Journal of Biotechnology 386 (2024) 10–18

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Primary microglia cell cultures in translational research: Strengths and limitations

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ARTICLE INFO

Keywords:
acute brain injury
microglia
neurodegeneration
neuroinflammation
primary cell culture

ABSTRACT

Microglia are the resident macrophages in the central nervous system, accounting for 10–15% of the cell mass in the brain. Next to their physiological role in development, monitoring neuronal function and the maintenance of homeostasis, microglia are crucial in the brain's immune defense. Brain injury and chronic neurological disorders are associated with neuroinflammation, in which microglia activation is a central element. Microglia acquire a wide spectrum of activation states in the diseased or injured brain, some of which are neurotoxic. The investigation of microglia (patho)physiology and therapeutic interventions targeting neuroinflammation is a substantial challenge. In addition to *in vivo* approaches, the application of *in vitro* model systems has gained significant ground and is essential to complement *in vivo* work. Primary microglia cultures have proved to be a useful tool. Microglia cultures have offered the opportunity to explore the mechanistic, molecular elements of microglia activation, the microglia secretome, and the efficacy of therapeutic treatments against neuroinflammation. As all model systems, primary microglia cultures have distinct strengths and limitations to be weighed when experiments are designed and when data are interpreted. Here, we set out to provide a succinct overview of the advantages and pitfalls of the use of microglia cultures, which instructs the refinement and further development of this technique to remain useful in the toolbox of microglia researchers. Since there is no conclusive therapy to combat neurotoxicity linked to neuroinflammation in acute brain injury or neurodegenerative disorders, these research tools remain essential to explore therapeutic opportunities.

1. Introduction

Neuroinflammation is a complex inflammatory response in the central nervous system (CNS) potentially with adverse consequences. Parenchymal immunity in the brain is mediated by microglia, the brain's organ specific, tissue-resident macrophage population. Microglia – as opposed to macrophages in the perivascular space, choroid plexus, and leptomeninges – are derived from myeloid progenitors in the yolk sac. Microglia are central to the maintenance of homeostasis and neuroplasticity in their capacity to survey the nervous tissue, sense neuronal activity or inhibition, prune synapses during development, phagocytose neuronal debris and waste, and modulate cerebral blood flow (Nimmerjahn et al., 2005; Kettenmann et al., 2011; Norris and Kipnis, 2019; Császár et al., 2022; Haruwaka et al., 2024). Microglia functional states are thus diverse in normal conditions. The microglia transcriptome reveals, for example, that microglia states and spatial distribution are

Abbreviations: CNS, central nervous system; CSF, colony stimulating factor; CSFR, colony stimulating factor receptor; CX3CL1, chemokine C-X3-C motif ligand 1, also known as fractalkine; DAMP, damage-associated molecular pattern; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorting; GWAS, genome-wide association studies; HMGB1, high-mobility group box 1; IFN, interferon; IFNγ, interferon gamma; IL-34, interleukin-34; IPSC, induced pluripotent stem cells; LPS, lipopolysaccharide; MACS, magnetic-activated cell sorting; NMDA, N-methyl-D-aspartate; OGD, oxygen-glucose deprivation; PAMP, pathogen-associated molecular pattern; QPCR, quantitative polymerase chain reaction; RAGE, receptor for advanced glycation end products; TGF-β, transforming growth factor-beta; TLR-4, toll-like receptor-4; TNFα, tumor necrosis factor alpha.

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https://doi.org/10.1016/j.jbiotec.2024.03.005

Received 8 February 2024; Received in revised form 13 March 2024; Accepted 13 March 2024

Available online 20 March 2024

driven locally by neuronal identity (Stogsdill et al., 2022).

In pathophysiological states, microglia become activated, proliferate, and are heavily implicated in profound responses to neuronal injury by producing cytokines, chemokines, reactive oxygen species and other mediators (Jurga et al., 2020; Wendimu and Hooks, 2022). The classic view holds that microglia reside in the physiologically intact nervous tissue, survey their microenvironment in a quiescent or resting state (M0), and acquire an activated state in response to endogenous danger signals or exogenous pathogens. The activation state of microglia is reflected by their morphologic phenotypes. As a rule, resting microglia are richly ramified and activated microglia acquire an ameboid shape. Further, activated microglia have been traditionally classified as pro-inflammatory or neurotoxic (M1) and anti-inflammatory or neuroprotective (M2). The M1-M2 polarization has been established by typical cell adhesion molecular patterns and the polarization-specific production profile of cytokines, chemokines, trophic factors and other mediators (Wendimu and Hooks, 2022). Further, the M1-M2 polarization has been refined as a spectrum with intermediate states (M2a, M2b, M2c and M1½), which may express characteristics partially overlapping between the M1 and M2 phenotypes, and shift dynamically between M1 and M2 (Jurga et al., 2020; Wendimu and Hooks, 2022). However, microglia states appear to be more complex, richer, and heavily context dependent, and the dualistic nomenclature has lately been challenged (Hickman et al., 2018). With the recent, rapid development of the transcriptomic and proteomic characterization of microglia, the classic divisions have proven to be too restrictive, and the use of a new terminology has been proposed using combinations of gene or protein markers to identify microglia states (Paolicelli et al., 2022).

