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Review Article

Human pluripotent stem cell (hPSC)-derived microglia for the study of brain disorders. A comprehensive review of existing protocols

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ABSTRACT

Microglia, resident immune cells of the brain that originate from the yolk sac, play a critical role in maintaining brain homeostasis by monitoring and phagocytosing pathogens and cellular debris in the central nervous system (CNS). While they share characteristics with myeloid cells, they are distinct from macrophages. In response to injury, microglia release pro-inflammatory factors and contribute to brain homeostasis through activities such as synapse pruning and neurogenesis. To better understand their role in neurological disorders, the generation of *in vitro* models of human microglia has become essential. These models, derived from patient-specific induced pluripotent stem cells (iPSCs), provide a controlled environment to study the molecular and cellular mechanisms underlying microglia-mediated neuroinflammation and neurodegeneration. The incorporation or generation of microglia into three-dimensional (3D) organoid cultures provides a more physiologically relevant environment

Abbreviations: AD, Alzheimer's disease; ADP, adenosine diphosphate; APLNR, apelin receptor; APOE, apolipoprotein E; ATAC-seq, assay for transposase-accessible chromatin with sequencing; ATP, adenosine triphosphate; Aβ, amyloid-beta peptide; BBB, blood-brain barrier; BIO, 6-bromoindirubin-3′oxime; BMP4, bone morphogenetic protein 4; BOr, brain organoids; BS, brain spheres; C/EBPβ, CCAAT/enhancer binding protein β; CCL2, C-C motif chemokine ligand 2; CD, cluster of differentiation; C/EBPA, CCAAT/enhancer-binding protein-alpha; CIQA, complement C1q A chain; CNS, central nervous system; CRISPRa, CRISPR activation; CRISPRi, CRISPR interference; CRYBB1, crystallin beta B1; CYBB, cytochrome b-245 beta chain; CSF1, colony stimulating factor 1; CSF1R, colony stimulating factor 1 receptor; CX3CL1, C-X3-C motif chemokine ligand 1; CX3CR1, C-X3-C motif chemokine receptor 1; CXCR4, CXC motif chemokine receptor 4; DAM, disease-associated microglia; DAP12, DNAX-activating protein of 12 kDa; DKK-1, Dickkopf-1; DS, Down syndrome; D-MG, dorsal spheroids with microglia-like cells; D-V-MG, -dorsal-ventral spheroids with microglia-like cells; EB, embryonic body; EMPs, erythro-myeloid progenitors; ESCs, embryonic stem cells; ETV2, ETS variant transcription factor 2; FACS, fluorescence-activated single-cell sorting; FBS, fetal bovine serum; FGF, fibroblast growth factor; FLI1, Friend leukemia integration 1; Flt3l, FMS-like tyrosine kinase 3 ligand; FVBOr, vascularized brain organoids; GAS6, growth arrest-specific protein 6; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GPR34, G-protein coupled receptor 34; GWAS, genome-wide association study; HexB, hexosaminidase subunit beta; HIV, human immunodeficiency virus; HLA, human leukocyte antigens; HMGs, xenotransplanted microglia; HPCs, hematopoietic progenitor cells; HPSC, human pluripotent stem cell; HTERT, human telomerase reverse transcriptase; I3LMNs, iPSC-derived lower motor neurons; IBA1, ionized calcium binding adaptor molecule 1; IFN, interferon; IHBO, hMGs-containing immunocompetent human brain organoid; IHPCs, induced hematopoietic progenitor-like cells; IL, interleukin; IMac, primitive-like macrophages; IMACs, iPSC-derived macrophages; IMGs, induced microglia induced microglial-like cells; IMicro, microglia-like cells; IPD, idiopathic Parkinson's disease; IPS-MGs, iPSC-derived microglia; IPSC, induced pluripotent stem cells; IRF8, interferon regulatory factor 8; ITGB, Integrin β; KDR, kinase insert domain receptor; Klf4, Krüppel-like factor 4; LPS, Lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MACS, magnetic-activated cell sorting; MAFB, MAF bZIP transcription factor B; MEA, multi-electrode array; MEF2C, myocyte enhancer factor 2 C; MERTK, MER proto-oncogene, tyrosine kinase; MG, -microglia-like cells; MGLs, microglia-like cells–; MHC, major histocompatibility complex; MITF, microphthalmia-associated transcription factor; MIXL1, mix paired-like homeobox; MPCs, mesenchymal progenitor cells; MRNA, messenger ribonucleic acid; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; NPCs, neural progenitor cells; Oct3/4, octamer-binding transcription factor 3/4; P2RY12, purinergic receptor P2Y12; PD, Parkinson's disease; PDGFα, platelet-derived growth factor subunit A; PFN1, profilin 1; PLO, poly-L-ornithine; PMGLs, -microglia-like cell precursors; PMPs, primitive macrophage progenitors; PROS1, protein S; QRT-PCR, quantitative reverse transcription polymerase chain reaction; ROCK, Rho-kinase; ROS, reactive oxygen species; RtTA, reverse tetracycline transactivator; RUNX1, RUNX family transcription factor 1; SALL1, spalt like transcription factor 1; SCF, stem cell factor; ScRNA-seq, single-cell RNA sequencing; SgRNA, single guide RNA; Sox2, SRY-box transcription factor 2; SPI1, Salmonella pathogenicity island 1; TAL1, TAL bHLH transcription factor 1, erythroid differentiation factor; TC, tissue culture; TfHO, tubular forebrain organoids; TGFβ1, transforming growth factor beta 1; TGFβR1, transforming growth factor beta receptor 1; TMEM119, transmembrane protein 119; TNF, tumor necrosis factor; TNFAIP3, TNF alpha induced protein 3; TPO, thrombopoietin; TREM2, triggering receptor expressed on myeloid cells 2; VEGF, vascular endothelial growth factor; VOr, vessel organoids; V-MG, ventral spheroids with microglia-like cells.

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that offers further opportunities to study microglial dynamics and disease modeling. This review describes several protocols that have been recently developed for the generation of human-induced microglia. Importantly, it highlights the promise of these *in vitro* models in advancing our understanding of brain disorders and facilitating personalized drug screening.

1. Introduction

Microglia are macrophages residing in the central nervous system (CNS) that play roles in brain homeostasis, neuroinflammation, and immune surveillance (Prinz and Priller, 2014). Unlike the rest of the body, the CNS lacks humoral immunity. Here, the microglia carry out the crucial task of surveying their environment to identify and phagocytose invading pathogens, cellular debris, and abnormal proteins from damaged or dying cells (Prinz and Priller, 2014). They release pro-inflammatory cytokines and chemokines as immune responses to injury or infection, in addition to mediating non-immune functions such as synapse pruning, neurogenesis, and the clearance of toxic metabolites, all of which contribute to brain homeostasis (Salter and Stevens, 2017).

Despite being originally classified as macrophages due to their morphological similarities and expression of several macrophage markers, microglia, and macrophages have different origins: microglia are derived from progenitor cells in the yolk sac, whereas macrophages are derived from the fetal liver or bone marrow (Ginhoux and Prinz, 2015; Kierdorf et al., 2013). During the early stages of development, microglia invade the embryonic brain rudiment, a process conserved across vertebrate species (Ginhoux and Prinz, 2015). Due to their mesodermal origin, microglia share several characteristics with other myeloid cell types in the body (Greter and Merad, 2013). Immunohistochemical studies performed approximately 30 years ago revealed phenotypic similarities between circulating monocytes, tissue macrophages, and microglia, as evidenced by the expression of macrophage markers such as F4/80, Fc receptor, and CD11b in mice and humans. Alternatively, CD39, an ectonucleoside triphosphate diphosphohydrolase-1, has recently been proposed as a specific marker for microglia that distinguishes them from their peripheral counterparts (Butovsky et al., 2012). Transcriptomic studies have also identified a cluster of microglia-specific transcripts encoding proteins involved in sensing endogenous ligands and microbes, collectively known as the sensome (Hickman et al., 2013). These markers, which represent activated or inactivated forms of microglia, serve as valuable tools for studying the innate functions of these cells in both healthy and pathogenic conditions in the brain.

