Journal of Neuroscience Methods 340 (2020) 108742

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Isolation, culture, and downstream characterization of primary microglia and astrocytes from adult rodent brain and spinal cord

Nilesh M. Agalave[1], Brandon T. Lane[1], Prapti H. Mody, Thomas A. Szabo-Pardi, Michael D. Burton*

Neuroimmunology and Behavior Laboratory, School of Behavioral and Brain Sciences, Center for Advanced Pain Studies, University of Texas at Dallas, Richardson, Texas, USA

ARTICLE INFO

Keywords:
Microglia
Astrocytes
Isolation
Culture
Immunocytochemistry
Flow cytometry
ELISA
IL-6

ABSTRACT

Background: Neuroimmunologists aspire to understand the interactions between neurons, microglia, and astrocytes in the CNS. To study these cells, researchers work with either immortalized cell lines or primary cells acquired from animal tissue. Primary cells reflect *in vivo* characteristics and functionality compared to immortalized cells; however, they are challenging to acquire and maintain.

New Method: Established protocols to harvest primary glia use neonatal rodents, here we provide a method for simultaneously isolating microglia and astrocytes from brain and/or spinal cord from adult rodents. We utilized a discontinuous percoll density gradient enabling easy discrimination of these cell populations without enzymatic digestion or complex sorting techniques.

Results: We found cells isolated from the percoll interface between 70 %−50 % were microglia, as they express ionizing calcium-binding adaptor molecule 1 (Iba1) in immunocytochemistry and $CD11b^{hi}$ and $CD45^{lo}$ using flow cytometry. Isolated cells from the 50 %−30 % interface were astrocytes as they express glial fibrillary acidic protein (GFAP) in immunocytochemistry and Glutamate aspartate transporter (GLAST)-1 using flow cytometry. Cultured microglia and astrocytes showed a functional increase in IL-6 production after treatment of lipopolysaccharide (LPS).

Comparison with Existing Methods: Our method allows for rapid isolation of both microglia and astrocytes in one protocol with relatively few resources, preserves cellular phenotype, and yields high cell numbers without magnetic or antibody sorting.

Conclusion: Here we show a novel, single protocol to isolate microglia and astrocytes from brain and spinal cord tissue, allowing for culturing and other downstream applications from the cells of animals of various ages, which will be useful for researchers investigating these two major glial cell types from the brain or spinal cord of the same rodent.

1. Introduction

Glial cells are non-neuronal cell populations that provide structural, nutritional, and functional support to neurons in the central and peripheral nervous systems (Parpura et al., 2012). Two of the major glial cell types in the central nervous system (CNS) are microglia and astrocytes. The ubiquitous involvement of these cells in normal and pathological physiology has been demonstrated during development, maintenance, and aging processes (Clarke and Barres, 2013; Molofsky et al., 2012; Njie et al., 2012; Paolicelli et al., 2011; Sofroniew and Vinters, 2010). Microglia are specialized antigen presenting cells that serve as the primary line of defense against infections in the CNS (Rock et al., 2004). These cells play a dynamic role in CNS function and can shift activation states in both healthy and diseased CNS tissue (Nimmerjahn et al., 2005). Microglia express pathogen recognition receptors (PRRs) and inflammasomes to regulate the innate immune response during exogenous and sterile infections (Graeber and Streit, 2010; Patel et al., 2013; Rock et al., 2004). Microglia are intimately

Abbreviations: CNS, Central nervous system; PNS, Peripheral nervous system; Iba-1, Ionizing calcium-binding adaptor molecule 1; CD, Cluster of differentiation; GFAP, Glial fibrillary acidic protein; GLAST-1, Glutamate aspartate transporter 1; FBS, Fetal bovine serum; SIP, Standard isotonic percoll

* Corresponding author at: Neuroimmunology and Behavior Laboratory, School of Behavioral and Brain Sciences, Center for Advanced Pain Studies, University of Texas at Dallas, Richardson, 75080, Texas, USA.

E-mail address: Michael.Burton@utdallas.edu (M.D. Burton).

[1] These authors contributed equally to this manuscript.

https://doi.org/10.1016/j.jneumeth.2020.108742
Received 20 January 2020; Received in revised form 10 April 2020; Accepted 14 April 2020
Available online 19 April 2020

involved in chronic and acute neurological and immunological diseases (Heindl et al., 2018; Wang et al., 2015; Wen et al., 2011). In the developing nervous system, microglia play an active role in neuronal apoptosis, synaptic pruning, and synaptogenesis (Bessis et al., 2007; Paolicelli et al., 2011). Healthy adult microglia survey the local microenvironment for pathogens and cellular debris to phagocytose, scavenge metabolic factors, maintain synaptic homeostasis, and regulate synaptic plasticity (Kettenmann et al., 2013; Nimmerjahn et al., 2005; Njie et al., 2012).

Astrocytes also carry out many functions in healthy and diseased CNS tissue. During development, these cells induce synaptogenesis, facilitate synaptic maturation, and direct growth of vasculature (Bozoyan et al., 2012; Christopherson et al., 2005; Clarke and Barres, 2013). In adults, astrocytes play an important functional support role in neuronal transmission by regulating glutamate homeostasis, coordinating local blood flow, and maintaining ion and pH homeostasis in the CNS microenvironment (Agulhon et al., 2008; Sofroniew and Vinters, 2010). As a consequence of their vital role in normal functioning, disruption of these astrocytic functions is linked to a number of developmental and neurodegenerative diseases (Maragakis and Rothstein, 2006; Molofsky et al., 2012). Moreover, astrocytes play a role in CNS infection and inflammation by regulating both innate and adaptive immune responses and production of pro- and anti- inflammatory cytokines and chemokines (Dong and Benveniste, 2001; Farina et al., 2007; Marella and Chabry, 2004).