The activation of microglia is a common pathological hallmark of chronic, prominent neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Bartels et al., 2020; Leng and Edison, 2021), and acute brain injury including ischemic or hemorrhagic stroke and neurotrauma (Loane and Byrnes, 2010; Planas, 2024). Microglia become activated in these conditions in response to damage-associated molecular patterns (DAMPs) or danger signals liberated from dying or injured cells or disrupted extracellular matrix. As such, adenosine and ATP released from stressed cells act on microglial ionotropic (X) or metabotropic (Y) purinergic P1 and P2 receptors to promote inflammasome activation and IL-1β release (Beamer et al., 2016; Cserép et al., 2020). The priming of microglia may also rest on Type I and Type II interferon (IFN) signaling, which, at elevated level, contribute to the transformation of microglia to the pro-inflammatory, neurotoxic phenotype (West et al., 2019; Kann et al., 2022; Wangler and Godbout, 2023). Nuclear protein high-mobility group box 1 (HMGB1) is also a DAMP released to the extracellular space upon injury, which binds to microglial toll-like receptor-4 (TLR-4) and the receptor for advanced glycation end products (RAGE), leading to the ultimate production of several pro-inflammatory cytokines (Paudel et al., 2020). In response to these stimuli, microglia have also been found to undergo metabolic reprograming with a switch from oxidative phosphorylation to glycolysis, which culminates in mitochondrial dysfunction and the exacerbation of the microglial pro-inflammatory response (Orihuela et al., 2016).

Activated microglia fulfill a highly complex role in the diseased or injured brain. Microglial activation has been implicated in protection and repair (Hanish and Kettenmann, 2007; Szalay et al., 2016), but more attention has been directed at microglia as key players in the pathogenesis of brain disease. With the advancement of experimental methods such as single-cell transcriptomics, an increasingly more nuanced microglia landscape is emerging in acute brain injuries. A longitudinal map of various transcriptomic states of microglia in experimental acute ischemic stroke suggests, for example, that phagocytic and proliferative states may dominate early after ischemia onset. Weeks later, microglia express genes typical of disease-associated microglia (Garcia-Bonilla et al., 2024).

Microglia-linked damage can be caused by a context driven spectrum of compromised physiological function, defective protection, exaggerated detrimental reactions or their causative interplay. Further, recent genome-wide association studies (GWAS) suggest that microglia may actively drive neurodegenerative disorders as some risk genes are expressed specifically in microglia (Efthymiou and Goate, 2017; Gosselin et al., 2017). Without attempting to be comprehensive, activated microglia have been recognized to secrete pro-inflammatory molecules that injure neurons either directly, or by inducing reactive astrocytes with neurotoxic potential (Liddelow et al., 2017). Further, excessive complement-mediated synaptic pruning by microglia has been implicated in progressive neurodegeneration (Bartels et al., 2020; Gomez-Arboledas et al., 2021). Next, microglia may contribute to glutamate excitotoxicity by releasing glutamate to the extra-synaptic compartment via their connexin hemichannels or reversed glutamate transport (Takeuchi et al., 2006; Domercq et al., 2007). The cerebrovascular system also receives input from by microglia (Császár et al., 2022). Pro-inflammatory states of microglia have been linked to the loosening of cerebrovascular endothelial tight junctions and the disruption of the blood-brain barrier (Ronaldson and Davis, 2020). Finally, and importantly, activated microglia recruit immune cells (macrophages, monocytes, neutrophil granulocytes and lymphocytes) from the periphery, which transmigrate through the blood-brain barrier and contribute to neuroinflammation and injury (Prinz and Priller, 2017).

Both the overwhelming complexity of microglial states in brain diseases and the goal of developing new therapeutic approaches justify the use of simple preclinical model systems. Primary microglial monocultures or co-cultures with neurons, astrocytes or oligodendrocytes are widely used in studies of microglial pathobiology because these tools allow reproducible, in-depth and high-throughput analysis of microglial states and function. The controlled manipulation of experimental factors, the standardized experimental setting and the possibility of focused interpretation of the results also make cell culture models essential for gaining valuable insights into microglial biology.