In vitro models, representing normal and dysfunctional microglia, are essential for studying their precise functions in brain homeostasis and neurological disorders. They provide critical insights into the molecular and cellular mechanisms underlying microglia-mediated neuroinflammation and neurodegeneration in a controlled and reproducible environment. Moreover, these models can be generated from patient-specific iPSCs, allowing for the study of disease-specific microglial phenotypes and personalized drug screening. In this review, we will present detailed protocols for the generation of *in vitro* microglia, with a special focus on human PSC-derived microglia, for the study of brain disorders.

2. Generation of two-dimensional (2D) *in vitro* models of human microglia

2.1. Human microglia cell lines

The limited availability of human brain tissue poses as a major challenge to the use of primary microglia for research purposes (Watkins and Hutchinson, 2014). An alternative approach is to generate immortalized microglia cell lines from various species, including humans, rats, mice, and macaques (Righi et al., 1989; Blasi et al., 1990; Timmerman et al., 2018; Blasi et al., 1985; Hovarth et al., 2008; Cheepsunthron et al., 2001; Nagai et al., 2005; Nagai et al., 2001; Janabi et al., 1998; Janabi et al., 1995; Martin et al., 2003; Garcia-Mesa et al., 2017). These cell lines are derived from primary microglia cultures and are rendered immortal by viral transduction with oncogenes (Aktories et al., 2022). For example, Nagai and colleagues developed HMO6 cell lines by infecting primary human microglial cultures derived from human embryonic telencephalic cells with a v-myc oncogene (Nagai et al., 2001). These cell lines express markers specific for microglia or macrophages, including Ricinus communis agglutinin-1 lectin (CD11b), class I MHC antigen HLA-ABC, class II MHC antigen HLA-DR, CD68, and CD86 (Nagai et al., 2005, 2001). Interestingly, these cell lines showed increased expression of cytokines such as interleukin *(IL)-8*, *IL-10*, *IL-12*, and tumor necrosis factor alpha (TNF-α) when treated with amyloid beta (Aβ)25–35/Aβ1–42 peptides (Nagai et al., 2001; Timmerman et al., 2018). Other immortalized human microglial cell lines used in research include HMC3, CHME-5 (Janabi et al., 1998, 1995), which is also referred to as CI3NJ (Martin et al., 2003). More recently, Garcia-Mesa and colleagues have developed a novel microglial cell line, Hμglia 1A1 (Garcia-Mesa et al., 2017), which was isolated from the cortical tissue of adult human brains and immortalized using human telomerase reverse transcriptase (hTERT) (Garcia-Mesa et al., 2017). However, significant differences have been observed in the gene expression profiles between primary microglia and *ex vivo* microglia, possibly due to artificial activation and increased proliferation rates resulting from oncogenic transformation (Dello Russo et al., 2018). These differences have made microglia cell lines less attractive for research purposes.

2.2. Generation of human-induced microglia from human pluripotent stem cells (hPSCs) via classical differentiation protocols

The use of human pluripotent stem cells (hPSCs) to generate microglia has many advantages, the most important being the ability to generate a high yield of microglia exhibiting adult phenotypes, which can be difficult to obtain using conventional methods. In addition, a wide variety of hPSC lines, including those derived from patients with various diseases of interest or rare genetic disorders, could be used to generate microglia. These advantages make hPSC-derived human microglia a valuable tool for studying neuroinflammatory and neurodegenerative diseases in detail.

Following the discovery in 2010 that microglia are derived from primitive yolk sac macrophages (Ginhoux et al., 2010), several protocols using hPSCs have been developed. These protocols are aimed at differentiating hPSCs into embryonic yolk sac macrophage precursors that are subsequently redirected to a microglia-like phenotype using growth factors or cytokines (Ginhoux and Prinz, 2015). Some of these protocols generate microglia-like cells (MGLs) by using small molecules to induce mesodermal differentiation into embryonic bodies (EBs) or differentiation of hematopoietic progenitor cells (HPCs). We refer to this approach as the classical differentiation method and report recent studies that have adopted this protocol.

Muffat et al. described the first comprehensive method to generate microglia directly from hPSCs, without an intermediate monocytic colony-forming stage (Muffat et al., 2016). By culturing hPSCs in a specific medium on murine fibroblast feeder layers under controlled oxygen conditions, they enzymatically passaged the cells to form EBs. These EBs, cultured in a serum-free neuroglia differentiation medium with *IL-34* and CSF1, exhibited two phenotypes: dense neuralized

spheroids and cystic EBs expressing early yolk sac myelogenesis markers. When plated individually on low-adhesion plates, the yolk-sac EBs yielded semi-adherent immature microglia-like cell precursors (pMGLs) after two weeks. Fluorescence-activated single-cell sorting (FACS) analysis confirmed the expression of PU.1 (encoded by SPI1), ionized calcium binding adaptor molecule 1 (IBA1), CD45 and CD11b by the pMGLs. After an additional 30 days in culture, pMGLs differentiated into mature cells expressing microglia markers and exhibiting a ramified morphology similar to *ex vivo* primary microglia. Their cytokine and chemokine profiles, analyzed under different conditions, also showed similarities to primary microglia. While the differentiation process took about 2–3 months with a relatively low yield of 1–8 million microglia, the transcriptomic analysis showed similarity to freshly isolated fetal human microglia. Although these MGLs can be maintained for several months, their renewal and proliferation capacity decreases over time. Following the protocol established by Muffat et al (Muffat et al., 2016)., Amos and colleagues (Amos et al., 2017) aimed to differentiate human ESCs and iPSCS into MGLs using a chemically undefined medium supplemented with cytokines and fetal bovine serum (FBS). Initial mesodermal differentiation was induced by treatment with basic fibroblast growth factor (bFGF) and bone morphogenetic protein 4 (BMP4), after which a medium containing bFGF, SB431542 and 6-bromoindirubin-3′-oxime (BIO) directed the cells to generate MGLs. The resulting EBs were cultured in microglia medium with different supplements for 20 days. FACS analysis at the first passage (P1) showed the highest expression of microglial markers triggering receptor expressed on myeloid cells 2 (TREM2), C-X3-C motif chemokine receptor 1 (CX3CR1) and CD11b. Reverse transcription polymerase chain reaction (RT-PCR) showed increased messenger ribonucleic acid (mRNA) expression of hexosaminidase subunit beta (HexB), CX3CR1, and purinergic receptor P2Y12 (P2RY12) in cystic EBs. These cystic EBs showed a higher tendency to generate MGLs with microglia-like morphology, and the yield decreased after each passage. Notably, the expression of transmembrane protein 119 (TMEM119) was undetectable, suggesting an immature microglial phenotype consistent with previous findings (Muffat et al., 2016). The generated MGLs exhibited Aβ phagocytosis and expressed inflammatory markers upon lipopolysaccharide (LPS) stimulation. However, a notable limitation of this protocol was a reduction in MGL yield, with only a small number of cystic EBs exhibiting microglia-like morphology and function. These limitations cautioned against replication of protocols without comparative studies using primary human microglia. Additionally, previous studies have shown that serum components can activate and influence the phenotype of microglia (Adams et al., 2007; Ransohoff and Perry, 2009; Perry and Gordon, 1987). Therefore, it is imperative to consider protocols that outline a serum-free methodology.

