petri dish was filled with 5ml of post-digestion solution and the liver was mechanically disrupted using a
11 scalpel blade. Liver tissue was collected and diluted with 30ml of post-digestion solution into a 50ml
12 conical tube on ice.

13
14 Non-parenchymal liver cell isolation

15 Fig 2A summarizes the procedure for non-parenchymal cell isolation. All procedures were conducted at
16 4°C. Liver suspension was passed through a 70µm cell strainer. The cell strainer was rinsed twice with 5ml
17 of post-digestion solution. The suspension was centrifuged once at 68xg (Rotixa 50 RS, Hettich Zentrifugen,
18 Tuttlingen, Germany) for 5 minutes to pellet parenchymal cells. The supernatant was harvested and
19 centrifuged at 600xg for 10 minutes. Meanwhile, tricine solution was mixed with Optiprep at a 1:2 ratio to
20 get a 40% working solution, according to manufacturer's instructions. The pellet was suspended into 20ml
21 17.6% Optiprep (8.5ml 40% working solution, 11.5ml tricine solution), and 20ml 8.2% Optiprep (4ml 40%
22 working solution, 16ml tricine solution) were carefully added at the top of cell suspension. The 8.2/17.6%
23 Optiprep gradient was centrifuged at 1400xg for 30 minutes, with acceleration and deceleration set to
24 minimal values. The first 15ml of the upper gradient were carefully discarded and the cells located at the
25 interface between the two density cushions were gently collected and suspended into 40ml MACS buffer.
26 The suspension was centrifuged at 780xg for 10 minutes. Cells were used for further purification, either by

short-term selective adherence, by CD146+ MACS or by CD11b- MACS combined with long-term selective adherence. Six mice were allocated to each purification method for outcome comparison.

LSEC purification from the non-parenchymal cell fraction using short-term selective adherence

Protocol for purification using short-term selective adherence was adapted from a previous publication by Cheluvappa [33] and is summarized by Fig 2B. Briefly, cells were suspended in 1.25ml of culture medium and seeded in 5 wells of a 24-well plate. After exactly 8 minutes of incubation at 37°C, cells were harvested by washing the wells thrice. Cells were centrifuged at 600xg for 10 minutes. LSEC were counted, suspended in endothelial cell medium at a density of 2×10^6 LSEC/ml and seeded on a non-coated 24-well plate at 500µl per well and on a non-coated 48-well plate at 250µl per well. The plates were centrifuged at 160xg for 2 minutes to sediment the cells and incubated at 37°C for 2 hours. The medium was changed once and the cells were kept at 37°C for purity assessment.

LSEC purification from the non-parenchymal cell fraction using CD146+ MACS

The technique for LSEC purification in mice using CD146+ MACS was previously described by Liu et al. [19]. As reported by Fig 2C, non-parenchymal cells obtained from gradient centrifugation were suspended in 80µl MACS buffer and incubated for 15 minutes with 20µl anti-CD146 microbeads at 4°C. Meanwhile, a MACS LS column was placed in the magnetic field of a MACS separator on a MACS platform and prepared by rinsing with 3ml MACS buffer. The suspension containing the CD146-labelled cells was diluted to 2ml and centrifuged at 300xg for 10 minutes, according to manufacturer's instructions. The pellet was suspended in 500µl MACS buffer and loaded on top of the MACS column. The column was rinsed thrice with 3ml MACS buffer. The column was removed from the magnetic field and placed on a 15ml tube. 5ml MACS buffer were pipetted in the column, which was immediately flushed out by pushing the plunger to harvest CD146+ cells (LSEC). Cells were centrifuged at 600xg for 10 minutes. LSEC were counted, suspended in endothelial cell medium at a density of 2×10^6 LSEC/ml and seeded on a non-coated 24-well plate at 500µl per well and on a non-coated 48-well plate at 250µl per well. The plates were centrifuged at

160xg for 2 minutes to sediment the cells and incubated at 37°C for 2 hours. The medium was changed once and the cells were kept at 37°C for purity assessment.