Several studies have shown that primary microglia cultures are essential and have been successfully utilized to understand various aspects of microglia biology and pathophysiology. To name a few, cell culture studies identified that activated microglia – rather than astrocytes – serve as a source of soluble interleukin-1 (Hetier et al., 1988). Primary microglia cultures have also been instrumental in identifying markers that distinguish microglia from peripheral macrophages. As such, a pattern of ion channels unique to microglia has been identified by electrophysiological means (Kettenmann et al., 1990). In research on neuroinflammation and neurodegeneration, microglia cultures have effectively complemented *in vivo* work. For example, the internalization of misfolded proteins or beta-amyloid aggregates by microglia was shown elegantly *in vitro* (Paresce et al., 1996; Leal-Lasarte et al., 2017). Microglia co-cultures with neurons have been successfully used to interrogate how microglia states that occur in neurodegenerative diseases modulate neuronal survival or cell death (Shi et al., 2017). Microglia cultures also offer a convenient platform to test therapeutic interventions against neuroinflammation (Redondo-Castro et al., 2018).

The goal of the current paper is to weigh the advantages against the limitations of primary microglia cultures in biomedical research, and to offer examples for the successful integration of primary microglia cultures among the experimental tools in pre-clinical research.

1.1. *Primary microglia cultures – strengths*

Primary microglia cultures are derived from donor brains. The tissue is mechanically or enzymatically dissociated to obtain a mixed cell suspension, which is then used to generate first mixed cultures grown as a confluent cell layer. Subsequently, microglia are mechanically separated to establish pure microglia monocultures (Giulian and Baker, 1986; Witting and Möller, 2011) (Fig. 1). These steps have been shown to yield a high number of cells and generate microglia cultures with a purity of >95–99% (Dulka et al., 2021). However, it is important to note that the samples may be contaminated with macrophages from the

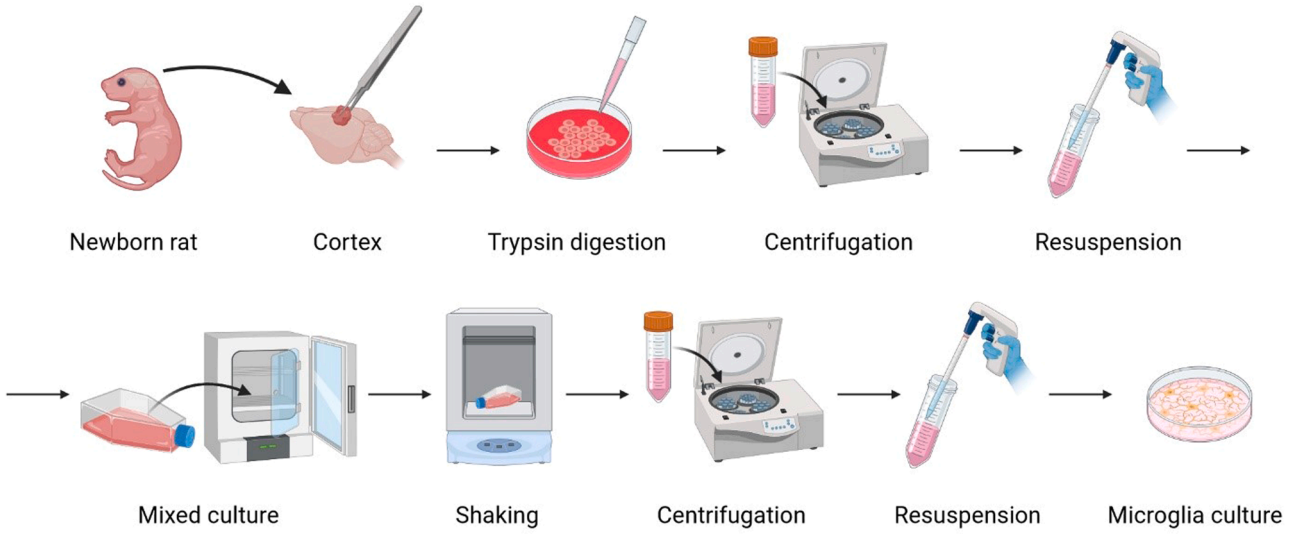

Fig. 1. Protocol to establish primary microglia monocultures with an initial step of primary mixed cell cultures. According to a routine used for many years in our lab (Szabo and Gulya, 2013; Kata et al., 2016; Dulka et al., 2021; Szabo et al., 2023), a tissue sample is obtained from the cerebral cortex of the newborn rodent brain and immersed in Dulbecco's Modified Eagle's Medium. The sample is supplemented with 0.25% trypsin and then centrifuged at 1000 g for 10 min. The pellet is resuspended, and the cells are seeded in poly-L-lysine-coated culture flasks to deliver mixed primary cortical cell cultures. The mixed cultures are incubated at 37 °C in humidified air supplemented with 5% CO_2. Microglia monocultures are then derived from the mixed cultures by shaking the flasks at 100 rpm in a platform shaker for 30 min at 37 °C. Microglia from the supernatant are collected by centrifugation at 3000 g for 8 min and resuspended. Finally, the cells are seeded in Petri dishes at densities required for subsequent morphological or functional assays. The illustration has been created in BioRender.