Abud et al. (2017) developed a two-step protocol to generate MGLs from hPSCs within five weeks. First, hPSCs were differentiated into induced hematopoietic progenitor-like cells (iHPCs) using fibroblast growth factor 2 (FGF2), BMP4, and Activin A under hypoxic conditions for the first four days. Subsequently, Activin A and BMP4 were replaced by additional growth factors, such as vascular endothelial growth factor (VEGF), stem cell factor (SCF), *IL-3*, and *IL-6* from days 4–10. $CD43^+$ iHPCs were isolated by FACS and subsequently cultured in serum-free media with macrophage colony stimulating factor (M-CSF), *IL-34*, and transforming growth factor beta 1 (TGFβ-1) for another 25 days. During the last 3 days, iHPCs were transformed into mature MGLs by culturing them in serum-free media supplemented with CX3CL1 and cluster of differentiation 200 (CD200). These mature MGLs exhibited a ramified morphology and expressed several proteins that are characteristic of human microglia (MER proto-oncogene, tyrosine kinase (MERTK), integrin subunit beta 5 (ITGB5), CX3CR1, transforming growth factor beta receptor 1 (TGFβR1), protein S (PROS1), PU.1, TREM2, and P2RY12. Functional analysis confirmed phagocytic ability and cytokine production. Notably, the MGLs exerted synaptic pruning. This protocol yielded 30–40 million MGLs from 1 million iPSCs, with a purity of approximately 97.2%, making them suitable for high-throughput screening studies of microglial functions. Based on their previous publication (Abud et al., 2017), McQuade and colleagues presented an adapted methodology that eliminates the need for hypoxic conditions and FACS isolation to generate $CD43^+$ iHPCs (McQuade et al., 2018). Instead, their protocol uses the STEMdiff Hematopoietic Kit to directly generate $CD43^+$ iHPCs from feeder-free human iPSCs. These iHPCs were then cultured in *IL-34* and TGFβ-1 for 25 days. Additional cytokines, CD200 and CX3CL1, were added to promote microglial maturation and homeostasis. Functional assays of the resulting MGLs could be performed after 28 days, with an optional 1–2-week extension if necessary. Long-term culture was not recommended due to reduced microglial proliferation rates, previously reported by Muffat et al. (2016). MGLs generated using this protocol showed comparable gene expression patterns to those generated by Abud et al. (2017). and primary human microglia. However, they expressed lower levels of CD235a, suggesting potential immaturity. Like the MGLs generated by Abud et al. (2017)., the MGLs in this study exhibited a ramified morphology, phagocytic capacity and expressed the microglial marker IBA1. Although the omission of FACS isolation slightly reduced the purity (90–94%) compared to the methods of Abud et al., the iHPCs obtained using this approach still showed significant purity for $CD43^+$ cells. This protocol yielded 125 million $CD43^+$ cells from 1 million iPSCs and provides a cost-effective solution by reducing defined factors and eliminating the need for FACS. However, it is important to consider the reduced MGL purity and potential phenotypic differences compared to the original method of Abud et al. Further studies are needed to evaluate the feasibility of using a commercially available kit as a substitute for defined factors and to assess the importance of generating pure iHPCs in MGL generation.

In addition to the protocol delineated by McQuade et al. (2018)., two alternative methodologies, derived from Abud et al. (2017). were introduced by Pandya et al. (2017). and Xu et al. (2019). Pandya et al.'s approach involves a similar two-step process to generate MGLs within a month. IPSCs were first differentiated into induced pluripotent stem cell-derived hematopoietic progenitor cells (iPS-HPCs) over a 4-day period using VEGF, BMP4, SCF and Activin A. Subsequently, VEGF and Activin A are withdrawn and granulocyte-macrophage colony stimulating factor (GM-CSF), FMS-like tyrosine kinase 3 ligand (Flt3l), *IL-3,* and *IL-6* are added for 15 days under hypoxic conditions to yield $CD34^-$ and $CD43^+$ iHPCs. Co-culturing with astrocytes and supplementation with *IL-3,* GM-CSF, and M-CSF for 1–2 weeks lead to the generation of microglia expressing various markers. However, this method is limited by relatively low microglia yield (2–3 million from 1 million iPSCs) and transcriptome profiles resembling other cell types such as dendritic cells and macrophages. Also, insufficient information on the source and characteristics of astrocytes used for co-culture raised concerns about the reproducibility of the protocol. Xu et al.'s protocol involves differentiating iPSCs into iHPCs using the STEMdiff Hematopoietic Kit. $CD43^+$ iHPCs are isolated on day 12 using magnetic-activated cell sorting (MACS) and further differentiated into MGLs over 25 days using a specific medium containing GM-CSF, M-CSF, *IL-34, TGFβ1* and insulin growth factor 1 (IGF-1). The resulting MGLs exhibit microglia markers and functional capabilities, with alterations observed in response to LPS stimulation. Additionally, the study (Xu et al., 2019) also compared MGLs generated from individuals with Alzheimer's disease (AD-MGLs) which demonstrated an enhanced inflammatory phenotype (secretion of interferon gamma (IFN-γ), TNF-α, *IL-6,* and *IL-10*). Taken together, the protocol established by Xu et al. allowed for the generation of MGLs from human iPSCs that exhibited microglia characteristics and showed that these characteristics differed between individuals with Alzheimer's disease (AD) and healthy controls.

Haenseler et al. (2017). presented a protocol to generate macrophage progenitors from iPSCs and differentiated them into MGLs by co-culturing with iPSC-derived neurons. iPSCs were cultured with

BMP4, VEGF, and SCF to form EBs, which were then transferred to a culture medium containing M-CSF and *IL-3* for 3–4 weeks. Harvested microglia-like cell (MG) precursors were then co-cultured with iPSC-derived cortical neurons for an additional 14–42 days. The resulting MGLs exhibited a ramified morphology and expressed CD11b, IBA1, and MERTK. While they showed weak expression for certain markers such as TMEM119, P2RY12, and CD11c, transcriptome analysis confirmed the expression of essential microglia specific genes. Co-cultured MGLs showed phagocytic ability and secreted inflammatory cytokines upon stimulation with a yield comparable to that reported by Abud et al. (2017)., and significantly higher than those reported by Muffat et al. (2016). and Pandya et al. (2017). Adapting from the protocol, Banerjee and colleagues introduced another methodology for generating MGLs (Banerjee et al., 2020). This method involves supplementing the culture medium with a conditioned medium obtained from human iPSC-derived cortical neural progenitor cells (NPCs). The protocol aimed to mimic the environmental signals received by yolk-sac derived erythromyeloid precursors during brain development and involves the differentiation of iPSCs into NPCs and myeloid progenitors using chemically defined media and specific growth factors. Initially, iPSCs were directed towards a neural fate using a medium containing N-acetylcysteine, activin inhibitor, and LDN19318, leading to the formation of neurospheres. These neurospheres were subsequently transferred to laminin-coated plates and cultured in a medium supplemented with FGF2 under hypoxic conditions. The NPC-conditioned medium, collected weekly after several passages, was introduced as a supplementation medium to the microglia medium to facilitate MGL differentiation. In this protocol, human iPSCs were differentiated into cystic EBs with yolk sac-like structures using BMP4, VEGF and SCF. Following sphere formation, myeloid progenitors were observed “floating” in the supernatant and harvested for differentiation into MGLs. Cell collection continued weekly for up to 16 weeks, with harvested cells maintained in microglia medium supplemented with *IL-34* and GM-CSF. The NPC-conditioned medium was gradually introduced over 15 days, after which MGLs were harvested for further characterization. The resulting MGLs exhibited a ramified morphology and expressed various markers including TMEM119, P2RY12, PU.1, and CD11. RNA-sequencing analysis revealed human microglia specific gene expression and increased expression of phagocyte-related genes compared to primary human microglia and iPSC-derived macrophages. Upon stimulation with LPS, MGLs underwent morphological alterations and expressed proinflammatory genes such as *IL-1B*. The total time required to generate MGLs using this protocol was approximately 45–50 days, with a yield of 65- to 70-fold from a single iPSC.

