Video Article

Characterization and Isolation of Mouse Primary Microglia by Density Gradient Centrifugation

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Abstract

Microglia, the resident immune cells in the brain, are the first responders to inflammation or injury in the central nervous system. Recent research has revealed microglia to be dynamic, capable of assuming both pro-inflammatory and anti-inflammatory phenotypes. Both M1 (pro-inflammatory) and M2 (pro-reparative) phenotypes play an important role in neuroinflammatory conditions such as perinatal brain injury, and exhibit differing functions in response to certain environmental stimuli. The modulation of microglial activation has been noted to confer neuroprotection thus suggesting microglia may have therapeutic potential in brain injury. However, more research is required to better understand the role of microglia in disease, and this protocol facilitates that. The protocol described below combines a density gradient centrifugation process to reduce cellular debris, with magnetic separation, producing a highly pure sample of primary microglial cells that can be used for *in vitro* experimentation, without the need for 2-3 weeks culturing. Additionally, the characterization steps yield robust functional data about microglia, aiding studies to better our understanding of the polarization and priming of these cells, which has strong implications in the field of regenerative medicine.

Video Link

The video component of this article can be found at https://www.jove.com/video/57065/

Introduction

Damage acquired during the perinatal period from inflammation, hypoxic-ischaemia and haemorrhage can have an array of long term sequelae. The complex pathophysiology of perinatal brain injury is theorized to involve inflammation and ischemia with ensuing neuronal and axonal death[1]. The innate immune response plays an important role in the cascade of events leading to injury[2].

Microglia, the resident immune cells within the central nervous system (CNS), are the first responders to injury[3]. Microglia are plastic cell types with the capacity to be both protective or toxic, dependent on the environment[4]. They are involved in chemotaxis, phagocytosis, antigen presentation and production of cytokines and reactive oxygen species[4,5]. Senescent microglia constantly survey the environment and are activated by the presence of a foreign or harmful substance[4]. Activation leads to a pro-inflammatory response, critical in CNS protection[4]. These M1 "pro-inflammatory" phenotype microglia are primarily involved in antigen presentation and death of pathogens[4]. Despite the crucial role of the inflammatory response in neuroprotection, uncontrolled or prolonged inflammation can be harmful and lead to neuronal damage[4]. However, when exposed to certain environmental stimuli, microglia can exhibit an anti-inflammatory phenotype. These pro-reparative M2 microglia have a critical role in wound healing and repair[6], releasing a range of cytokines and other soluble mediators that downregulate inflammation, increase phagocytosis and promote repair[4,7]. The roles of microglia are diverse and include driving oligodendrocyte differentiation during re-myelination[8], protecting neurons during oxygen and glucose depletion in stroke models[9] and promoting neurite outgrowth in spinal cord injury models[10].

The study of these glial cells represents an important aspect in understanding and manipulating the response to neuroinflammation. The described protocol allows for further investigation into the therapeutic potential of microglia modulation in neuroinflammatory disorders.

The modulation of microglial activation towards a neuroprotective role has been observed in a range of conditions[11,12,13]. Thus, improving current understanding and further studying modulation of microglial activation is critical, requiring the use of various models including both *in vitro* and *in vivo*. *In vitro* studies represent an important tool due to their greater efficiency, lower cost and ability to investigate an isolated cell population.

There are a range of protocols described in the literature for the isolation of microglia from murine brains, the challenge to efficiently produce a high yield sample with good viability and high purity. Commonly used methods of isolation of primary microglia are by magnetic separation and prolonged shaking of mixed glial cultures. Through personal experience, it was found that there was a high degree of cellular debris which obstructed the magnetic column. Thus, the following protocol was utilized, which incorporates an initial density gradient centrifugation step

followed by CD11b magnetic separation. The protocol described below has been optimized to produce a highly pure sample in sufficient quantity. It is advantageous due to its high purity and the short time period — one can perform assays within 2 days without having to culture for 2-3 weeks. This protocol can potentially be adapted for the isolation of primary murine astrocytes.

Protocol

The following procedures have been approved by the Animal Ethics Committee at the Monash University. Healthy untreated neonate C57Bl6/J P3-6 mice were used to generate the representative results.

1. Enzymatic Digestion

NOTE: It is important to consider sterility when isolating and culturing primary cells. Whilst ensuring the environment is as sterile as possible, the initial dissection and harvest of murine brains can be completed outside of a laminal flow hood, with all subsequent steps performed within a laminar flow hood.

