Modelling microglial function with induced pluripotent stem cells: an update

Jennifer M Pocock* & Thomas M Piers*

Department of Neuroinflammation, University College London Institute of Neurology, London, UK.

*email: j.pocock@ucl.ac.uk; thomas.piers@ucl.ac.uk

Abstract| It is becoming increasingly apparent that microglia, the immune cells of the CNS, and their peripheral counterparts, macrophages, have a major role in normal physiology and pathology. Recent technological advances in the production of particular cell types from induced pluripotent stem cells (iPSCs) have led to an interest in applying this methodology to the production of microglia. Here we will discuss recent advances in this area and describe how they will aid our future understanding of microglia.

[H1] Introduction

Both microglia and macrophages have been increasingly recognized for their significant roles in brain function. Under physiological conditions, microglia are exquisite sensors of changes in their micro-environments, detecting infection or damage and responding by migrating, proliferating, phagocytosing or producing cytokines and neurotrophins to protect, defend, and maintain a homeostatic environment[1] (Fig. 1). Such responses are also observed in a wide range of neurodegenerative conditions (Fig. 1) as well as in acute conditions such as stroke; however in these instances, the microglial response may become dysregulated and chronic[2]. In certain conditions, monocyte-derived macrophages that are usually restricted to the periphery are thought to enter the brain, where they have been shown to be capable of assuming a microglial-like phenotype[3,4].

A number of factors have pushed microglia into the forefront of neuroscience research in recent years. These include the growing appreciation of the role of these cells in the development of neurological conditions, particularly dementia (an escalating societal and economic problem in western society, with an urgent need for therapeutics) and the findings of recent human genetic studies (and in particular of genome-wide association studies (GWAS) and whole genome sequencing studies) that have highlighted a clear genetic link between dementia and inflammation[5,6,7]. In addition, the well-established role of neuroinflammation in multiple sclerosis[8] as well as increasing evidence for its role in other neurodegenerative diseases, including Parkinson disease, Alzheimer disease and amyotrophic lateral sclerosis[9,10,11] have helped to drive an increasing focus on microglia.

The lack of successful disease-modifying therapeutic developments in neurological diseases has brought into question the capacity of animal models to recapitulate the human condition[12,13]. This, coupled with a growing opposition in western society to the use of animals in research because of ethical and welfare concerns[14], has led to the search for better 'humanised' models of neurological function and disease. The use of 'primary' human tissue provides one possible solution; however, this material is often derived from contentious sources, including foetal tissues, that also raise ethical concerns. In addition, although primary tissue is highly informative and vital for ongoing research, issues including post-mortem artefacts, the influence of underlying diseases (such as epilepsy) present in the individuals from whom the tissue is derived and the obvious difficulties in obtaining sufficient study material invalidate this as a source of material for long term mechanistic studies of the early physiological changes that occur in neurological diseases.

These caveats have led to the development of approaches for the long-term culture of human cells. These have included the generation of microglial-like models directly from human monocytes[15,16] and a number of cell biology companies have been established that specialize in the production of such cells. However, due to the rarity of many of the genetic risk factors associated with neurodegenerative diseases, cells from these sources are unlikely to express the single-nucleotide polymorphisms or mutations that would enable targeted studies of these diseases. These risk factors can of course be introduced by new genome editing technologies; however, to study the mutations in the context of disease it is necessary to acquire cells directly from a patient.

To address these concerns, recent advances in iPSC methodologies that were initially developed to generate primitive and definitive hematopoietic cells and neurons[17,18] are now being applied to the production of macrophages and microglia. The advantages of this approach include the ability to produce large numbers of human-derived cells with an adult phenotype that is very difficult to obtain through traditional methods and the provision of a source of cells expressing particular genetic mutations linked to disease, allowing in depth disease-relevant study of pathological mechanisms. This is especially advantageous when the number of patients expressing these gene mutations are very rare, as is the case for the variants in the genes encoding receptors such as triggering receptor expressed on myeloid cells 2 (TREM2) that are linked to increased risk of developing late onset Alzheimer disease[5,7].

