Chapter 7

Isolation and Phenotyping of Adult Mouse Microglial Cells

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Abstract

Microglia are the resident macrophages of the central nervous system parenchyma and fulfill crucial roles in brain development, homeostasis, and inflammation. The isolation of a pure microglia population from brain tissue enables the examination of microglial phenotypes without the interference of other cell populations. Microglial extractions from the neonatal brain have been described in various protocols, yet the more established and complex adult mouse brain poses a greater challenge. Here we describe a refined protocol including enzymatic and mechanical dissociation of adult mouse brain tissue and removal of myelin by Percoll density gradient. Microglial cells were subsequently extracted by an immunomagnetic approach. This isolation procedure enables the use of functionally viable cells for various applications such as cell culture, flow cytometry, functional assays including bacteria- or bead-based phagocytosis, stimulation assays, and transcriptome profiling techniques such as qRT-PCR and microarray/RNA sequencing.

Key words Microglia, Enzyme digestion, Percoll gradient, CD11b immunomagnetic beads

1 Introduction

Microglia, specially adapted tissue-resident macrophages of the central nervous system (CNS), are highly dynamic and vital players in CNS physiology and neuroimmune function. Their multifunctional role begins during embryonic and early postnatal development sculpting the developing CNS [1] by supporting synaptic maturation of active neurons through synaptic pruning and remodeling [2-4]. In adulthood under steady-state conditions microglia function further extends to include maintenance of brain homeostasis by continuous surveillance of their local environment and neural parenchyma, examining synaptic activity [5], and phagocytic removal of cell debris [6]. As one of the earliest responders to sterile and microbial inflammatory stimuli, activated microglia migrate to sites of injury/infection and initiate neuroimmune and inflammatory responses involving the internalization of damaged cells or invading pathogens, and the synthesis of a variety of immunoregulatory and inflammatory components [7-9] for the protection and repair of the brain [10, 11].
Dysregulation of microglial activity is also increasingly implicated in a range of neurological conditions [12, 13].

Yolk sac-derived microglia migrate into the brain during early development and are evident from embryonic day (E) 9.5 in mice [14] and soon after birth these cells comprise 5–12% of the total number of cells [15]. The investigation of the phenotypes and crucial functions of microglia in response to distinct physiological and pathological conditions, including ageing, has been constrained by the considerable challenge to directly isolate pure populations of microglia from the adult brain. Over the last 20 years, a range of methods have evolved from initial in vitro culture systems of microglia purification from rodent neonates [16, 17] and early postnatal brains [18] to adult microglia in rats [19, 20] and humans [21, 22] as well as more recently the generation of microglia derived from induced pluripotent stem cells (iPSC) [23, 24]. Due to the low abundance of these cells the strategy of microglial isolation has become crucial to achieve sufficient numbers and with minimal deviation from the microglial in vivo signature. The latter is crucial as it has been reported that a few hours after isolation and culture the unique signature of microglia is downregulated and ultimately their functional phenotype altered [25, 26]. However, efforts are made to identify key factors, which promote an in vivo phenotype in culture [27, 28] after the isolation as described in this protocol. In addition to the investigation of whole-brain microglia, the isolation of cells was furthermore applied to study discrete brain regions [29] and single cells [30] to identify discrete microglia phenotypes and their contribution to age-related neurodegenerative diseases.

In general, the isolation of microglia from the adult mouse brain as described here is composed of three steps. The brain tissue is first dissociated with enzymes and subsequently homogenized by a Dounce homogenizer. To facilitate the separation of microglia, myelin is removed by a two-layer density gradient, followed by the labeling of microglia with CD11b immunomagnetic beads and magnetic separation of CD11b-positive microglia from the remaining brain cell suspension. Once isolated and cell purities and yield are confirmed, extracted microglial cells are suitable for a range of downstream phenotyping and functional applications including flow cytometry, cell culture, stimulation and phagocytosis assays, and -omics profiling.

# 2 Materials

1. Perfusion pump.
2. Tissue culture hood.
3. Incubating oven.
4. 15 mL Dounce homogenizer.
5. Falcon tubes and microtubes.
6. 5, 10, and 20 mL stripettes.
7. Centrifuge.
8. LS columns (Miltenyi Biotec).
9. MACSmix Tube Rotator.
10. MidiMACS separator and MultiStand.