perivascular space, choroid plexus, and leptomeninges, despite the removal of the meninges and large cerebral vessels. Additionally, astrocytes, oligodendrocyte progenitor cells, oligodendrocytes, and vascular mural cells may sporadically occur in the preparations (Cadiz et al., 2022). Therefore, to achieve more targeted purification, cell sorting techniques are often employed. Antibody-coated magnetic beads are mixed with the dissociated cell suspensions, and the magnetic beads selectively bind to targeted cells, which are then separated using magnetic-activated cell sorting (MACS) (Pan and Wan, 2020; Zelenka et al., 2022). Similarly, cells can be labeled with fluorescent antibodies and isolated using fluorescence-activated cell sorting (FACS) (Bohlen et al., 2019; Pan and Wan, 2020). The main advantage of cell sorting is the acquisition of pure cell populations, although this comes at the cost of low cell numbers and the need for a higher number of animals (Aktories et al., 2022).

Primary microglia cultures are traditionally derived from mouse and rat pups on postnatal day 1–3 (Giulian and Baker, 1986; Witting and Möller, 2011; Lian et al., 2016). However, the mature murine microglia phenotype is not established until postnatal day 15–20 (Harry, 2013). Furthermore, microglia acquire a primed phenotype with aging, which is implicated in the brain's increased vulnerability to age-related disorders (Niraula et al., 2017). Clearly, neurodegenerative diseases with ongoing neuroinflammation typically manifest with advancing age, and aging is the most significant independent risk factor of acute ischemic stroke. These considerations have warranted the use of adult rodent brains as donor organs for primary microglia cultures (Gaikwad and Heneka, 2013; Agalave et al., 2020). Finally, while murine and human microglia share conserved core properties across evolution, recent research has identified species-specific features in microglia (patho) physiology (Smith and Dragunow, 2014). Distinct differences have been identified between murine and human microglia in their activation state in the normal brain (Lassmann, 2020), homeostatic signature (Healy et al., 2020), and metabolic reprogramming (Sabogal-Guáqueta et al., 2023). Efforts have been made to create primary microglia cultures from human brain tissue to more accurately replicate the human condition and increase translational potential (Warden et al., 2023). Human microglia can be cultured from postmortem samples, perioperative brain resections, or fetal brain tissue available after abortion (Warden et al., 2023).

Although human microglia are preferred, there are distinct advantages of using murine primary microglia cultures. These advantages include genetic homogeneity of the animals, a pathogen-free and aseptic breeding environment, and controlled ante-mortem conditions and post-mortem delay (Timmerman et al., 2018). Also, the use of primary microglia derived from transgenic mice allows for the identification of the role of specific genes or intracellular signaling pathways in microglia activation. (Apolloni et al., 2013; Zeyen et al., 2020; Dukay et al., 2021; Zhang and Cui, 2021).

Microglia cell lines from various species immortalized by viral transduction with oncogenes are also available for *in vitro* research (Stansley et al., 2012; Timmerman et al., 2018). However, the application of primary microglia cultures are preferable to the use of virus-infected immortalized cell lines for several reasons including the loss of differentiation of immortalized microglia, their phenotype altered by the viral infection, and a range of genetic and functional differences (Stansley et al., 2012; Timmerman et al., 2018).

The activation of microglia in culture has been successfully applied in mechanistic studies that interrogate the signaling cascades underlying microglial inflammatory responses (Shi et al., 2010). Various triggers can stimulate primary microglia cultures (Fig. 2). The most widely used approach to activate microglia in culture is the addition of lipopolysaccharide (LPS) to the culture medium (Witting and Möller, 2011). LPS is an outer membrane component of the cell wall of gram-negative bacteria and is a pathogen-associated molecular pattern (PAMP) that initiates immune responses. LPS has been recognized as the archetypical activator of TLR-4, which is also a microglial target of DAMPs that trigger neuroinflammation in brain injury. Microglia activation in culture targeting TLR-4 is a valid approach to recapitulate microglia states in neurological disorders. For instance, the activation of microglia TLR-4 was found to be crucial in the progression of injury following experimental ischemic stroke (Parada et al., 2019) or subarachnoid hemorrhage (Islam et al., 2022), and proved to attenuate post-impact seizure development in models of traumatic brain injury (Ping et al., 2021; Radpour et al., 2022).

