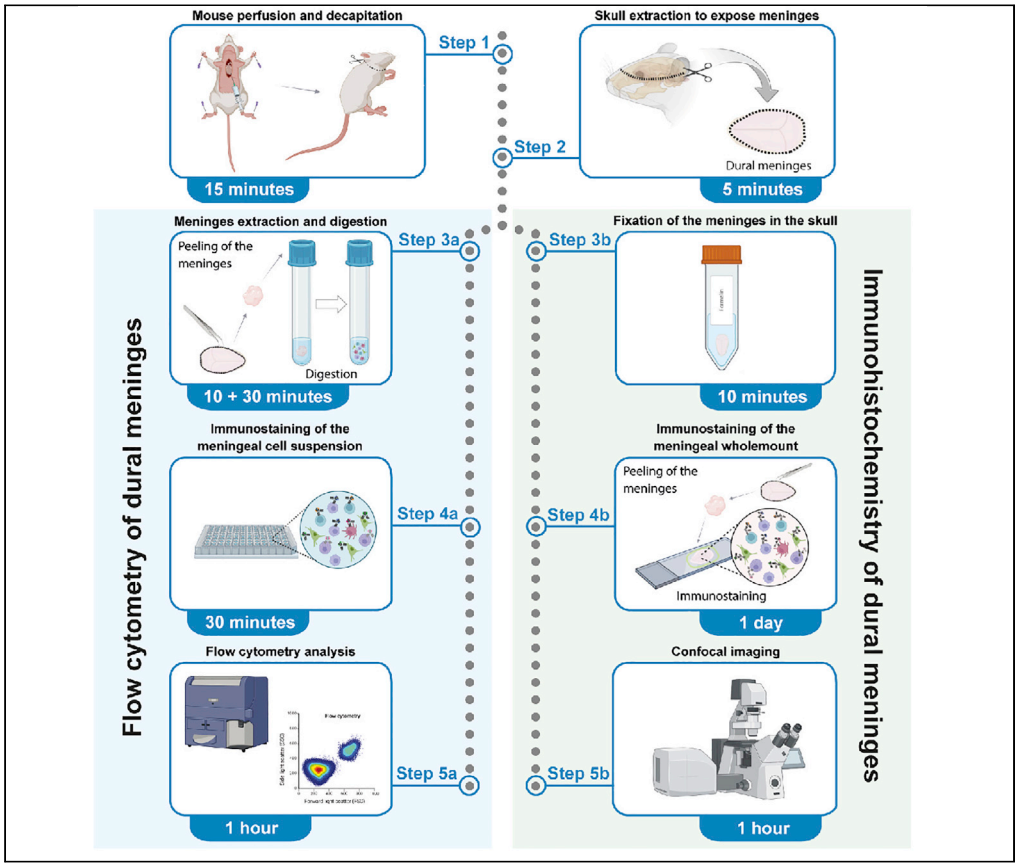
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Flow cytometry and immunohistochemistry of the mouse dural meninges for immunological and virological assessments



The highly vascularized meninges protect the surface of the central nervous system and contain a dense network of immune cells controlling neuroinfection and neuroinflammation. Here, we present techniques for the immunological and virological assessment of mouse dural meninges. We describe steps for immunophenotyping including meninges extraction and digestion, immunostaining, and flow cytometry. We then describe viral assessment upon lymphocytic choriomeningitis virus infection including steps for fixation of the meninges in the skull, whole-mount immunohistochemistry, and confocal imaging.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Annie Roussel-Queval, Julie Rebejac, Elisa Eme-Scolan, Laurie Arnaud Paroutaud, Rejane Rua

roussel@ciml.univ-mrs.fr (A.R.-Q.)
rua@ciml.univ-mrs.fr (R.R.)

Highlights

Step-by-step procedure for meningeal processing using two different techniques

Flow cytometry panel design for global immunophenotyping of the meningeal landscape

Immunohistochemistry of LCMV viral protein in the meninges

Roussel-Queval et al., STAR Protocols 4, 102119
March 17, 2023
https://doi.org/10.1016/j.xpro.2023.102119

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Flow cytometry and immunohistochemistry of the mouse dural meninges for immunological and virological assessments

Annie Roussel-Queval,[1,2,*] Julie Rebejac,[1] Elisa Eme-Scolan,[1] Laurie Arnaud Paroutaud,[1] and Rejane Rua[1,3,*]

[1]Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, Inserm, CNRS, Marseille, France

[2]Technical contact

[3]Lead contact

*Correspondence: roussel@ciml.univ-mrs.fr (A.R.-Q.), rua@ciml.univ-mrs.fr (R.R.)
https://doi.org/10.1016/j.xpro.2023.102119

SUMMARY

The highly vascularized meninges protect the surface of the central nervous system and contain a dense network of immune cells controlling neuroinfection and neuroinflammation. Here, we present techniques for the immunological and virological assessment of mouse dural meninges. We describe steps for immunophenotyping including meninges extraction and digestion, immunostaining, and flow cytometry. We then describe viral assessment upon lymphocytic choriomeningitis virus infection including steps for fixation of the meninges in the skull, whole-mount immunohistochemistry, and confocal imaging.