LSEC purification from the non-parenchymal cell fraction using MACS-based CD11b cell depletion in combination with long-term selective adherence

Procedure for LSEC purification using CD11b- MACS combined with long-term selective adherence is summarized by Fig 2D. Cells were suspended in 120µl MACS buffer and incubated with 60µl anti-CD11b microbeads for 15 minutes at 4°C. The cell suspension was diluted with 2ml MACS buffer and centrifuged at 300g for 10 minutes. Meanwhile, 2 MACS LS columns placed on a MACS separator were rinsed with 3ml MACS buffer each. Cells were suspended in 500 µl MACS buffer and passed through the first column. The column was rinsed once with 4x 500µl MACS buffer. The flow-through was collected and passed through the second column by adding 500µl at a time on top of the column. The second column was rinsed once with 4x 500µl MACS buffer. The flow-through was centrifuged at 600xg for 10 minutes. LSEC were counted, suspended in endothelial cell medium at a density of 2×10^6 LSEC/ml and seeded on a non-coated 24-well plate at 500µl per well and on a non-coated 48-well plate at 250µl per well. The plates were centrifuged at 160xg for 2 minutes and incubated at 37°C for 2 hours. The medium was discarded and the wells were rinsed thrice with Dulbecco's phosphate buffered saline to remove non-adherent cells. Each well was then filled with endothelial cell medium and the cells were kept at 37°C for purity assessment.

Determination of cell yield and viability

LSEC yield and viability were assessed by count in a Neubauer chamber. Briefly, cells collected after MACS or short-term selective adherence but before plate washing or long-term selective adherence (before LSEC adherence to culture plate) were suspended in 1ml MACS buffer. One sample of cell suspension was mixed with 0.4% trypan blue solution and viable and dead cells were counted. A volume corresponding to an equivalent of 1×10^5 cells was centrifuged at 600xg for 5 minutes. Cells were suspended in flow cytometry buffer (Dulbecco's phosphate buffered saline, 2% foetal bovine serum), incubated with Fc-receptor

1 blocking anti-mouse CD16/32, then incubated with 0.02µg allophycocyanin-conjugated anti-mouse F4/80
2 and 1µg Alexa Fluor 488-conjugated anti-mouse stabilin-2 for 20 minutes at 4°C, washed and fixed. Cells
3 were acquired using an Accuri C6 flow cytometer (BD Biosciences, Bedford, USA) and analysed on Flowjo
4 version 10 (Tree Star Inc., Ashland, USA). After gating for the cell population using forward-scattered (FSC-
5 H) and side-scattered (SSC-H) parameters, expressions of stabilin-2 and F4/80 were analysed. Antibodies
6 concentrations (S2A Fig) and thresholds of positivity using isotype controls (S2B Fig) were set in preliminary
7 experiments. The yield of LSEC obtained from one liver was calculated by multiplying the total number of
8 cells estimated by the counting chamber with the proportion of stabilin-2+ F4/80- cells (LSEC) obtained
9 using the flow cytometry analysis.

10

11 Purity assessment by flow cytometry using phenotypic and functional assays

12 Purity assessment using phenotypic markers specific to LSEC was performed from cells in culture in 24-well
13 plates after plate washing or long-term selective adherence. The culture medium was discarded and the
14 wells washed once with Dulbecco's phosphate buffered saline. 50µl Trypsin-EDTA solution was added to
15 each well and the plates were incubated at 37°C for 3 minutes. When the cells appeared round and
16 detached from the surface, 500µl flow cytometry buffer were added to each well. Cells were harvested and
17 each well was intensively washed with the same buffer. We were able to successfully trypsinize all cells
18 from uncoated culture plates for flow cytometry analyses. Plates were checked for remaining cells, as
19 macrophages were reported to be more difficult to harvest compared to LSEC [35]. We would like to
20 emphasize that trypsin was not part of the isolation procedure and was only used to harvest cells for
21 analyses.

