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DEVELOPING SYSTEMS FOR STUDYING MICROGLIA EXTRACELLULAR TRAPS

3.1 ABSTRACT

Microglia are resident innate immune cells of the Central Nervous System (CNS). Microglia play a critical role during development, in maintaining homeostasis and during infection or injury (Lenz and Nelson 2018, Li and Barres 2018). Several independent research groups have highlighted the central role that microglia play in autoimmune diseases, autoinflammatory syndromes and cancers (Loane and Kumar 2016, Hansen, Hanson et al. 2017, Gutmann and Kettenmann 2019, Voet, Prinz et al. 2019). The activation of microglia in some neurological diseases may directly participate in pathogenic processes. Primary microglia are a powerful tool to understand the immune responses in the brain, cell-cell interactions and dysregulated microglia phenotypes in disease. Primary microglia mimic in vivo microglial properties better than immortalized microglial cell lines. Human adult microglia exhibit distinct properties as compared to human fetal and rodent microglia. Along with studying the formation of microglia extracellular traps in BV2 mouse microglia cell line, we wanted to explore these traps in primary human microglia. For this we developed a cost effective and robust protocol for isolating adult human microglia. This protocol provides an efficient method for isolation of primary microglia from adult human brain. Studying these microglia can provide critical insights into cell-cell interactions between microglia and other resident cellular populations in the CNS including, oligodendrocytes, neurons and astrocytes. Additionally, microglia from different human brains may be cultured for characterization of unique immune responses for personalized medicine and a myriad of therapeutic applications.

3.2 INTRODUCTION

The Central nervous system (CNS) is constructed of a complex network of neurons and glia (Allen and Barres 2009). Among the glial cells, microglia function as the innate immune cells of the CNS (Gordon, Plüddemann et al. 2014, Lenz and Nelson 2018). Microglia are responsible for maintaining homeostasis in the healthy CNS (Li and Barres 2018). Microglia also play an important role in neurodevelopment, by pruning synapses (Lenz and Nelson 2018). Microglia are central to the pathophysiology of several neurological diseases including but not restricted to; Alzheimer's disease (Hansen, Hanson et al. 2017), Parkinson's disease (Tremblay, Cookson et al. 2019), stroke (Qin, Zhou et al. 2019), multiple sclerosis (Voet, Prinz et al. 2019), traumatic brain injury (Loane and Kumar 2016), neuropathic pain (Inoue and Tsuda 2018), spinal cord injury (Bellver-Landete, Bretheau et al. 2019) and brain tumours such as gliomas (Gutmann and Kettenmann 2019).

Studies related to CNS homeostasis and diseases utilize rodent microglia due to a dearth of cost efficient and time efficient human primary microglia isolation protocols (Smith and Dragunow 2014). Rodent microglia resemble primary human microglia in expression of genes such as Iba-1, PU.1, DAP12 and M-CSF receptor and have been effective in understanding normal as well as diseased brain (Smith and Dragunow 2014). Interestingly, the expression of several immune related genes such as TLR4, MHC II, Siglec-11 and Siglec-33 varies between human and rodent microglia (Smith and Dragunow 2014). The expression of several genes also varies in temporal expression and in neurodegenerative diseases in both species (Galatro, Holtman et al. 2017, Friedman, Srinivasan et al. 2018). These significant differences make human microglia an essential model to study microglia function in homeostasis and disease. Primary human microglia can also be an effective tool for preclinical screening of potential drug candidates (Rustenhoven, Smith et al. 2018). The above mentioned reasons underline the growing need of cost effective protocols for isolation of primary human microglia.