For complete details on the use and execution of this protocol, please refer to Rebejac et al. (2022).[1]

BEFORE YOU BEGIN

This protocol describes the steps to determine the immune landscape of the mouse dural meninges, as well as their level of infection following neurotropic viral injection.

The brain has long been thought to be immunoprivileged and devoid of most immune cell types apart from microglia.[2] Brain protection was thought to be mainly achieved by the blood-brain barrier that efficiently blocks the entry of toxic compounds/pathogens.[3] However, recently, we and others have shown that the borders of the brain have an active immune system, and include a diversity of immune cells such as mast cells, macrophages, B cells, T cells and NK cells.[4–10] Indeed, most immune cell diversity is found in the meninges that envelop the brain, and not in the brain parenchyma itself. The enrichment of immune cells in the meninges protects against infection but can also foster neuroinflammation.[4] This revolutionizes our view of neuro-immune communication and brain protection.

Meninges are composed of 3 layers: the dura mater which is the outer collagenous layer attached to the skull, the arachnoid mater, and the pia mater which is the inner layer (the arachnoid and pia being collectively referred as leptomeninges).[11,12] Our protocol describes the surgical isolation of adult mouse dural meninges, their preparation for flow cytometry using single cell suspension, as well as their preparation for immunohistochemistry using whole mount imaging.

This protocol can be extended to other experimental approaches that aim to measure the immunophenotyping of dural meninges and their spatiotemporal infection/inflammation.

Institutional permissions

All animal experiments were approved and performed in accordance with the limiting principles for the use of animals in research (the three Rs: replacement, reduction, and refinement) and approved by the French Ministry of Higher Education and Research. Animal experiments were done in accordance with institutional animal care and ethical committees and French and European guidelines for animal care. Experiments with LCMV were performed in the biosafety level 3 laboratory (BSL3) of CIML by authorized, trained personnel. BSL3 facility operations are overseen by a Biosecurity Officer.

The day before: Prepare solutions and buffers

Timing: 1 h

1. Prepare solutions as described in materials and equipment.
   a. Prepare Phosphate Buffer Saline 1× (PBS 1×).
   b. Prepare the stock of the enzymatic solutions: DNAse I and collagenase D.
   c. Prepare the ketamine/xylazine cocktail solution for mouse euthanasia.
   d. Prepare Fluorescence Activated Cell Sorting (FACS) Buffer for staining and washing steps.
   e. Prepare ImmunoHistoChemistry (IHC) Buffer Triton-X100 for staining and washing steps.
   f. Prepare Formalin 5%.

The day of the experiment: Prepare digestion mix and staining buffers

Timing: 30 min

2. Prepare solutions as described in materials and equipment for mononuclear cell isolation by flow cytometry of mouse dural meninges.
   a. Prepare 5 mL tubes with 1 mL digestion buffer for each sample, keep it on ice.
   b. Turn on the water bath to 37°C.
   c. Prepare blocking buffer solution (50 µL /sample).
   d. Prepare antibody mix for staining (50 µL /sample): dilute antibody needed for the gating strategy in FACS Buffer.
3. Prepare the solutions as described in materials and equipment for meningeal whole mount immunohistochemistry of mouse dural meninges.
   a. Prepare blocking buffer.
   b. Prepare antibody mix: dilute each antibody needed for your cell observation in FACS Buffer Triton-X100.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BUV 737 mouse anti mouse CD45.2 (LY-5.2) (clone 104) (used at 1:200 dilution)	BD Biosciences	Cat#612779; RRID: AB_2870107
Brilliant Violet 570(TM) rat monoclonal anti-mouse/human CD11b (clone M1/70) (used at 1:200 dilution)	BioLegend	Cat# 101233, RRID: AB_10896949
BUV395 rat monoclonal anti-mouse CD90.2/Thy1.2 (clone 53-2.1) (used at 1:200 dilution)	BD Biosciences	Cat# 565257, RRID: AB_2739136
Brilliant Violet 711 rat monoclonal anti-mouse Ly-6G (clone RA3–6B2) (used at 1:200 dilution)	BioLegend	Cat# 127643, RRID: AB_2565971