Researchers assessed the role of these cells by *in vitro* examination of either immortalized cell lines or by isolating primary cells directly from animals or donors (Timmerman et al., 2018). Immortalized microglia cell lines, BV2 (Blasi et al., 1990) and HAPI (Cheepsunthorn et al., 2001) as well as astrocyte cell lines MG5 (Kanazawa et al., 2002) and A7 (Geller and Dubois-Dalcq, 1988), had been deemed easier to work with than freshly isolated primary cells (Timmerman et al., 2018). Though these cell lines are more proliferative and easier to maintain than primary cells, immortalized cells are not appropriate for all applications (Galland et al., 2019). Numerous cell lines have been cultivated from neonatal animals, but young cells may not resemble the phenotype of adult or aged cells, as they have not yet undergone maturation (Timmerman et al., 2018). Additionally, the immortalization process can notably alter cells and disrupt normal physiological functioning (Das et al., 2016; Horvath et al., 2008). Primary cells, on the other hand, while more difficult to acquire and maintain, are not impacted by inserted DNA vectors or oncogenes. As such, primary cells are considered more physiologically normal than those derived from an immortal line (Pan et al., 2009). Acquisition of pure, viable cells in sufficient quantity is one of the core challenges of working with primary cells. There are a variety of protocols in the literature to isolate glial cells from CNS tissue of rodents, but the challenge is to produce pure, high yield samples in a timely, efficient manner (Cardona et al., 2006; Foo et al., 2011; Hamby et al., 2006; Moussaud and Draheim, 2010).

Typically, methods for isolation of primary microglia and astrocytes rely on enzymatic digestion, immuno-panning, or magnetic sorting to isolate the target cells (Cardona et al., 2006; Foo et al., 2011; Orre et al., 2014; Tamashiro et al., 2012). The method described here can be used to circumvent prolonged and expensive techniques like mechanical shaking, magnetic, or fluorescent separation, by utilizing a discontinuous percoll gradient to separate both astrocytes and microglia from other CNS elements. Additionally, this method does not require enzymatic digestion that may alter cell surface proteins and further disrupt cellular viability (Garaud et al., 2014). Here, we demonstrate the use of a percoll density gradient to isolate primary microglia and astrocytes from adult mice and rats (Fig. 1).

2. Materials and methods

2.1. Animals

Adult B6.129P2(Cg)-Cx3cr1$^{tm2.1(cre/ERT2)Litt}$/WganJ mice (10–14 weeks old), stock number 021,160 (Cx3cr1^{CreER}) and normal WT mice purchased from the Jackson Laboratory and maintained in our animal facility at the University of Texas at Dallas were used for this study. In brief, we utilized the IRES-YFP fused element as a reporter tag in microglia that is independent to *Cre* induction. Adult F1 generation bred Fischer 344 × Brown Norway rats between 12 and 16 weeks were used. These animals were purchased from Charles Rivers and bred in the animal facility at the University of Texas at Dallas. Fischer 344 females were bred with Brown Norway males. All animals were housed under a reverse-phase 12 -h light-dark cycle at 22 °C and allowed access to food and water *ad libitum*. All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee at University of Texas at Dallas, Richardson (TX) USA.

2.2. Reagents

Isotonic percoll dilutions were made by diluting stock percoll (GE-healthcare, 17 − 0891 − 01) at a 9:1 ratio with 10X phosphate buffered saline (PBS) to make 1.12 g/mL stock isotonic percoll (SIP), which we designate 100 % SIP. The remaining layers were created by diluting the 1.12 g/mL SIP with 1X Dulbecco's phosphate buffered saline (DPBS) to make a 1.08 g/mL (70 % SIP), a 1.06 g/mL (50 % SIP), and a 1.05 g/mL (35 % SIP). All gradient layers were prepared and allowed to reach room temperature before use. Moreover, for cell culture experiments, we autoclaved all reagents for sterility.

2.3. Isolation of cells

Rodents were deeply anesthetized using isoflurane and decapitated per the University of Texas at Dallas IACUC approval 2 measures. Rodents in these experiments were not perfused, though perfusion may be used to reduce the presence of blood mononuclear cells. The skull cap was removed to expose and extract the brain. Spinal column was cut at the level of the iliac crest and a 21-gauge needle (18-gauge in rats) was used to hydro-extrude the spinal cord with sterile 1X DPBS. Both tissues were collected and transferred in sterile 1X DPBS on ice until homogenization. To maximize cell yield when investigating CNS glia, we combine both tissues for homogenization. However, spinal cord and brain may be separately used for extracting target cells if region-specific cell populations are more relevant to the experiment.

Tissues were mechanically homogenized and passed through a 70 μm nylon cell strainer (Millipore Sigma, Z742103 − 50EA) with approximately 10 − 15 mL of sterile 1X DPBS supplemented with 0.2 % glucose (Millipore Sigma, G7528) into a 50 mL conical tube. The use of excessive force in this step can damage both the tissue and the cell strainer and result in low yield. The homogenate was then centrifuged at 600 × g for 6 min at room temperature (RT). All of the resulting supernatant was decanted carefully and the pellet resuspended in 6 mL of sterile 70 % SIP. The resuspended homogenate was transferred to a sterile 15 mL polypropylene conical tube and 3 mL of sterile 50 % SIP was carefully layered over. Another 3 mL of sterile 35 % SIP was carefully layered on top of the 50 % SIP layer, and 2 mL of sterile 1X DPBS was layered on top of the 35 % layer. Droplets of liquid falling from any distance will compromise the gradient, so it is recommended to keep the tip of the transfer pipette as close to the surface of the underlying layer as possible while overlaying the next layer. Alternatively, an underlay method may be used in which each layer is inserted below the previous layer in the reverse order.

The prepared 15 mL conical tubes were then centrifuged at 2000 × g for 20 min at RT without brake. Three discrete layers were established