### 2.1 Reagents

1. 1× Hanks’ balanced salt solution (HBSS) without calcium and magnesium.
2. 10× HBSS.
3. Percoll.
4. CD11b human/mouse MicroBeads.
5. Purified anti-mouse CD16/CD32 (Fc block).
6. Anti-mouse antibodies against CD11b, CD45, and F4/80.

### 2.2 Solutions

1. Physiological saline: 0.9% Saline treated with 0.1% diethyl pyrocarbonate (DEPC), store at 4 °C.
2. Enzyme cocktail: 10 mL of enzyme cocktail contains the following ingredients and stock concentrations: 50 μL of 50 U/mL collagenase D, 10 μL of 100 µg/mL Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride, 50 μL of 5 U/mL DNaseI, and 250 μL of 340 U/mL dispase in 9.64 mL HBSS. Prepare stock concentration of enzymes according to the manufacturer’s instruction. Enzyme mix was prepared fresh or stored in appropriate aliquots at −20 °C.
3. 10% Fetal bovine serum (FBS): Make up 10% FBS in HBSS. Prepare fresh on the day or freeze in aliquots at −20 °C.
4. Isotonic Percoll: Prepare isotonic Percoll by adding 1 volume of 10× HBSS to 9 volumes of Percoll. Prepare fresh each time.
5. 35% Percoll: 16 mL is required per whole brain. Add 5.6 mL of isotonic Percoll to 10.4 mL 1× HBSS. Prepare fresh each time.
6. 0.5 M Ethylenediaminetetraacetic acid (EDTA) stock: Add 56 g NaOH to 800 mL water. Weigh 186.12 g of EDTA disodium salt (or 146.2 g of EDTA anhydrous) and add to NaOH solution. Let dissolve, adjust pH to 7.5, and top up to 1 L with water.
7. Separation buffer: 1× PBS, pH 7.2, 0.5% bovine serum albumin (BSA, low endotoxin), 2 mM EDTA. Add 0.5 g BSA to 100 mL PBS (pH 7.2) and add 400 µL of 0.5 M EDTA stock solution. Store at 4 °C.
8. Flow cytometry buffer: Add 0.1 g BSA (low endotoxin) to 100 mL PBS (without calcium and magnesium). Store at 4 °C.
3 Methods

Carry out all procedures at 4 °C and use ice-cold reagents unless otherwise specified (see Note 1). The isolation procedure is summarized in Fig. 1.

3.1 Perfusion and Isolation of Mouse Brain

1. Perfuse animals transcardially with physiological saline (10 mL/min) until exudate runs clear (see Note 2).

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**Fig. 1** Schematic workflow of the microglia isolation process
2. Remove brain and transfer into 50 mL Falcon tube containing 10 mL HBSS either as whole brain, hemisphere, or brain region.
3. Finely mince brain tissue with a round-edge blade scalpel to allow fast cooling of the tissue.
4. Spin minced brain tissue for 5 min at 400 × g and aspirate supernatant.

### 3.2 Enzymatic Dissociation and Homogenization

1. Add enzyme cocktail to the minced whole-brain tissue (10 mL) or brain hemisphere/brain region (5 mL) (see Note 3).
2. Incubate for 1 h at 37 °C under gentle rotation (see Note 4).
3. Transfer the digested brain tissue to a 15 mL Dounce homogenizer and dissociate on ice with 20 passes using the large clearance pestle (see Note 5).
4. Transfer the homogenized brain cell suspension to an equal volume of 10% FBS.
5. Centrifuge for 5 min at 4 °C and 400 × g (no brake) and remove the supernatant.

### 3.3 Myelin-Free Mixed Brain Cell Suspension by Density Gradient

1. Resuspend the cell suspension from whole brain in 16 mL or brain hemisphere/region in 8 mL of 35% Percoll (see Note 6).
2. Split only the whole brain in 2 × 8 mL.
3. Carefully overlay each sample with 5 mL 1× HBSS and leave samples to rest for 5 min on ice (see Note 7).
4. Spin samples for 45 min at 4 °C and 800 × g (no brake) and subsequently different layers can be observed (see Fig. 1).
5. Aspirate the supernatant including the myelin layer carefully, leaving only the pelleted mixed brain cells.
6. Wash the cell pellet in 1 mL 1× HBSS and transfer into a new tube containing 4 mL 1× HBSS.
7. Spin for 5 min at 4 °C and 400 × g and remove supernatant.