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PE-CF594 rat monoclonal anti-mouse Ly-6C (clone AL-21) (used at 1:200 dilution)	BD Biosciences	Cat# 562728, RRID: AB_2737749
FITC rat monoclonal anti-mouse CD206 (MMR) (clone C068C2) (used at 1:200 dilution)	BioLegend	Cat# 141704, RRID: AB_10901166
Brilliant Violet 421 rat monoclonal anti-mouse I-A/I-E (clone M5/114.15.2) (used at 1:400 dilution)	BioLegend	Cat#107632 ; RRID: AB_2650896
Alexa Fluor 700 rat monoclonal anti-mouse CD8a (clone SK1) (used at 1:200 dilution)	BioLegend	Cat# 100730, RRID: AB_493703
BV786 rat monoclonal anti-mouse CD4 (Clone RM4-5) (used at 1:200 dilution)	BD Biosciences	Cat# 563727, RRID: AB_2728707
APC mouse monoclonal anti-mouse NK-1.1 (clone S17016D) (used at 1:200 dilution)	BioLegend	Cat# 108710, RRID: AB_313397
Brilliant Violet 605 mouse monoclonal anti-mouse CD64 (FcgammaRI) (clone X54–5/7.1) (used at 1:200 dilution)	BioLegend	Cat# 139323, RRID: AB_2629778
PE-Cyanine5.5 Armenian hamster monoclonal anti-mouse CD11c (clone N418) (used at 1:200 dilution)	Thermo Fisher Scientific	Cat# 35-0114-82, RRID: AB_469709
Rat monoclonal anti-mouse CD16/CD32 (used at 1:100 dilution)	BD Biosciences	Cat# 553141, RRID: AB_394656
InVivo mAb anti-LCMV nucleoprotein (clone VL-4) (used at 1:500 dilution)	Bio X Cell	Cat# BE0106, RRID: AB_10949017
Goat anti rat secondary antibody Alexa Fluor 568 (used at 1:500 dilution)	Thermo Fisher Scientific	Cat A-11077 RRID: AB2534121
Chemicals, peptides, and recombinant proteins		
Formalin solution, neutral buffered, 10%	Sigma-Aldrich	Cat# HT501128
DPBS 10×	Thermo Fisher Scientific	Cat# 14200067
Xylazine (ROMPUN 2%)	Elanco	N/A
Ketamine (IMALGENE)	Boehringer Ingelheim Animal Health France	N/A
RPMI Medium 1640 1× (+ L-Glutamine)	Thermo Fisher Scientific	Cat# 21875034
LiveDead fixable Blue Cell Staining kit (used at 1:1000 dilution)	Thermo Fisher Scientific	Cat# L23105
Collagenase D Roche	Sigma-Aldrich	Cat# 11088866001
DNase I Roche	Sigma-Aldrich	Cat# 11284932001
Triton X-100	Sigma-Aldrich	Cat# X100-100ML
Experimental models: Cell lines and Organisms/strains		
CD16/32 clone 2.4G2 cells hybridoma	American Type Culture Collection	HB-197
Mouse : B6 : C57BL/6J (used at 6 weeks of age; male or female)	The Jackson Laboratory	RRID: IMSR_JAX:000664
Software and algorithms		
FlowJo	Tree Star Inc.	https://www.flowjo.com/
Prism 9.1.2.	GraphPad	https://www.graphpad.com/scientific-software/prism/
Imaris 9.6.0	Oxford Instruments	https://imaris.oxinst.com/versions/9-6
Other		
BD FACS Symphony A5 Cell Analyzer	BDBiosciences	N/A
Dumont #7 Forceps	Fine Science Tools	Cat# 11271-30
20 mL syringe	Terumo	Cat# SS+20L1
26G × $^{1}/_{2}$-inch needle	Terumo	Cat# AN*2613R1
23G × 1-inch needle	Terumo	Cat# AN*2325R1
5 mL tube with cell strainer cap	Falcon	Cat# 352235
5 mL tube	Falcon	Cat# 352054

MATERIALS AND EQUIPMENT

Euthanasia solution (ketamine/xylazine cocktail)		
Reagent	Final concentration	Amount for 10 mice
Ketamine (100 mg/mL)	10 mg/mL	1 mL
Xylazine (20 mg/mL)	1 mg/mL	0.5 mL
PBS 1×	N/A	8.5 mL
Total	*N/A*	*10 mL*
Keep at 4°C for up to one week.		

PBS 1×		
Reagent	Final concentration	Amount
Sterile PBS 10×	1×	200 mL
MilliQ Water	N/A	1,800 mL
Total	*N/A*	*2 L*
Keep at 25°C for up to 1 month.		

⚠ CRITICAL: This stock has to be sterile, therefore prepare it under a laminar flow hood.

Collagenase D stock solution (40×)		
Reagent	Final concentration	Amount
Collagenase D *(Clostridium histolyticum)*	100 mg/mL	500 mg
Sterile RPMI	N/A	5 mL
Total	*N/A*	*5 mL*
Keep at −20°C for up to 6 months.		

⚠ CRITICAL: This stock has to be sterile, therefore prepare it under a laminar flow hood.

DNAse I stock solution (40×)		
Reagent	Final concentration	Amount
DNAse I (bovine pancreas)	4 mg/mL	100 mg
Sterile RPMI	N/A	25 mL
Total	*N/A*	*25 mL*
Keep at −20°C for up to 6 months.		

⚠ CRITICAL: This stock has to be sterile, therefore prepare it under a laminar flow hood.