1. Using sterile instruments, euthanize the mouse by cervical dislocation, decapitate the animal and rinse with 100% ethanol. Using small sterile scissors, make small incisions along the right and left sides of the head. At this age, the skin peels away easily. Using the tips of the curved forceps, gently slide the tissue towards the rostrum to expose the skull, including the Bregma.
2. Insert the tip of scissors in the opening of the spinal canal and make a lateral incision to the ear canal. Make an incision along the sagittal suture to the Bregma, gently pointing the tip of the scissors upwards to prevent damaging the brain. Insert tip of scissors at Bregma and make lateral incisions to the right and left sides of the head along the coronal suture.
3. Using forceps, gently peel away the skull to expose the brain. To do so, grasp the edges of skull exposed by the lateral incision, and gently pull the skull to the side. The skull should peel away easily. Using curved forceps, gently scoop under the brain to remove it.
4. Place the brain in a 5 mL sterile container, wash 2-3 times with ice-cold wash media (low glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin), approximately 5 mL media per wash, to remove blood.
5. In a laminar airflow hood, place the contents of the 5 mL container in a small petri dish (35 mm), resting on ice. Using a sterile scalpel blade or sterile scissors, remove the cerebellum and olfactory bulb. Make a midline cut to separate the two hemispheres.
6. Carefully peel away the meningeal layer with fine forceps, taking care not to damage the cortices. Identify the meningeal layer as a very thin layer of cells with a red tinge on the surface of the brain, with visible blood vessels. Keeping the brain cold is essential for proper meninges removal. If the meningeal layer breaks, continue peeling away the torn fragments until it is completely removed.
7. Transfer the hemispheres to a new small petri dish (on ice) and fill with wash media. Chop the brain into small pieces with sterile scalpel blade/scissors.
   NOTE: These should be ~1 mm^2 in size, and care should be taken not to cut them into too small pieces as this may reduce yield.
8. Add 100 µL papain (17 U/mg stock) and 150 µL DNase I into the media and incubate for 30 min at 37 °C
9. Following digestion, triturate tissue using a P1000 pipette. If tissue pieces are too large to enter the pipette, consider using a pair or sterile scissors to widen the pipette tip. During this process, take care not to introduce bubbles into the media as this may reduce cell viability.

2. Myelin Debris Removal

1. Under a laminar flow hood use sterile equipment, prepare a 50-mL conical tube with a 100 µm cell strainer, for each brain. Pour contents of the petri dish, containing the digest medium and brain pieces onto a strainer. Push through the pieces of brain by using the plunger of a sterile 3 mL syringe in a grinding motion until there is no more tissue visible. Following digestion, the brain tissue should fall apart easily.
2. Continuously wash the filter with the wash media, by topping up the cell strainer throughout this process. This will wash through any cells trapped in the strainer.
   NOTE: This should be done until there is approximately ~15 mL in the tube. This is to ensure that the strainer is sufficiently washed through and to maximize the amount of cells collected.
3. **Centrifuge the single cell suspension at 500 x g for 5 min at 4 °C. During this time, prepare the density gradient medium as described.**
   1. Prepare the stock isotonic percoll (SIP), which is the density gradient medium used. Do this by adding 9:1 ratio of density gradient medium to 10x sterile Hanks balanced salt solution (HBSS).
   2. Prepare gradients as 30% SIP in DMEM and 70% SIP in 1x HBSS. For example, to prepare 10 mL of 30% SIP, add 3 mL of SIP to 7 mL DMEM.
4. Aspirate the supernatant from the conical tubes and resuspend the cell pellet with 8 mL of 30% SIP in DMEM. Transfer the full volume to a fresh 15 mL conical tube.
5. Underlay the 70 % SIP solution. To do so, fill a transfer pipette with 70% SIP and carefully push through the transfer pipette to the bottom of the conical tube. Once the tip is close to the bottom, gently push through the contents of the transfer pipette.
   NOTE: Do this slowly so as not to disrupt the 30/70 interface. There must be a clear line separating the two layers.
6. Centrifuge the SIP layers, including the cells, at 650 x g with brake 0 and acceleration 4, for 25 min at room temperature.
   NOTE: It is essential to complete the spin with brake OFF and to allow the centrifuge to slowly come to a halt, as application of brake will disrupt the interphase and dramatically reduce the cell collection between the SIP layers.
7. Aspirate the cellular debris at the top of the tube, and remove approximately 4 mL media from the top. This will ease the removal of the mononuclear cells in the following step. Using a P1000 pipette, carefully lower the pipette tip towards the interphase. Isolate the mononuclear cells from the 30/70 density gradient medium interface. Collect approximately 3 mL from the cloudy interface and transfer to a new 15 mL conical tube. Following this, dilute the mixture with 9 mL of HBSS to aid removal of the density gradient medium.