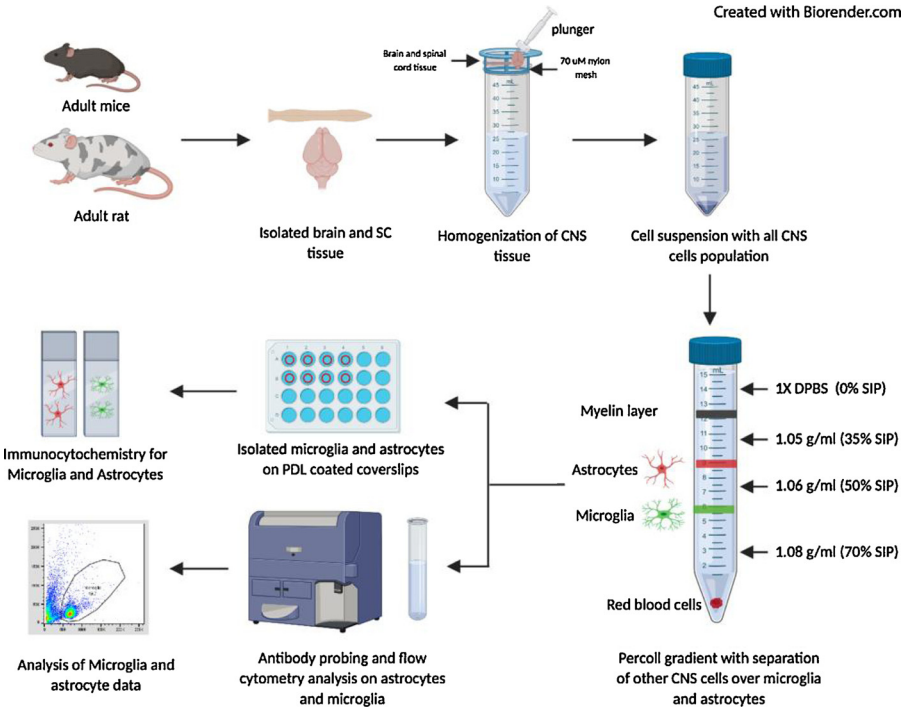

Fig. 1. An illustration showing the experimental procedure for extracting microglia and astrocytes from rodent brain and spinal cord. Whole brain and spinal cord were collected from rodents and homogenized by passing through a 70 µm nylon mesh. Cell homogenates were subjected to centrifugation in percoll gradients and cell populations were collected from 70 % – 50 % and 50 % – 35 % interfaces. These extracted cell populations were then characterized by immunocytochemistry, flow cytometry, or ELISA. (Illustration created with Biorender.com).

Table 1
List of antibodies used for immunocytochemistry and their information.

Antibody	Host	Dilution	Vendor	Cat. No.
Iba1	Rabbit	1:1000	Wako	019 – 19741
GFP	Chicken	1:1000	Aves Labs	GFP-1010
GFAP	Rabbit	1:1000	Dako	Z0334

after centrifugation. The top layer of myelin was discarded and cells from the interface between 70 % – 50 % SIP and 50 % – 35 % SIP were collected in separate conical tubes. Isolated cells were resuspended in sterile 1X DPBS and centrifuged at 600 x g for 6 min at RT to remove any remaining percoll. Washed cells were immediately subjected to used for downstream applications: cell culture (Immunohistochemistry and IL-6) and flow cytometry analysis.

2.4. Cell culture

The two isolated cell populations were washed with sterile 1X DPBS, and resuspended in DME/F12 media (Hyclone, SH30023.01) supplemented with 10 % fetal bovine serum (FBS) (Hyclone, SH30088031R) and 1 % Penicillin/Streptomycin (ThermoFisher, 15,070,063). Cells were counted using 0.5 % Trypan blue dye (Lonza, 17 – 942E) and a hemocytometer, and 100,000 cells per well were seeded onto 12 mm coverslips previously coated with poly-D-lysine hydrobromide (Millipore Sigma, Cat No. P0899) in a 24-well plate. Poly-D-lysine was used to avoid potential proteolytic degradation seen when using poly-L-lysine with some cells. Cells were maintained in a humidified incubator

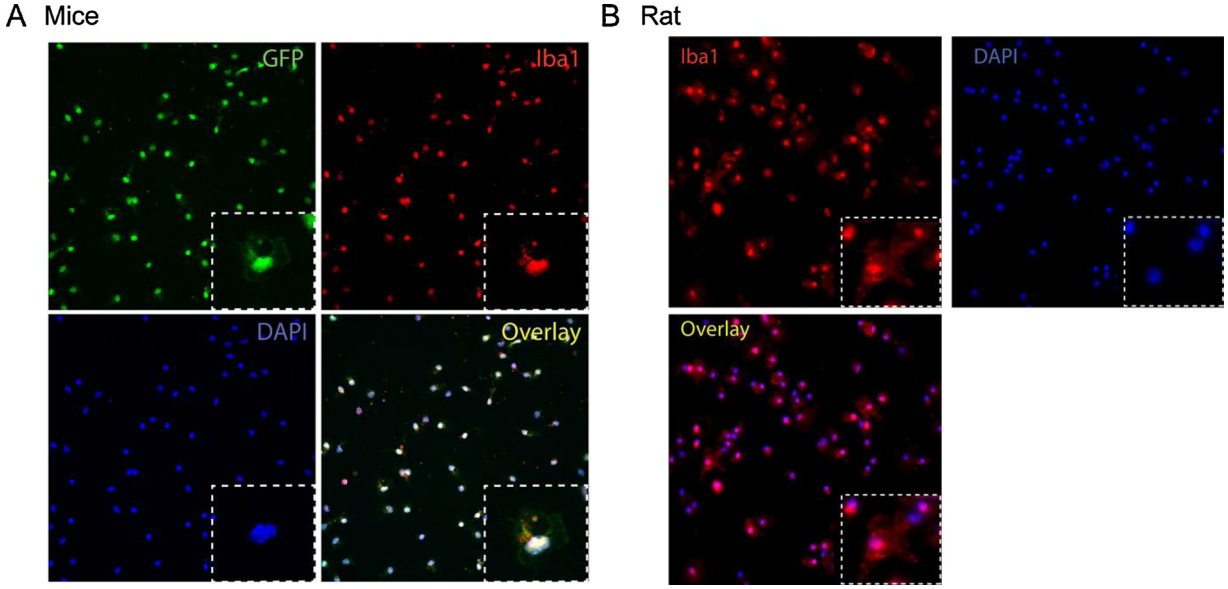

Fig. 2. Immunocytochemistry for Iba1 (microglial marker) on cells isolated from 70 % – 50 % SIP interface. A) Immunofluorescent labeling of Iba1, GFP, and DAPI in cells isolated from mice. (B) Immunofluorescent labeling for Iba1 and DAPI in cells isolated from rats.