These advances may enable future production of ‘disease in a dish’ models, which may allow us to better understand the consequences of genetic predisposition to dementias and other chronic neurological diseases in which microglia are thought to play a role. The sophistication of these models can be altered depending on the users’ needs. Cells can be derived in isolation or new culturing techniques, including organoid development, can be utilised to allow users to introduce microglia or microglial precursors into three dimensional tissues of neurons, astrocytes and oligodendrocytes (all with controlled phenotypes) to investigate cell autonomous and non-cell autonomous responses. These cells can also be used to inform us of changes in the physiology of human microglia in the healthy nervous system during development and in genetic-associated diseases.

In this article we will discuss the properties of microglia generated from iPSCs and consider the advantages and caveats of such models. To provide context to the history iPSC-derived microglia methodologies, we shall also briefly discuss the generation of iPSC-derived macrophages.

[H1] Protocols and phenotypes

A number of methods for the generation of iPSC-derived microglia-like or macrophage-like cells from both human and rodent tissue have been published in the last five years (**Table 1**). These methods have allowed large numbers of cells to be produced and, in general, the phenotype of these cells has been described as being similar either to that of peripheral blood monocyte (PBM)-derived macrophages or to that of tissue resident macrophages and CNS-localised microglia. To date, all published protocols follow a similar path, based largely on cellular ontogeny. Donor cells are initially de-differentiated to produce iPSCs. These cells are then supplemented with growth factors associated with mesodermal specification, leading to the development of hemangioblasts and primitive hematopoietic progenitors, and this is followed by continued maturation along the myeloid lineage with further growth factor cocktails.

A number of established protocols can produce microglia-like cells from human monocytes. However, the use of monocyte-derived microglia as a human model of CNS microglia remains contentious because of the differences in the proposed origins of CNS microglia from those of peripheral macrophages *in vivo*. Microglia are thought to be erythromyeloid progenitor (EMP)-derived, arising from the yolk-sac[19,20], whereas PBM-derived macrophages arise from circulating bone marrow-derived cells[21]. However, tissue-resident macrophages, including the Langerhans cells of the skin, alveolar macrophages of the lung and Kupffer cells of the liver[22], are also derived from EMPs. These cells therefore do not have a monocytic progenitor and are maintained independently of the bone marrow, like microglia[19,23,24] . Furthermore, it has been shown that microglia and tissue-resident macrophages, but not PBM-derived macrophages, are derived from precursors whose development depends on the transcription factors PU.1 and runt-related transcription factor 1 (RUNX1) but does not require the transcriptional activator MYB, indicating different embryonic sources of these cells[19,20,25,26]. Such discoveries have enabled researchers to refine iPSC-derived models to generate the cell-specific progenitors required for the development of true microglial-like cells. This allows for more phenotypically accurate studies, potentially more translatable to *in vivo* conditions.

There is considerable debate in the literature about what certain iPSC microglial differentiation protocols actually produce in terms of their phenotype. Recent advances in iPSC methodologies have found that a microglial-like phenotype can be induced by incubation of human iPSC-derived microglial and/or macrophage progenitors with various combinations of factors: these include high levels of colony-stimulating factor 1 (CSF1) and interleukin 34 (IL-34)[27]; IL-34 and granulocyte macrophage colony-stimulating factor (GM-CSF)[28]; IL-3, IL-34, and GM-CSF[29], and IL34, CSF1, transforming growth factor-β (TGFβ1), fractalkine (CX3CL1) and CD200[30]. These protocols are able to generate cells that exhibit similar gene expression patterns to those of human primary microglia[28,30] , express known microglial markers[27,28,29,30], perform microglial functions (including phagocytosis and secretion of cytokines)[27,28,29,30] and respond to ADP or ATP via P2Y purinoceptor 12 (P2RY12) to produce intracellular calcium transients[28,30].