We have developed a protocol for isolation of primary human microglia from adult human brain tissue collected as a result of surgical window created for tumor resections or other surgical resections. Our method is considerably different from the existing methods. We were able to isolate and culture microglia after a transit time of about 75 minutes from the tissue collection site to starting the isolation protocol in our laboratory. We have used the supernatant of L929 fibroblast cells to promote the growth of isolated microglia. Our method specifically focuses on the culture and development of only primary microglia. The resulting culture prepared is 80% microglia. While other protocols provide a enriched culture of microglia by density gradient centrifugation, flow cytometry and magnetic beads, our protocol is a rapid, simple, robust and cost effective way to culture primary human microglia (Sierra, Gottfried-Blackmore et al. 2007, Rustenhoven, Park et al. 2016, Mizee, Miedema et al. 2017, Spaethling, Na et al. 2017). The ability to utilize surgically removed live adult brain tissue instead of fixed brain tissues from cadavers proves an added advantage of this method in contrast to existing procedures (Olah, Raj et al. 2012, Mizee, Miedema et al. 2017).

3.3 PROTOCOL

Note: All tissues were acquired after ethical clearance from the institute ethics committees of Indian Institute of Technology Jodhpur and All India Institute of Medical Sciences (AIIMS) Jodhpur.

3.3.1 Tissue Acquisition and Processing (Day 0)

1. Collect the tissue, in a 50 ml tube containing 10 ml of ice cold artificial cerebrospinal fluid (aCSF) (2 mM – $CaCl_2.2H_2O$, 10 mM – Glucose, 3 mM – KCl, 26 mM – $NaHCO_3$, 2.5 mM – NaH_2PO_4, 1 mM – $MgCl_2.6H_2O$, 202 mM – Sucrose) (Spaethling, Na et al. 2017). Ensure that the tube is kept on ice if the tissue needs to be transferred to a different location.
   Note: Prepare aCSF in autoclaved distilled water. Filter it with 0.22 µm syringe filter in the laminar hood. This can be stored for 1 month at 4 °C.
2. Wipe the collection tube carefully with 70% alcohol and transfer to an aseptic laminar air flow chamber.
3. Discard aCSF carefully and weigh tissue in aseptic condition. Tissue weight is essential to calculate the volume of trypsin-EDTA needed for subsequent steps.
4. Keep the tissue in fresh warm aCSF at 37 °C for 5 minutes. This step is critical to avoid cell death.
5. Discard aCSF and wash tissue once with 1 x PBS (Phosphate-buffered saline) at 37 °C. Ensure all blood is washed away with repeated PBS washes (as needed).
6. Incubate the tissue in warm PBS, at 37 °C, for 5 minutes.
7. Discard PBS carefully and transfer tissue to a sterilized Petri dish. PBS may be removed with a pipette. This will prevent any loss of tissue.
8. Dice the tissue into small (at-least 1 mm^3) pieces using a sterile scalpel. Finely diced tissue provides higher tissue surface area for tissue dissociation by trypsin-EDTA ensuring higher yield.
9. Transfer the diced tissue to a 50 ml falcon tube containing 10 ml/g tissue of 0.25% trypsin-EDTA and mix by pipetting through a 10 ml serological pipette. Add 2 ml trypsin-EDTA to petri dish and wash the plate thoroughly with the help of pipette. Add this trypsin back to the falcon tube. This minimizes loss of tissue and cells while dicing.
10. Incubate the tube on a shaker for 30 minutes at 37 °C at 250 rpm. This step increases the dissociation of cells from tissue.
11. At the end of the incubation, add 10 ml of neutralizing medium (50% DMEM/50% F12 with glutamine, 1% penicillin-streptomycin, 10% FBS) to neutralize trypsin. Mix with a 10 ml serological pipette. The amount of neutralizing media added should be equal to the amount of trypsin used.
12. Centrifuge the tube at 2000 x g at 4 °C for 10 minutes.
13. Discard the supernatant and re-suspend the pellet in 1 ml of culture medium (50% DMEM/50% F12 with glutamine, 1% penicillin-streptomycin, 20% L929 supernatant, 10% FBS). Note: L929 cells are culture in DMEM (DMEM with glutamine, 1% penicillin-streptomycin, 10% FBS). ATCC recommended culture method should be followed for cell culture. Supernatant must be collected from the culture flask which is at least 75% confluent. It can be collected in bulk and stored at -80°C to prevent degradation of growth factors. It is recommended to add L929 supernatant separately in flasks instead of adding to the stock culture medium.
14. Plate the cells in a T-25 flask, suited for adherent cells, and add 4 ml of additional culture medium. Incubate the flask at 37 °C with 5% CO_2. Carefully shake the flask to

homogenously disperse the tissue. Avoid bringing the media to the neck of the flask, while shaking, as this may increase the chances of contamination.