8. Centrifuge the diluted density gradient medium interphase, containing the mononuclear cells at 500 x g for 5 min. Aspirate supernatant and resuspend with 1 mL growth medium (DMEM supplemented with 10 % fetal bovine serum and 1% antibiotics). Following this, stain the resuspended cells with trypan blue and perform a cell count using a haemocytometer.

3. Magnetic Activated Cell Sorting

NOTE: These steps are modified from manufacturers' protocol.

1. Centrifuge the collected mononuclear cells at 300 x g for 10 min at 4 °C to remove the growth medium as this will interfere with the magnetic isolation. Using the same cell count obtained from Step 2.8, resuspend at 1 x 10^8 nucleated cells/mL in PBS (Ca^{++} and Mg^{++} free) containing 2 % FBS and 1 mM EDTA, within a volume range of 0.1-2.5 mL.
2. Add the full volume of nucleated cells in PBS from the previous step to a fresh 5 mL (12 x 75 mm) polystyrene round bottom tube. Add 50 µL CD11b PE labelling reagent per 1 mL of sample. Incubate at room temperature for 15 min protected from light.
3. Add 70 µL of selection cocktail (a combination of monoclonal antibodies against CD11b in PBS) per 1 mL of sample. Incubate at room temperature for 15 min protected from light.
4. Mix magnetic particles by pipetting up and down more than 5 times. Add 50 µL/mL to the sample. Incubate at room temperature for 10 min protected from light.
   NOTE: These particles then form a tetrameric complex with the antibodies and will get attached to the magnetic column.
5. If the total volume of cell mixture is less than 2.5 mL, top up to this volume with PBS (Ca^{++} and Mg^{++} free) containing 2 % FBS and 1 mM EDTA, and mix by gently pipetting 2-3 times. Place the tube (without lid) into the magnet and incubate at room temperature for 5 min.
6. In one continuous motion, fully invert the magnet containing the tube for 2-3 s, pouring off the supernatant. Return the magnet to an upright position, and remove the tube from the magnet. Prepare to wash the remaining cells out from the column.
   NOTE: The supernatant contains unlabelled and unwanted cells, which are removed by inverting the tube while still in the magnet. The tube contains the attached $Cd11b^+$ cells.
7. Wash the cells by repeating steps 3.5 and 3.6 twice more. If the sample is greater than 1 mL, a further repeat of steps 3.5 and 3.6 is recommended.
8. Resuspend the cells in a desired growth medium. Rinse the side of the collection vessel as well (*e.g.* a 15 mL tube) to collect cells from the sides of the tube and maximize yields.

4. Verification of Microglia Purity

NOTE: The primary microglia isolated from 3 L (n = 5 animals total) were verified via fluorescent activated cell sorting (FACS) to determine purity for the representative results.

1. Culture cells in a T-25 flask in the growth medium containing 10% FBS overnight, seeding at a density of 1 - 2 x 10^6 cells per flask.
2. Wash the cells in the T-25 flasks with PBS 3x for 5 min to remove any remaining growth media which might interfere with the trypsinization.
3. Add 2 mL of 0.25% trypsin to detach the cells from the flasks. Ensure that the volume of trypsin is sufficient to cover the entire surface of the flask. Following gentle swirling of the flask to ensure uniform coverage of trypsin, incubate for 5 min at 37 °C.
4. Quench the trypsinization process by adding 2 mL of growth media containing 10% FBS into the flask.
5. Collect the supernatant containing the trypsinized cells and centrifuge at 500 x g for 5 min at 4 °C to collect cells.
6. Remove the supernatant containing the growth medium and trypsin and resuspend the pellet in 500 µL FACS buffer. Perform the cell count using trypan blue.
7. Centrifuge at 500 x g for 5 min at 4 °C. Perform Fc receptor block by adding 50 µL FACS buffer + 1 µL FcR blocker per 5 x 10^6 cells. Incubate for 15 min at 4 °C.
   NOTE: The Fc receptor block is a critical step in the staining procedure to prevent non-specific binding of immunoglobulins to Fc receptors in immune cell populations. This blockade does not affect downstream binding of other antibodies.
8. Terminate the blocking process by diluting with 500 µL FACS buffer. Following this, centrifuge the mixture containing the cells at 500 x g for 5 min at 4 °C.
9. Resuspend in fresh 500 µL FACS buffer.
10. Place the majority of cells (*e.g.* if resuspending the cells in 500 µL of FACS buffer, use 400 µL) into a sample tube. Distribute the remaining cells amongst the control tubes and top up to 100 µL per control tube *(e.g.* for 6 control tubes, put 16.67 µL into each control tube and top up with 83.33 µL FACS buffer). Make the groups (control tubes in bold) as Unstained, single stained (CD45, CD11b), single live/dead stain, FMOs, and sample tubes.
11. Pellet the cells in all tubes by centrifuging at 500 x g for 5 min at 4 °C.
12. Stain the cells in the tube with 1 µL antibody per 100 µL FACS buffer per 5 x 10^6 cells. Incubate for 20 min at 4 °C.
    NOTE: Use 50 µL antibody cocktail if less than 2 x 10^6 cells are used.
13. Wash the cells with 500 µL FACS buffer. Centrifuge the cells at 500 x g for 5 min at 4 °C.
14. Resuspend the cells in 300 µL FACS buffer.
    NOTE: Use ~100 µl if cell count is less than 2 x 10^6.
15. Using flow cytometry analysis, quantify the cells that are $CD45^{low}$ and positive for CD11b staining. This percentage corresponds to the isolated primary microglia.