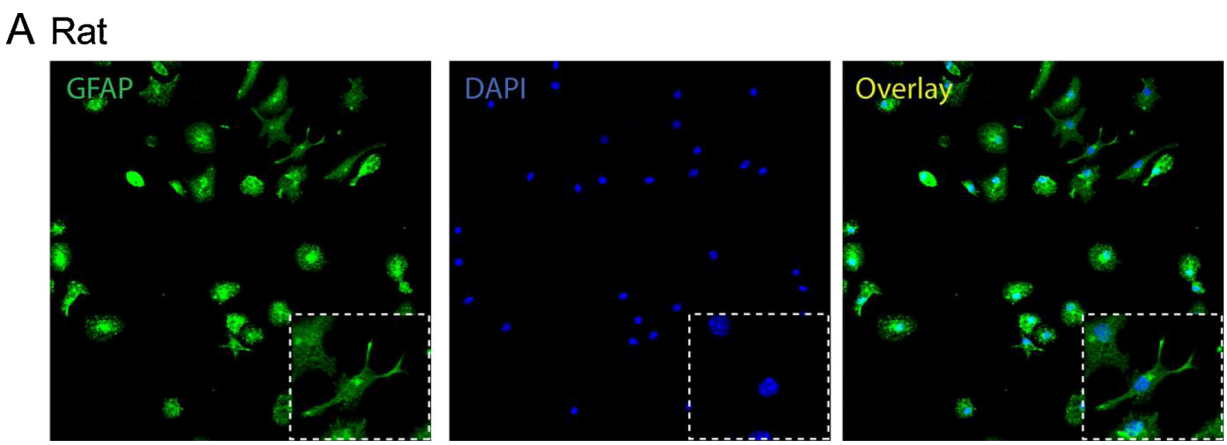

Fig. 3. Immunocytochemistry for GFAP (astrocyte marker) on cells isolated from 50 % – 35 % SIP interface. A) Immunofluorescent labeling of GFAP and DAPI in cells isolated from rats.

at 37 °C with 5 % CO_2. Culture media was changed every other day until the end of the experiment. Cells are ready to use after replacing media on Day 1. Immunohistochemistry and ELISA experiments was performed on Day 3 or 4 for both cell types.

2.5. Immunocytochemistry

Post-culture day 3 (microglia) and post-culture day 5 (astrocytes), the cells were incubated with 1X PBS twice for 10 min each before being fixed with 4 % paraformaldehyde at RT for 15 min. Cells were washed with 1X PBS containing 0.05 % Tween20, to remove leftover fixative. Cells were blocked in blocking solution (3 % Normal goat serum, 2 % Bovine serum albumin, 1 % Triton-X100, 1 % Tween-20, and 0.05 % sodium azide in 1X PBS) for one hour. Primary antibodies (Table 1) were added to the respective wells and incubated overnight at 4 °C.

After primary antibody incubation, the cells were washed with 1X PBS containing 0.05 % Tween-20, then incubated in respective secondary antibodies for 2 h at RT. The cells were washed with 1X PBS before a one-minute incubation with DAPI nuclear stain (Millipore Sigma, D9542). Finally, the cells were washed with 1X PBS and mounted onto the glass slide with Gelvatol mounting media (Sigma, P8136). Images were acquired on an Olympus confocal microscope (Olympus, Tokyo, Japan).

2.6. Flow cytometry

Isolated cells from the interface between 70 % and 50 % SIP were subjected to microglia-specific staining and cells from 50 % to 35 % SIP interface were stained for astrocyte markers. Cells were washed twice with 1X PBS, centrifuged at 600 x g followed by resuspension in flow buffer (0.5 % bovine serum albumin + 0.02 % glucose in 1X PBS). Cells were incubated in blocking buffer (Cd16/32 antibody, eBioscience, 16 – 0161-85) for 10 min to block the Fc receptor and avoid non-specific binding. Primary antibody cocktails were prepared for anti-mouse Cd11b-APC-cy7 (Life Technology, A15390), anti-rat Cd11b/c-PE (BD Pharminogen, 554,862), anti-mouse Cd45-Brilliant violet 421 (eBioscience, 103,133) for microglia and anti-mouse GLAST-1-APC (Miltenyi Biotech, 130 – 095-814) for astrocytes at 1:400 dilution. Microglia and astrocytes were incubated in primary antibody cocktails for 40 min at 4 °C in the dark. Stained cells were examined using the BD Fortessa flow cytometer (BD Bioscience, San Diego, CA) and analyzed using FlowJo software (San Carlos, CA).

2.7. IL-6 ELISA

After 3 days, media without FBS (serum-starved) was added to the culture for approximately 14 – 18 h. Microglia were stimulated with 1 μg/mL and astrocytes were stimulated with 0.5 μg/mL of LPS (a potent toll like receptor 4 ligand) or vehicle and incubated for 24 h in a humidified incubator at 37 °C with 5 % CO_2. After 24 h, media supernatants were collected and subjected to IL-6 analysis according to the manufacturer's instructions (R&D system, Cat no- DY 406).

2.8. Recommendations for isolation procedure

Some critical aspects of this protocol must be given special attention in order to obtain pure, viable cells. This protocol is sensitive to deviations in percoll density due to temperature and incomplete centrifugation, decantation, or aspiration. If clear and distinct cell layers are not obtained after gradient centrifugation, then the yield and perhaps purity of the isolation will be unsatisfactory. Care must be taken to ensure that the isolation is completed within five hours from the start of tissue collection. Prolonging the protocol will result in diminished cell viability.

3. Results

3.1. Expression of microglia (Iba1) and astrocyte (GFAP) in cells isolated from the interface between 70 % – 50 % and 50 % – 35 % SIP, respectively

To identify the cell populations from the discrete interfaces, we used immunofluorescent staining on cultured cells. Microglia can be distinguished from other CNS elements by their expression of ionized calcium binding protein (Iba1) (Ito et al., 1998). Cells isolated from the interface between the 70 % – 50 % were subjected to immunolabeling using an antibody against Iba1 as well as an antibody against GFP reporter tag. All DAPI positive cells present on the coverslip were positive for Iba1 and GFP staining for both mice (Fig. 2A) and rats (Fig. 2B).

Cells collected from the interface between the 50 % – 35 % SIP layers were subjected to GFAP staining, a known astrocyte marker (Cahoy et al., 2008). Cells isolated from 50 % – 35 % SIP interface were positively stained for GFAP and DAPI for mice (data not shown) and rats (Fig. 3).

3.2. Flow cytometry analysis shows the expression of Cd11bhi and Cd45low positive cells in interface between 70 % – 50 % SIP

To further validate the distinct cell populations, we used flow cytometry to characterize the cells at the different percoll interfaces.

