Isolation and Phenotyping of Adult Mouse Microglial Cells

Chapter 7

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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].

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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 *N*α-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**).

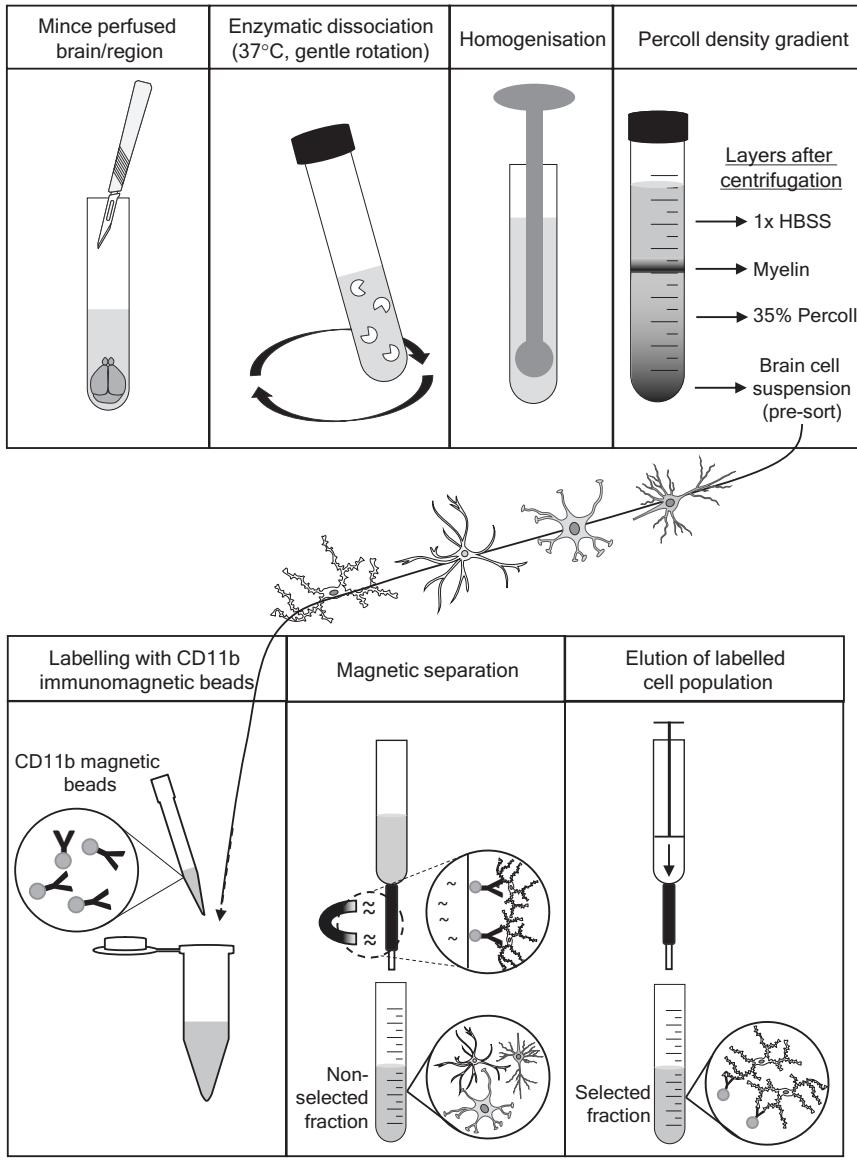

Fig. 1 Schematic workflow of the microglia isolation process

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

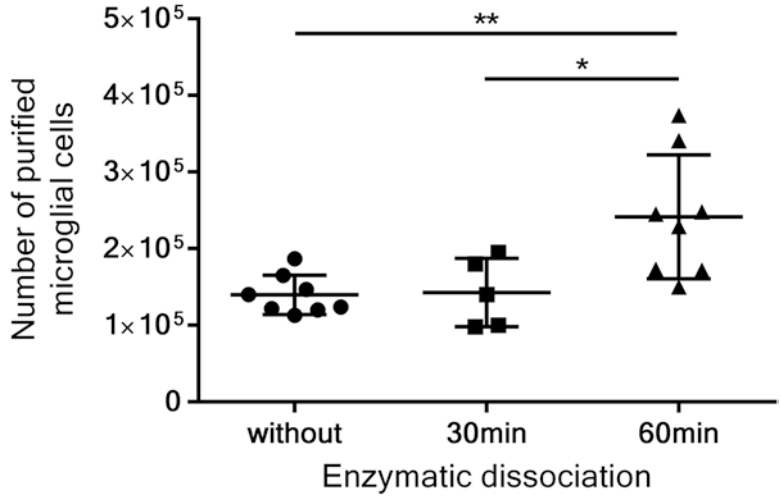

Fig. 2 Effect of enzymatic treatment. The application and duration of the enzymatic digestion of whole-brain tissue resulted in variance in the yield of extracted microglial cells