22 Cells were centrifuged at 600xg for 10 minutes and prepared for flow cytometry using stabilin-2 and F4/80
23 stainings, as previously described. After gating for the cell population, expressions of stabilin-2 and F4/80
24 were analysed. Purity was calculated as the ratio of stabilin-2+ F4/80- cells (LSEC) on the total number of
25 gated cells. Procedure is summarized by Fig 3A.

1 The purity of the final cell preparation was cross-checked using flow cytometry for the well-described Ac-
2 LDL uptake functional assay [30, 35-38] in combination with staining for macrophages from cells in culture
3 in 48-well plates. After 6 hours in culture, 0.1µg Alexa Fluor 488-conjugated Ac-LDL was added to each
4 well. After 18 hours in culture [22, 23], cells were trypsinized, harvested and centrifuged as previously
5 described. Cells were prepared for flow cytometry using F4/80 labelling alone. After gating for the cell
6 population, positivity for Ac-LDL and expression of F4/80 were analysed. A 99% filter was applied on the FL-
7 1 channel (for Alexa Fluor 488) to reduce the intensity of signal. Purity was calculated as the ratio of Ac-
8 LDL+ F4/80- cells (LSEC) on the total number of gated cells. Procedure is summarized by Fig 3B.

9 10 Determination of the number of cells removed by CD11b-MACS

11 To determine the number and the proportion of macrophages retained by CD11b- MACS, each column was
12 removed from the magnetic field at the end of the procedure and placed on a 15ml tube. 5ml MACS buffer
13 were pipetted into the columns, which were immediately flushed out by pushing the plungers. Cells were
14 centrifuged at 600xg for 10 minutes. The pellet was suspended in 1ml MACS buffer and cells were counted
15 in a Neubauer chamber. A volume corresponding to an equivalent of 1×10^5 cells was centrifuged at 600xg
16 for 5 minutes. Cells were prepared for flow cytometry using F4/80 labelling, as previously described. After
17 gating for the cell population, expression of F4/80 was analysed. The number of macrophages was
18 calculated by multiplying the total number of cells estimated by the counting chamber to the proportion of
19 cells positive for F4/80 obtained by the flow cytometry analysis.

20 21 Immunofluorescence

22 Isolated cells in culture were fixed, rinsed, blocked using 0.5% bovine serum albumin and incubated
23 overnight with Alexa Fluor 488-conjugated rat anti-mouse stabilin-2. Then, cells were rinsed and incubated
24 with Alexa Fluor 488-conjugated chicken anti-rat IgG (H+L) antibody for 1 hour. Cells were rinsed,
25 incubated with Hoechst fluorescent dye and rinsed. Pictures were acquired using a LSM 700 confocal
26 microscope (Carl Zeiss AG, Oberkochen, Germany).

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Scanning electron microscopy

LSEC were processed and HDMS-dried according to the method described by Braet et *al.* [39], with small modifications. Briefly, LSEC were cultured on uncoated glass cover slips in 48-well plates for 24 hours. Cells were washed with Dulbecco’s phosphate buffered saline, fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer with 0.1M sucrose overnight, and treated with 1% tannic acid in 0.2M sodium cacodylate buffer for 1 hour, followed by 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour. Cells were dehydrated in ethanol solutions of 70, 80, 90% and thrice 100% for 10 minutes each. Cover slips were immersed in 100% HDMS for 3 minutes and transferred to a dessicator for 25 minutes. Cover slips were mounted on studs, sputter-coated with 10nm gold using a sputter coater apparatus (SPI Supplies, West Chester, USA). Cells were analysed using a JSM-6010LA scanning electron microscope (JEOL, Tokyo, Japan). Random fields of view were checked for the presence of fenestrations organized in sieve plates.

Statistical analysis

Results were expressed as medians, or as means when specified. 95% confidence intervals and standard deviations were reported, when appropriate. Differences between the purification techniques in terms of outcomes (yield, purity and viability) were compared using the non-parametric Mann-Whitney rank-sum test. The null hypothesis was rejected at $p < 0.05$. GraphPad Prism version 6 (GraphPad Software Inc, La Jolla, USA) was used for statistical analyses.