Enzymatic digestion solution		
Reagent	Final concentration	Amount (for 1 sample)
Collagenase D stock solution (100 mg/mL)	2.5 mg/mL	25 µL
DNAse I stock solution (4 mg/mL)	0.1 mg/mL	25 µL
Sterile RPMI	N/A	950 µL
Total	*N/A*	*1 mL*

⚠ CRITICAL: This solution should be prepared right before the experiment, and used within 5 h. Once the solution is ready, keep it on ice until the meninges are added for digestion.

Staining/Washing Buffer solution for flow cytometry (FACS Buffer)		
Reagent	Final concentration	Amount
Fetal bovine serum	2%	1 mL
PBS 1×	N/A	49 mL
Total	*N/A*	*50 mL*
Keep at 4°C for up to 1 week.		

Staining/Washing buffer solution for immunohistochemistry (FACS Buffer Triton 0.5%)		
Reagent	Final concentration	Amount
Triton-X100	0.5%	250 µL
FACS Buffer	N/A	49.75 mL
Total	*N/A*	*50 mL*
Keep at 4°C for up to 1 week.		

Blocking Buffer solution for flow cytometry and immunohistochemistry		
Reagent	Final concentration	Amount
FACS Buffer	N/A	180 µL
CD16/32 (clone 2.4G2) hybridoma supernatant	1%	20 µL
Total	*N/A*	*200 µL*
Keep at 4°C for up to 1 week.		

Formalin 5%		
Reagent	Final concentration	Amount
Formalin 10%	5%	25 mL
PBS 1×	N/A	25 mL
Total	*N/A*	*50 mL*
Keep at 4°C for up to a week.		

⚠ CRITICAL: This solution has to be prepared under a chemical fume hood.

Antibody mix for flow cytometry (2×)		
Reagent	Final concentration	Amount (for 10 samples)
FACS Buffer	N/A	471.25 µL
Brilliant Violet 605 mouse monoclonal anti-mouse CD64 (FcgammaRI) (clone X54–5/7.1) (0.2 mg/mL)	1 µg/mL	2.5 µL
PE-Cyanine5.5 Armenian hamster monoclonal anti-mouse CD11c (clone N418) (0.2 mg/mL)	1 µg/mL	2.5 µL
BUV 737 mouse anti mouse CD45.2 (LY-5.2) (clone 104) (0.2 mg/mL)	1 µg/mL	2.5 µL
Brilliant Violet 570 (TM) rat monoclonal anti-mouse/human CD11b (clone M1/70) (0.2 mg/mL)	1 µg/mL	2.5 µL
BUV395 rat monoclonal anti-mouse CD90.2/Thy1.2 (clone 53-2.1) (0.2 mg/mL)	1 µg/mL	2.5 µL
Brilliant Violet 711 rat monoclonal anti-mouse Ly-6G (clone RA3–6B2) (0.2 mg/mL)	1 µg/mL	2.5 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount (for 10 samples)
PE-CF594 rat monoclonal anti-mouse Ly-6C (clone AL-21) (0.2 mg/mL)	1 μg/mL	2.5 μL
FITC rat monoclonal anti-mouse CD206 (MMR) (clone C068C2) (0.2 mg/mL)	1 μg/mL	2.5 μL
Brilliant Violet 421 rat monoclonal anti-mouse I-A/I-E (clone M5/114.15.2) (0.2 mg/mL)	0.5 μg/mL	1.25 μL
Alexa Fluor 700 rat monoclonal anti-mouse CD8a (clone SK1) (0.2 mg/mL)	1 μg/mL	2.5 μL
BV786 rat monoclonal anti-mouse CD4 (Clone RM4-5) (0.2 mg/mL)	1 μg/mL	2.5 μL
APC mouse monoclonal anti-mouse NK-1.1 (clone S17016D) (0.2 mg/mL)	1 μg/mL	2.5 μL
Total	*N/A*	*500 μL*
Keep at 4°C in the dark and use the same day.		

Viability marker mix		
Reagent	Final concentration	Amount (for 10 samples)
FACS Buffer	N/A	499.5 μL
LiveDead fixable Blue Cell Staining kit	1/1000	0.5 μL
Total	*N/A*	*500 μL*
Keep at 4°C in the dark and use the same day.		

Antibody mix for primary staining of immunohistochemistry		
Reagent	Final concentration	Amount (for 1 sample)
FACS Buffer Triton 0.5%	N/A	196.6 μL
Anti-LCMV nucleoprotein (clone VL-4) (0.5 mg/mL)	1 μg/mL	0.4 μL
Total	*N/A*	*200 μL*
Keep at 4°C in the dark and use the same day.		

Antibody mix for secondary staining of immunohistochemistry		
Reagent	Final concentration	Amount (for 1 sample)
FACS Buffer Triton 0.5%	N/A	196.6 μL
Goat anti rat secondary antibody Alexa Fluor 568 (2 mg/mL)	4 μg/mL	0.4 μL
Total	N/A	200 μL
Keep at 4°C in the dark and use the same day.		