Adapting from a previous protocol outlined by Yanagimachi and colleagues to differentiate iPSCs towards a myeloid lineage using a serum- and feeder-free protocol (Yanagimachi et al., 2013), Douvaras et al. (2017). detailed a similar protocol for the generation of myeloid progenitors from human iPSCs, followed by their differentiation into MGLs. The protocol, which mimics embryonic development, involves culturing with BMP4 for the first 4 days to promote primitive hemangioblast formation, followed by a transition to bFGF-, SCF-, and VEGF-containing medium from day 4–6. Specific growth factors, including SCF, thrombopoietin (TPO), *IL-3*, M-CSF, and Flt3l were used until day 25, with M-CSF, GM-CSF, and Flt3l maintained in the media. $CD14^+$ or $CD14^+CX3CR1^+$ progenitors were isolated from day 25 to day 50 and then differentiated into MGLs using GM-CSF and *IL-34*. These MGLs expressed microglia markers, exhibited phagocytic capabilities, and responded to external stimuli like primary microglia. However, the efficiency of the protocol was relatively low (68%) and the yield was unexpectedly modest, generating only 200,000 MGLs from 100,000 plated iPSCs.

Takata et al. (2017). presented a procedure to generate MGLs from human iPSCs. Human iPSCs were cultured with CHIR99021, BMP4, and VEGF for 2 days, followed by culturing with FGF2 for another 2 days. Subsequently, further modifications to the protocol were made until day 25 to induce their differentiation into human iPSC-derived macrophages (iMACs). Human iMACs were isolated and co-cultured with human-derived neurons, resulting in MGLs expressing IBA1. Moreover, since the MGLs generated through this protocol could not be characterized, the efficiency of the protocol was not clear. Trudler et al. (2021). reported a rapid and straightforward protocol to generate high-purity MGLs and study their role in neuroinflammation associated with Parkinson’s disease (PD). The protocol mimics the natural course of microglia development by directing cells into the yolk sac, which results in MGLs expressing specific markers in just 21 days. Human iPSCs were plated as small clumps in an ultra-low attachment plate and cultured under hypoxic conditions. A medium supplemented with bFGF and BMP4 was used for the first 4 days. Floating cystic EBs, previously described in other studies (Muffat et al., 2016; Amos et al., 2017; Haenseler et al., 2017), were harvested on day 4 and transferred to a medium containing bFGF, VEGF, *IL-6*, *IL-3*, and SCF, and maintained in hypoxic conditions. At days 8–15, bFGF was withdrawn and TPO was introduced, and cells were maintained under normoxic conditions. On day 15, cystic EBs were harvested and cultured in microglia differentiation medium containing *IL-34* and GM-CSF until day 21. At this point, the MGLs were fully adherent to the plate and ready for further experiments. The different stages of microglia development were demonstrated through the expression of specific markers. Mix paired-like homobox 1 (MIXL1) and apelin receptor (APLNR) genes were expressed on day 4, while genes such as kinase insert domain receptor (KDR), platelet derived growth factor subunit A (PDGFα), ETS variant transcription factor 2 (ETV2), RUNX family transcription factor 1 (RUNX1), and TAL bHLH transcription factor 1, erythroid differentiation factor (TAL1) were expressed from days 4–11. From days 17–21, mature microglia markers such as colony stimulating factor 1 receptor (CSF1R), interferon regulatory factor 8 (IRF8), crystallin beta 1 (CRYBB1), MAF bZIP transcription factor B (MAFB), and CD14 were highly expressed. On day 21, MGLs showed signs of maturation, including reduced CSF1 levels, a highly ramified shape with irregular processes, and expression of IBA1, TMEM119, and P2RY12, characteristic of homeostatic microglia in the human brain. More importantly, the MGLs displayed phagocytic capabilities and an inflammatory response upon LPS stimulation. Interestingly, a downregulation in TREM2 and CD33 expression was noted upon LPS stimulation, which has previously been associated with brain inflammation in AD.

An efficient and cost-effective protocol was developed by Lanfer et al. (2022). to generate MGLs from human iPSCs within a short span of 28 days. The protocol employed the STEMdiff Hematopoietic Kit to differentiate iPSCs into non-adherent HPCs in small colonies. On days 12 and 14, HPCs were harvested and cultured in a T75 flask containing RPMI media supplemented with GM-CSF. HPCs collected on day 14 could be cryopreserved without compromising viability upon thawing, although long-term storage is not recommended. On day 15, HPCs were cultured on uncoated glass-bottomed plates and maintained in RPMI supplemented with *IL-34* and GM-CSF for an additional 2 weeks, to induce their differentiation into MGLs. The use of glass-bottomed plates resulted in better adherence and a higher yield of MGLs. The protocol ensured a relatively pure population of HPCs expressing the pan-hematopoietic marker $CD45^+$ and the leukocyte marker $CD43^+$, at rates of approximately 90% and 50%, respectively. The isolation of HPCs before differentiation into MGLs was optional. Resulting MGLs expressed IBA1 and TREM2 and exhibited a ramified morphology that is typical of microglia. They also demonstrated phagocytic capabilities and upregulated inflammatory genes such as *IL-6*, *IL-1B*, and TNF, upon treatment with LPS. This protocol presents advantages in terms of cost-effectiveness and reduced time requirements compared to other methodologies outlined in this review and a notable advantage is the high yield of MGLs generated (80-fold). An accelerated protocol to generate MGLs from human iPSCs was developed by Funes and Bosco (Funes and Bosco, 2022), which first differentiated iPSCs into primitive macrophage progenitors (PMPs). This streamlined approach takes

approximately one month. Briefly, iPSCs were dissociated into single cells and cultured in an EB medium with BMP4, SCF, and VEGF from day 0–4. Upon the formation of spherical EB structures, they were transferred to Matrigel-coated plates and maintained in a PMP medium with *IL-3* and M-CSF for 7 days. On day 28, large, round floating cells, called PMPs, were collected from the supernatant, a process that could be repeated every 4–7 days for several months. The collected PMPs were then cultured in a microglia differentiation medium with *IL-34*, M-CSF, and TGF-β for another 10–12 days, during which they acquired microglia-like morphologies. High expressions of the microglia markers IBA1 (more than 95%), TMEM119, and P2RY12 (about 90%), were reported in the generated MGLs. The authors also developed a method to study the phagocytic capacity of MGLs using synaptosomes from human iPSC-derived lower motor neurons (i3LMNs). Live cell imaging demonstrated the ability of MGLs to phagocytose human synaptosomes. However, this protocol has several limitations. The time required to generate MGLs using this protocol is approximately 6–8 weeks, and the yield of MGLs is relatively low, at approximately 1 million cells per 100, 000 plated iPSCs. However, the purity of MGLs remains unclear. Additional functional studies beyond phagocytosis assays are needed to determine the suitability of MGLs generated by this protocol for further studies.