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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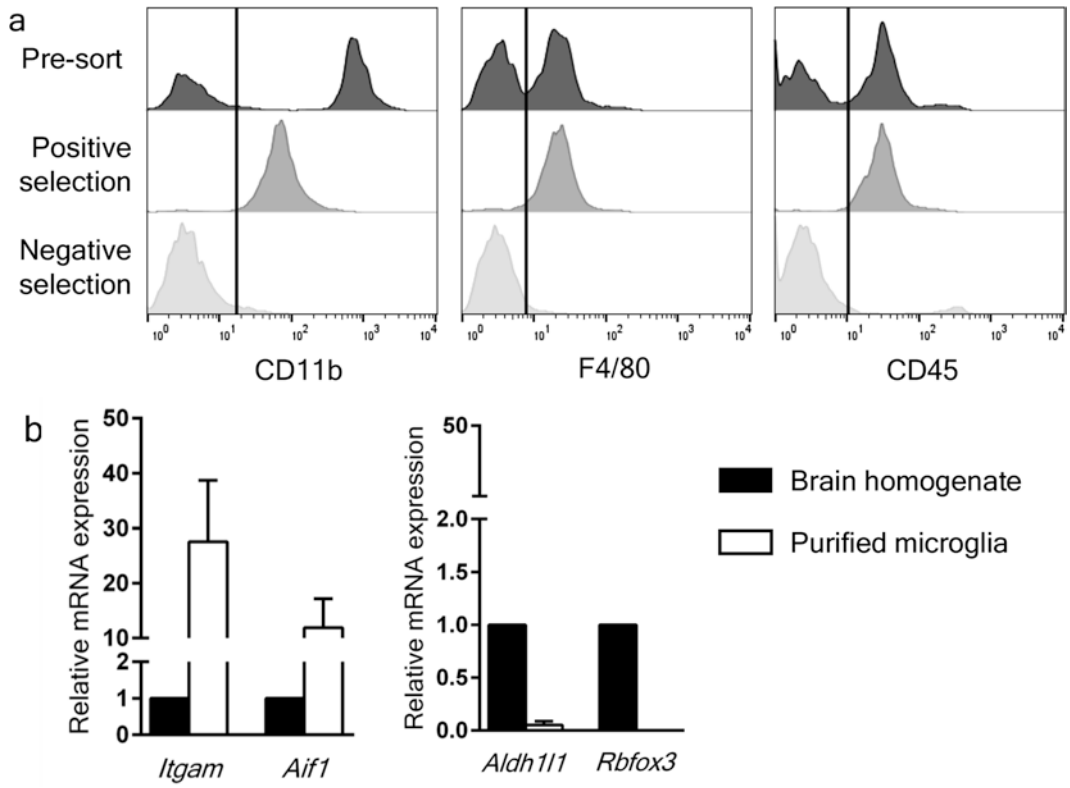

Fig. 3 Purity of adult microglia isolated from mouse whole brain. (**a**) Flow cytometric comparison of positive selected microglia with pre-sorted and nonselected samples demonstrates highly pure CD11b^{+} F4/80^{+} and CD45^{low} positive selected microglia and 1 log higher CD11b positivity in microglia before bead separation. Nonselected samples display a negligible loss. (**b**) Relative mRNA expression of microglial (*Itgam*, *Aif1*), astrocyte (*Aldh1l1*), and neuronal (*Rbfox3*) marker in whole-brain homogenates versus isolated microglia

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