Purification and Culture Methods for Astrocytes

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Introduction

Inflammatory responses are a major part of all CNS insults, including acute trauma, infection, and chronic neurodegenerative disease (Sofroniew, 2015). In trauma and infection, the principle culprits in initiating and propagating this inflammatory response are circulating bone-marrow-derived leukocytes. In chronic neurodegenerative disease, the concept of neuroinflammation has evolved and implies an inflammatory process thought to originate primarily from CNS cell types. Chief among these CNS glial cells are microglia, the resident myeloid cells of the brain. It is also becoming apparent, however, that this response involves astrocytes. Microglia and astrocytes have both pro-inflammatory and anti-inflammatory functions, depending on the mode of injury (Zamanian et al., 2012; Anderson et al., 2016; Crotti and Ransohoff, 2016; Liddelow et al., 2017). Acute trauma, chronic infection, and other diseases of the CNS trigger a coordinated multicellular inflammatory response that involves glia as well as neurons and other nonneuronal CNS cells.

As techniques for astrocyte purification and visualization have improved, recent advances have shown that astrocytes are able to respond to a vast array of CNS insults. Such insults include, but are not limited to, traumatic brain injury, spinal cord injury, stroke, brain tumor, inflammation, and a wide range of neurodegenerative diseases (for references, see Sofroniew, 2015; Liddelow and Barres, 2017). These injuries coincide with robust activation of astrocytes as well as microglia and other peripheral immune cells, and therefore it has been difficult to discern the relative importance and function of individual cell-type responses. We now know that the astrocyte response machinery includes phagocytosis of synapses, changes in the secretion of neurotrophins, clearance of debris and dead cells, repair of the blood–brain barrier (BBB), and formation of a scar to enclose the necrotic lesion of such injuries or infection. These effects benefit the CNS, but as we will discuss, mounting evidence points to negative outcomes of reactive astrocyte responses as well.

The large number of cell types involved in inflammatory responses in CNS injury and disease, as well as the complex cell–cell interactions among these and other neural cell types, has hampered mechanistic understanding of glial reactivity. The main focus of recent work has been to address the lack of appropriate models for studying the mechanisms of glial dysfunction. How heterogeneous is the glial response to injury and disease, and how is this heterogeneity induced? Are reactive glia helpful or harmful and, if so, how are their effects mediated? Advances in these areas have implications for the development of new therapies for CNS injury and disease.

This chapter will run through the most commonly employed methods to purify and culture astrocytes *in vitro*. The main goal is to begin comparing the new suites of cell purification and culturing methods that have been developed and to highlight key areas in which they could be improved in the future.

Purification and Cell Culture Methods to Study Astrocyte Function

Cell purification provides a powerful method that enables the study of the intrinsic properties of a cell type in isolation, as well as enabling the investigation of interactions between different cell types. Despite their abundance in the CNS, the study of astrocytes has been hindered by the lack of appropriate methods for their purification and culture. This section briefly reviews the main methods for astrocyte purification. It should be noted that this list is not exhaustive, and there are many alterations to each of these methods. What should be considered at all times is which method is going to most appropriately enable you to distinguish the astrocyte function under investigation. Equally important is whether the model you choose is an appropriate proxy for this function, as observed in astrocytes *in vivo*—either in rodents or, more specifically, in humans. A brief overview of each method, including pros and cons, is provided in Figure 1.

The McCarthy and de Vellis astrocyte model

The MD-astrocyte model, so named for its authors Ken McCarthy and Jean De Vellis (1980), was the first *in vitro* system to allow for the widespread study of isolated astrocytes. These MD astrocytes have been extremely powerful and useful but have several shortcomings. First, purification takes several weeks and lends itself to considerable contamination of other CNS cell types, including microglia and progenitor cells. Second, these cultures are maintained in serum-containing media, and owing to the presence of the BBB, serum components are usually excluded from the CNS (except in instances of trauma or vascular distress following stroke). Serum exposure appears to alter astrocyte transcriptomes and morphology in various ways, leading to fewer processes and larger hypertrophied cell bodies akin to reactive astrocytes or fibroblasts *in vivo* (Foo et al., 2011). Although MD cells are largely used as the