Despite the phenotypic similarities between these iPSC-derived cells and endogenous microglia, the laboratories responsible for their production and others have questioned the simplicity of such models and have provided evidence that more complex culture conditions are necessary for the generation of more authentic models of microglia[29,30,31,32]. Indeed, some prescribe the use of neuronal co-cultures to further enhance the microglial 'signature' — that is, the gene expression, cytokine release in response to stimulants, phenotypic responses to exogenous or endogenous insults and cellular morphology — of their generated cells[27,30,31,32]. Although a number of the recently published protocols provide strong phenotypic evidence for the production of microglial-like cells, it is clear that there is not yet a definitive method for the production of a completely satisfactory microglial phenotype from iPSCs and that such a protocol is a work in progress.

The protocols described above differ from those used for the production of macrophages from human iPSCs, in which the use of macrophage/monocyte colony-stimulating factor (M-CSF) and IL-3 have been used[33,34]. In the presence of M-CSF alone, human iPSCs generated cells with phenotypes that resemble those of anti-inflammatory, regenerative 'M2-type' macrophages, including long filopodia, large intracellular vacuoles and the expression of classical macrophage markers such as CD45, CD14, CD163 and CD86[34]. These cells were also able to phagocytose latex beads and produce a number of cytokines in response to lipopolysaccharide, including tumour necrosis factor α (TNF-

α), monocyte chemoattractant protein 1, IL-6, IL-8 and IL-10[34]. The ability to secrete IL-6 does, however, suggest that these cells consisted of a somewhat mixed phenotype, because this cytokine has been found to be secreted by inflammatory 'M1-type' macrophages and M2-type macrophages[35]. The high level of TNFα secretion by iPSC-derived macrophages is also similar to PBM-derived M1-type macrophages[34]. One possibility, which should be explored, is that there may be an influence of priming on these findings: that is, pre-exposure to a stimulus, such as serum factors in undefined media compositions, may convert the cells into an alerted, activated state as described by the M1/2 classification. It may therefore be necessary to mature the cells for longer in culture in order to ensure that they are in a more down-regulated state, similar to that found *in vivo*.

[H1] Microglia or macrophages?

The variations in protocol and phenotype reported by the studies described above raise an important question: what defines an iPSC-derived microglial cell and separates this from an iPSC-derived tissue-resident macrophage or monocyte-derived macrophage?

Recent transcriptomic studies of the differences between human iPSC-derived microglia and PBM-derived macrophages have proposed that microglia express the purinergic receptor P2RY12 and transmembrane protein 119 (TMEM119)[27,28], whereas peripheral PBM-derived macrophages do not[36]. Similarly, two recent studies also used transcriptomics to separate iPSC-derived microglia from other myeloid cells including dendritic cells, monocytes, and macrophages[29,30]. Calcium responses to ADP, which occur via P2RY12 stimulation, are evoked in iPSC-derived microglia but not in PBM-derived macrophages[28]. Other microglial-specific markers that have been shown to be expressed on human iPSC-derived microglia, but not PBM-derived macrophages include allograft inflammatory factor 1 (AIF1, also known as IBA-1), CD11b, CD11c and CX3C chemokine receptor 1 (CX3CR1)[28]. However, further study of these putative markers is required as the expression levels of receptors are likely to be affected by the particular cocktail of growth factors or cytokines used. Microglia are capable of expressing a huge repertoire of receptors[37,38] that could influence their phenotype. However, a number of receptors expressed on human adult microglia, including P2RY12, CD64 and tyrosine-protein kinase Mer (MERTK) can become down-regulated in microglia upon activation[39]. Therefore any of these proteins in isolation cannot be used as a reliable marker for microglia derived from iPSC cells.