Because LPS is an exogenous agent, researchers may choose to use a tumor necrosis factor alpha (TNFα)/interferon gamma (IFNγ) cocktail to

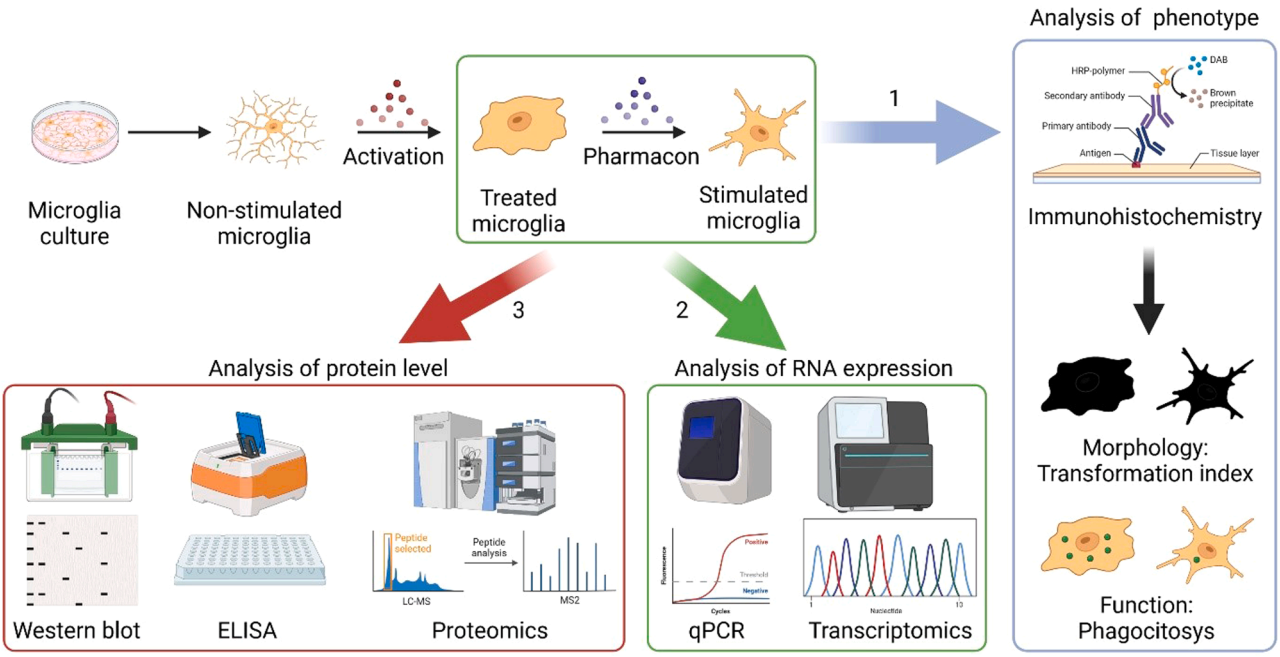

Fig. 2. Assays to provide insight into microglia states in cell culture. Microglia activation in culture is achieved by the addition of stimulating agents to the medium such as LPS, TNFα/INFγ, or NMDA. Oxygen-glucose deprivation (OGD) is a challenge to recapitulate conditions in cerebral ischemia. The phenotypic characterization of microglia morphology and the functional analysis estimating phagocytotic activity are technically traditional approaches (1). The assessment of changes in RNA expression (2) underlies the screening of microglia secretome from the culture media or the analyses of surface marker patterns on the cell membrane (protein assays) (3). The resolution of the morphologic and functional characterization of microglia states has been greatly advanced by state-of-the-art transcriptomic and proteomic analyses (2, 3). Abbreviations: ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction. The illustration has been created in BioRender.

activate cultured microglia, which more closely approximates an activating stimulus in a stressed environment in the injured brain (Lively and Schlichter, 2018). A comparative analysis of LPS and TNFα/IFNγ stimulation *in vitro* revealed apparent differences in microglial responses to the two stimuli. Although both stimuli reliably activated microglia, LPS exposure resulted in a stronger pro-inflammatory response with upregulated expression of more pro-inflammatory mediators compared to TNFα/IFNγ (Lively and Schlichter, 2018).

Microglia in culture can also be activated by N-methyl-D-aspartate (NMDA) to achieve glutamatergic stimulation (Kaindl et al., 2012). Microglia express NMDA receptors (non-selective ionotropic glutamate receptors) in both the rodent and human brain, making them susceptible to supraphysiological extracellular glutamate concentrations that occur during acute brain injury (Andrew et al., 2022). NMDA receptor activation of microglia in culture has been proposed to elucidate the glutamate-dependent, selective contribution of microglia to injury progression. Indeed, cultured microglia switch to an activated state and produce pro-inflammatory cytokines and reactive oxygen species in response to NMDA (Kaindl et al., 2012). Microglia exposed to metabolically stressed neurons have also been shown to become activated by sensing glutamate through their metabotropic glutamate receptors (Kaushal and Schlichter, 2008).

Oxygen-glucose deprivation (OGD) is an established method to mimic cerebral ischemia *in vitro* and is proving to be a useful tool to recapitulate some aspects of microglial activation states in the ischemic brain. While prolonged OGD injures microglia (Yenari and Giffard, 2001; Rabenstein et al., 2020), microglia have been found to proliferate in response to sublethal OGD (Ziabska et al., 2022) and exhibit both M1- and M2-like polarization as evidenced by their cytokine and chemokine expression profile (Barakat and Redzic, 2015; Du et al., 2017). These latter observations are consistent with the notion that microglia in culture form heterogeneous populations even at rest, prior to any stimulation. Microglia in primary culture can be divided into several subpopulations based on their transcriptomic signatures (Cadiz et al., 2022), and it is highly likely that the activation state of microglia in culture is also heterogeneous.