STEP-BY-STEP METHOD DETAILS

Mouse intracardiac perfusion

Timing: 15 min

To eliminate circulating blood cells, the euthanized mouse is intracardially perfused. Of note, histochemistry also helps distinguish cells located in the dura mater versus cells within the blood vessel lumen.

1. Euthanize the mouse.
   a. Use an intraperitoneal injection (i.p.) of ketamine and xylazine cocktail. Inject 0.9 mL of cocktail for a 30 g mouse (ketamine 300 mg/kg body weight and xylazine 30 mg/kg of body weight) with a syringe equipped with a 26G × $^{1}/_{2}$ inch needle.

b. Wait for the mouse to be unresponsive to a toe pinch. This should take around 5 min.
c. Place the mouse on its back and pin each limb down to the dissection board. Spray ethanol 70% on the mouse abdomen to clean the area.

2. Perform intracardiac perfusion.
   a. Open the mouse abdomen with scissors and cut up to the thorax. Carefully expose the heart without cutting any major blood vessels.
   b. Once you have exposed the heart, make a cut in the right atrium to let the blood flow.
   c. Inject the left ventricle of the heart with a 20 mL syringe equipped with a 23G × 1 inch needle filled with ice-cold PBS 1×. At the end of the perfusion, the liver should have whitened.

Isolation of mouse dural meninges

Timing: 5 min

In this section, we describe how to recover the mouse dural meninges from the skull.

3. Decapitate the mouse.
4. Using scissors, cut and remove the scalp until the skull is fully visible.
5. Cut the skull in the longitudinal plane from the back of the head to the nose, without damaging the brain.
   a. The skull cap should be extracted without difficulty.
   b. The brain should come with the skull cap.
6. Gently extract the brain from the skull using forceps without scraping the inside of the skull. Dural meninges are now accessible inside the skull.
7. For flow cytometry, go to step 8 and for meningeal whole mount immunochemistry, go to step 20.

Flow cytometry of dural meninges

Timing: 2 h

In this section, we describe how to obtain single-cell suspension from mouse dural meninges and how to process and analyze the cells by flow cytometry.

8. Gently extract the meninges from the skull with fine-tipped forceps. For flow cytometry, the meninges should come in one piece (Figure 1) and can be put in the enzymatic mix on ice until digestion.

⚠ CRITICAL: be careful to take the entire dura and no bone or brain tissue. Keep the dura hydrated using PBS 1× frequently.

9. Digest the meningeal tissue in the enzymatic mix at 37°C during 30 min in the water bath.
10. Gently shake the tube every 10 min.

⚠ CRITICAL: Over-digestion will lead to reduced cell viability.

11. After 30 min, stop the digestion.
    a. Dissociate the tissue by 20 recurrent aspirations through a 1,000 µL tip.
    b. Pass the cellular suspension through a 35-µm pore cell strainer capped tube.
    c. Spin at 450 g at 4°C for 2 min, then remove most of the supernatant and leave 200 µL of cell suspension.
12. Transfer this suspension in a 96-V bottom well plate for staining and wash two times with 100 µL of FACS Buffer (spin at 450 g at 4°C for 2 min, then remove supernatant).

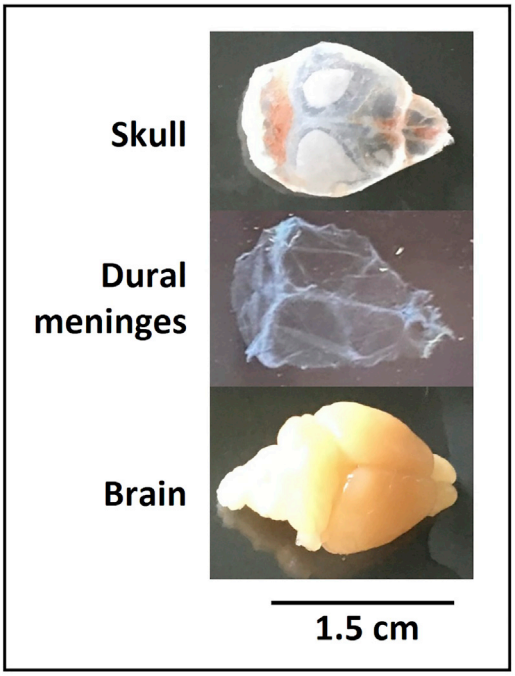


Figure 1. Separation of mouse skull, fresh dural meninges and brain

⚠ CRITICAL: The pellet can be hard to see. Process carefully to avoid losing cells.

13. Stain with the viability marker for 15 min at 4°C. Wash with FACS Buffer (spin at 450 g at 4°C for 2 min, then remove supernatant).
14. Block cell suspension with 50 µL of blocking solution for 10 min on ice.
15. Add 50 µL of the antibody cocktail (2×) for 30 min on ice and keep in the dark.
16. Wash two times with FACS Buffer (spin at 450 g at 4°C for 2 min, then remove supernatant).
17. Resuspend in 150 µL of FACS Buffer.
18. Acquire the samples on the cytometer.