Results

Higher yields of LSEC were obtained using MACS-based purification techniques compared to short-term selective adherence

1 To determine the numbers of cells yielded by the three purification methods, the proportion of LSEC
2 obtained using each purification method was multiplied by the total number of cells counted at this step,
3 as previously described. Short-term selective adherence gave LSEC yields of 0.988 ± 0.660 million per liver,
4 while CD146+ and CD11b- MACS allowed to obtain 2.941 ± 1.281 and 7.066 ± 3.802 million LSEC,
5 respectively. The MACS technique was therefore more efficient compared to the technique of short-term
6 selective adherence. No difference could be found between CD146+ and CD11b- cell sorting techniques
7 ($p=0.0931$). These results are summarized by Fig 4A and Fig 4D.

8

9 **The CD146+ MACS purification technique yielded cells with lower viability**

10 Cell viability was high in cell populations obtained using short-term selective adherence or CD11b- MACS
11 combined with long-term selective adherence, as shown by Fig 4A and Fig 4D ($95.23 \pm 2.31\%$ and
12 $95.66 \pm 1.43\%$, respectively). However, cells purified using CD146+ MACS presented a lower viability
13 ($84.73 \pm 16.06\%$), suggesting that magnetic columns either induced cell death or retained dead cells. Of
14 note, viability of cells isolated using CD11b- MACS associated with long-term selective adherence was
15 maintained after 12, 24 and 48 hours in culture (S3 Fig).

16

17 **Negative selection of LSEC by CD11b- MACS combined with long-term selective adherence resulted in** 18 **highly pure LSEC population**

19 To assess purity using stabilin-2 as a marker for LSEC, cells were harvested and labelled using anti-stabilin-2
20 and anti-F4/80 antibodies, before being analysed by flow cytometry. The proportion of LSEC (stabilin-2+,
21 F4/80- cells) was found to be clearly weak when non-parenchymal cells were purified using short-term
22 selective adherence ($47.95 \pm 9.82\%$, Fig 4B, Fig C and Fig D). In contrast, CD146+ and CD11b- MACS
23 techniques allowed to obtain purities of 86.65 ± 3.26 and $95.10 \pm 2.58\%$, respectively (Fig 4B, Fig C and Fig D).
24 Immunofluorescence staining of cells isolated using CD11b- MACS associated with long-term selective
25 adherence validated the expression of the LSEC-specific marker stabilin-2 on isolated cells (S4 Fig).

1 In addition to phenotypic characteristics, LSEC have historically been identified using their unique
2 endocytic capacities. Therefore, we cross-checked purity of the same cell preparations using the functional
3 Ac-LDL uptake assay. Cells in culture were incubated for 18 hours in the presence of Ac-LDL before being
4 harvested and processed for flow cytometry. Considering the ability of macrophages to ingest Ac-LDL *in*
5 *vivo* [40] and *in vitro* [41-43], a counter-staining for macrophages was performed using F4/80. LSEC were
6 defined as Ac-LDL⁺ F4/80⁻ cells. The proportions of LSEC obtained using both short-term selective
7 adherence and CD146⁺ MACS were found to be similar ($81.30 \pm 12.33\%$ *versus* $83.50 \pm 4.17\%$, $p=0.5714$),
8 whereas the CD11b⁻ MACS technique combined with long-term selective adherence gave a higher purity of
9 $97.50 \pm 0.88\%$ (Fig 4C and Fig 4D). Further, the final cell preparation was almost completely free of
10 contaminant cells. . For instance, the proportion of macrophages and unidentified cells were only of
11 $1.87 \pm 0.77\%$ and $3.10 \pm 2.69\%$, respectively. Stellate cells were not identified in the final cell preparation
12 using violet laser stimulation on a flow cytometer (as described in [44]) (not shown). In contrast, CD146⁺
13 MACS isolated LSEC showed contaminations of $4.85 \pm 1.11\%$ by macrophages and of $9.20 \pm 2.63\%$ by
14 unidentified cells, whereas LSEC isolated by short-term selective adherence presented cell contaminations
15 of $6.16 \pm 2.55\%$ and $46.65 \pm 12.65\%$, respectively (Fig 4C and Fig 4D).