5. Immunohistochemical Staining of Primary Microglia

1. Plate the cells in a 96-well plate at a density of 1 x 10^5 cells with the growth medium and incubate overnight.
2. Remove the supernatant and wash 3 times with PBS.
3. Fix the cells with 4% paraformaldehyde in PBS for 15 min. Remove the PFA with a P1000 pipette, then wash them 3 times with PBS + 0.1 % Triton-X.

4. Block for non-specific binding with 3% bovine serum albumin for 1 h at room temperature.
5. Incubate with rabbit Iba-1 (1:100) for 24 h at 4 °C. Following this, remove the primary antibody mixture and wash 3 times with PBS.
6. Incubate with secondary anti-rabbit GFP conjugate (1:200) for 1 h at room temperature. Following this, remove the secondary antibody mixture and wash 3 times with PBS.
7. Mount slides with a fluorescent mounting medium and capture images using a fluorescent microscope.

6. Quantification of Microglia Using the pHrodo Assay

NOTE: The pHrodo assay allows for identification of levels of phagocytosis in cultured cells. Upon uptake via endocytosis, internalization into the more acidic environment increases levels of fluorescence of the bioparticle conjugates. Fluorescence levels can then be quantified by FACS. The following steps are modified from manufacturers' protocol.

1. Culture the cells in a T-25 flask in the growth medium overnight, seeding at a density of 1 - 2 x 10^6 cells per flask. Wash the cells in T-25 flasks with PBS 3 times for 5 min.
2. Detach the cells using 2 mL 0.25% trypsin. Incubate for 5 min at 37 °C.
3. Quench the trypsinisation process by adding 2 mL of growth media containing 10% FBS into the flask.
4. Centrifuge the cell suspension at 500 x g for 5 min at 4 °C to pellet cells.
5. Remove the supernatant and resuspend the pellet containing the primary microglia in culture medium at 10^6 cells/mL.
   NOTE: Alternatively, plate cells in a 96-well plate at least a day before and aim for 1 x 10^5 viable cells per well on the day the assay is to be performed.
6. Plate cells into 96-well plate at 1 x 10^5 cells/well, 100 µL per well. Plate controls and experimental wells in triplicate, and leave one empty well per positive control for a no-cell control background subtraction.
7. Add 100 µL of growth media to the wells left empty for no-cell background subtraction.
   NOTE: Like any other *in vitro* assay, appropriate controls are vital for the accuracy of the readouts. Alongside the no-cell control (background reading), also include the negative control well (pHrodo conjugate added, but placed on ice).
8. Cover the plate and incubate for 1 h in an incubator with 5 % CO_2 at 37 °C.
9. Prepare experimental wells by adding stimulant and treatment. Add vehicle controls (growth media only) to untreated wells.
   NOTE: Lipopolysaccharide 1 µg/mL [stimulant] and human amnion epithelial cell (hAEC)-conditioned media [treatment] were used to generate the representative results.
10. Thaw a vial of conjugated dye, add 2 mL uptake buffer and briefly vortex. Transfer the tube contents into a clean glass tube and sonicate for 5 min, to ensure that particles are evenly dispersed.
11. Aspirate the culture medium from microplate wells and quickly replace with 100 µL of dye suspension. Cover and incubate at 37 °C for 1 h.
    NOTE: Remember to stain the control tubes as well.
12. Using flow cytometry analysis, quantify cells staining positive for the conjugated pHrodo beads and present as a percentage (of parent) of actively phagocytosing microglia.