3.3.2 CELL CULTURE (DAY 2)

1. Collect the media from the T-25 culture flask prepared on day 0 in three 1.5 ml centrifuge tubes by collecting equal volume of media in each tube. Wash the flask once with 1 x PBS. Shake the flask gently to remove any remnant tissue fragments left. Avoid harsh shaking of the flask as any remnant fragments will not adversely affect the culture. Add 5 ml fresh culture media to the flask.
2. Centrifuge the collected media at 1466 x g at 4 °C for 4 minutes.
3. Discard the supernatant from each tube and add 1 ml of culture medium to one of the tubes. Mix thoroughly with pipette. Serially add the mixed media with cells to other tubes. Mix thoroughly with pipette and pool the cells in one tube.
4. Plate the cells in a separate T-25 flask, suited for adherent cells. Add 4 ml of culture medium and incubate the flask at 37 °C with 5% CO_2.

3.3.3 CELL CULTURE (DAY 4)

1. Discard the media from both flasks and add fresh 5 ml culture media to the flask.
2. Incubate the flask at 37 °C with 5% CO_2 for 2 days.

3.3.4 CELL CULTURE (DAY 6)

1. Cells will be ready for further experiments.

3.4 RESULTS AND DISCUSSION

Microglia ensure homeostasis in the normal brain and play central roles in the pathophysiology of various neurological diseases (Li and Barres 2018). Microglia are central to neurodevelopment and formation of synapses (Lenz and Nelson 2018). Microglial studies have proven pivotal in understanding the development and progression of diverse neurological diseases (Li and Barres 2018). Rodent microglia are the prevalent model of choice for primary microglial studies, even though, rodent microglia are different from primary human microglia in key aspects (Smith and Dragunow 2014). Development of cost effective, high-yielding, protocols for isolation of primary human microglia may help bridge this gap. By using this protocol (Figure 3.1) we were able to isolate primary human microglia from live surgically resected brain, adult, human brain tissue. Cultured cells were stained with *Ricinus communis agglutinin-1* (RCA-1) lectin for microglia (Green) and with Glial fibrillary acidic protein (GFAP) for astrocytes (Red) (Figure 3.2) as previously described (Arnett, Mason et al. 2001, Arnett, Hellendall et al. 2002, Plant, Iocca et al. 2007, Jha, Srivastava et al. 2010, Freeman, Guo et al. 2017). 4′, 6-diamidino-2-phenylindole (DAPI) was used to stain nuclei (Blue). On the sixth day from the starting of the experiment the cells were ready for further experiments. Stained cells were counted blind for microglia and astrocytes present in the culture. We were able to achieve microglial purity of about 80% (Figure 3.2).