16 To determine the importance of performing a second CD11b⁻ MACS step, cells retained in the magnetic
17 columns were counted and analysed by flow cytometry following staining with anti-F4/80 antibody. While
18 the first magnetic sorting step allowed to remove a median ($\pm$ SD) of 1.945 (± 0.410) million macrophages,
19 we found that the second passage still retained 0.215 (± 0.124) million macrophages (not shown).
20 Therefore, a double sorting step was considered to be necessary in order to efficiently remove
21 contaminating macrophages.

22 However, the flow-through after the MACS step still contained a significant proportion of non-adherence
23 CD11b-negative contaminating cells. These non-adherent cells were efficiently removed after 2 hours of
24 adhesion of the LSEC population and washing the culture plates thrice (long-term selective adherence), as
25 illustrated by S5A Fig (from left to right). In comparison, cells obtained after CD146⁺ MACS contained less

1 non-adherent cells, but these cells were not sufficiently removed by medium's change, as illustrated by
2 S5B Fig (from left to right).

3
4 **Cells isolated using MACS-based CD11b cell depletion combined with long-term selective adherence**
5 **presented the characteristic morphological features of LSEC**

6 Cells obtained using the CD11b- MACS purification technique constituted a confluent monolayer of cells
7 with cobblestone morphology after 2 hours in culture (S5A Fig, right). After 24 hours in culture, they
8 adopted a fusiform shape (not shown).

9 The current cell markers used to define LSEC in mice are still controversial [18]. However, the specific
10 morphologic features of these cells, a non-diaphragmed fenestrated membrane, is well recognized [30]. In
11 order to validate the identity of the isolated cells, we performed scanning electron microscopy on cells
12 after 24 hours in culture. As shown in figure 5, the cultured cells presented typical fenestrations organized
13 in sieve plates (Fig 5C and Fig D), therefore confirming their identity as *bona fide* LSEC.

14
15 **Discussion**

16
17 The prevalent method for non-parenchymal cell isolation, which had been adapted by many laboratories,
18 consists in liver tissue dispersion [16] by liver perfusion [17] with a calcium-free solution followed by
19 perfusion with a calcium-supplemented collagenase solution. However, although liver perfusion in rodents
20 is commonly performed by catheterising the portal vein and sectioning the inferior *vena cava* for
21 anterograde efflux, this method shows practical limitations inherent to the portal vein anatomy. Indeed,
22 the short length of this relatively large vein complicates a rapid and efficient installation of the catheter for
23 a successful liver perfusion. For rats, a more convenient retrograde vascular access had been described
24 where the infra-hepatic portion of the inferior *vena cava* was cannulised with a 16-gauge catheter, its
25 supra-hepatic portion ligated and the portal vein sectioned [38]. We therefore developed a retrograde

1 approach associating the cannulation of the supra-hepatic portion of the inferior *vena cava* through the
2 right atrium with the section of the portal vein. This procedure provided a more convenient and efficient
3 access to the liver microvasculature and allowed us to wash the blood out of the tissue almost instantly, as
4 we observed an immediate discoloration of the liver following perfusion with a calcium-free solution (not
5 shown). Access via the heart prevented leakage of the solution and allowed to maintain an optimal
6 perfusion pressure. This perfusion step was shown to be crucial for subsequent liver cell dispersion, as it
7 was demonstrated to prevent the formation of thrombi in the sinusoids and to remove calcium from liver
8 vasculature, both factors that might contribute to poor liver tissue dispersion [45]. Accordingly, we
9 performed liver perfusion with a calcium-free solution supplemented with heparin for 5 minutes, followed
10 by collagenase and DNase infusion under a warming lamp for 5-7 more minutes. This step allowed us to
11 obtain optimal liver cell dispersion in a minimal amount of time. Moreover, digestion of the liver tissue is
12 highly dependent on the collagenase enzyme concentration and batch. For instance, we observed in
13 preliminary experiments that concentrations lower than 0.05% generally resulted in poor liver cell
14 dispersion and therefore lower cell yields (not shown).