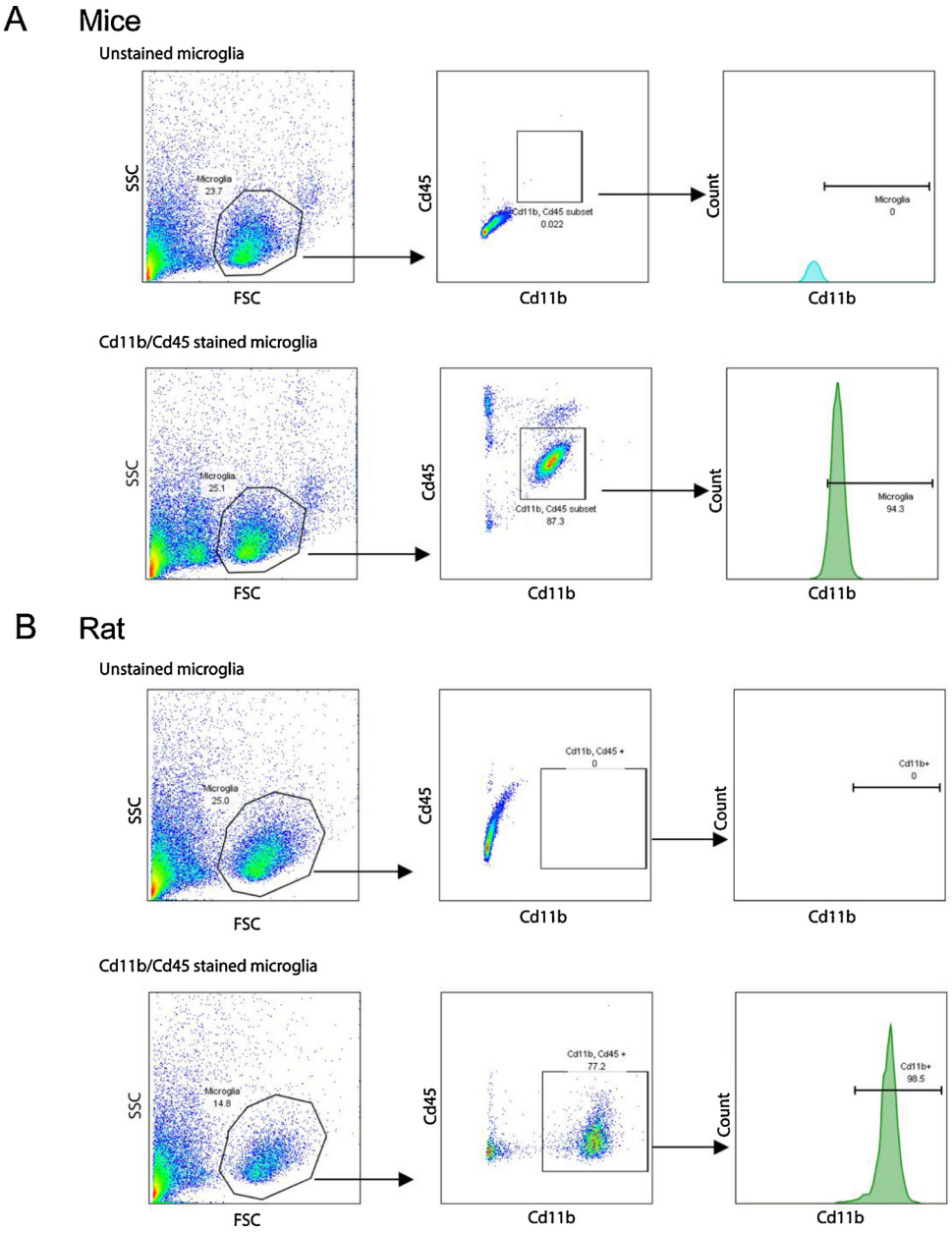

Fig. 4. Flow cytometry analysis on cells isolated from 70 %−50 % SIP interface. Isolated cells from mice (A) and rats (B) shows expression of $Cd11b^{+}$ and $Cd45^{low}$ (microglial marker) compared to respective unstained control.

Isolated cells from the 70 %−50 % SIP interface were subjected to antibody labeling using CD11b and CD45. We found a distinct $CD11b^{hi}$ and $CD45^{low}$ cell population in mice (Fig. 4A) as well as in rats (Fig. 4B). CD11b expression is higher in microglia than perivascular macrophages, supraependymal macrophages, and other phagocytic cells (Ford et al., 1995; Martin et al., 2017). Infiltrating monocyte-lineage cells show high CD45 expression, while resident microglia express low levels of CD45 (Greter et al., 2015). The flow cytometry data indicate that the cell population at the interface between 70-%-50 % SIP layers are microglia.

3.3. Flow cytometry analysis shows the expression of GLAST-1 positive cells in the interface between 50 % and 35 % SIP

In addition to GFAP, Glutamate Aspartate Transporter 1 (GLAST-1) is a commonly used marker to identify astrocyte cell populations, as astrocytes are major glutamate regulators in the CNS (Perego et al., 2000; Shibata et al., 1997). We stained the cell population from the 50 %−35 % interface with GLAST-1 and found that cells from that layer were positive for GLAST-1 in mice (Fig. 5A) and in rats (Fig. 5B). Flow cytometry data indicates that cells from the 50 %−35 % SIP interface were astrocytes.

3.4. Isolated microglial and astrocytes show production of interleukin 6 after LPS stimulation

To validate functionality of the isolated microglia and astrocytes, we performed an ELISA to check the capability of these cells to produce cytokines after stimulation. Microglia (1 μg/mL) and astrocytes (0.5 μg/mL) were stimulated with LPS for 24 h. Media supernatants were collected and subjected to an IL-6 ELISA. We found a statistically significant increase in IL-6 level after LPS treatment in microglia (9699 ± 300.6 vs 2669 ± 1056, n = 4 per group, unpaired *t*-test $***p < 0.0001$) (Fig. 6A) and astrocytes (222.5 ± 26.49 vs