7. Quantification of Microglia Apoptosis Following Inflammatory Insult

1. Repeat the steps 1-7 from "Verification of microglia purity" section.
2. Place the majority of cells (*e.g.* if you resuspended the cells in 500 µL of FACS buffer, use 400 µL) into a sample tube. Distribute the remaining cells amongst the control tubes and top up to 100 µL per control tube (*e.g.* for 6 control tubes, put 16.67 µL into each control tube and top up with 83.33 µL FACS buffer). Groups (control tubes in bold): Unstained, single stains (AnnexinV, Propidium iodide), single live/dead stain, FMOs, sample tubes.
3. Pellet the cells in all the tubes by centrifuging at 500 x g for 5 min.
4. Incubate with 1 µL antibody/100 µL FACS buffer/5 x 10^6 cells for 20 min at 4 °C.
   NOTE: Use 50 µL if less than 2 x 10^6 cells.
5. Wash the cells by adding 500 µL FACS buffer and centrifuge at 500 x g for 5 min.
6. Resuspend the cell pellet in 300 µL FACS buffer.
   NOTE: ~100 µL if cell count is less than 2 x 10^6.
7. Quantify cells within each quadrant (Q1: necrotic, Q2: late apoptotic, Q3: viable, Q4: early apoptotic).

Representative Results

Using the methods outlined here, pure populations of microglia can be isolated and can be ready for characterization using *in vitro* and FACS analysis. To begin with, up to 18 animals can be used per cull, with an expected yield of approximately 450,000 - 600,000 microglial cells. It is crucial to first confirm the purity of the isolated cells, and to do so FACS analysis was performed by staining for the two markers CD45 and CD11b. Identification of microglia can prove troublesome, as many markers expressed by microglia are also expressed by macrophages, and the use of these two markers allows accurate and reliable quantification. Specifically, microglia have a low expression of CD45, compared to the high expression seen in CNS and peripheral macrophages, and are also positive for CD11b (**Figure 1**). From this particular isolation, a purity of 89.3 % was reported. After staining for CD11b, we note that our primary microglia share similar morphological features, with a range of morphologies from the distinctly unramified (large spherical cell body with little or no extended processes) to the more ramified (with smaller, more oval somata and typically up to secondary order processes) correlating to a range of activation states, as expected to see *in vivo* (**Figure 2**).

Following this, two characterization assays for microglia function and survival were run. Microglia are the major phagocytic cell in the CNS, and the pHrodo assay allows quantification of this particular functional property. A near 3-fold increase in phagocytic function after 24-h co-culture with hAEC conditioned medium (**Figure 3**) was noted, as measured by the quantification of fluorescent pHrodo particles. Finally, an AnnexinV-PI staining following co-culture was performed to identify survival of microglia following an inflammatory insult. A reduction in microglia apoptosis after treatment with conditioned medium (**Figure 4**) was observed. These findings suggest that hAEC conditioned medium protects microglia and enhances their phagocytic activity, which might have therapy in perinatal brain injury and neuroinflammatory disorders.

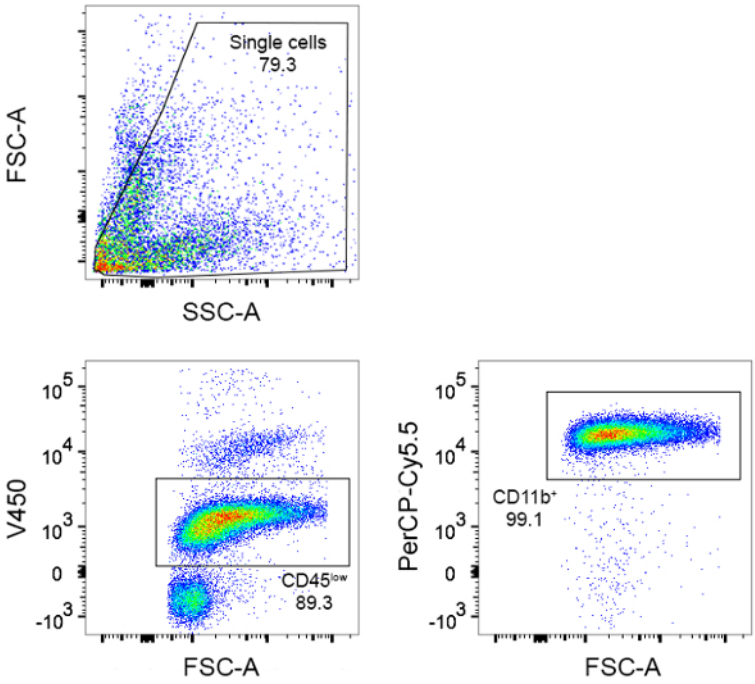

Figure 1: Expression of CD45 and CD11b by microglia. Each dot represents a labelled cell and FACS analysis allows elucidation of separate cell populations. Microglia have a lower expression of the CD45 antigen compared to peripheral monocyte-derived macrophages and are positive for CD11b. Numbers below antibody label indicate the proportion of gated cells expressed as percentage of the parent population. Please click here to view a larger version of this figure.