The activated cultures provide a preclinical tool to screen the effect of drug candidates on microglial activation with a view to neuroinflammation (Chao et al., 1992; Suzumura et al., 1999; Kata et al., 2016; McKee et al., 2023; Szabo et al., 2023). This simple approach allows a rough estimation of the effects of drug candidates selectively on microglia, without the need to filter out pharmacological effects on other CNS cell types or the interaction between other affected CNS cells and microglia. Microglia cultures are also an appropriate model system for screening pharmacological agents after *in silico* molecule design and before *in vivo* testing (Sebastian-Valverde et al., 2021). In addition, the use of microglia cultures is a useful tool to confirm the effects of anti-inflammatory drugs targeting microglia *in vivo* (Greco et al., 2003; Braatz et al., 2023).

Microglia cultures allow the assessment of a wide range of read-outs (Fig. 2). Phenotypic characterization can capture morphological features of immunostained cells corresponding to either inactivated (arborized) or activated (ameboid) states. Several algorithms have been developed to calculate a ramification or transformation index that reflects the degree of arborization. These algorithms are derived from Scholl analysis or use a ratio of cell perimeter to surface area (Faulkner et al., 2011; Szabo and Gulya, 2013; Maguire et al., 2022). In addition, automated analysis of microglial morphology using a deep learning approach has been shown to be an elegant solution (Dukay et al., 2021).

Functional phenotypes can also be distinguished focusing on migration, endocytosis, phagocytosis, autophagy or metabolic program. The ability of microglia to migrate is assessed by specific assays. The transwell migration assay using chemoattractants appears to be the most appropriate for primary microglial monocultures (Rumianek and Greaves, 2020; Maguire et al., 2022). Estimation of endocytotic and phagocytotic activity can be accurately determined by adding specific fluorescent cargoes to the culture medium and detecting their internalization by microglia. Labeled transferrin or epidermal growth factor

is used to detect endocytosis, while the uptake of E. coli, zymosan bioparticles (a complex carbohydrate derived from yeast), or latex beads (synthetic polymer microspheres) indicates phagocytosis (Kata et al., 2016; Maguire et al., 2022). Autophagy is a process by which long-lived proteins, cytoplasmic debris or organelles are isolated and delivered to lysosomes for degradation. LC3 (microtubule-associated protein 1 A/1B light chain 3) has been implicated in the formation of autophagosomes (Tanida et al., 2008). The association of LC3 with autophagosomes allows the quantification of LC3-positive particles in a cell or the ratio of cytosolic and membrane-bound LC3. Furthermore, the intensity of autophagy can be estimated with the LC3 turnover assay (Mizushima et al., 2010; Plaza-Zabala and Sierra, 2024). Finally, the metabolic program and activation state of microglia are interrelated, and microglial metabolism is emerging as a target to modulate microglial activity (Sabogal-Guáqueta et al., 2023). Microglial metabolic programming is assessed by measuring oxygen consumption rate and extracellular acidification rate using a Seahorse Extracellular Flux Analyzer, which characterizes mitochondrial respiration and glycolysis (Montilla et al., 2020).

Microglial states can also be identified by protein detection or gene expression profiling (Fig. 2). Proteins, such as cytokines secreted by microglia, are identified and quantified from culture media using single-protein ELISA or multiplex ELISA to assay multiple proteins simultaneously in a single experiment (Maguire et al., 2022). Mass spectrometry for a more comprehensive analysis of the protein content of the medium is feasible if the medium is serum-free. In addition, extracellular vesicles isolated from the culture medium and their contents can also be identified by mass spectrometry (Santiago et al., 2023). Proteins in the microglial cell membrane, within cellular organelles and in the cytosol are effectively measured by Western blot for a few selected proteins (Lam et al., 2017) or by mass spectrometry for a complete proteomic screening (Flowers et al., 2017). Microglial protein surface markers are used to sort microglia into subtypes by flow cytometry (Milner et al., 2022). In addition to using quantitative polymerase chain reaction to quantify specific nucleic acid sequences, it has become standard practice in microglia research to obtain a comprehensive gene expression profile using single-cell RNA sequencing (Dumas et al., 2021). Finally, chromatin accessibility across the genome is determined using an assay for transposase-accessible chromatin with sequencing (Grandi et al., 2022). Preferably, the combination of these methods provides a good estimation of the activation state of microglia in culture. These techniques are suitable to characterize the microglial response to stimulation and the efficacy of pharmacological treatment to inhibit the neurotoxic or enhance the neuroprotective state of microglia.