There are also subtle points of overlap between the phenotypes of microglia and those of tissue resident macrophages, which depend on their level of activation (and thus overall gene and protein expression). Human microglia express *C1QA,* macrophage colony-stimulating factor 1 receptor (*CSF1R*) and *CD34* genes, as do other tissue resident macrophages. Similarly TAM-related genes such as *MERTK*, vitamin K-dependent protein S (*PROS1*) and growth arrest-specific protein 6 (*GAS6*), expressed on human macrophages, are also expressed in human microglia[23]. A number of genes regarded as providing a specific 'signature' for rodent microglia were recently identified; these include *Mertk*, G-protein coupled receptor 34 (*Gpr34*), *Pros1*, *C1qa*, *Gas6* and *P2ry12*[23] . These genes were also found to be expressed in human iPSC-derived microglia that were co-cultured with human iPSC-derived cortical neurons or astrocytes[29,31] and to differ from the genes expressed by blood-derived monocytes. However, the expression of some of these genes in other human tissue resident macrophages does suggest that a microglial-specific signature for human cells is yet to be fully defined, and temporal changes in expression levels and an environment-specific context must be taken into account[32].

As outlined above, transcriptomic studies that have compared mouse iPSC-derived microglia and macrophages have been used both to indicate the genes and proteins that it may be important to consider when investigating mouse models of disease and to produce a possible genetic signature that is relevant for human microglia[23]. In addition, a recent study that compared gene expression in tissue-resected primary human microglia with that of isolated primary mouse microglia, has led to the most comprehensive genetic microglial signature to date[40]. The authors generated a comprehensive picture of the transcriptomic and epigenetic landscapes of the primary isolated microglia to provide a detailed overview of human microglial identity. Interestingly, the authors found that there is extensive down-regulation of microglia-specific genes when the cells were placed in a tissue culture environment, albeit using undefined serum containing medium. The identification of such down-regulation is a significant caveat when trying to develop iPSC-derived microglia *in vitro,* and lends further support to the importance of the environmental context that these cells are matured in[32].

The type of information provided by transcriptomic studies enables fine-tuning of iPSC-microglia methodologies to provide a more realistic phenotype. However, it will be imperative that, in the future, such comparisons are attempted with fully adult human microglia. Studies that use microglia resected from children have so far provide unparalleled data on primary human microglial identity[40]. However, transcriptomic data from microglia derived from aged individuals, however small the cohort, may provide further information on temporal genetic changes, which may be relevant to the study of disorders of old age.

Many phenotypic changes are known to occur in microglia with age. Broadly speaking, early postnatal microglia focus on synaptic pruning[41] and the refinement of CNS connectivity, whereas adult microglia exhibit a surveillant phenotype[42]. How then do the new iPSC-derived microglia compare with these differing microglia phenotypes? To date, only one study has directly compared iPSC-derived microglia to both fetal and adult human microglia. This study used whole-transcriptome differential gene expression analysis to reveal increased expression of nearly 2000 genes in iPSC microglia when compared to foetal microglia, and just over 1000 genes when compared to adult microglia. Furthermore, enrichment analysis provided an insight into how iPSC-derived microglia compare to foetal and adult microglia[30]. Three independent studies[23,43,44] appeared to show a strong similarity in the differences in gene expression levels observed between iPSC-derived microglia and primary human microglia. However the age of the primary microglia was not reported[28]. Thus, further analysis of the published protocols is required to gauge how comparable the iPSC-derived cells are to early or late primary microglia. This will not be an easy task due to limited availability of primary cell material; however, putting more emphasis on comparing these models to primary microglia rather than other myeloid cells should be a priority.

As shown above, a number of iPSC protocols produce good models of macrophage-like cells. The involvement of peripheral cellular dysfunction in diseases such as Alzheimer disease should not be ignored[45,46,47] and thus it is likely that these cells can be used to inform us of the contributions of peripheral changes that can influence central disease. In addition macrophages derived from the cells of human patients will express the variants implicated in disease as will their central ‘cousins’, the microglial cells. It is critical that both cell types are investigated with regard to disease as their responses may be different[48] and it may be advantageous to target one over the other when considering future therapeutic interventions.