15 We further timely optimized the method using low-speed centrifugation to discard parenchymal cells.
16 Slightly increasing the centrifugation speed compared to the ones usually reported in the literature (usually
17 30-50xg) allowed us to discard most of the parenchymal cells within 5 minutes. In comparison, procedures
18 involving up to 3 centrifugations [46] or centrifugations of 10 minutes [39, 47, 48] were sometimes
19 reported. Non-parenchymal cells were further enriched using an 8.2/17.6% Optiprep gradient, as reported
20 elsewhere [19]. This gradient centrifugation was a critical step to prevent saturation and to increase the
21 specificity of the magnetic columns by removing most of undesired cells (not shown). However, gradient
22 centrifugation alone remained unsuccessful in separating LSEC from resident macrophages, as these cell
23 populations present almost identical densities. We further purified LSEC from the non-parenchymal cell
24 fraction, which contained macrophages, using MACS-based CD11b cell depletion associated with long-term
25 selective adherence. This approach had so far not been described for this application and appeared to be
26 highly efficient for the removal of contaminating macrophages.

1 Further, difficulties persist in finding a suitable marker for the identification of LSEC. In rats, the SE-1
2 antibody, which recognizes the antigen CD32b, was shown to specifically target LSEC [31, 49]. The
3 expression of CD32b was described to be associated with the hyaluronan receptor stabilin-2 [50]. Stabilin-2
4 was found to be specific for the discontinuous endothelium in various organs, including the liver [51-53]
5 and is currently used to characterize LSEC in rats [50, 54]. Recently, Falkowski et *al.* described stabilin-2 to
6 be specifically expressed on LSEC in mice [34]. Therefore, in our study, stabilin-2 was used as a reliable
7 phenotypic marker for LSEC to compare the outcome of our new purification procedure with the outcomes
8 from the common short-term selective adherence and CD146+ MACS purification techniques. The method
9 described here allowed to increase the yield and the purity of LSEC compared to previous techniques used
10 in mice. Importantly, contamination by macrophages was low when using MACS-based CD11b cell
11 depletion, an important prerequisite for experiments such as proteomic analyses or secretion assays,
12 where high cell purities are required. These findings were confirmed using the usual Ac-LDL uptake assay
13 combined with counter-staining for macrophages. Ac-LDL was reported to be uptaken from endothelial
14 cells irrespective of their sinusoidal origin [95] and by macrophages [92][2, 93, 94], but remained widely
15 used to analyse LSEC purity, especially when combined to a counter-staining for macrophages [18].
16 The limitation of short-term selective adherence for rodent LSEC isolation has been described earlier. For
17 instance, Tokairin et *al.* showed in rats that LSEC isolation using gradient centrifugation associated with
18 short-term selective adherence yielded 24.3 ± 0.5 million cells with a purity of $92.0 \pm 0.8\%$, according to
19 immunohistochemistry using a SE-1 antibody. Moreover, contaminations by macrophages ($2.3 \pm 0.5\%$) and
20 stellate cells ($0.7 \pm 0.2\%$) were reported. In contrast, a SE-1+ MACS-based technique allowed to obtain
21 10.7 ± 0.5 million cells with a purity of $97.6 \pm 0.9\%$ [46]. Similarly, Braet et *al.* described short-term selective
22 adherence to yield 70% purities in rats when assessed by transmission electron microscopy [39, 47].
23 Moreover, Cheluvappa, adapted a protocol for short-term selective adherence from rats [55] to mice and
24 reported outcomes to be highly dependent on technical features. LSEC purities ranging from 75 to 95%,
25 which correspond to the purities we obtained using this short-term selective adherence purification
26 technique in mice, were considered to be “acceptable”[33]. Therefore, magnetic sorting might be more