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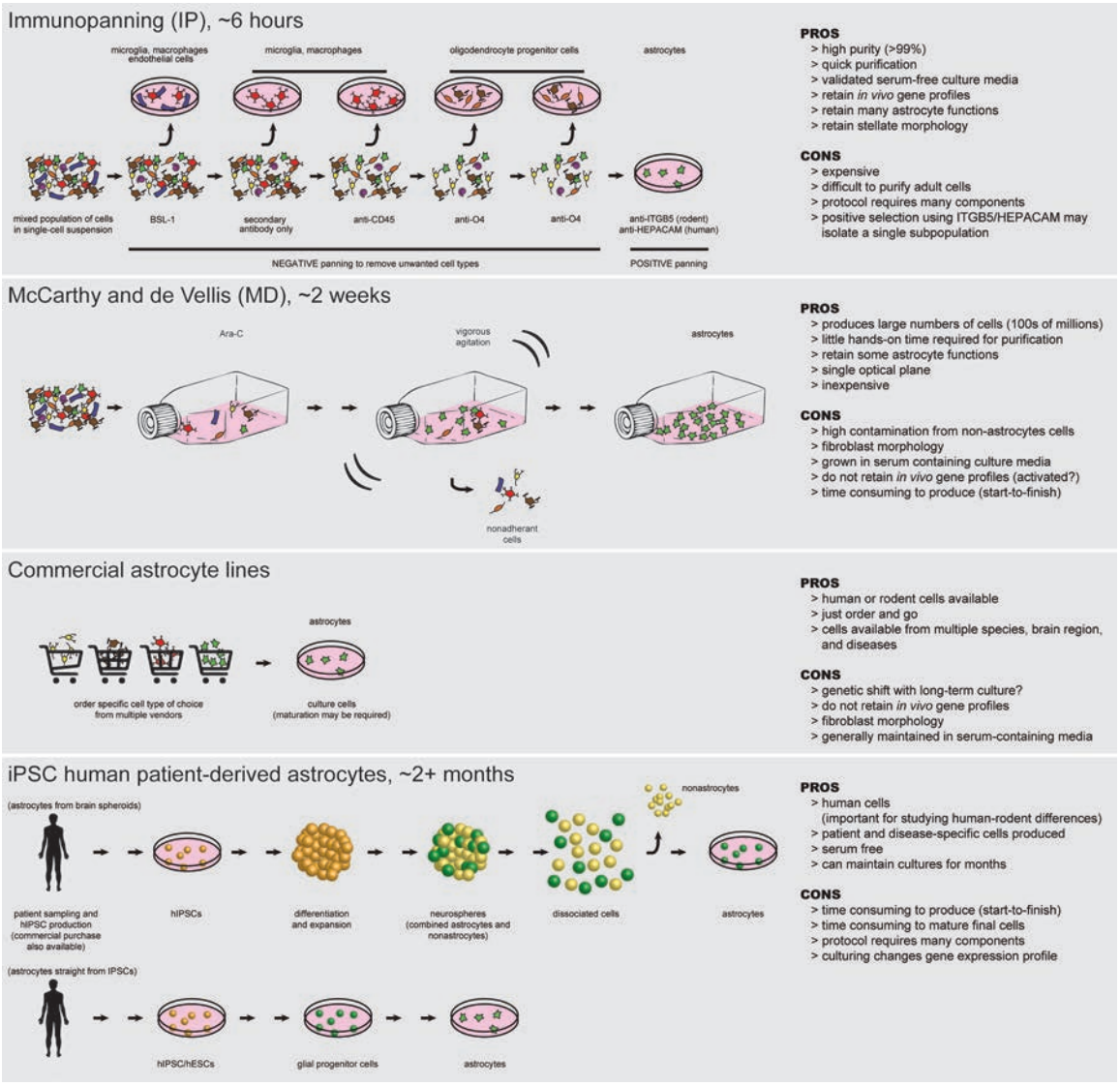

Figure 1. Methods for purifying and culturing postnatal astrocytes. Top row, IP reliably provides high-yield, high-viability cells in three steps: (1) enzymatic preparation of a cell suspension, (2) passing this suspension over a series of antibody-coated panning (Petri) dishes, and (3) removing the purified cells from the final dish. Second row, the MD-astrocyte model (McCarthy and De Vellis, 1980) comprises mainly (1) the death of neurons in cultures prepared from postnatal rat cerebra; (2) the rapid proliferation of astrocytes and oligodendrocytes in culture; and (3) the selective detachment of the overlying oligodendrocytes when exposed to sheer forces generated by shaking the cultures. Third row, commercially available cell lines grown in serum-containing media, from patients with verified disease states. Bottom, human patient–derived embryonic stem cells or adult fibroblasts are retro-engineered to IPSC states that can then be enticed to differentiate into astrocytes. BSL-1, Griffonia (Bandeiraea) Simplicifolia Lectin 1; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell.

premiere astrocyte purification and culture system of choice, it is becoming more apparent that these cells do not adequately model many aspects of *in vivo* astrocytes. Additionally, because these MD astrocytes can be isolated only from neonatal brain, they are highly mitotic, unlike mature astrocytes *in vivo*. Hence, it is speculated that these cells may be more akin to radial glia or astrocyte progenitor cells. Recent transcriptome databases of astrocytes purified by fluorescence-activated cell sorting (FACS) show that MD astrocytes highly express hundreds of genes that are not normally expressed *in vivo* (Cahoy et al., 2008). In addition, their profiles indicate that they may consist of a combination of reactive and developing astrocytes (Zamanian et al., 2012; Zhang et al., 2016) as opposed to resting, mature astrocytes.