In addition to molecular markers, morphological phenotypes are often used to define microglia versus macrophages; however, although it is true to say that surveillant microglia in the brain exhibit a highly ramified, motile phenotype compared with more bipolar phenotypes observed in macrophage cultures, this rapidly changes upon activation and is therefore not a particularly robust marker to distinguish a microglial cell from a macrophage. We would suggest that one morphological marker of a microglial phenotype is that of domain sensitivity and contact inhibition (see Fig. 2). Surveillant microglia very rarely touch each other *in vivo* or *in vitro* and only when in an activated state do the cells display non-contact inhibition[49] (Fig. 2). Furthermore, human macrophages (particularly regulatory macrophages, which are akin to human surveillant microglia) *in vitro* do not display contact-inhibition[50,51]. We therefore argue that a number of markers, including genes, proteins and morphology need to be considered when defining a microglial cell and that no single marker will be sufficiently robust.

[H1] Culture types

Many recent studies of human iPSC-derived microglia have tended to focus on two dimensional cultures. This approach has both advantages and disadvantages for the user. For example, it is possible to study a cell type without the interference of signals from other cell types; however the user is likely to be losing vital information from non-cell autonomous signalling. Therefore, many protocols are now focussing on the development of more complex systems, through the introduction of the iPSC-derived microglia progenitors to *in vivo* tissue models or to co-cultures, in a drive to produce cells that are more like microglia[27,29,30,31,32] .

Co-culturing iPSC-derived microglia with neurons seems to produce microglial like cells with ramifications similar to those present *in vivo*[27,31,32], although a ramified motile morphology has also been observed in mono-cultures[27,28,30]. In these cultures there also appears to be a need for the CSF1R ligand IL-34, as well as low levels of GM-CSF or M-CSF, to be provided in order to maintain microglial survival. This is not unexpected, given that the signals from neurons and other glia in the brain have a major influence on the survival and function of microglia and the genes and proteins that they express.

If one is to attempt a more complex culture approach, it seems likely that it is important to include as many different brain cell types as possible. Indeed, the assumption that culturing iPSC-derived microglia solely with neurons goes some way to approaching a more realistic microglial phenotype may be somewhat naïve, given the complexity of brain structure and organisation. Furthermore, the relative proportions of each cell type will require consideration, as will the specific neuronal cell type used. Indeed, recent work shows that in a complex culture environment there may be no requirement of growth factor supplementation[32]. In this study, iPSC-derived macrophages transferred to a new born brain further differentiated into microglia in a manner that relied solely on brain-specific cues[32]. These culture conditions are thus said to recapitulate the development and maturation of microglia in the brain, resulting in highly ramified cells expressing a number of microglial markers.

A recent study found that the transference of iPSC-derived human microglia into rat hippocampal co-cultures, transgenic mice modelling aspects of Alzheimer disease, or human 3D brain organoids (containing neurons, astrocytes and oligodendrocytes) resulted in the production of cells with a more microglial-like phenotype than was

observed when the iPSC-derived microglia were cultured alone[30]. Furthermore, culturing iPSC-derived microglia with factors such as CX3CL1, CD200 and TGFβ, which are typically produced by other brain cells (such as neurons and astrocytes) in the intact brain, produced cells with a transcriptome profile that is highly similar to that of human adult and foetal microglia and distinct from monocytes and blood dendritic cells[30].

The use of organoid cultures will also allow the influence of microglial risk factors on other cells in the brain, including radial glia and neurons, to be examined[30,52], as well as the effects of macrophages on peripheral function[53]. This is important because microglia do not exist in isolation in the brain and, as previously mentioned, non-cell autonomous effects cannot be observed in two dimensional cultures of isolated cell types. Although each type of cell culture for investigating iPSC-microglial and macrophage function (mono-culture, co-culture or organoid) has its own advantages, each too comes with its own disadvantages, and these must be factored into models when interpreting results.

[H1] The future

There is little doubt that, over the next few years, we will see an increasing refinement in the protocols for the production of human iPSC-derived microglia and, hopefully, a consensus on the proteomic and genetic signatures of these cells (when compared with tissue-resident macrophages and adult primary microglia) will arise. The production of large numbers of macrophage-like and microglial-like cells from iPSCs is a major step towards understanding how human microglia and macrophages behave, given that the majority of published studies on microglia have used rodent cells. Furthermore, human iPSC-derived microglia hold promise for understanding the functional consequences of an increasing number of disease-associated risk factors linked to these cells[7]. This will be important for translational studies and drug screening, and for the development of individualised gene-therapy methodologies[54]. Future research directions could involve the use of human iPSC-generated microglia in transplantation therapies, as has been described for rodent models of such diseases as obsessive-compulsive behaviour, CNS lysosomal storage diseases and Parkinson disease[55,56].