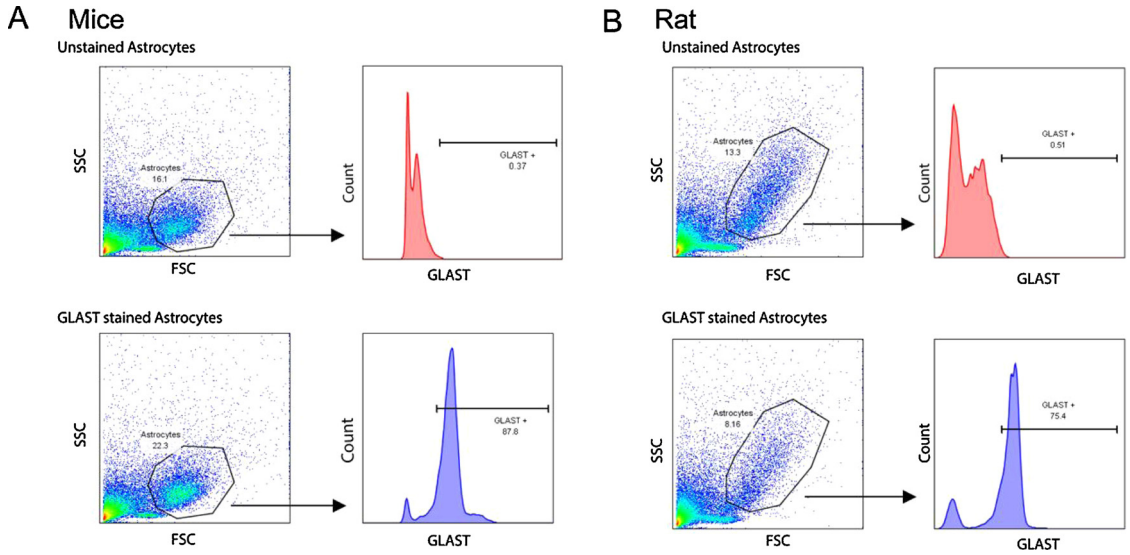

Fig. 5. Flow cytometry analysis on the cells isolated from 50 %−35 % SIP interface. Isolated cells from mice (A) and rats (B) shows the expression of GLAST compared to respective unstained control.

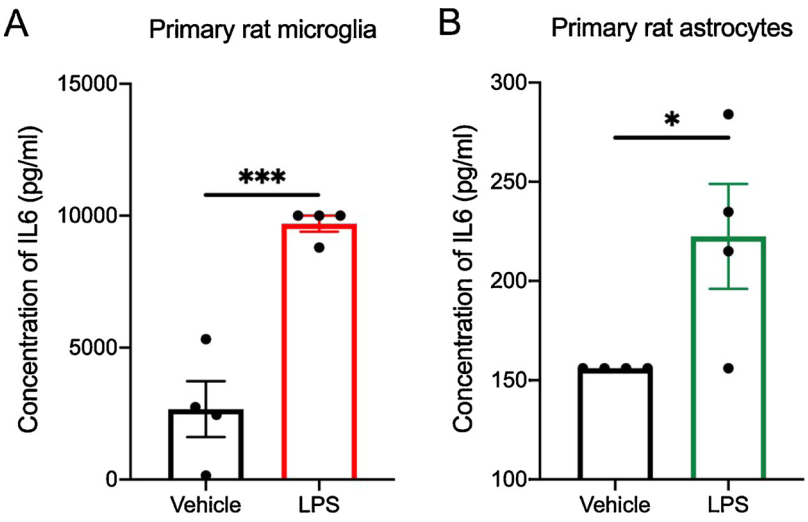

Fig. 6. Isolated primary microglia and astrocytes cells stimulated with LPS, to assess functionality. (A) Microglia produce significant increase in level of IL-6 after LPS stimulation (1 µg/mL, n = 4 animals per group, unpaired *t*-test ***$p < 0.0001$. (B) Astrocytes produce significant increase in level of IL-6 after LPS stimulation (0.5µg/mL, n = 4 animals per group, unpaired *t*-test *$p < 0.05$).

156.0 ± 0.00, n = 4 per group, unpaired *t*-test *$p < 0.05$) (Fig. 6B). These data indicate that isolated microglia and astrocytes from our protocol are functional and can be used to perform downstream assays.

4. Discussion

We have developed a protocol for isolating microglia and astrocytes simultaneously from homogenized CNS tissue of adult rats and mice by density-based percoll separation. Unlike immunopanning methods which target a single cell population, density gradients allow for isolation of both microglia and astrocytes from one animal with a single procedure (Collins and Bohlen, 2018). Previously, other groups have used a similar strategy to isolate microglia and mixed glial cells using a different gradients of SIP (Cardona et al., 2006; Martin et al., 2017). Cardona et al. used 70 %−37 %−30 % SIP to isolate microglia (Cardona et al., 2006), whereas Moussaud et al., used 20 % SIP to isolate all glial cells from whole CNS suspension (Moussaud and Draheim, 2010). We have modified the concentration of stock SIP and changing the concentration gradient to 70 %−50 %−35 % to discretely isolate microglia and astrocytes separately from the whole CNS cell suspension. These discrete populations can be acquired without the expense of fluorescent antibodies or magnetic beads, and without the risk of cellular activation from antibody binding or the comparatively harsh sorting procedure (Dugas et al., 2008). Previous protocols have employed enzymatic digestion steps to dissociate glial cells from CNS tissue (Cardona et al., 2006; Ford et al., 1995; Martin et al., 2017). However, it has been reported that enzymatic treatment has a mild toxic effect on live cells that affects cell viability. Addition to this, enzymatic treatment to dissociate these cells damages cell surface epitopes, which can interfere with downstream applications (Greter et al., 2015; Perego et al., 2000; Shibata et al., 1997). We dissociate the CNS tissue by passing tissue samples through a 70 µm nylon mesh which protects the cell surface markers from being damaged and increases the viability of cells. Using this procedure, we can exclude/eliminate extra steps such as enzymatic digestion, immunopanning, and magnetic sorting techniques to isolate these cell populations are unnecessary.

Isolated microglia and astrocytes were characterized using immunocytochemistry and flow cytometric analysis. Immediately after isolation, microglia and astrocytes were subjected to flow cytometry and were found to express appropriate levels of characteristic surface proteins. In addition, cells not used in flow cytometry experiments were kept in culture for 3 or 5 days before immunolabelling to demonstrate the continued usability of the cells. Functionality of the cells were determined by treating both populations with LPS to verify if these cells

had intact receptor signaling. We found, microglia and astrocytes stimulated with LPS showed significant increases in IL-6 production. As in previously published data from our group, cells were indeed healthy after isolation and here we demonstrate that both microglia and astrocytes are able to mount a robust response to LPS treatment with production of IL-6 (Burton et al., 2016, 2013; Elmore et al., 2014). Important to note, time in culture and serum levels can dictate levels of basal activation from microglia and astrocytes. Initial optimization and appropriate experimental controls will need to be utilized to assess differences in basal activation for downstream applications.