This original method of purifying "astrocytes" is based on three main steps: (1) the death of neurons in cultures prepared from postnatal rat cerebra; (2) the

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rapid proliferation of astrocytes and oligodendrocytes in culture; and (3) the selective detachment of the overlying oligodendrocytes when exposed to sheer forces generated by shaking the cultures (Fig. 1). These three steps leave a highly proliferative, dense monolayer of astrocyte-like cells that can be replated and passaged to provide an enormous number of additional cells.

Several alterations to the original MD protocol go some way to improving the method. Each amendment has been produced to improve a specific readout of astrocyte function. Several three-dimensional matrices of MD astrocytes using high concentrations of HB-EGF (heparin-binding epidermal growth factor, an astrocyte trophic support molecule) (Foo et al., 2011) have yielded cultures with far more processes-bearing morphologies (Puschmann et al., 2013; Placone et al., 2015). Follow-up studies, however, showed that such high concentrations of HB-EGF can cause these astrocytes to de-differentiate (Puschmann et al., 2014). Alternative approaches that use the original MD purification steps but then grow the acquired cells in serum-free media have also been used. Morita and colleagues (2003) first used serum-free media for growing astrocytes. These produced thin processes and glutamate-inducible, but not spontaneous, Ca^{2+} fluctuations (spontaneous astrocyte Ca^{2+} fluctuations occur in brain slices) (Nett et al., 2002; Foo et al., 2011). Further additions to the serum-free approach included other growth factors (e.g., EGF and TGF-α); however, these largely produced "reactive" astrocytes with increased GFAP immunoreactivity (Tsugane et al., 2007). The latest alteration to the MD protocol, so-called AWESAM (a low-cost easy stellate astrocyte method), has proven better for measuring Ca^{2+} dynamics (Wolfes et al., 2016). Unfortunately, the authors did not present a transciptome analysis of their astrocyte cultures, so it is unknown whether these are representative of more "normal" astrocyte functions.

What is unknown is whether the MD method produces astrocytes that are irreversibly changed from their *in vivo* counterparts—and whether they can perhaps be enticed to change back into a nonreactive, process-bearing form.

Immunopanning astrocytes

The use of immunopanning (IP) to purify CNS cells was developed by Ben Barres in the 1980s (Barres et al., 1988) and has been modified continuously for multiple CNS cell types in the decades since. Once proficiency is achieved, IP reliably provides high-yield, high-viability cells. Panning is, at its heart, rather trivial and involves only three steps: (1) enzymatic preparation of a cell suspension, (2) passing this suspension over a series of antibody-coated panning (Petri) dishes, and (3) removing the purified cells from the final dish. Having said this, although purification of cells by panning is simple, it does take practice, as every step needs to be done correctly to achieve high viability by the end of the procedure. A detailed outline of the major pitfalls of IP, key tips for producing personalized panning protocols, and references to IP protocols for multiple CNS cell types are provided elsewhere (Barres, 2014).

In a typical IP purification, cell-type-specific antibodies are adsorbed to the surface of a Petri dish, and a cell suspension from the tissue sample of interest is then consecutively passed over several of these coated IP dishes (Fig. 1). The first "negative panning" dishes deplete unwanted cell types, such as microglia, and the final "positive" dish selects for the cell type of interest (e.g., astrocytes). Because the protocol is based on prospectively catching your cell of interest, there is a requirement for a cell-type-specific cell-surface antigen to which an appropriate antibody has been raised. There are many searchable cell-type-specific transcriptome databases; for example, mouse and human CNS glia datasets are freely accessible and downloadable at http://www.brainseq.org (Cahoy et al., 2008; Zhang et al., 2016). More brain-region-specific astrocyte transcriptome databases can be accessed at http://astrocyternaseq.org (Chai et al., 2017).