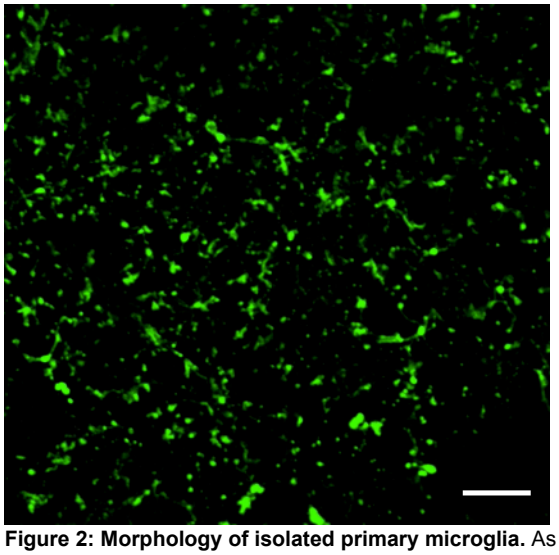

Figure 2: Morphology of isolated primary microglia. As can be seen in this figure, microglia retain their spherical cell body and distinct ramified structure. Scale bar = 20 µm.

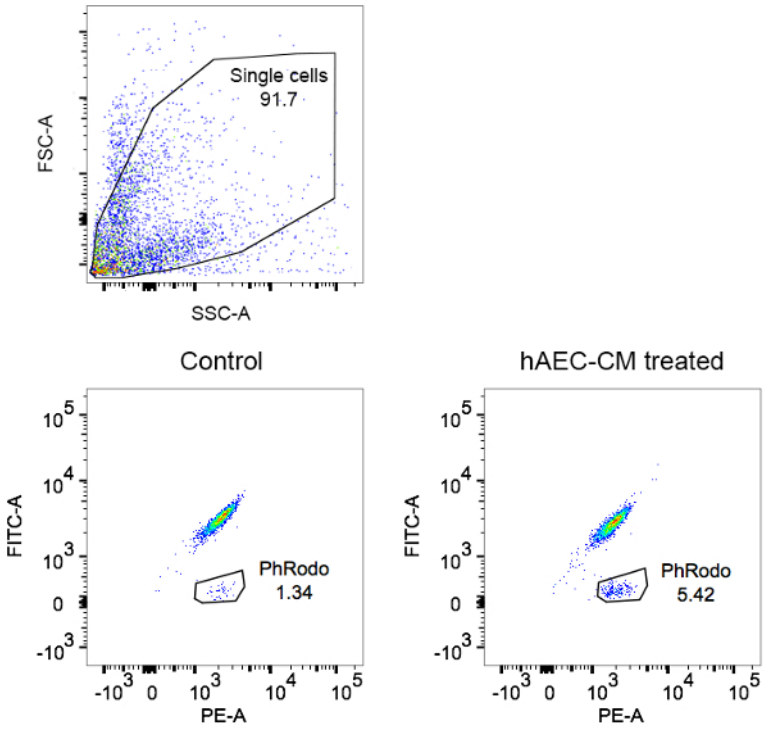

Figure 3: Quantification of phagocytic function in isolated primary microglia. pHrodo-labelled particles only fluoresce when in acidic environments, such as in cellular endosomes following phagocytosis. Here, an increase in pHrodo particle uptake in microglia treated with hAEC conditioned medium is observed. Please click here to view a larger version of this figure.