1 suited to specifically target a cell type in a mixed cellular population. Nevertheless, this technique also
2 encounter limitations regarding the specificity of surface antigens expressed on LSEC, as previously
3 discussed. The surface antigen CD146+ MACS was described for the isolation of mouse LSEC populations
4 [19]. However, the comparison of the LSEC populations obtained by CD146+ MACS to our technique
5 showed that significant higher proportions of macrophages ($4.85\pm1.11\%$) and unidentified cells
6 ($9.20\pm2.63\%$) were still present in the final cell population using the CD146+ MACS technique. To increase
7 purity of LSEC isolated using this method, some authors optimized the procedure by adding a CD45-
8 magnetic sorting to remove blood cells [29, 56]. Further, we observed a decreased viability in the
9 population obtained by the CD146+ MACS technique, suggesting that the positive sorting either led to a
10 non-specific retention of dead cells or decreased cell viability. Indeed, the effect of cell labelling by
11 magnetic beads and cell sorting on cell viability remain unknown.

12 The approach presented here, based on a MACS CD11b cell depletion combined with long-term selective
13 adherence, does not present any of the sensitivity and specificity issues mentioned above. The negative
14 sorting does not lead to the selection of LSEC subpopulations [18] and allows to remove LSEC-macrophage
15 doublets, contaminating macrophages [57] and some non-adherent immune cells [58-60]. The introduction
16 of the second sorting step was essential in reaching that aim, as we have shown that one round of CD11b-
17 MACS was not efficient enough in removing the complete pool of macrophages. The importance of
18 macrophage removal [27], as well as the necessity of performing two negative cell sorting steps to reach
19 satisfying purities [61], were already reported in other studies. Afterwards, remaining contaminating non-
20 adherent cells were efficiently removed by long-term selective adherence [37-39, 47, 62]. The association
21 of magnetic sorting and long-term selective adherence was previously reported [23, 61]. Finally, the
22 efficiency of our optimized protocol in isolating LSEC was validated by the identification of typical
23 fenestrations organized in sieve plate on all cells observed, further confirming their identity as LSEC.

24 In conclusion, exploiting the latest updates regarding LSEC characteristics, we demonstrated that the
25 described procedure constituted a highly efficient new approach for LSEC isolation in mice. Using this

- 1 optimized protocol will allow the study of LSEC complex functions and biology and will help to extend our
- 2 knowledge in the field of liver pathologies and liver regeneration.

3

4

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2

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6

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Figure captions

FIG 1. Procedure for liver digestion.

A: Schematic representation of the mouse heart and liver anatomy. B: Summary of the steps for liver digestion.

FIG 2. Protocols for liver sinusoidal endothelial cell isolation and purification.

Non-parenchymal cells were isolated from digested liver tissue by filtration, low-speed centrifugation and purification in an 8.2/17.6% Optiprep gradient (A). LSEC were further purified by means of short-term selective adherence (B), CD146+ MACS (C) or CD11b- MACS in combination with long-term selective adherence (D).

FT = flow-through; P = pellet; SN = supernatant

FIG 3. Methods for determining the purity of the final cell preparation.

A. Following medium's change or long-term selective adherence, cells were labelled using allophycocyanin-conjugated anti-mouse F4/80 and Alexa Fluor 488-conjugated anti-mouse stabilin-2 and processed for flow cytometry. After gating for cells using side-scattered light (SSC-H) and forward-scattered light (FSC-H) parameters, expressions of stabilin-2 and F4/80 were analysed. LSEC were defined as stabilin-2+ F4/80- cells, macrophages as F4/80+ cells and stabilin-2- F4/80- cells were considered as unidentified. Purity was determined as the proportion of stabilin-2+ F4/80- events relative to the total number of gated events. B. After 18 hours in culture in the presence of Alexa Fluor 488-conjugated Ac-LDL (left picture: phase-contrast light microscopy combined with immunofluorescence of the same cells labelled with Alexa Fluor 488-conjugated Ac-LDL), cells were labelled using allophycocyanin-conjugated anti-mouse F4/80 and processed for flow cytometry. LSEC were defined as Ac-LDL+ F4/80- cells, macrophages as F4/80+ cells and Ac-LDL-

1 F4/80- cells were considered as unidentified. Purity was determined as the proportion of Ac-LDL+ F4/80-
2 events relative to the total number of gated events.
3 Figures were obtained from LSEC purified using the short-term selective adherence technique. Numbers
4 represent the respective proportions of cells in %. Phase-contrast pictures were acquired using a Leica
5 DFC320 microscope (Wetzlar, Germany) and are represented in grayscale.