Unlike the weeks-long MD purification methods, IP rapidly purifies astrocytes from postnatal rodent brain in < 1 d (Foo et al., 2011). Panning for astrocytes is possible in the rodent using antibodies to the cell-surface antigen ITGB5 (Foo et al., 2011, 2013) and in the human using HEPACAM (Zhang et al., 2016). Rodent cells can also be grown in serum-free conditions (a minimal base media with the addition of the astrocyte trophic factor HBEGF), which enables them to retain their *in vivo* gene profiles for extended periods. In addition to retaining gene profiles, IP rodent astrocytes maintain their distinct tiling domains in culture, are multiprocess bearing, have polarized aquaporin 4 (AQP4) protein localization, conduct Ca^{2+} transients, are connected via gap junctions, and maintain many other normal physiological functions (Foo et al., 2011; Liddelow et al., 2017). Although human astrocytes can easily be purified using HEPACAM antibodies (Zhang et al., 2016), maintaining their nonactivated transcriptome profiles remains elusive.

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To date, IP astrocytes (and other IP purified cells discussed in Barres, 2014) remain the best way to obtain highly pure populations of cells that can be maintained in a nonactivated state. Although expensive, the data obtained from these culture methods are largely reproducible in *in vivo* models, making the difficulties of setting up cultures and maintaining a serum-free culture system well worth the effort.

Recently, we used base IP methods to develop a new model system that enables pure neuroinflammatory reactive (A1) astrocytes to be studied in a culture dish (Liddelow et al., 2017). This was possible thanks to our ability to rapidly purify astrocytes from the uninjured postnatal brain, grow them in serum-free cultures, and finally supplement these cultures with a reactive astrocyte–inducing, microglial-derived cytokine cocktail. Microglial activation (by either acute CNS injury or systemic lipopolysaccharides injection) induces A1 reactive astrocytes both *in vitro* and *in vivo*. We found that microglia induce these A1s by releasing three cytokines: interleukin 1 alpha (IL-1α), tumor necrosis factor alpha (TNF-α), and the complement component subunit 1q (C1q), which together are sufficient *in vitro* to induce A1 reactive astrocytes whose gene profiles closely mirror that of A1 reactive astrocytes *in vivo* (Liddelow et al., 2017). The resulting cultures of pure A1 reactive astrocytes provide a powerful tool with which to investigate their functions. Using this model, we found that A1s have a striking loss of most major astrocyte functions: a decreased ability to induce synapse formation and function, diminished ability to phagocytose synapses, and a loss of ability to promote neuronal survival and growth. In an improvement to GFAP staining as a marker for reactivity, single-cell data showed that the complement component C3 was specifically upregulated in A1 reactive astrocytes (and not in resting or ischemic "A2" reactive astrocytes). This marker now provides a way to distinguish among different activation states of reactive astrocytes in both rodent and human tissue. Surprisingly, the A1 reactive astrocytes also exhibited a new function in which they secreted a yet to be identified neurotoxin that induced apoptosis of neurons and oligodendrocytes but no other CNS cell types. Important to note, when validating these findings *in vivo*, we found that A1 reactive astrocytes were rapidly induced after CNS injury and were responsible for the death of axotomized CNS neurons. When A1 formation was prevented genetically or pharmacologically, the death of the axotomized CNS neurons was entirely prevented. Interestingly, on their own, the activated microglia used for inducing A1s were insufficient to induce the death of neurons or oligodendrocytes.

This study highlights the importance of using the correct purification and culture system to model your disease, injury, or dysfunction of interest. If the model you are using does not recapitulate the human disease state of your cell of interest, it is not the best system to answer your question.

Commercially available cell lines

Using commercially available cells is an easy way to acquire a range of cell types from patients with verified disease states. These cells also provide a quick way for laboratories that are new to cell separation techniques to gain access to cells and start experiments rapidly. Care should be taken, however, as many of these lines either are irreversibly activated, contain many precursor cell types, or are contaminated with other cell types. Additionally, like most cell culture methods, these cells are grown in serum-containing media, which as outlined above, is not a normal contributor to the tightly controlled CNS milieu (serum leakage into the brain is characteristic of ischemic injury).

Another caveat is that cell lines can change over time in culture even without any external contamination from cells or bacteria. As they grow generation after generation, chromosomal duplications and/or rearrangements, mutations, and epigenetic changes can alter their original phenotype. These changes often go undetected because cells from different sources can be morphologically similar. It unfortunately seems inevitable that cell-line alteration will occur (Lorsch et al., 2014), which is ultimately problematic. For these reasons, it is generally better for mechanistic studies of astrocytes to be performed in primary cells or for culture-line purity to be routinely tested.