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Author Contributions

J.M.P. made a substantial contribution to discussions of the content of the article. J.M.P and T.M.P researched data for the article, wrote the article and reviewed and/or edited the manuscript before submission.

Competing interests

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Fig. 1. The physiological and pathological functions of microglia. The schematic image illustrates some of the physiological functions of microglia that should be considered when using induced pluripotent stem cells to model these cells. Microglia contribute to healthy nervous system physiology in several ways. They provide cues and remove inappropriate synapses during development and they secrete neurotrophins and cytokines to support and maintain neural networks in the mature nervous system[42]. In addition, they rapidly sense ATP signalling via receptors such as P2Y purinoceptor 12 (P2RY12)[37,38,50] and migrate to areas of damage, where they proliferate and phagocytose apoptotic cells and any other damaged tissue to aid repair. Indeed activation of microglia following CNS damage or disease induces a respiratory burst, necessary for an efficient innate immune response[38,50]. Recent research has focussed on the role of lipid signalling in microglia in neurological diseases, including Alzheimer disease (AD). Such signalling is mediated by putative membrane associated receptors including triggering receptor expressed on myeloid cells 2 (TREM2), mutations in which are genetic risk factors for AD[7]. Microglia also influence nervous system pathology in a number of disorders. They attack the myelin sheath of oligodendrocytes and Schwann cells, phagocytose myelin and attempt repair in multiple sclerosis and become reactive in white matter diseases such as leukodystrophies. Microglia migrate to and surround amyloid beta plaques in AD, in an attempt either to phagocytose this aberrant protein or to corral and contain it to prevent neuronal damage. Microglia also become reactive in Huntington disease, dementia with Lewy bodies, Parkinson disease and Creutzfeldt Jakob disease. In the ageing brain[7,9,10], microglia appear dystrophic and become reactive, senescent and dysfunctional. In addition, their numbers are altered during ageing, decreasing in some areas of the brain and increasing in others (see REF.57 for review). ADP, adenosine diphosphate; IBA1, allograft inflammatory factor 1; TMEM119, transmembrane protein 119.

Fig. 2. iPSC-derived microglia phenotype characterisation: basic and comprehensive. Current protocols use a myriad of assays to assess the phenotypes of iPSC-derived microglia. Basic gene signatures, surface protein expression, the expression of cell specific markers, the phagocytosis of microspheres, *Eschericia coli* or amyloid-β (Aβ), and the induction of cytokine release are routinely investigated[27,28,29,30,31,32]. More comprehensive phenotype checking consists of RNA sequencing analysis of the iPSC-derived cells[28,29,30]. Comparative analyses of the cells' transcriptomic signatures to those recorded from primary microglia data sources will be important to improve capacity of iPSC-derived microglia to model their endogenous counterparts. The ability of the cells to generate calcium transients and respiratory bursts in response to stimuli are also fundamental microglial phenotypes that should be present[28,29,30]. Integration into iPSC-derived organoids or other tissue sources enables researchers to critically assess microglial morphology[27,29,30,32]. Contact inhibition, including tiling *in vitro,* is another classical microglial phenotype that should also be assessed[49].