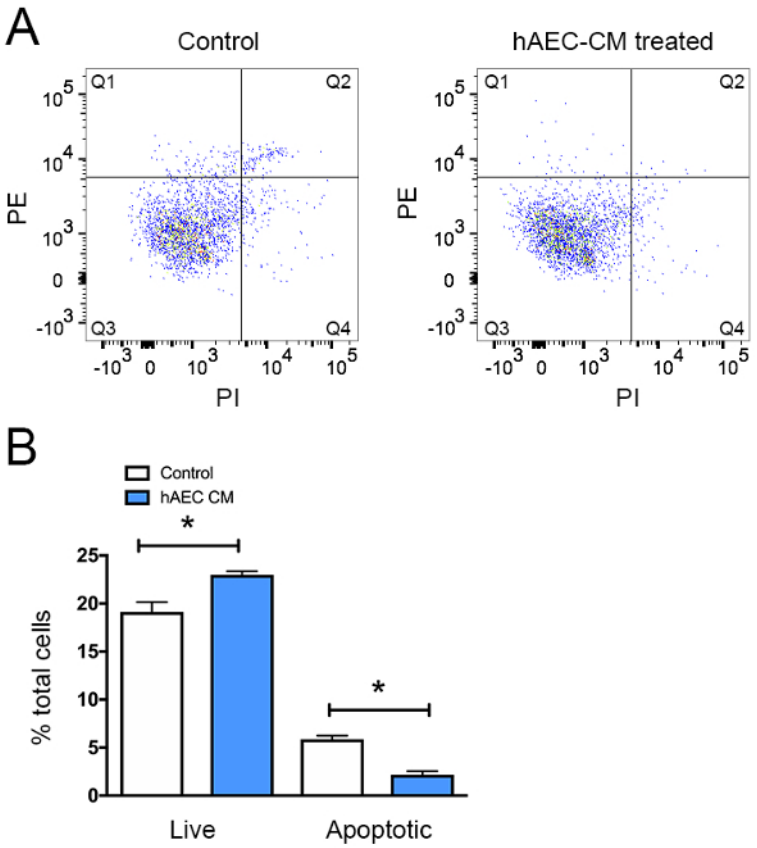

Figure 4: Analysis of microglia survival after inflammatory insult. (**A**) Representative FACS plots of isolated microglia. The combined staining of AnnexinV and propidium iodide allows categorisation into either apoptotic, necrotic, or live cells after stimulation with LPS. (**B**) hAEC conditioned medium significantly reduced microglia apoptosis relative to controls, suggesting a form of protection on this cell type. * signifies p <0.05, student's t-test. Please click here to view a larger version of this figure.

Wash medium
Low Glucose Dulbecco's Modified Eagle Medium
1% v/v penicillin-streptomycin
Digest medium (per brain)
150 µL DNase I
100 µL Papain (17U/mg stock)
3 ml wash medium
Growth medium
Low Glucose Dulbecco's Modified Eagle Medium
10% foetal bovine serum
1% v/v penicillin-streptomycin

Table 1: Table of solutions.

Discussion

Microglia have the ability to be both pro- and anti-inflammatory, altered by environment stimuli. Previous studies have shown the modulation of microglia activation can confer neuroprotection. Their ability to provide protection to neurons and repair injury necessitates more research to further the current understanding of these complex cells. Thus, isolation of high purity primary microglia is an important and useful technique. This is a relatively quick method to obtaining highly pure primary microglia ready for *in vitro* experimentation within 2 days.

There are a range of protocols described in the literature for the isolation of primary microglia from murine brains. The principal challenge is to efficiently produce sufficient sample, high in viability and purity. The main advantage of this protocol is the yield of a highly pure sample without the need for the time in culture. Thus, the method is shortened from 2 weeks to 2 days, this facilitates fast results. The characterization steps yield robust functional data about microglia, enhancing current understanding of these complex cells. The protocol combines two current approaches - density gradient centrifugation and magnetic separation. Magnetic separation has been well validated in the literature for isolation

of microglia in a relatively gentle manner compared to other isolation techniques[14,15,16]. Whilst there are undoubtedly changes to the functional characteristics of the isolated primary microglia compared to in their native state *in vivo*, any changes to microglia properties as observed in the assays developed above are calculated from a post-digestion baseline, and as such are reflective of direct modulation of this immune subset.

Whilst the protocol is relatively straightforward, care during critical steps will help to ensure a good yield. Firstly, it is vital to use low glucose media to support glial growth. Furthermore, the murine brains must be kept ice cold at all times during the isolation. Thus, it is recommended to pre-chill all medium, as well as instruments if possible, and perform the procedure on ice. Importantly, prolonged isolation times will lead to poorer cell health and yield, thus it is critical to work quickly. Moreover, proper removal of the meningeal layer is a crucial step when working with adult mice, otherwise the fibroblasts will outcompete the glial cells in terms of growth. During the enzymatic digestion of the brains, it is important not to cut the brains into too small pieces as this can increase cell death, ideally aim for 1 mm^2 pieces of tissue. When grinding the tissue through the cell strainer, thoroughly rinse with media every few minutes to ensure all cells are washed through and do not get trapped in the strainer, additionally it is recommended to use a 100 µm cell strainer, smaller filters result in a reduced yield. Another critical step is the underlaying of the 70% density gradient medium layer, performing this step carefully and slowly so as to ensure a clear interphase is seen is essential, the use of a Pasteur pipette is recommended. Also, the use of 15 mL conical tubes is recommended as the separation of cells was less effective when 50 mL conical tubes were utilized. Lastly, the density gradient centrifugation must be performed at room temperature, as temperature can affect the density gradient.