2.3. Generation of microglia using transcription factors-based, directed differentiation protocols

Classical differentiation protocols, as discussed earlier, rely on the use of extrinsic factors, such as morphogens, to direct the differentiation process toward a desired cell fate. These protocols aim to mimic the natural regionalization processes that occur during development. However, a major challenge in generating specific cell types using these classical protocols is the time-consuming nature of the differentiation process (Muffat et al., 2016; Amos et al., 2017; Abud et al., 2017; Banerjee et al., 2020; Douvaras et al., 2017; Takata et al., 2017; Lanfer et al., 2022; Washer et al., 2022; McQuade et al., 2018; Pandya et al., 2017; Xu et al., 2019; Haenseler et al., 2017; Funes and Bosco, 2022). Furthermore, these protocols often require complex multi-step growth factor regimens, which result in highly heterogeneous cultures that need to go through additional sorting steps to yield pure populations of microglia. Another important issue is the autocrine secretion of morphogens during the fate differentiation process, which exacerbates the heterogeneity of the culture (Abud et al., 2017; Xu et al., 2019; Douvaras et al., 2017; Takata et al., 2017). Considering that morphogen-based cell specification typically relies on the activation of specific transcriptional programs, the discovery of the Yamanaka factors spurred the use of an alternative approach that involves the use of distinct transcription factors to direct cell fate acquisition (Takahashi and Yamanaka, 2006; Zhang et al., 2013; Sun et al., 2019; Sun et al., 2016). Here, we chronologically present a summary of directed differentiation protocols developed recently.

Human-induced microglia-like cells (hiMGs) were successfully generated from hiPSCs by Chen et al (Chen et al., 2021). through the forced expression of two transcription factors, PU.1 and CCAAT/enhancer binding protein alpha (C/EBPα). Notably, this method bypassed the progenitor stage of primitive development. Firstly, a set of seven candidate transcription factors known to play a critical role in microglia development were selected. Following this, two or three transcription factors were expressed simultaneously based on data from extensive literature reviews, and the most effective combination—one that resulted in the highest percentage of $CD11B^{+}$ and $IBA1^{+}$ cells—was identified. Remarkably, robust expression of microglia markers was observed after just 9 days of induction. These hiMGs displayed physiological functionality, including inflammatory properties, phagocytic abilities, and responses to adenosine diphosphate/adenosine triphosphate (ADP/ATP)-evoked signaling and migration. Importantly, these features were comparable to those of EB-derived microglia and progenitor cells.

The method for rapid generation of hiMGs outlined in the study by Speicher et al. (2022). involves inducible expression of PU.1 and CCAAT enhancer binding protein beta (CEBP*β*). The process, which was carried out in a basal medium previously described by Muffat et al. (2016)., resulted in MGL precursors expressing several common myeloid surface markers such as CD11b, CD14, CD45, and CX3CR1 within 16 days. Upon culturing for an additional 10 days, the generated MGLs exhibited higher expression of established microglia signature genes compared to macrophages, monocytes, and dendritic cells. These MGLs displayed core microglia characteristics, including phagocytosis and cytokine secretion in response to IFN-γ stimulation. However, LPS stimulation did not induce cytokine secretion in these cells. Mass sγpectrometry analysis of MGL lysate revealed proteins associated with key microglia processes, including immune response, oxidative stress response, and IFN-γ, *IL-1,* and TGF-*β* signaling pathways. Although this protocol generated MGLs in a significantly shorter timeframe (approximately 20 days) compared to classical protocols, the yield was only about twice the number of initially seeded iPSCs. The cost-effectiveness of this approach is hampered by the extensive use of cytokines and small molecules required to mimic the entire microglia developmental process, from posterior primitive streak development to primitive hematopoiesis and primitive yolk sac macrophage maturation.

A streamlined method was presented by Sonn et al. (2022). for generating homogeneous populations of MGLs from iPSCs within approximately 30 days. They induced expression of the PU.1 transcription factor using an inducible SPI1 plasmid and a non-viral approach involving the reverse tetracycline transactivator (rtTA) and piggybac transposase. PU.1 overexpression was successfully achieved by selection with hygromycin and puromycin. To drive MGL differentiation, iPSCs were cultured in media supplemented with BMP4 and doxycycline for the first two days, with doxycycline maintained until day 18. VEGF and FGF2 were added on day 2, while BMP4 was removed on day 4. Cells were maintained under hypoxic conditions until day 6 and then transferred to normoxic conditions. From day 6 to day 12, the media were supplemented with VEGF, FGF2, *IL-3*, *IL-6*, and the WNT pathway inhibitor, IWR-1-endo. Notably, IWR-1-endo significantly increased the population of CD235a/KDR-positive mesodermal cells. The resulting HPCs, which were then cultured until day 18, showed bubble-like structures. On day 18, MGLs were replated and further differentiated in a microglia differentiation medium for another 7 days. Compared to classical protocols that omitted doxycycline, these protocols yielded MGLs with over 90% CD34 and CD43 expression in HPCs. However, PU.1 overexpression resulted in over 90% CD11b/CD45 positive cells, whereas the classical protocol resulted in 60% positivity. MGLs generated by PU.1 overexpression showed higher expression of myeloid, and microglia specific markers compared to THP-1 cells. Immunohistochemical staining confirmed the expression of microglia specific markers in MGLs. Gene expression analysis revealed that PU.1 overexpression downregulated inflammatory and antigen-responsive genes without affecting neurodegenerative disease-associated genes. Functional studies showed that MGLs from both protocols exhibited phagocytosis and cytokine secretion in response to LPS stimulation and formed an inflammasome under inflammatory conditions induced by Aβ peptide and LPS co-treatment. Notably, PU.1 overexpression facilitated the generation of highly pure MGL cultures without FACS/MACS, thus resulting in a high yield—up to 120 times higher than the originally seeded iPSCs. This approach could serve as a valuable tool for studying microglia dysfunction in neurodegenerative diseases.

A novel rapid method to generate MGLs from iPSCs was reported by Drager et al. (2022). The protocol involved developing a doxycycline-inducible iPSC line that expressed six specific transcription factors highly expressed in microglia: PU.1, MAFB, C/EBPα, C/EBPβ, IRF5, and IRF8. MGLs were efficiently generated within 8 days by inducing the transcription factors and supplementing the culture medium with specific growth factors (GM-CSF, *IL-34*, M-CSF, and TGF-β) on

day 2 and day 4. By day 8, MGLs exhibited a fully ramified morphology, expressed microglia specific markers (P2RY12, CSF1R, cytochrome b-245 beta chain (CYBB), and CD14), and exhibited functional properties like synaptosome phagocytosis and inflammatory responses. Interestingly, upon LPS stimulation, these MGLs secreted *IL-6* and *IL-8*, confirming their activation. Co-culture with iPSC-derived glutamatergic neurons enhanced the ramified morphology of MGLs. CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) platforms identified microglia specific genes and modifiers of survival, phagocytosis, and inflammatory activation in the MGLs.

Liu, S. et al. proposed an innovative approach for single-cell transcription factor screening, identifying SPI1, C/EBPα, C/EBPβ, IRF8, friend leukemia integration 1 (FLI1), and myocyte enhancer factor 2 C (MEF2C) as a potent combination to differentiate human iPSCs into MGLs in only 4 days. Notably, the protocol, which involves a single medium change, used mTESR Plus medium supplemented with doxycycline and Y-27632 ROCK inhibitor to eliminate the need for frequent media changes and additional factors. The generated MGLs expressed key microglia proteins such as CD11b, P2RY12, and CX3CR1 and demonstrated phagocytic ability. These cells were responsive to ADP stimulation, which is indicative of microglia activation (Inoue, 2008; Di Virgilio et al., 2009). This protocol offers a rapid and economical approach for generating functional MGLs suitable for various research applications.