Table 1: Published methods for the generation of induced pluripotent stem cell-derived macrophages and microglia

Generated cell type stated*	Species	Tissue and /or cellular origin	Brief methodologies	Cellular phenotypes	Refs
ESC-derived microglia	Mouse	ESCs from 129/Sv Gat mice	Retinoic acid induced embryoid body differentiation	$ITGAM^+$ and galactin-3^+	58
ESC-derived microglia	Mouse	ESCs from 129/Sv CP mice; Green ES FM260 cells	$Nestin^+$ selective cell expansion, growth on laminin with bFGF followed by GM-CSF exposure	• $MHCI^+$ and /or $MHCII^+$, $CD40^+$, $CD80^+$, $CD86^+$, IFN-γR^+ • Selective accumulation in the brain after IV injection. • Ramified morphology in vivo	59
ESC-derived microglia precursor	Mouse	C57BL/6-ATCC ESCs	Growth on laminin with FGF2, followed by laminin withdrawal and optional addition of GM-CSF	• $IBA1^+$, $ITGAM^+$, $ITGAX^+$, $CD45^+$, $CD68^+$, $CD80^+$, $CD86^+$, $CD115^+$, $F4/80^+$, $CD49d^+$, $CD29^+$. • LPS and/or IFNγ-mediated upregulation of pro-inflammatory cytokines • Phagocytosis of microsphere bead • In vivo engraftment induced a classic microglial phenotype and morphology	60,61
iPSC monocyte-derived macrophage	Human	Fibroblast	Embryoid body formation: addition of BMP4, SCF, VEGF. Progenitor formation: M-CSF, IL-3. Maturation: M-CSF	• $CD45^+$ and/or $CD14^+$, $CD16^+$, $CD163^+$, $CD86^+$, $MHCII^+$ • Phagocytic and HIV-1 infectable • LPS, IFNγ- and/ or IL4 mediated altered cytokine profiles	62
iPSC-derived microglia-like cell	Human	Fibroblast	Embryoid body formation using a defined in house media formulation, with M-CSF and IL-34	• $PU.1^+$, $ITGAM^+$, $IBA1^+$ • Phagocytic and highly motile • Morphologically similar to primary microglia • $P2RY12^+$, $TMEM119^+$ • LPS-mediated pro-inflammatory cytokine induction • Comparative transcriptional signature to primary microglia, enhanced with neural co-cultures • Integration into organotypic neural cultures • Dynamically motile and able to rapidly respond to cellular damage	27
iPSC-derived microglia	Human	Fibroblast	Progenitor formation: sequential addition of BMP4, bFGF, SCF, VEGF, IL-3, TPO, M-CSF, FLT3L, GM-CSF followed by sorting for CD14 and/or $CX3CR1^+$	• $CD14^+$, $CD45^+$, $CX3CR1^+$ • highly motile extending processes similar to microglia in vivo • $IBA1^+$, $ITGAX^+$, $TMEM119^+$, $P2RY12^+$, $ITGAM^+$, $CX3CR1^+$ • Deep RNA sequencing showed cells clustering with human microglia	28