Ona final note, the use of primary microglial cultures serves the "Three Rs" principle (Replacement, Reduction and Refinement), a recommendation to conduct animal research in a humane manner and to minimize the use of laboratory animals in scientific procedures.

1.2. *Primary microglia cultures – limitations*

In general, some of the strengths of rodent primary microglia cultures listed above may also be perceived as limitations. These include the lack of heterozygosity due to inbreeding and the pathogen-free housing conditions (Timmerman et al., 2018). As detailed above, the divergence from human microglia is also understood to block clinical translation (Smith and Dragunow, 2014), but the technical possibility to bring human microglia into primary cultures resolves this limitation. With the detailed transcriptomic, epigenetic, and proteomic characterization of microglia in culture, more focused concerns are being raised to guide the refinement of culture conditions.

Microglia isolated from the newborn rodent brain are not mature. Furthermore, the loss of interaction with other cell types in the brain microenvironment has long been recognized as an important limitation of primary microglial cultures. In the healthy brain, microglia continuously monitor neuronal activity and integrity. Neuronal signals such as the chemokine CX3CL1 (also known as fractalkine) and the neuronal membrane proteins CD200 and CD22, which maintain the quiescent state of microglia (Biber et al., 2007), are absent in microglia monocultures (Biber et al., 2014). Some of these limitations are overcome by culturing microglia together with other cells in mixed 2D co-cultures. Co-culturing microglia with astrocytes provides a supportive environment for microglia and allows the study of complex microglia-astrocyte interactions in various disease contexts (Akhmetzyanova et al., 2024; Warden et al., 2023). This is confirmed by findings described below that astrocyte-derived molecular patterns are essential to support microglia differentiation (Bohlen et al., 2017). Co-culturing microglia with neurons may also offer distinct advantages for studying cell-cell interactions (Roqué and Costa, 2017). For example, neuronal viability can be assessed in a shared environment with microglia of different states (Shi et al., 2017). In a similar manner, microglia-oligodendrocyte co-culture systems are suitable for dissecting the mechanistic role of microglia in myelin synthesis (Hamilton and Rome, 1994).

The importance of the presence of astrocytes – or at least substances of astrocyte origin – have been demonstrated in more detail. Recent investigations have shown that microglial differentiation and survival in culture can be supported by the addition of astrocyte-derived growth factors and cytokines to the medium (Bohlen et al., 2017). Microglia in the CNS require activation of the colony stimulating factor receptor (CSFR) for survival (Elmore et al., 2014). In addition to its ligand CSF-1, interleukin-34 (IL-34), a cytokine that activates CSFR, is critical for microglial differentiation (Wang et al., 2012) and has been used together with CSF-1 to supplement culture media (Bohlen et al., 2017). Furthermore, transforming growth factor beta (TGF-β) is a cue for microglia to acquire their adult molecular signature (Butovsky et al., 2014) and has also been found to be essential for microglial survival in culture (Bohlen et al., 2017). In addition to growth factors and cytokines, cholesterol has been identified as another lipid supplement to maintain cell cultures (Bohlen et al., 2017). Taken together, a finely balanced composition of the culture medium must instruct microglia to acquire a phenotype that approximates the physiological state of microglia in the CNS (Bohlen et al., 2017).

Due to the lack of microenvironmental input, microglia in primary cultures are proliferative, acquire an amoeboid, phagocytotic phenotype, and have a low inflammatory profile (Witting and Möller, 2011; Stansley et al., 2012). Furthermore, some key genes involved in microglial activation have been found to be upregulated (Cadiz et al., 2022). Despite the activated phenotype, the "non-stimulated" state of microglia proves suitable to study some effects of activation with LPS or other triggers (Witting and Möller, 2011). Furthermore, activation of freshly isolated microglia can resolve within hours to days. However, some changes are more persistent and have been linked in part to the composition of the culture medium (Bohlen et al., 2017; Cadiz et al., 2022). Culture media are often supplemented with serum to support cell survival. Microglia cultured in the presence of serum resemble microglia exposed to blood-borne substances after blood-brain barrier disruption in the injured brain (Bohlen et al., 2017). To circumvent this obstacle and achieve a more ramified, quiescent state of microglia in culture, a protocol using serum-free medium has recently been developed and shared (Collins and Bohlen, 2018).

In parallel with the acquisition of an activated state, some key microglia-specific homeostatic markers are downregulated in culture (Gosselin et al., 2017; Cadiz et al., 2022). Downregulated microglial signature genes include Sall1, which encodes a transcriptional regulator (Buttgereit et al., 2016), Tmem119, a Type I transmembrane protein gene (Bennett et al., 2016), and P2ry12, which encodes the microglia-specific P2Y12R purinergic receptor (Cserép et al., 2020). TGF-β1 limited the downregulation, although it did not fully restore the expression of these genes (Gosselin et al., 2017). Furthermore, casual network analysis identified the immune and microglial gene C1qc as one of the key drivers of the shift in gene expression from homeostatic to activated state markers (Cadiz et al., 2022). In support of this notion,

C1qc knockdown downregulated microglial activation genes (Cadiz et al., 2022).