3. 3D organoid models of human microglia

3.1. Co-culture of microglia cells with brain organoids

To study the interaction between cultured microglia and the human brain environment, Abud et al. published the first protocol for co-culturing organoids with hiPSCs-derived iMGLs (Abud et al., 2017). The mature microglia were able to integrate into a 3D environment and elicit functional responses towards injuries. Lin et al. used a similar co-culture system to study the effects of APOE4, a risk factor for Alzheimer's Disease, on microglia function (Lin et al., 2018). Interestingly, Abreu et al. integrated an SV-40 immortalized human microglia cell line into human brain spheres (BS) and showed that inflammatory responses to host-pathogen interactions could only be induced in BS with microglia (μBS) (Abreu et al., 2018). However, the μBS can only be maintained for a maximum duration of one week due to the proliferative nature of the immortalized microglia cells, thus limiting the utility of this protocol. To improve efficiency and scalability, Speicher et al. optimized a protocol for inducing MGLs with transcription factors such as PU.1 and C/EBPβ for 16 days, and co-culturing these cells with D30 cerebral organoids for an additional 30 days (Speicher et al., 2022). Popova et al. incorporated primary microglia from mid-gestation brain into human brain organoids to study brain-wide consequences and rubella virus infectivity (Popova et al., 2023). Several other studies have also used these neuroimmune organoids to study host-virus interactions of dengue virus, Zika virus, and HIV (Abreu et al., 2018; Muffat et al., 2018; Gumbs et al., 2022; Dos Reis et al., 2023; Dos Reis et al., 2020). Collectively, these studies suggest that microglia cells that have been immortalized or induced chemically or with transcription factors can integrate into 3D brain organoids and assume functional features.

Although many reports have suggested that 2D-induced microglia recapitulate their human counterparts, many sought to establish similar findings in microglia-incorporated organoids. A study by Sankowski et al. mapped the microglia spatiotemporal landscape in healthy and diseased human brains using single-cell RNA sequencing and mass cytometry by time of flight (CyTOF) (Sankowski et al., 2019). They showed eight different transcriptional cell states across microglia with different functional states. More recently, Popova et al. generated a "microglia report card" by characterizing the transcriptomic profiles of microglia across different models including primary fetal microglia, cultured primary microglia, cultured iMGs, 3D organoid-engrafted primary microglia, and xenotransplanted iMGs (Popova et al., 2021). While microglia transplanted into the mouse brain, as well as microglia from 3D organoids, exhibited transcriptomic similarities to primary human microglia, only microglia from 3D organoids developed immune signatures comparable to the developing brain, whereas microglia transplanted into the mouse brain induced only moderate immune signatures. Several microglia populations that were specific to cultured microglia exhibiting enhanced expression of metallothionine genes were also identified. This study not only provided valuable resources to the scientific community in the form of an interactive browser, but it also highlighted the differences between the mouse and human brain environment to better inform experimental model decisions. Speicher et al. reported a higher expression of primary human microglia genes in 3D brain organoid microglia, compared to 2D cultured microglia; 3D brain organoid microglia mapped to all clusters defined by Sankowski et al., whereas 2D culture microglia mapped to only four clusters (Speicher et al., 2022). Simultaneously, the study was able to validate reports by Popova et al.

The impact of region-specific heterogeneity of microglia has gained increasing attention recently. Several research and review articles highlighted the developmental regulation (Tan et al., 2020; De Baise and Bonci, 2019; Li et al., 2022; Brandi et al., 2022) and functional responses of region-specific microglia (Lee et al., 2022; Bussy et al, 2015; Abellanas et al., 2019; Bollinger et al., 2017). To the best of our knowledge, only Yan Li's group has examined the effects of MG in region-specific spheroids. By co-culturing dorsal and ventral forebrain spheroids with MG (D-MG, V-MG, D-V-MG), Song elucidated the differences in migratory ability and immunological responses such as TNF-α expression and ROS production. Transcriptomic analysis of MG and D-MG co-cultures revealed differences in metabolic pathways such as glycolysis and hypoxia, respectively (Song et al., 2019). In a separate article, the group further dissected the metabolic differences between MG and D-MG, revealing the ability of microglia to remodel the immune profile in organoids (Bejoy et al., 2019). However, the group did not further dissect the metabolic differences between D-MG and V-MG.

3.2. Co-culture of mesodermal progenitors with brain organoids

Since microglia are of mesodermal origin, a few labs have investigated the idea of incorporating mesodermal progenitors into organoids that would first differentiate into PMPs, migrate to the developing brain, and subsequently differentiate into microglia. In this regard, Wörsdörfer et al. were the first to develop a method that co-cultured Brachyury$^+$ hiPSCs-derived mesodermal progenitors with neural spheroids to generate vascularized neural organoids containing CD31$^+$ vessel-like structures (Wörsdörfer et al., 2019). While the study objective was to generate vascularized organoids, Iba1$^+$ microglia-like cells were surprisingly noted.

Fagerlund and colleagues incorporated day 8 hiPSC-derived CD41$^+$ EMPs into cerebral organoids and observed the migration and maturation of Iba1$^+$ microglia in these organoids (Fagerlund et al., 2021). In-depth immunohistochemical and electrophysiological analysis of these Iba1$^+$ cells demonstrated interactions with synapses and enhanced neuronal maturation (Fagerlund et al., 2021). Similarly, Park et al. generated microglia-enriched brain organoids by co-culturing the organoids with primitive-like macrophages (iMac), which differentiated into microglia-like cells (iMicro) in the organoids. The authors showed that iMac responded to the brain-like microenvironment of the brain organoids and that iMicro promoted neuronal maturation via cholesterol transfer mechanism (Park et al., 2023).

Sabate-Soler et al. were the first to characterize hiPSC-derived microglia in midbrain organoids (Sabate-Soler et al., 2022). The authors derived microglia using a protocol by Haenseler et al. (2017). and co-cultured them with midbrain organoids in an optimized culture medium. snRNA-seq revealed eight distinct cell types, including microglia and A9 and A10 dopaminergic neurons. These microglia

showed canonical immune profiles; oxidative stress and synaptic remodeling gene signatures were altered in other cell types, suggesting additional roles for microglia in these cellular mechanisms.

3.3. Co-culture of myeloid progenitors with NPCs to generate human brain organoids

Researchers have also explored an alternative method to generate microglia-containing brain organoids by co-culturing of MPCs with NPCs as the latter can self-organize into 3D brain organoids. This strategy is particularly valuable for studying the interactions between microglia and neurons during brain development. Xu et al. developed a protocol that co-cultured $CD43^{+}/CD235^{+}$ PMPs and NPCs to generate brain region-specific microglia-containing organoids (Xu et al., 2021). The microglia in these organoids promoted neural maturation and exhibited phagocytic capabilities such as the removal of apoptotic cells, synaptic pruning, and immune responses. Furthermore, Jin et al. used this method to show that Down syndrome (DS) microglia had increased synaptic pruning capabilities in both control and DS organoids (Jin et al., 2022).