This protocol describes a reliable method of producing highly pure primary microglia cells as shown by both high expression of CD45 and positive expression of Cd11b (as compared to low CD45 expression seen in peripheral monocytes). Cells can also be isolated relatively quickly, thus it has the scope to significantly increased understanding of these glia cells. It can be used to identify different microglia populations from different backgrounds, allowing for studies which may reveal differences between microglia isolated from diseased/injured and healthy animals. Through isolation of a pure microglia population, therapeutic modulation of this immune cell population can be assessed. For instance, it was found that hAEC-conditioned media increased phagocytosis, as well as improved microglia survival during an inflammatory stimulus, thus affording therapeutic benefits[17]. This correlated to reduced apoptotic debris and thus suggests clearance of these debris may have neuroprotective effects.

The protocol can be applied to both neonatal or adult mice, however older mice will produce a reduced yield. Furthermore, it may be adapted to isolate primary astrocytes from neonatal or adult mice, again older mice will result in a decreased yield. Additionally, the modification of the mechanical dissociation step with a custom-made nylon mesh with larger pore sizes as opposed to the 100 µm cell strainer, might further improve cell yield[18].

Limitations of this protocol include the lower yield and the time required for this protocol. The yield is approximately 150,000 - 200,000 cells per cull of six animals, versus the millions that can be obtained through weeks of cell culture. Additionally, whilst cells can be obtained within two days, the procedure on the first day is quite time-consuming, taking approximately six hours.

Nonetheless, this method is a highly useful tool in understanding the role of microglia in disease, capable of isolating and characterising the cells within two days. It produces high purity primary microglia cells, facilitating accurate and efficient research.

Disclosures

The authors have nothing to disclose.

References

1. Volpe, J.J. Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. (Report). *Lancet Neurology.* **8** (1), 110 (2009).
2. Saliba, E., & Henrot, A. Inflammatory Mediators and Neonatal Brain Damage. *Biology of the Neonate.* **79** (3-4), 224-227 (2001).
3. Uwe-Karsten, H., & Helmut, K. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature Neuroscience.* **10** (11), 1387 (2007).
4. Cherry, J.D., Olschowka, J.A., & O'Banion, M.K. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *Journal of neuroinflammation.* **11**, 98 (2014).
5. Michell-Robinson, M.A. *et al.* Roles of microglia in brain development, tissue maintenance and repair. *Brain.* **138** (5), 1138-1159 (2015).
6. Guohua, W. *et al.* Microglia/macrophage polarization dynamics in white matter after traumatic brain injury. *Journal of Cerebral Blood Flow & Metabolism.* **33** (12), 1864 (2013).
7. Neumann, H., Kotter, M.R., & Franklin, R.J.M. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain.* **132** (2), 288-295 (2009).
8. Veronique, E.M. *et al.* M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nature Neuroscience.* **16** (9), 1211 (2013).
9. Hu, X. *et al.* Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. *Stroke.* **43** (11), 3063 (2012).
10. Kigerl, K.A. *et al.* Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *The Journal of Neuroscience : the official journal of the Society for Neuroscience.* **29** (43), 13435 (2009).
11. Suzuki, T. Microglial α7 nicotinic acetylcholine receptors drive a phospholipase C/IP3 pathway and modulate the cell activation toward a neuroprotective role. *Journal of neuroscience research.* **83** (2006).
12. Bedi, S.S. Intravenous multipotent adult progenitor cell therapy attenuates activated microglial/macrophage response and improves spatial learning after traumatic brain injury. *Stem cells translational medicine.* **2** (2013).
13. Tran, T.A., McCoy, M.K., Sporn, M.B., & Tansey, M.G. The synthetic triterpenoid CDDO-methyl ester modulates microglial activities, inhibits TNF production, and provides dopaminergic neuroprotection. *Journal of neuroinflammation.* **5**, 14 (2008).

14. Grützkau, A., & Radbruch, A. Small but mighty: How the MACS®-technology based on nanosized superparamagnetic particles has helped to analyze the immune system within the last 20 years. *Cytometry Part A.* **77A** (7), 643-647 (2010).
15. Nikodemova, M., & Watters, J.J. Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. *Journal of Neuroinflammation.* **9**, 147-147 (2012).
16. Holt, L.M., & Olsen, M.L. Novel Applications of Magnetic Cell Sorting to Analyze Cell-Type Specific Gene and Protein Expression in the Central Nervous System. *PLOS ONE.* **11** (2), e0150290 (2016).
17. Leaw, B. *et al.* Human amnion epithelial cells rescue cell death via immunomodulation of microglia in a mouse model of perinatal brain injury. *Stem cell research & therapy.* **8** (1), 46 (2017).
18. Moujalled, D. *et al.* TDP-43 mutations causing amyotrophic lateral sclerosis are associated with altered expression of RNA-binding protein hnRNP K and affect the Nrf2 antioxidant pathway. *Human Molecular Genetics.* **26** (9), 1732-1746 (2017).