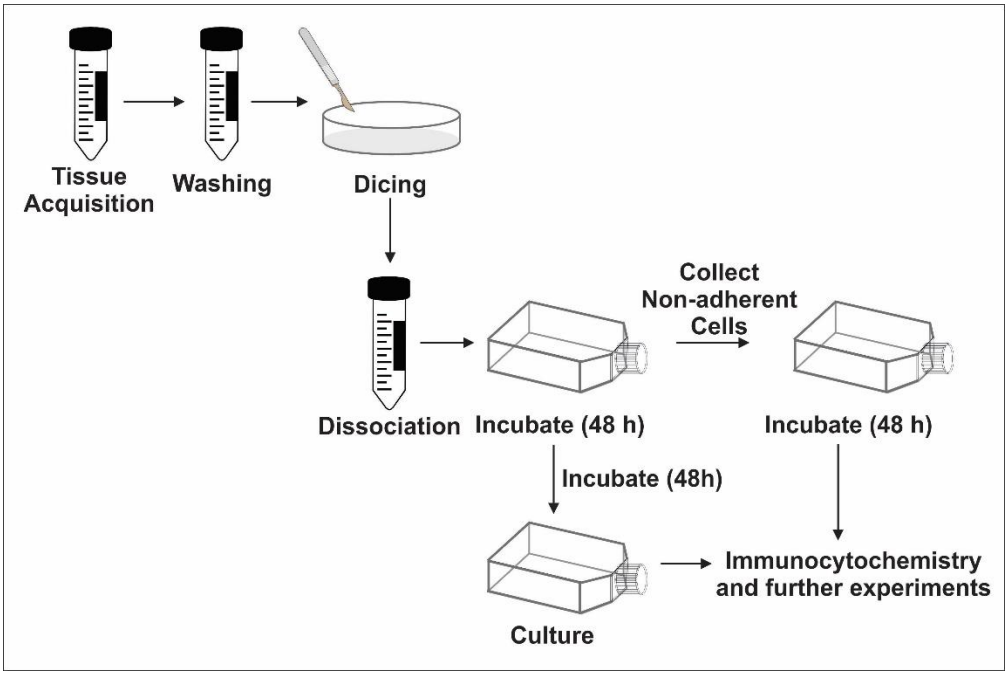
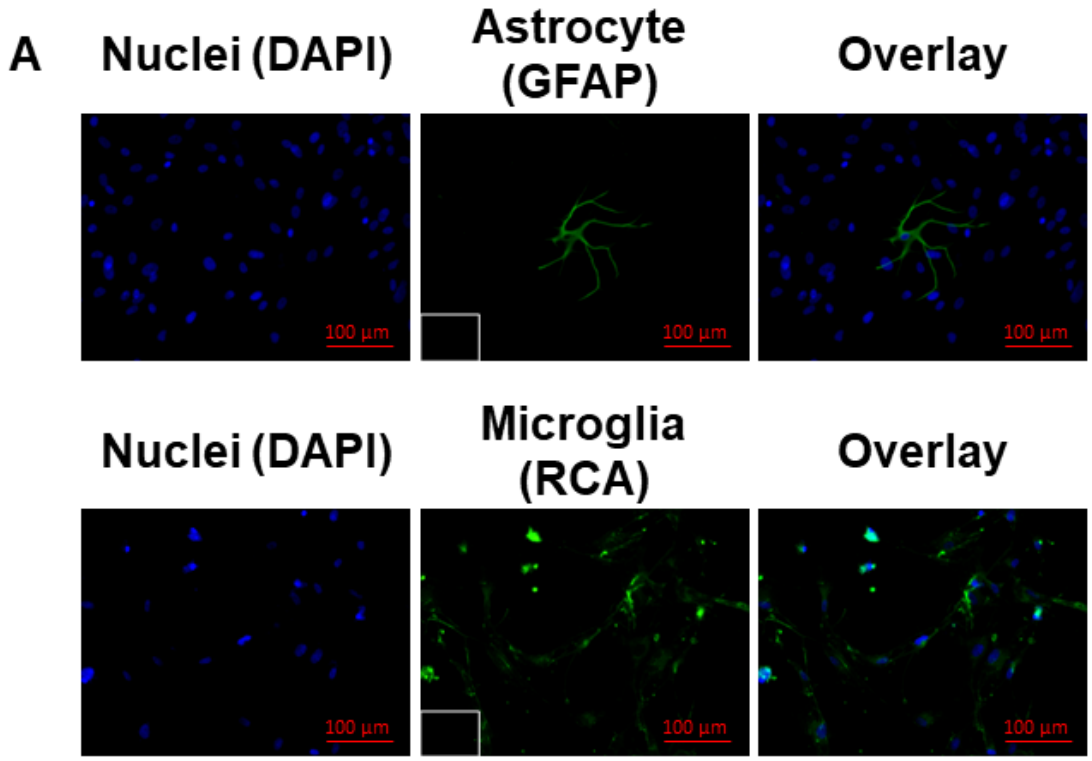


Figure 3.1 : Schematic of primary microglia isolation from adult brain. Surgically removed tissue was collected in ice cold 10 ml aCSF in a 50 ml falcon tube and transferred to laboratory. Tissue was washed with aCSF and PBS respectively and finely diced, dissociated with the help of trypsin-EDTA and plated in a T-25 flask. On the second day the media was collected and centrifuged. Pellet was mixed in fresh media and plated in a T-25 flask. Fresh media was added to the first flask. Media was changed in both the flasks on alternate days. Cells were ready for further experiments on day 6.