Note: Make sure to use a cytometer compatible with the fluorochromes used in this protocol.

19. Analyze the results (gating strategy in Figure 2A and cell counts in Figure 2B).

Whole mount immunohistochemistry of mouse dural meninges

Timing: 2 days

In this section we describe how to stain the dural meninges for the viral nuclear protein of LCMV. This has to be done on different samples than the ones used for flow cytometry.

20. Fix the skull containing the dural meninges for 10 min with formalin 5% at 25°C.

Note: This low fixation time is used for LCMV staining and should be adapted depending on the targeted epitope.

21. Wash 3 times with 1 mL of PBS 1×. Each wash should be left 1 min.
22. Incubate dural meninges in 1.5 mL of blocking solution at 25°C for 15 min.
23. Extract gently the meninges from the skull with fine-tipped forceps (Methods video S1).
24. Spread out and flatten the meninges on a glass slide in PBS 1× (Methods video S2).

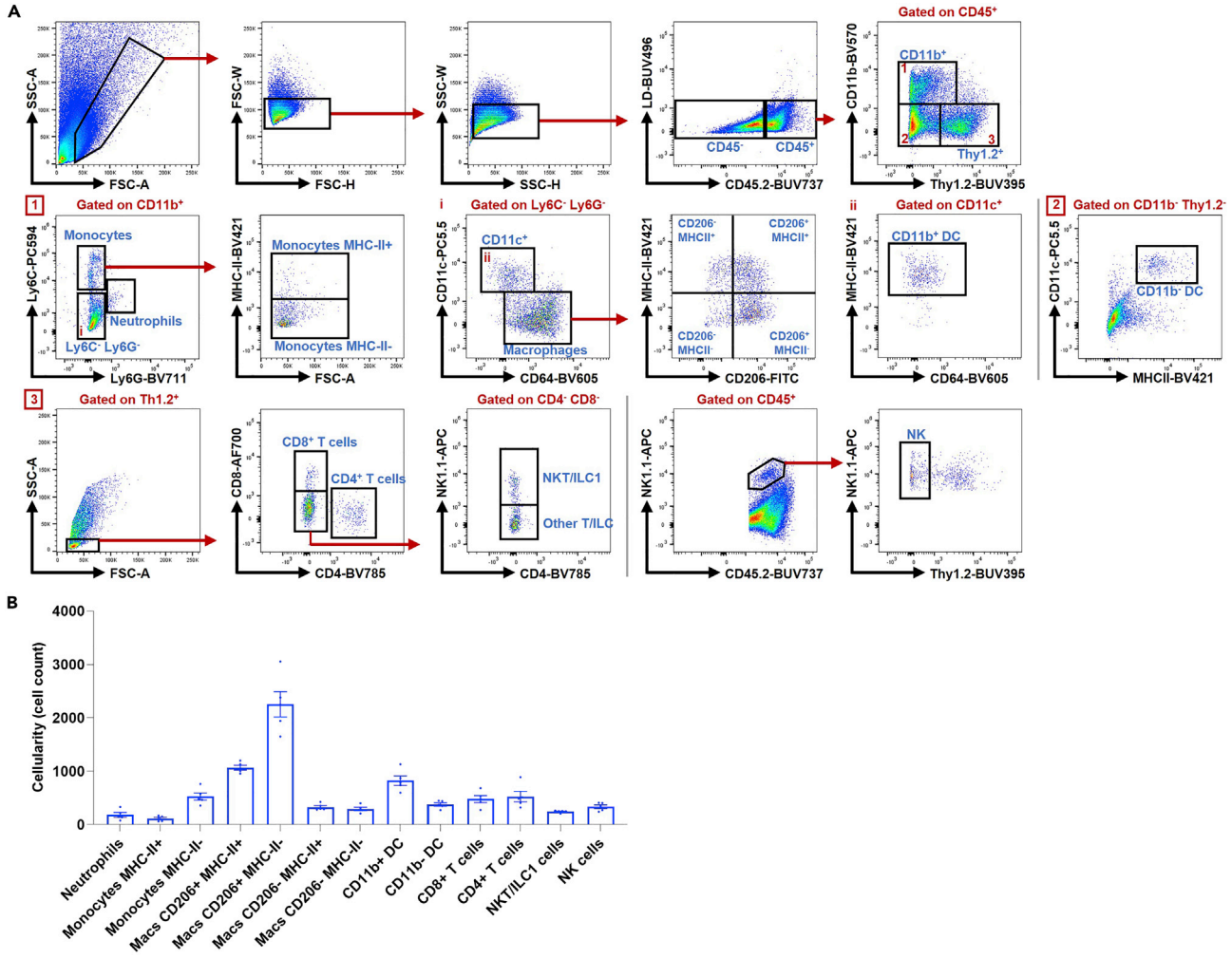


Figure 2. Flow cytometry analysis of dural meninges

(A) Gating strategy for dural meninges by flow cytometry. This allows to identify neutrophils, monocytes, macrophages (macs), CD11b+ DC, CD11b- DC, CD8+ T cells, CD4+ T cells, NKT/ILC1 cells and NK cells.