Induced pluripotent stem cells: monocultures and brain balls

Most recently, the proliferation of newer methods of producing astrocytes (or astrocyte-like cells) from human patient–derived samples has exploded. Multiple methods are now available that begin with different starting materials, be they embryonic stem cells or adult fibroblasts retro-engineered to induced pluripotent stem cell (IPSC) states that can then be enticed to differentiate into astrocytes (Krencik and Ullian, 2013; Santos et al., 2017). Each of these methods produces equally pure monolayered populations of astrocytes that have highlighted some key differences between rodent and human astrocytes, as well as providing new insights into the genetic differences in astrocytes between healthy and diseased individuals.

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An alternative approach is to produce spheroids of either pure neurons or a mixture of glia and neurons (Paşca et al., 2015; Sloan et al., 2017). These systems provide patient-derived human cells with the added benefit of including multiple CNS cell types, being more akin to the *in vivo* setting, and allow for investigations of cell–cell interactions. For example, one can coculture nondiseased neurons with diseased astrocytes (or vice versa) to help ascertain the relative contributions that individual cell types make to disease. Of additional benefit is the fact that at the end of growing such spheres, other purification methods (e.g., IP, FACS) can be used to separate astrocytes for further culturing or sequencing analyses (Paşca et al., 2015; Sloan et al., 2017). Notably, recent advances in cortical neurospheres have shown that astrocytes in these organoids undergo maturation akin to that which occurs during normal human development (Sloan et al., 2017).

Like all methods, however, there are several pitfalls that must be considered (Fig. 1). The availability of originating human samples can be difficult at some institutions, and the length of time required (several months) for producing and maturing such spheroids can be both cost- and time-prohibitive for some researchers. In addition, the long culture times required can lead to increased instances of contamination if sterile culture protocols are not adhered to.

What about our little friends, the microglia?

Purification and culture systems for microglia have largely lagged behind the successes of astrocyte culture systems. This has been a major impediment to further investigations into how they might interact with astrocytes in both physiological and reactive settings, whether this activation results from infection, disease, or trauma. In addition, the difficulty of producing appropriate transcriptome databases of resident microglia (as distinct from circulating peripheral immune cells) has meant that a "baseline" to aim for in the production of new culture methods has been difficult. Recent advances have shown that TMEM119 (transmembrane protein 119) can be used as an appropriate marker to delineate CNS and peripheral immune cells (Bennett et al., 2016). This baseline microglial transcriptome database has provided key validation of newer methods for studying these cells *in vitro*. It shows that TGF-β signaling (Butovsky et al., 2014; Bohlen et al., 2017), in addition to IL-34 (colony-stimulating factor 1) and cholesterol (Bohlen et al., 2017), are required to mitigate the upregulation of traditional microglial reactivity markers generally seen in previous microglia cultures. What has been surprising in these studies is that, although the reactivity can be minimized in serum-free media, a rapid and sustained downregulation of microglial signature genes still occurs when the cells are placed in culture (Bohlen et al., 2017; Gosselin et al., 2017). This suggests that improvements can be made to ensure that the transcripts that delineate microglia from peripheral immune cells are not lost when removing the cells from the CNS and placing them in culture.

Things to keep in mind

It has been very difficult to distinguish the contributions of astrocytes from those of other CNS cells. This is particularly difficult with interactions between astrocytes and microglia because they usually become reactive in concert and are both involved in neuroinflammation. These delicate interactions are difficult to study in *in vivo* systems because many of the key proteins and genes are present or expressed by multiple cell types. As a result, it is generally better to use culture systems to complete such mechanistic investigations. But how does one address such questions, given the multitude of methods available to purify and culture these cells? Each method has pros and cons that need to be taken into account when choosing the most appropriate methods for your investigation (Fig. 1).

For instance, there is evidence that growing astrocytes on a three-dimensional polymer matrix might be even more appropriate than methods outlined above. These astrocytes show less upregulation of *Gfap* than cells grown in a two-dimensional monolayer, and their many branching processes make them morphologically complex (Puschmann et al., 2014). However, even these astrocytes are prepared according to the original MD-serum-containing methods of the 1980s, and as such, they are probably transcriptomically different from astrocytes present in the normal healthy CNS. The variety of purification and growth paradigms suggests that perfect culture systems for modeling a "normal" *in vivo* astrocyte are likely to require a combination of features, including specific trophic support, the correct substrate, and possibly other unknown factors.

Final Remarks

Improved methods for producing highly purified astrocytes and microglia will allow their relative contributions and highly coordinated interactions to be better dissected and understood. In addition, the validation of available animal models of

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neurodegenerative disease will be essential. Most of these models were produced to model particular aspects of neuronal dysfunction in disease. If these models are to be used to investigate immune–glial interactions in these diseases, we must ensure that they correctly recapitulate the glial dysfunction seen in human patients.

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