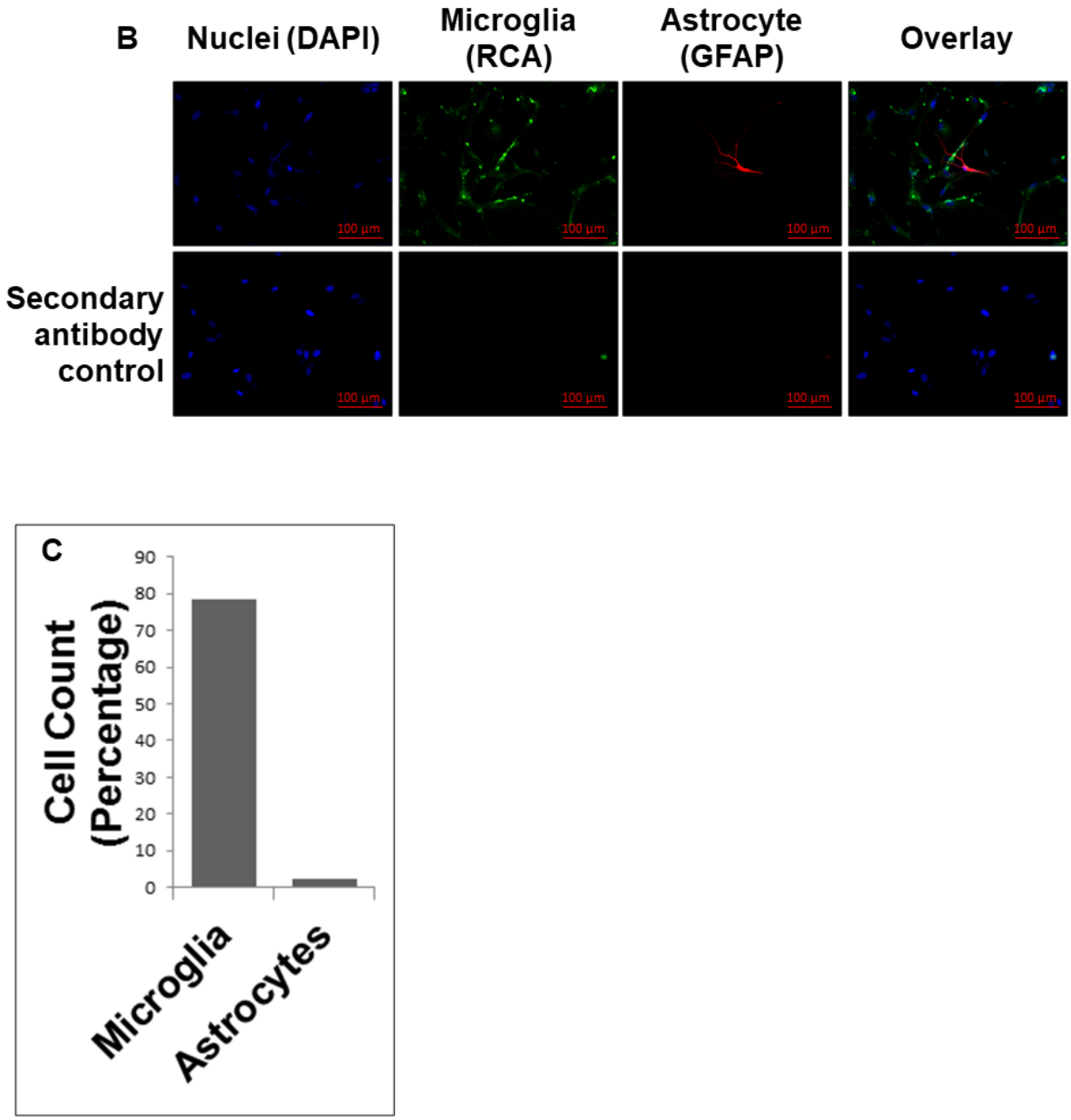


Figure 3.2 : Immunocytochemistry of isolated primary human microglia. (A) Isolated cells were plated in a two well chamber slide and were stained with GFAP for astrocytes (Green-first panel) or RCA for microglia (Green-second panel). Nuclei were stained blue with DAPI. Control for RCA and secondary antibody control for GFAP is shown in inset. (B) Isolated cells were plated in a two well chamber slide and were stained with RCA for microglia (Green) and GFAP for astrocytes (Red). Second row shows the control for RCA and secondary antibody control for GFAP. Nuclei were stained blue with DAPI. (C) Cells were counted by blinded control. Quantification is representative of counting by one blinded control. About 80% of the cells were microglia.

One of the most critical steps of the protocol was the transportation of acquired tissue to the laboratory for processing. As the transit time was about 75 minutes, it was probable that we may not be able to isolate any cells. We managed this by using 50 ml flacon tube with only 10 ml of aCSF. aCSF provided the required nutrients and the remaining space in the tube helped aerate the aCSF and tissue. There is possibility that there was considerable death of neurons and other cells during the transition period. While this helps us with the isolation of microglia, this protocol

may not be efficient for isolation of other neurological cells. We were able to isolate microglial cells from 268 mg of dissected tissue. We were also able to achieve significant purity of microglia by also avoiding the coating of flask by Poly-D-Lysine. While this may have resulted in some loss of microglia, this also avoided other glial population from adhering to the flask. Additionally, this avoided an extra step of shaking the flask and collecting microglia. It was possible that some of the cells might have not adhered in the flask prepared on day 0. We collected non adherent cells from the initial culture and plated it again in another flask on day 2 which also yielded microglia cells. It should be noted that finely dicing the tissue is important as it will increase the surface the area of the tissue. This will allow trypsin to access most of the tissue and dissociate more cells.