(B) Expected counts of the cell populations in 6 weeks old B6 mice, based on the previous gating strategy. Bar graphs show the mean ± SEM.

a. Circle the meninges with a hydrophobic pen to keep the solution from spreading on the slide.
b. For the duration of the stain, keep your slices away from light and in a moisturized chamber. This will prevent the meninges from drying.

⚠ CRITICAL: The meningeal tissue can easily be lost if moved around too much as it is very small and almost transparent. Ensure the meningeal tissue stays flattened on the slide.

25. Stain the meninges with the primary antibody mix described in materials and equipment using the purified rat anti-LCMV antibody, 12 h at 4°C.
26. Wash 3 times with 200 µL of PBS 1×. Each wash should be left 1 min.

⚠ CRITICAL: Be really cautious to keep the integrity of the meninges.

27. Stain with the secondary antibody goat anti-rat Alexa Fluor 568 for 2 h at 25°C.
28. Wash 3 times with 200 µL PBS 1×. Each wash should be left 1 min.

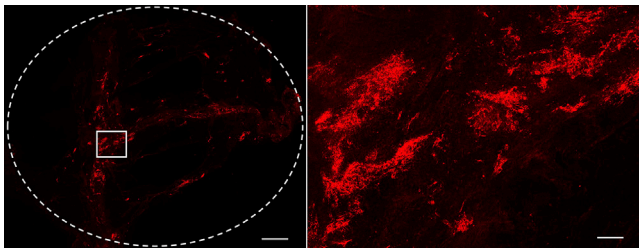


Figure 3. Maximal intensity projection of meningeal whole-mount stained for LCMV (red) showing clusters of infected areas

Indeed, upon depletion of meningeal macrophages, LCMV peripheral infection readily spreads in the meninges. The dotted line delineates the meningeal whole mount (right), and the inset is zoomed in (right). Scale bars = 1 mm (left) and 0.1 mm (right).

⚠ CRITICAL: be really cautious to keep the integrity of the meninges.

29. Finally, mount the meninges with a cover-slip and mounting medium.
30. Put a dot of nail polish at each corner of the cover-slip to stabilize it.
31. Let the meninges dry at 25°C 2 h before imaging.

EXPECTED OUTCOMES

Recovery of dural meninges

The meninges should be recovered whole as seen in Figure 1. The perfusion step is essential for eliminating circulating blood cells and good perfusion can be seen when the meninges are clear and white.

Flow cytometry of dural meninges

For flow cytometry data analysis, we implemented a strategy to reliably distinguish different cell populations based on identified cell surface markers. We first gated on cells using a dot plot depicting forwards and side scatter parameters. We then excluded cell doublets using forward scatter-height and side scatter height. Using a viability dye, we made sure that the selected cells were alive. We then plotted the different immune cells populations (CD45+ cells) using the gating strategy described below (Figure 2).

Confocal imaging of whole mount dural meninges

Fluorescent microscopy of the infected dural meninges should show spots of infected areas. When the meninges are flattened accordingly, we should observe the distribution of LCMV nucleoprotein in different parts of the tissue (Figure 3). Upon depletion of meningeal macrophages, LCMV spots multiply in the sinuses of the meninges.

LIMITATIONS

The mouse dural meninges are small, thin and fragile, therefore sensitive to excessive mechanical manipulation. It is crucial to be delicate when removing them from the skull. The meninges are easier to remove from the skull of a young adult mouse (1 month–6 months old). After this age, removing the meninges in one piece requires more practice.

In this protocol we focused on the mouse dural meninges. For preparation of mouse leptomeninges and of primate meninges, this protocol has to be adapted (for more information contact us).

TROUBLESHOOTING

Problem 1

Poor tissue recovery.

The meninges can be ripped or can desiccate (steps 8, 23, 25, 27).

Potential solution

- Use forceps adapted to this protocol (Key data table) and extract gently the meninges from the skull.
- Frequently hydrate the meninges in PBS 1×.

Problem 2

Small yield of cells for flow cytometry.

- A longer digestion can reduce cell viability (step 9).
- Cells can be lost during filtration if the tissue is not properly digested (step 11b).
- The pellet can be lost during washes (steps 12, 13, 16).

Potential solution

- Respect the indicated time of the digestion.
- Slowly and carefully aspirate the supernatant.

Problem 3

Poor tissue integrity in immunohistochemistry.

- The meninges can be ripped or can desiccate (steps 23, 24).
- Floating meninges can fold on the slide (step 24).

Potential solution

- Handle the meninges delicately, and delicately unfold them using appropriate forceps.

Problem 4

Possibility to lose the tissue in immunohistochemistry.

- Hydrophobic pen can be washed away with FACS Triton 0.5% (step 24).

Potential solution

- Be careful and prepare the hydrophobic slides beforehand if necessary.

Problem 5

Low immunohistochemistry staining.