5. Conclusion

Many previously established protocols rely on enzymatic digestion of CNS homogenates, which can negatively impact cell viability and yield (Cardona et al., 2006; Moussaud and Draheim, 2010; Sedgwick et al., 1991). Other isolation methods utilize magnetic or fluorescent sorting techniques that are time-consuming and resource intensive (Orre et al., 2014). The protocol provided allows for the isolation of both microglia and astrocytes in one procedure without the use of enzymes or sorting. Brain and spinal cord homogenates were used here to maximize cell yield, which is typically 500,000–1,000,000 of microglia and over 5,000,000 astrocytes per animal, but yield will vary depending on the tissues included. The minimal amount of resources needed, coupled with the purity and viability of the resultant isolated primary cells, make this protocol suitable for several downstream applications in addition to cell culture, immunocytochemistry, and flow cytometry demonstrated here.

Author contributions

All authors have read, discussed, and approved the manuscript for submission. NMA, BL, and TSP optimized the isolation protocol. NMA and BL conducted immunocytochemistry and flow cytometry experiments. TSP conducted the ELISA. NMA, BL, TSP, and PHM formatted figures and analyzed data. NMA, BL, PHM, and TSP have written the manuscript. MDB had major oversight over the entire project: design, optimization, data acquisition, analysis, and writing.

CRediT authorship contribution statement

Nilesh M. Agalave: Methodology, Data curation, Formal analysis, Writing - original draft. **Brandon T. Lane:** Methodology, Data curation. **Prapti H. Mody:** Data curation, Formal analysis. **Thomas A. Szabo-Pardi:** Methodology, Data curation, Formal analysis. **Michael D. Burton:** Conceptualization, Methodology, Data curation, Formal analysis, Writing.

Declaration of Competing Interest

The authors have declared no conflict of interest.

Acknowledgements

This work was supported by NIH grant K22NS096030 (MDB), American Pain Society Future Leaders Grant (MDB), Rita Allen Foundation Award in Pain (MDB) and The University of Texas System STARS program research support grant (MDB)

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jneumeth.2020.108742.

References

Agulhon, C., Petravicz, J., McMullen, A.B., Sweger, E.J., Minton, S.K., Taves, S.R., Casper, K.B., Fiacco, T.A., McCarthy, K.D., 2008. What is the role of astrocyte calcium in neurophysiology? Neuron 59, 932–946.
Bessis, A., Bechade, C., Bernard, D., Roumier, A., 2007. Microglial control of neuronal death and synaptic properties. Glia 55, 233–238.
Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., Bistoni, F., 1990. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J. Neuroimmunol. 27, 229–237.
Bozoyan, L., Khlghatyan, J., Saghatelyan, A., 2012. Astrocytes control the development of the migration-promoting vasculature scaffold in the postnatal brain via VEGF signaling. J. Neurosci. 32, 1687–1704.
Burton, M.D., Rytych, J.L., Freund, G.G., Johnson, R.W., 2013. Central inhibition of interleukin-6 trans-signaling during peripheral infection reduced neuroinflammation and sickness in aged mice. Brain Behav. Immun. 30, 66–72.
Burton, M., Rytych, J., Johnson, R.A., 2016. Dietary luteolin reduces proinflammatory microglia in the brain of senescent mice. Rejuvenation Res. 19.
Cahoy, J.D., Emery, B., Kaushal, A., Foo, L.C., Zamanian, J.L., Christopherson, K.S., Xing, Y., Lubischer, J.L., Krieg, P.A., Krupenko, S.A., Thompson, W.J., Barres, B.A., 2008. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J. Neurosci. 28, 264–278.
Cardona, A.E., Huang, D., Sasse, M.E., Ransohoff, R.M., 2006. Isolation of murine microglial cells for RNA analysis or flow cytometry. Nat. Protoc. 1, 1947–1951.
Cheepsunthorn, P., Radov, L., Menzies, S., Reid, J., Connor, J.R., 2001. Characterization of a novel brain-derived microglial cell line isolated from neonatal rat brain. Glia 35, 53–62.
Christopherson, K.S., Ullian, E.M., Stokes, C.C., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., Barres, B.A., 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120, 421–433.
Clarke, L.E., Barres, B.A., 2013. Emerging roles of astrocytes in neural circuit development. Nat. Rev. Neurosci. 14, 311–321.
Collins, H.Y., Bohlen, C.J., 2018. Isolation and culture of rodent microglia to promote a dynamic ramified morphology in serum-free medium. J. Vis. Exp.
Das, A., Kim, S.H., Arifuzzaman, S., Yoon, T., Chai, J.C., Lee, Y.S., Park, K.S., Jung, K.H., Chai, Y.G., 2016. Transcriptome sequencing reveals that LPS-triggered transcriptional responses in established microglia BV2 cell lines are poorly representative of primary microglia. J. Neuroinflammation 13, 182.
Dong, Y., Benveniste, E.N., 2001. Immune function of astrocytes. Glia 36, 180–190.
Dugas, J.C., Mandemakers, W., Rogers, M., Ibrahim, A., Daneman, R., Barres, B.A., 2008. A novel purification method for CNS projection neurons leads to the identification of brain vascular cells as a source of trophic support for corticospinal motor neurons. J. Neurosci. 28, 8294–8305.
Elmore, M.R., Burton, M.D., Conrad, M.S., Rytych, J.L., Van Alstine, W.G., Johnson, R.W., 2014. Respiratory viral infection in neonatal piglets causes marked microglia activation in the hippocampus and deficits in spatial learning. J. Neurosci. 34, 2120–2129.
Farina, C., Aloisi, F., Meinl, E., 2007. Astrocytes are active players in cerebral innate immunity. Trends Immunol. 28, 138–145.
Foo, L.C., Allen, N.J., Bushong, E.A., Ventura, P.B., Chung, W.S., Zhou, L., Cahoy, J.D., Daneman, R., Zong, H., Ellisman, M.H., Barres, B.A., 2011. Development of a method for the purification and culture of rodent astrocytes. Neuron 71, 799–811.
Ford, A.L., Goodsall, A.L., Hickey, W.F., Sedgwick, J.D., 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.
Galland, F., Seady, M., Taday, J., Smaili, S.S., Goncalves, C.A., Leite, M.C., 2019. Astrocyte culture models: molecular and function characterization of primary culture, immortalized astrocytes and C6 glioma cells. Neurochem. Int. 131, 104538 epub ahead of print.
Garaud, S., Gu-Trantien, C., Lodewyckx, J.N., Boisson, A., De Silva, P., Buisseret, L., Migliori, E., Libin, M., Naveaux, C., Duvillier, H., Willard-Gallo, K., 2014. A simple and rapid protocol to non-enzymatically dissociate fresh human tissues for the analysis of infiltrating lymphocytes. J. Vis. Exp.
Geller, H.M., Dubois-Dalcq, M., 1988. Antigenic and functional characterization of a rat central nervous system-derived cell line immortalized by a retroviral vector. J. Cell Biol. 107, 1977–1986.
Graeber, M.B., Streit, W.J., 2010. Microglia: biology and pathology. Acta Neuropathol. 119, 89–105.
Greter, M., Lelios, I., Croxford, A.L., 2015. Microglia versus myeloid cell nomenclature during brain inflammation. Front. Immunol. 6, 249.
Hamby, M.E., Uliasz, T.F., Hewett, S.J., Hewett, S.J.A., 2006. Characterization of an improved procedure for the removal of microglia from confluent monolayers of primary astrocytes. J. Neurosci. Methods 150, 128–137.
Heindl, S., Gesierich, B., Benakis, C., Llovera, G., Duering, M., Liesz, A., 2018. Automated morphological analysis of microglia after stroke. Front. Cell. Neurosci. 12, 106.
Horvath, R.J., Nutile-McMenemy, N., Alkaitis, M.S., Deleo, J.A., 2008. Differential migration, LPS-induced cytokine, chemokine, and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures. J. Neurochem. 107, 557–569.
Ito, D., Imai, Y., Ohsawa, K., Nakajima, K., Fukuuchi, Y., Kohsaka, S., 1998. Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res. Mol. Brain Res. 57, 1–9.
Kanazawa, H., Ohsawa, K., Sasaki, Y., Kohsaka, S., Imai, Y., 2002. Macrophage/microglia-specific protein Iba1 enhances membrane ruffling and Rac activation via