### 3.4 Immunomagnetic Bead Separation

1. Resuspend the cell pellet in 90 μL separation buffer and transfer into a microtube.
2. Add 10 μL anti-CD11b MicroBeads and incubate the cell-bead mix for 15 min at 4 °C under gentle rotation (see Note 8).
3. Meanwhile place LS single-use columns in magnet and wash through with 3 mL of separation buffer.
4. Add 500 μL separation buffer to bead-cell-suspension, apply onto LS column, and collect flow-through (see Note 9).
5. Wash the columns three times with 3 mL separation buffer (see Note 10).
6. Remove LS columns from the magnet and place into a 15 mL tube.

7. Add 5 mL of separation buffer and flush out bead-bound microglial cells by firmly pushing the plunger.

8. Pellet the cells for 5 min at 400 g and 4 °C.

### 3.5 Flow Cytometric Analysis

1. Resuspend purified microglia in flow cytometry buffer and incubate with 1 μg/mL anti-mouse CD16/CD32 for 20 min at room temperature (see Note 11).

2. Spin at 400 × g for 5 min and remove supernatant.

3. Resuspend cells in fluorophore-conjugated antibodies for the protein of interest (here CD11b, CD45, F4/80) for 20 min in the dark at room temperature (see Note 12).

4. Wash samples for 5 min at 400 g and resuspend samples in an appropriate volume of flow cytometry buffer.

### 4 Notes

1. All procedures should be performed under the tissue culture hood, particularly if intending to use isolated cells for subsequent cell culture.

2. By performing perfusions blood is removed from the circulation and minimizes the contamination with red blood cells, which has been observed to reduce the efficiency of the bead-based microglia separation from brain cell suspension, and other immune cells such as CD11b⁺ leukocytes.

3. To shorten the time frame of the overall procedure the brain tissue digest was performed without and with different durations of enzyme mix. Our results (see Fig. 2) demonstrated that an enzymatic digestion improved yield of microglia.

4. Rotating movement is not necessarily required; the pieces of brain tissue should be under gentle movement to avoid pelleting and insufficient enzymatic digestion.

5. Brain cell suspension should have a homogenous and milky appearance after Dounce homogenization.

6. To achieve a good separation of mixed brain cells from the myelin the ratio between tissue and Percoll volume is crucial. A surplus of tissue in the gradient will impair the yield and purity of microglial cells.

7. Resting the samples enables the settling and strengthening of the different layers.

8. The application of CD11b MicroBeads (clone: M1/70) will lead to the reduced binding capacity of any CD11b flow cytometric antibodies if the clone of both antibodies is the same.
Fig. 2 Effect of enzymatic treatment. The application and duration of the enzymatic digestion of whole-brain tissue resulted in variance in the yield of extracted microglial cells and therefore competing for binding positions. Difference in a lower CD11b staining of positive selected microglia is evident when compared to pre-sorted microglia within the mixed brain cell suspension after myelin removal (see Fig. 3). In view of recent data [27, 31, 32] other antibodies (e.g., TMEM119 or FCRLs) could be applied for microglia isolation as CD11b does not necessarily label microglia only, particularly in inflamed conditions.

9. Flow-through containing depleted fraction can be collected to confirm negligible loss of microglia or to collect information from remaining cells contained in the brain cell suspension.

10. Let wash buffer run through completely each time for maximum washing efficiency.

11. Nonselected and pre-sorted samples can be used for flow cytometry to compare to purified microglia and for the validation of specificity.

12. Depending on the protein of interest, intracellular staining or a secondary antibody may be required.

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References

1. Bilimoria PM, Stevens B (2015) Microglia function during brain development: new insights from animal models. Brain Res 1617:7–17
2. Paolicelli RC, Bolasco G, Pagani F et al (2011) Synaptic pruning by microglia is necessary for normal brain development. Science 333:1456–1458
3. Schafer DP, Lehrman EK, Kautzman AG et al (2017) Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74:691–705
4. Tremblay M-È, Stevens B, Sierra A et al (2011) The role of microglia in the healthy brain. J Neurosci 31:16064–16069
5. Wake H, Moorhouse AJ, Jinno S et al (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29:3974–3980