3.4. Spontaneous and induced generation of microglia in organoids

Unguided protocols without SMAD inhibition have been used to generate organoids with neuroectodermal lineage cells as well as with mesodermal progenitors such as myeloid and microglial cells. Ormel et al. were the first to generate cerebral organoids with innate microglia by simply reducing the concentration of heparin from 1 ug/mL to 0.1 ug/mL in the culture media and delaying the embedding of the organoids in the Matrigel (Ormel et al., 2018). Microglia were generated as early as day 24, and exhibited a ramified morphology, expressed microglia-specific markers such as IBA1, and elicited functional immune responses. Bodner et al. slightly modified this protocol to recapitulate the spontaneous generation of microglia in the organoids (Bodnar et al., 2021; Samudyata et al., 2022; Hong et al., 2023). Variability and reproducibility were critical aspects of such spontaneous generation of microglia in the brain organoids. Therefore, Cakir et al. sought to develop a protocol that could yield a controllable proportion of microglia (Cakir et al., 2022). They combined hESCs overexpressing the myeloid-specific transcription factor PU.1 and normal hESCs in a 1:9 ratio to generate brain organoids. scRNA-seq analysis of MGLs within these organoids revealed a transcriptomic profile similar to the mid/late stage of fetal microglia. Furthermore, the MGLs shared more similarities with human primary microglia than with microglia generated by Ormel et al (Ormel et al., 2018; Cakir et al., 2022).

3.5. Xenotransplanted model of microglia

It has been debated that neither 2D nor 3D cultures of microglia can fully recapitulate brain-wide interactions, especially in the context of functional recapitulation of neurological disease manifestations (Bohlen et al., 2017; Giandomenico and Lancaster, 2017). In this regard, chimeric models have been developed to study human microglia in the mouse brain. For instance, Abud et al. transplanted mature iMGLs into the cortex of MITRG mice and observed engraftment and long-term survival (Abud et al., 2017). They showed that both iMGLs and human fetal microglia transplanted into the hippocampus of AD mice had phagocytotic responses against fibrillar Aß. Hasselmann et al. showed that iPSC-derived hematopoietic progenitors transplanted into mice differentiated into human microglia (xMGs) *in vivo* and acquired human microglial gene signatures and immune responses to acute and chronic stimuli (Hasselmann et al., 2019). Remarkably, these xMGs also exhibited responses to Aβ plaques, a characteristic feature of human microglia. These findings were replicated in a similar study by Mancuso et al (Mancuso et al., 2019).

Following successful studies with xenotransplantion models such as those described above and previous reports of progenitor cell transplantation (Chen et al., 2016; Han et al., 2013), several groups have examined the transplantation of hPSC-derived microglial progenitors and hiPSC-derived PMPs into neonatal mouse brains (Jin et al., 2022; Svoboda et al., 2019; Xu et al., 2020). Svoboda et al. reported that transplanted microglia acquired human brain-like gene expression and morphology, a phenotype that is difficult to recapitulate in *in vitro* microglia (Svoboda et al., 2019). Xu and colleagues closely monitored the differentiation of the transplanted PMPs and showed that they proliferated for 6 months. In addition, they found that these microglia were concentrated around blood vessels and used the vessels to guide their migration, resulting in their widespread distribution in various brain regions. Jin et al. showed that the transplantation of DS microglia into the mouse brain resulted in less convoluted morphologies compared to control microglia (Jin et al., 2022). Fattorelli et al. further optimized the xenotransplantation protocol due to the high variability in microglia yield, time point, and engraftment efficiency reported in previously published studies (Fattorelli et al., 2021). In their modified protocol, mesodermal induction was performed in EB for 4 days, followed by plating and culturing in defined media until day 18, a time point at which microglial progenitors were harvested for xenotransplantation. Accordingly, approximately 30% of chimerism was present at 17 days post-xenotransplantation and increased up to 80% at 3–6 months. More recently, Schafer et al. developed a protocol for the xenotransplantation of microglia (hMGs)-containing immunocompetent human brain organoids (iHBO), allowing the study of microglia under physiological conditions *in vivo* (Schafer et al., 2023). They showed that the human brain-like environment supported the development of hMGs and inhibited their non-physiological activation. Transcriptomic and functional studies showed that these hMGs displayed human-specific gene signatures and responded to injury and inflammatory stimuli.

4. Utilizing human-induced MGLs for modeling the microglia dysfunction theory of neurodegenerative diseases

Aberrant microglial function has been linked to the pathogenesis of several neurological disorders (Salter and Stevens, 2017; Hickman et al., 2018). GWAS studies have identified certain myeloid-specific genes as risk factors for AD (Salter and Stevens, 2017; Abud et al., 2017; McQuade et al., 2020), sparking the hypothesis that microglial dysfunction drives AD pathogenesis rather than being a secondary effect of disease-associated neuronal death. One well-known AD genetic risk factor is the TREM2 (triggering receptors expressed on myeloid cells 2) receptor expressed on the microglia cell surface that is important for chemo-sensing and signal transduction (McQuade et al., 2020; Jonsson et al., 2013; Lambert et al., 2013). While the key functions of this receptor have been discovered in mouse models (Bemiller et al., 2017; Cheng-Hathaway et al., 2018; Leyns et al., 2017; Ulrich et al., 2014), whether it is functionally conserved in human microglia remains unknown. Using human iPSC-derived microglia, McQuade et al. demonstrated that TREM2-knockout resulted in gene expression and functional deficits relevant to AD progression (McQuade et al., 2020). The authors differentiated microglia from isogenic, CRISPR-modified TREM2-KO human iPSCs, and found that TREM2-KO microglia exhibited impaired survival and phagocytosis of APOE, fibrillar Aβ, and human synaptosomes. Xenotransplantation of TREM2-KO microglia into the brains of AD model mice revealed reduced amyloid plaque association, while impaired migration toward Aβ plaques or aged Aβ-producing neurons and astrocytes was observed *in vitro*. Impaired migration towards AD neurons and astrocytes in TREM2-KO microglia resulted from lack of the CXC motif chemokine receptor 4 (CXCR4) co-receptor necessary for microglial migration. Single-cell RNA-seq of transplanted TREM2-KO microglia revealed their intrinsically hypo-active state and inability to transit into disease-associated microglia (DAM).

Similar to the clearance of Aβ plaques in AD, microglia's phagocytic function is implicated in the clearance of alpha-synuclein aggregates in

Table 1
Summary of published hPSC-derived MGLs models.