phospholipase C-gamma -dependent pathway. J. Biol. Chem. 277, 20026–20032.
Kettenmann, H., Kirchhoff, F., Verkhratsky, A., 2013. Microglia: new roles for the synaptic stripper. Neuron 77, 10–18.
Maragakis, N.J., Rothstein, J.D., 2006. Mechanisms of Disease: astrocytes in neurodegenerative disease. Nat. Clin. Pract. Neurol. 2, 679–689.
Marella, M., Chabry, J., 2004. Neurons and astrocytes respond to prion infection by inducing microglia recruitment. J. Neurosci. 24, 620–627.
Martin, E., El-Behi, M., Fontaine, B., Delarasse, C., 2017. Analysis of microglia and monocyte-derived macrophages from the central nervous system by flow cytometry. J. Vis. Exp.
Molofsky, A.V., Krencik, R., Ullian, E.M., Tsai, H.H., Deneen, B., Richardson, W.D., Barres, B.A., Rowitch, D.H., 2012. Astrocytes and disease: a neurodevelopmental perspective. Genes Dev. 26, 891–907.
Moussaud, S., Draheim, H.J., 2010. A new method to isolate microglia from adult mice and culture them for an extended period of time. J. Neurosci. Methods 187, 243–253.
Nimmerjahn, A., Kirchhoff, F., Helmchen, F., 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308, 1314–1318.
Njie, E.G., Boelen, E., Stassen, F.R., Steinbusch, H.W., Borchelt, D.R., Streit, W.J., 2012. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. Neurobiol. Aging 33 (195), e1–12.
Orre, M., Kamphuis, W., Osborn, L.M., Melief, J., Kooijman, L., Huitinga, I., Klooster, J., Bossers, K., Hol, E.M., 2014. Acute isolation and transcriptome characterization of cortical astrocytes and microglia from young and aged mice. Neurobiol. Aging 35, 1–14.
Pan, C., Kumar, C., Bohl, S., Klingmueller, U., Mann, M., 2009. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. Mol. Cell Proteomics 8, 443–450.
Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., Ragozzino, D., Gross, C.T., 2011. Synaptic pruning by microglia is necessary for normal brain development. Science 333, 1456–1458.
Parpura, V., Heneka, M.T., Montana, V., Oliet, S.H., Schousboe, A., Haydon, P.G., Stout Jr., R.F., Spray, D.C., Reichenbach, A., Pannicke, T., Pekny, M., Pekna, M., Zorec, R., Verkhratsky, A., 2012. Glial cells in (patho)physiology. J. Neurochem. 121, 4–27.
Patel, A.R., Ritzel, R., McCullough, L.D., Liu, F., 2013. Microglia and ischemic stroke: a double-edged sword. Int. J. Physiol. Pathophysiol. Pharmacol. 5, 73–90.
Perego, C., Vanoni, C., Bossi, M., Massari, S., Basudev, H., Longhi, R., Pietrini, G., 2000. The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal co-cultures. J. Neurochem. 75, 1076–1084.
Rock, R.B., Gekker, G., Hu, S., Sheng, W.S., Cheeran, M., Lokensgard, J.R., Peterson, P.K., 2004. Role of microglia in central nervous system infections. Clin. Microbiol. Rev. 17, 942–964 table of contents.
Sedgwick, J.D., Schwender, S., Imrich, H., Dorries, R., Butcher, G.W., 1991. Ter Meulen V. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proc Natl Acad Sci U S A 88, 7438–7442.
Shibata, T., Yamada, K., Watanabe, M., Ikenaka, K., Wada, K., Tanaka, K., Inoue, Y., 1997. Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. J. Neurosci. 17, 9212–9219.
Sofroniew, M.V., Vinters, H.V., 2010. Astrocytes: biology and pathology. Acta Neuropathol. 119, 7–35.
Tamashiro, T.T., Dalgard, C.L., Byrnes, K.R., 2012. Primary microglia isolation from mixed glial cell cultures of neonatal rat brain tissue. J. Vis. Exp. e3814.
Timmerman, R., Burm, S.M., Bajramovic, J.J., 2018. An overview of in vitro methods to study microglia. Front. Cell. Neurosci. 12, 242.
Wang, W.Y., Tan, M.S., Yu, J.T., Tan, L., 2015. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann. Transl. Med. 3, 136.
Wen, Y.R., Tan, P.H., Cheng, J.K., Liu, Y.C., Ji, R.R., 2011. Microglia: a promising target for treating neuropathic and postoperative pain, and morphine tolerance. J. Formos. Med. Assoc. 110, 487–494.