			progenitors. Maturation: IL-34 and GM-CSF	• Expression of selected microglia 'signature genes' • Similar cytokine profile to primary microglia • Phagocytosis of microspheres • Calcium transients induced upon stimulation.	
iPSC-derived microglia-like cell	Human and mouse	*Cx3cr1*$^{Gfp/+}$ mouse fibroblasts and human cord blood-derived CD34^{+} cells	Progenitor formation in hypoxic conditions, addition of VEGF, BMP4, SCF, activin A, SCF, FLT3L, IL-3, IL-6, GM-CSF, BMP4 followed by sorting for CD34, CD45 and CD43^{+} progenitors. Addition to astrocytic co-cultures followed by sorting for CD39^{+} iPSC-derived microglia. Maturation: IL-3, GM-CSF and M-CSF.	• ITGAM^{+}, IBA1^{+}, HLA-DR^{+}, CD45^{+}, TREM2^{+}, CX3CR1^{+} • Microarray data showed cells clustering with human foetal microglia • High expression levels of microglial 'signature' genes • Phagocytosis of *E.coli* • LPS-mediated pro-inflammatory cytokine induction • Induce ROS upon stimulation.	29
iPSC-derived microglia-like cell	Human	Fibroblast or PBMC	Progenitor formation in hypoxic conditions: D0: FGF2, BMP4, Activin-A, RI, LiCl; D2: FGF2, VEGF; D4, 6, and 8: FGF2, VEGF, TPO, SCF, IL-6, IL-3. CD43+ progenitor sort. Maturation: M-CSF, IL-34, TGFβ-1, + 1CD200, CX3CL1 from D35	CX3CR1^{+}, P2YR12^{+}, MERTK^{+}, PROS1^{+}, TGFBR1^{+}, ITGB5^{+}, TREM2^{+}; cytokine secretion upon inflammatory stimuli (LPS, IFNγ, IL-1β), migrate and undergo calcium transients, and phagocytic capacity for CNS substrates including fibrillar Aβ); comparative transcriptional signature to primary foetal and adult microglia that is responsive to a neuronal environment (rat primary hippocampal cultures and 3D brain organoids); cells transplanted into transgenic mice or human organoids resemble microglia *in vivo.*	30
iPSC-derived microglia generation in neuronal co-culture	Human	Fibroblast	Embryoid body formation: BMP4, SCF and VEGF. Progenitor formation: M-CSF and IL-3. Maturation: IL-34, GM-CSF and neuronal co-culture	• Express *P2RY12, GPR34, MERTK, C1QA, PROS1, GAS6, TMEM119, TREM2* • Express key surface protein markers • Develop highly dynamic ramifications • Phagocytic • Become amoeboid and cluster upon LPS exposure • Release microglia-relevant cytokines, and upregulate homeostatic function pathways • Promote a more anti-inflammatory response than monocultures	31

iPSC-derived microglia generation in neuronal co-culture	Human and Mouse	Human or mouse fibroblasts	Progenitor formation in hypoxic conditions with addition of BMP4, VEGF, CHIR99021, FGF2, SCF, DKK1, IL-6, IL-3 followed by normoxic conditions with FGF2, SCF, IL-6, IL-3. Sorting for CD45, ITGAM,CD163, CD14 and CX3CR1⁺ 'hiMacs' for addition to neuronal co-cultures	• hiMacs: $CD45^+$, $CD14^+$, $ITGAM^+$ $CD163^+$, $CX3CR1^+$; • hiMicro: $IBA1^+$; $CX3CR1^+$ • Phagocytosis of beads and Aβ.	32

The table provides a brief overview of the current published methods of early iPSC-myeloid cell generation and subsequent techniques for the establishment of iPSC derived macrophages and microglia. Please refer to the citations within for full comprehensive methodologies. * If murine/human protocols are both described: methodologies and derived cell phenotypes are described for the human protocol. Aβ, amyloid-beta; bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; CHIR99021, glycogen synthase kinase 3 inhibitor; CSF1, colony-stimulating factor 1; CX3CR1, CX3C chemokine receptor 1; DKK1, dickkopf-related protein 1; ESC, embryonic stem cell; FGF2, fibroblast growth factor 2; FLT3L, FMS-like tyrosine kinase 3 ligand; GAS6, growth arrest-specific protein 6; GM-CSF, granulocyte macrophage colony-stimulating factor; GPR34, probable G-protein coupled receptor 34; hiMacs, (human iPSC-derived primitive macrophages); hiMicro, (human iPSC-derived Microglia-like cells) HIV-1; human immunodeficiency virus 1; HLA-DR, human leukocyte antigen – antigen D related; IBA1, allograft inflammatory factor 1; IFN-γ, interferon-γ; IFN-γR, interferon-γ receptor; IL-3, interleukin-3; iPSC, induced pluripotent stem cell; ITGAM, integrin alpha-M; ITGAX, integrin alpha-X; LPS, lipopolysaccharide; M-CSF, macrophage/monocyte colony-stimulating factor; MERTK, tyrosine-protein kinase Mer; MHC1, major histocompatibility complex class I molecules; P2RY12, P2Y purinoceptor 12; PROS1, vitamin K-dependent protein S; ROS, reactive oxygen species; SCF, stem cell factor; TMEM119, transmembrane protein 119; TPO, thrombopoetin; TREM2, triggering receptor expressed on myeloid cells 2 ; VEGF, vascular endothelial growth factor;