- After scanning on the microscope, LCMV staining can appear low.

Potential solution

- Respect the time of fixation and the concentration of the fixative indicated (step 20).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rejane Rua (rua@ciml.univ-mrs.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol did not generate any data sets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102119.

ACKNOWLEDGMENTS

These studies were supported by the ATIP-AVENIR young group leader program (INSERM), the Marie Curie Reintegration Fellowship (EU), the Federation pour la Recherche sur le Cerveau, the Region Sud Provence-Alpes-Côte d'Azur (co-financed by Innate Pharma S.A.), the European Research Council, and the AMIDEX (Aix-Marseille Université) grants, as well as by institutional funding from the Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, and Aix-Marseille-Université as well as Marseille-Immunopole. Microscopy facilities are supported by l'Agence Nationale de la Recherche (grant ANR-10-INBS-04-01 France Bio Imaging). We acknowledge the CIML Flow Cytometry Core, as well as the Imaging and Histology platforms of the CIML for technical assistance.

AUTHOR CONTRIBUTIONS

A.R. and J.R. performed the experiments and wrote the paper with the help of E.E.S. and L.A.P. R.R. supervised the study and reviewed and edited the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Rebejac, J., Eme-Scolan, E., Arnaud Paroutaud, L., Kharbouche, S., Teleman, M., Spinelli, L., Gallo, E., Roussel-Queval, A., Zarubica, A., Sansoni, A., et al. (2022). Meningeal macrophages protect against viral neuroinfection. Immunity *55*, 2103–2117.e10. https://doi.org/10.1016/j.immuni.2022.10.005.

2. Filiano, A.J., Gadani, S.P., and Kipnis, J. (2015). Interactions of innate and adaptive immunity in brain development and function. Brain Res. *1617*, 18–27. https://doi.org/10.1016/j.brainres.2014.07.050.

3. Upadhyay, R.K. (2014). Drug delivery systems, CNS protection, and the blood brain barrier. BioMed Res. Int. *2014*, 869269. https://doi.org/10.1155/2014/869269.

4. Rua, R., and McGavern, D.B. (2018). Advances in meningeal immunity. Trends Mol. Med. *24*, 542–559. https://doi.org/10.1016/j.molmed.2018.04.003.

5. Rua, R., Lee, J.Y., Silva, A.B., Swafford, I.S., Maric, D., Johnson, K.R., and McGavern, D.B. (2019). Infection drives meningeal engraftment by inflammatory monocytes that impairs CNS immunity. Nat. Immunol. *20*, 407–419. https://doi.org/10.1038/s41590-019-0344-y.

6. Kwong, B., Rua, R., Gao, Y., Flickinger, J., Jr., Wang, Y., Kruhlak, M.J., Zhu, J., Vivier, E., McGavern, D.B., and Lazarevic, V. (2017). T-bet-dependent NKp46(+) innate lymphoid cells regulate the onset of TH17-induced neuroinflammation. Nat. Immunol. *18*, 1117–1127. https://doi.org/10.1038/ni.3816.

7. Van Hove, H., Martens, L., Scheyltjens, I., De Vlaminck, K., Pombo Antunes, A.R., De Prijck, S., Vandamme, N., De Schepper, S., Van Isterdael, G., Scott, C.L., et al. (2019). A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and tissue environment. Nat. Neurosci. *22*, 1021–1035. https://doi.org/10.1038/s41593-019-0393-4.

8. Prinz, M., Erny, D., and Hagemeyer, N. (2017). Ontogeny and homeostasis of CNS myeloid cells. Nat. Immunol. *18*, 385–392. https://doi.org/10.1038/ni.3703.

9. Salvador, A.F., de Lima, K.A., and Kipnis, J. (2021). Neuromodulation by the immune system: a focus on cytokines. Nat. Rev. Immunol. *21*, 526–541. https://doi.org/10.1038/s41577-021-00508-z.

10. Mrdjen, D., Pavlovic, A., Hartmann, F.J., Schreiner, B., Utz, S.G., Leung, B.P., Lelios, I., Heppner, F.L., Kipnis, J., Merkler, D., et al. (2018). High-dimensional single-cell mapping of central nervous system immune cells reveals distinct myeloid subsets in health, aging, and disease. Immunity *48*, 380–395.e6. https://doi.org/10.1016/j.immuni.2018.01.011.

11. Protasoni, M., Sangiorgi, S., Cividini, A., Culuvaris, G.T., Tomei, G., Dell'Orbo, C., Raspanti, M., Balbi, S., and Reguzzoni, M. (2011). The collagenic architecture of human dura mater. J. Neurosurg. *114*, 1723–1730. https://doi.org/10.3171/2010.12.JNS101732.

12. Balin, B.J., Broadwell, R.D., Salcman, M., and el-Kalliny, M. (1986). Avenues for entry of peripherally administered protein to the central nervous system in mouse, rat, and squirrel monkey. J. Comp. Neurol. *251*, 260–280. https://doi.org/10.1002/cne.902510209.