The high-resolution assays of transcriptomics and proteomics have greatly increased the understanding of the similarities and differences of microglial states in the *in vivo* microenvironment and in *in vitro* model systems. This provides an unprecedented opportunity to further refine microglial cultures for translational biomedical research. It is also expected that the knowledge gained will improve the cautious interpretation of *in vitro* findings.

2. Outlook

The distinct strengths, together with the limitations of *in vitro* approaches to study microglia has spurred the invention of new *in vitro* model systems. Human induced pluripotent stem cells (iPSC) differentiated to microglia have been introduced into the field of microglia biology, which has become a powerful platform (Sabogal-Guáqueta et al., 2020). The human genetic background and the possibility to obtain, reprogram and differentiate cells from individuals suffering from neurological disorders offers the opportunity to study the impact of specific genetic variants associated with neuroinflammatory diseases. Inherently, these models are less effective to explore neuroinflammation in sporadic forms of CNS disorders. iPSC systems prove to be superior to primary cell cultures from a technical point of view, as well, namely that iPSCs are capable of timeless self-renewal to generate a consistent supply of microglia (Hedegaard et al., 2020; Stöberl et al., 2023).

Like primary microglia cultures, iPSC-derived microglia are studied in monocultures and co-cultures. The recent advances in the field aimed to recapitulate the brain microenvironment and cell-cell interactions by creating 3D scaffolds and 3D organoids (Hedegaard et al., 2020; Stöberl et al., 2023). Cerebral organoids containing microglia are at the forefront of the development of new tools to study microglia. Since the organoids themselves self-assemble from cells of ectodermal origin, these 3D structures must be populated with microglia, which can be achieved by the addition of iPSC-derived microglia (Zhang et al., 2023). Although organoid systems are often employed to study brain development because they recapitulate certain events of brain organogenesis (Sabate-Soler et al., 2022), their use to dissect mechanisms of brain pathology has recently gained momentum (Hong et al., 2023). Finally, xenotransplantation of human iPSC-derived microglia into the rodent brain has opened new ways to investigate human microglia in its brain tissue environment, exposed to all variety of brain cells and the extracellular matrix (Hedegaard et al., 2020; Stöberl et al., 2023). These new technologies carry great potential to gain further insight into microglia function in human neuroinflammatory disorders and acute brain injury, and are expected to narrow the translational gap between rodent and human microglia research.

3. Conclusions

Primary microglia cultures have been part of the toolbox of microglia research for the last 35–40 years and are still considered and used as a model system to interrogate microglia biology. The cultures are easy to maintain, are a consistent source of cells, offer high cell numbers, are easy to manipulate under controlled conditions, deliver samples suitable for a wide variety of assays, and generate reproducible data. Building on the isolation of microglia from the postnatal rodent brain, it is now possible to bring human microglia into primary culture from perioperative brain resections, or to reprogram human iPSC to microglia. With the fast development of new assays and analytical methods, microglia states in cell cultures are characterized increasingly more precisely. The detailed analyses of microglia states with transcriptomics and proteomics have been informative to appreciate a great variety of microglia states in cultures. Also, this knowledge has been instrumental to raise awareness of differences between microglia states acquired *in vitro* and *in vivo*. Moreover, the accumulating new information have urged the constant revision and progressive improvement of the *in vitro* model systems to represent microglia states in the brain microenvironment more accurately.

The stimulation of microglia cultures contributes to the understanding of the molecular mechanisms of microglia activation. Further, the administration of pharmacological agents to stimulated cultures serves as a first pre-clinical step in drug development against the deleterious aspects of neuroinflammation. Regrettably, there is no conclusive therapy to treat acute brain injury or neurodegenerative disorders. Therefore, these research tools remain essential and complementary to *in vivo* investigations to explore therapeutic opportunities to combat the neurotoxic consequences of neuroinflammation associated with brain diseases.

Funding

The EU's Horizon 2020 research and innovation program grant number 739593; the National Research, Development and Innovation Office of Hungary (grant number K146725); the Ministry of Innovation and Technology of Hungary and the National Research, Development and Innovation Fund (grant number TKP2021-EGA-28 financed under the TKP2021-EGA funding scheme); and the National Brain Research Program 3.0 of the Hungarian Academy of Sciences.

CRediT authorship contribution statement

István Pesti: Conceptualization, Methodology, Writing - original draft. **Ádám Légrádi:** Methodology, Writing - review & editing. **Eszter Farkas:** Conceptualization; Writing - original draft, Writing - review & editing; Funding acquisition.

Declaration of Competing Interest

The authors have no competing interests to declare.

Data Availability

No data was used for the research described in the article.

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