Fig. 3 Purity of adult microglia isolated from mouse whole brain. (a) Flow cytometric comparison of positive selected microglia with pre-sorted and nonselected samples demonstrates highly pure CD11b⁺ F4/80⁺ and CD45⁺⁺ positive selected microglia and 1 log higher CD11b positivity in microglia before bead separation. Nonselected samples display a negligible loss. (b) Relative mRNA expression of microglial (Itgam, Aif1), astrocyte (Aldh111), and neuronal (Rbfox3) marker in whole-brain homogenates versus isolated microglia.
6. Neumann H, Kotter MR, Franklin RJM (2009) Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 132:288–295
7. Streit WJ (2002) Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40:133–139
8. Ransohoff RM, Perry VH (2009) Microglial physiology: unique stimuli, specialized responses. Annu Rev Immunol 27:119–145
9. Kettenmann H, Hanisch U-K, Noda M, Verkhratsky A (2011) Physiology of microglia. Physiol Rev 91:461–553
10. Szalay G, Martinecz B, Lénárt N et al (2016) Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. Nat Commun 7:11499
11. Chen Z, Jalabi W, Hu W et al (2014) Microglial displacement of inhibitory synapses provides neuroprotection in the adult brain. Nat Commun 5:4486
12. Andreasson KI, Bachstetter AD, Colonna M et al (2016) Targeting innate immunity for neurodegenerative disorders of the central nervous system. J Neurochem 138:653–693
13. Colonna M, Butovsky O (2017) Microglia function in the central nervous system during health and neurodegeneration. Annu Rev Immunol 35:441–468
14. Ginhoux F, Greter M, Leboeuf M et al (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330:841–845
15. Lawson LJ, Perry VH, Dri P, Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39:151–170
16. Marek R, Caruso M, Rostami A et al (2008) Magnetic cell sorting: a fast and effective method of concurrent isolation of high purity viable astrocytes and microglia from neonatal mouse brain tissue. J Neurosci Methods 175:108–118
17. Daniele SG, Edwards AA, Maguire-Zeiss KA (2014) Isolation of cortical microglia with preserved immunophenotype and functionality from murine neocortices. J Vis Exp 83:e51005
18. Harms AS, Tansey MG (2013) Isolation of murine postnatal brain microglia for phenotypic characterization using magnetic cell separation technology BT - microglia: methods and protocols. In: Joseph B, Venero JL (eds), Humana Press, Totowa, NJ, pp 33–39
19. Ford AL, Goodsall AL, Hickey WF, Sedgwick JD (1995) Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. J Immunol 154:4309–4321
20. Frank MG, Wieseler-Frank JL, Watkins LR, Maier SF (2006) Rapid isolation of highly enriched and quiescent microglia from adult rat hippocampus: immunophenotypic and functional characteristics. J Neurosci Methods 151:121–130
21. Olah M, Raj D, Brouwer N et al (2012) An optimized protocol for the acute isolation of human microglia from autopsy brain samples. Glia 60:96–111
22. Rustenhoven J, Park TI-H, Schweder P et al (2016) Isolation of highly enriched primary human microglia for functional studies. Sci Rep 6:19371
23. Abud EM, Ramirez RN, Martinez ES et al (2017) iPSC-derived human microglia-like cells to study neurological diseases. Neuron 94:278–293.e9
24. Muffat J, Li Y, Yuan B et al (2016) Efficient derivation of microglia-like cells from human pluripotent stem cells. Nat Med 22:1358–1367
25. Beutner C, Linnartz-Gerlach B, Schmidt SV et al (2013) Unique transcriptome signature of mouse microglia. Glia 61:1429–1442
26. Gosselin D, Skola D, Coufal NG et al (2017) An environment-dependent transcriptional network specifies human microglia identity. Science 356:eaal3222
27. Butovsky O, Jedrychowski MP, Moore CS et al (2014) Identification of a unique TGF-[beta]-dependent molecular and functional signature in microglia. Nat Neurosci 17:131–143
28. Bohlen CJ, Bennett FC, Tucker AF et al (2017) Diverse requirements for microglial survival, specification, and function revealed by defined-medium cultures. Neuron 94:759–773
29. Grabert K, Michoel T, Karavolos MH et al (2016) Microglial brain region-dependent diversity and selective regional sensitivities to aging. Nat Neurosci 19:504–516
30. Keren-Shaul H, Spinrad A, Weiner A et al (2017) A unique microglia type associated with restricting development of Alzheimer’s disease. Cell 169:1276–1290
31. Bennett ML, Bennett FC, Liddelow SA et al (2016) New tools for studying microglia in the mouse and human CNS. Proc Natl Acad Sci 113:1738–1746

32. Satoh J, Kino Y, Asahina N et al (2016) TMEM119 marks a subset of microglia in the human brain. Neuropathology 36:39–49