To promote the growth of microglia in our culture we have conditioned our culture medium with supernatant of L929 cells (Boltz-Nitulescu, Wiltschke et al. 1987, Trouplin, Boucherit et al. 2013). This provides a rich source of Granulocyte-macrophage colony stimulating factor (GM-CSF) as supplement which enhances macrophage proliferation (Englen, Valdez et al. 1995, Trouplin, Boucherit et al. 2013). This helped in cutting the cost for additional expensive growth supplements that are a mainstay of several microglial primary isolation protocols. Adding L929 supernatant is crucial for the efficient isolation and growth of microglia in our protocol. However, for labs without L929 cell culture this becomes a limiting step considering the overall cost of the protocol as additional growth supplements will be needed. We were able to get microglial population of about 80% in our culture conditions. This is less than some published protocol but this can be overcome by having an additional round of isolation through specific protocols like using magnetic beads for specific microglial markers. At 80% culture purity the protocol is efficient for many experiments like immunocytochemistry. However for doing experiments like protein purification, protein identification and western blotting additional purification of the culture might be needed. Even with high purity of the primary cultures there is always a possibility that other cells present in the culture might increase with longer culture duration. We have successfully cultured isolated microglia for 9 days by passaging them once. While the culture conditions in our protocol favors the isolation and growth of microglia, presence of other cells should be considered when maintaining the culture for longer duration.

3.5 CONCLUDING REMARKS

Our protocol for isolating primary microglia is effective, robust and cost efficient. Such protocols for isolation of primary human microglia from adult brain tissue will enable timely research on immune functions, cell physiology and disease responses in the adult brain. Additionally, patient derived primary microglia may aid in developing personalized, future therapeutics.