6
7 FIG 4. Characteristics of the final cell preparations obtained by the three different purification methods.

8 A, C. Box-plots reporting the main outcomes (LSEC yield, viability, purity and contamination) measured
9 from the 3 assessed purification methods. Whiskers represent 95% confidence intervals. P-values were
10 obtained using the Mann-Whitney rank-sum test to compare groups. Six mice were included per group.

11 B. Flow cytometry representations of LSEC purified using short-term selective adherence (in green, left),
12 CD146+ MACS (in blue, middle) and CD11b- MACS combined with long-term selective adherence (in red,
13 right). Numbers represent the respective proportions of cells in %. D. Numbered summary of the results.

14 * $p < 0.05$; ** $p < 0.001$

15
16 FIG 5. Scanning electron microscopy of isolated cells.

17 Pictures of cells purified using CD11b- MACS combined with long-term selective adherence and processed
18 for scanning electron microscopy by HDMS-drying. After 24 hours in culture, isolated cells formed a
19 monolayer of confluent cells (A, B). Cytoplasm of isolated cells presented fenestrations organized in sieve
20 plates (C, D), a typical morphological characteristic of LSEC. *Thick arrows indicate nuclei; thin arrows*
21 *indicate fenestrations.*

A **B**

I. Cardiac puncture

II. Wash solution perfusion

III. Digestion solution perfusion

IV. Liver explantation

A **B**

D

Median $\pm$ SD	Selective adherence	CD146+ MACS	CD11b+ MACS
LSEC yield, $n \times 10^6$	0.988 $\pm$ 0.660	2.941 $\pm$ 1.281	7.066 $\pm$ 3.802
Viability, %	95.23 $\pm$ 2.31	84.73 $\pm$ 16.06	95.66 $\pm$ 1.43
Purity (Stablin-2+, F4/80-), %	47.95 $\pm$ 9.82	86.65 $\pm$ 3.26	95.10 $\pm$ 2.58
Purity (Ac-LDL+, F4/80-), %*	81.30 $\pm$ 12.33	83.50 $\pm$ 4.17	97.50 $\pm$ 0.88
Macrophages, %	6.16 $\pm$ 2.55	4.85 $\pm$ 1.11	1.87 $\pm$ 0.77
Unidentified cells, %	46.65 $\pm$ 12.65	9.20 $\pm$ 2.63	3.10 $\pm$ 2.69

TABLE 1. Solutions for liver sinusoidal endothelial cell isolation.

Media				Reagents		
Wash solution	Calcium-free Hank's Balanced Salt Solution	<i>ml</i>	25	Penicillin	<i>UI</i>	2'500
				Heparin	<i>UI</i>	125
				40% Glucose	μl	62.5
				HEPES	μmol	625
				EGTA	μmol	12.5
				Streptomycin	μg	2'500
Digestion solution	Iscoe's Modified Dulbecco's Medium with Glutamax	<i>ml</i>	25	Collagenase type 4	<i>mg</i> (<i>UI</i>)	12.5 (4'000)
				DNase type 1	μg	2
Post-digestion solution	Dulbecco's Modified Eagle Medium with Glutamax	<i>ml</i>	50	Penicillin	<i>UI</i>	5'000
				Foetal bovine serum	<i>ml</i>	2.5
				Streptomycin	μg	5'000
MACS buffer	Calcium-free Dulbecco's Phosphate Buffered Saline	<i>ml</i>	100	Foetal bovine serum	<i>ml</i>	0.5
				EDTA	μmol	200
Tricine solution				Dulbecco's phosphate buffered saline	<i>ml</i>	50
				Tricine	μg	269
				Sodium chloride	μg	425

Quantities and volumes of reagents are indicated for one mouse. All solutions were prepared on the day of the isolation procedure. Origins of media and reagents are cited in the text and in S1 Table.