Publication	Time required for MGL generation.	Overview of protocol	Pros (+)	Cons (-)
Muffat et al., 2016	Approximately 51 days	EBs were generated and cultured in a serum-free medium, specifically - a neuroglia differentiation medium supplemented with IL-34 and CSF-1. Cystic EBs were harvested and further differentiated into mature MGLs.	(+) Microglia signature genes resembling that of freshly isolated fetal human microglia	(-) Low yield of approximately ~0.5–4% (1 million – 8 million MGLs from 2 million iPSCs)
Abud et al., 2017	Approximately 5 weeks	Follows a two-step process: iHPC differentiation: iPSCs are differentiated into iHPCs in a culture medium containing FGF2, BMP4, and Activin A under hypoxic conditions. From day 4–10, BMP4 and Activin A are replaced by VEGF, SCF, IL-3 and IL-6. The isolation of $CD43^{+}$ iHPC and generation of MGL: CD43+ iHPCS are sorted using FACS. The isolated CD43+ progenitors are cultured in a differentiation medium containing M-CSF, IL-34, and TGFβ-1 for another 25 days. Subsequently, iHPCs transit into mature MGLs when cultured in a serum-free medium supplemented with CX3CL1 and CD200.	(+) High yield of approximately 30–40% (30 million – 40 million MGLs from 1 million iPSCs) (+) MGLs capable of performing synaptic pruning (+) Similar gene expression profiles to primary fetal human microglia and adult microglia	(-) Hypoxic condition required to differentiate iPSCs into iHPCs (-) Requires FACS sorting
Pandya et al., 2017	Approximately 1 month	The protocol similar to Abud et al., 2017, follows a two-step process: iHPC differentiation: iPSCs are differentiated into iHPCs by supplementing media with VEGF-A, Activin A, SCF, and BMP-4 for the initial 4 days. Subsequently, VEGF and Activin A are removed, and the culture medium is supplemented with GM-CSF, Flt3l, IL-3, and IL-6 for the next 15 days under hypoxic conditions MGL generation: iHPCs are co-cultured with astrocytes and maintained in a medium containing IL-3, GM-CSF, and M-CSF for the generation of MGLs.		(-) Hypoxic conditions required to differentiate iPSCs into iHPCs (-) Requires co-culturing with astrocytes (source of astrocytes not stated in the protocol) (-) Low yield of approximately 2–3% (2 million to 3 million MGLs from 1 million iPSCs) (-) Transcriptomic profile similar to dendritic cells and macrophages in addition to human fetal brain microglia
Haenseler et al., 2017	Approximately 74 days	The protocol involves the generation of macrophage progenitors from iPSCs and their subsequent differentiation into MGLs. Macrophage progenitors were first generated by forming EBs from iPSCs, using BMP4, VEGF, and SCF for 4 days. These progenitors were then cultured in X-VIVO15 medium supplemented with M-CSF and IL-3. The MGLs were then generated by co-culturing the precursors with iPSC-derived cortical neurons in a microglia medium supplemented with IL-34 and GM-CSF.	(+) Relative high yield of approximately 30–40% (similar to that reported by Abud et al., 2017)	(-) Requires co-culturing with iPSCs-derived cortical neurons (-) Co-cultured MGLs demonstrated weak expression levels of TMEM119, P2RY12, and CD11c
Douvaras et al., 2017	More than 50 days	Primitive hemangioblasts (KDR+CD253a+) were induced using BMP4, which was later replaced by bFGF, SCF, and VEGF from days 4–6. Subsequently, the culture medium included SCF, TPO, IL-3, M-CSF, and Flt3l for the next 8 days, with M-CSF, GM-CSF, and Flt3l remaining until day 25. CD14+ or CD14+CX3CR1+ progenitors were isolated using FACS/MACS and maintained in a chemically defined environment containing GM-CSF and IL-34 for the generation of GMLs.	(+) Resembles human fetal microglia	(-) Requires FACS/MACS sorting for CD14+ or CD14+CX3XR1+ progenitors (-) Low efficiency (67%) (-) Low yield of approximately 2% (200,000 MGLS from 100,000 iPSCs)
Takata et al., 2017	Approximately 25 days	iPSCs were guided towards a mesodermal lineage through a sequential culture protocol. Initially, CHIR99021, BMP4, and VEGF were employed for the first 2 days to induce mesodermal lineage and hemangioblast-like cell formation. Subsequently, CHIR99021 was replaced with FGF2 for an additional 2 days. From days 4–6, BMP4 was omitted and the medium was enriched with VEGF, FGF2, SCF, IL-6, IL-3, and DKK-1. Floating cells were sorted for CD45+CD11b+CD163+CD14+CX3CR1+hiMACs. These cells were co-cultured with human-derived neurons in a medium containing FGF2, SCF, IL-3, and IL-6 and later transitioned to SF-Diff commercial medium supplemented with CSF1 for the generation of MGLs.		(-) Primary characterization with mouse iPSCs (-) Requires FACS sorting (-) Initial hypoxic conditions and switched to normoxic conditions (-) Yield unknown
Amos et al., 2017	More than 50 days	Following a methodology similar to Muffat et al., 2016, EBs were formed by subjecting ESCs or iPSCs to bFGF, and BMP4 to induce mesodermal differentiation. The transition to bFGF, SB431542, and 6-BIO facilitated the generation of MGLs from these EBs. Subsequently, the EBs were transferred to a microglia medium supplemented with bFGF, GM-CSF, M-CSF, and TGFβ-1.		(-) Immature microglia phenotype (TMEM119 expression was undetectable) (-) Low yield (not all EBs exhibited microglia-like morphology and function; exact yield is unknown)
McQuade et al., 2018	Approximately 28 days	The STEMdiff Hematopoietic kit was utilized to generate CD43+ HPCs from human iPSCs. These CD43+ HPCs were cultured for 25 days in a microglia medium containing M-CSF, IL-34, and TGFβ-1. Subsequently, CD200 and CXCL3 were introduced to facilitate the maturation and homeostasis of microglia.	(+) Elimination of hypoxic conditions and requirement for FACS sorting (+) High yield of approximately 125% (125 million CD43+ cells from 1 million iPSCs) (+) Cost-effective	(-) Reduced purity of MGLs generated (90% - 94%)
Xu et al., 2019	Approximately 40 days	STEMdiff Hematopoietic kit was utilized to facilitate the differentiation of iPSCs into induced HPCs. On day 12, CD43+ cells were isolated using MACS and subsequently differentiated into MGLs. The	(+) Observable differences between wild-type and diseased MGLs	(-) Requires sorting with MACS (-) Yield unknown

(*continued on next page*)

Table 1 (*continued*)

Publication	Time required for MGL generation.	Overview of protocol	Pros (+)	Cons (-)
		differentiation process involved the use of a medium containing GM-CSF, M-CSF, IL-34, IGF-1, and TGFβ-1.		
Banerjee et al., 2020	45–50 days	Human iPSCs-derived cortical neuronal progenitor cells (NPCs) condition medium was utilized for MGL generation. iPSCs were initially differentiated into NPCs, maintained in a medium containing N-acetylcysteine, activin inhibitor, and LDN19318, resulting in neurosphere formation. The NPCs were cultured in hypoxic conditions with FGF2 supplementation. Simultaneously, myeloid progenitors were differentiated into EBs, with BMP4, VEGF, and SCF supplementation and subsequently differentiated into MGLs in a medium supplemented with IL-34 and GM-CSF. The NPC-conditioned medium was gradually introduced over 15 days before harvesting.	(+) High yield of MGLs (approximately 65–70%)	(-) Requires con-current generation of NPCs for medium supplementation
Trudler et al., 2021	21 days	iPSCs were cultured under hypoxic conditions and supplemented with bFGF, VEGF, IL-6, IL-3, and SCF. From day 8–15, bFGF was removed and TPO was introduced. Following the harvesting of EBs, cells underwent further maintenance in a microglia differentiation medium containing IL-34 and GM-CSF.	(+) Rapid protocol (+) Mimics natural progression of microglia development	(-) Hypoxic conditions required to maintain iPSCs (-) Inconclusive results to determine if MGLS represent human microglia (-) Yield unknown
Lanfer et al., 2022	28 days	The differentiation of iPSCs into non-adherent HPCs was achieved using the STEMdiff Hematopoietic Kit. Subsequently, on days 12–14, the harvested HPCs were cultured in RPMI media and supplemented with GM-CSF and IL-34 to facilitate the generation of MGLs.	(+) Ability to cryopreserve HPCs without compromising viability although long-term storage is not recommended (+) Cost-effective (+) High yield (80-fold)	
Funes and Bosco, 2022	6–8 weeks	The production of MGLs involves the initial differentiation of iPSCs into primitive macrophage progenitors (PMPs) and is achieved through the formation of EBs in a medium supplemented with BMP4, SCF, and VEGF. Subsequently, PMPs are cultured in a specific PMP medium containing IL-3 and M-CSF. Harvested PMPs are then subjected to further differentiation in a microglia differentiation medium, which includes IL-34, M-CSF, and TGF-β.	(+) Mimics the development of yolk sac macrophages	(-) Low yield of approximately 10% (~1 million MGLs from 100,000 iPSCs) (-) Long duration required to generate MGLs (-) Additional functional studies are required to determine the suitability of MGLs

PD (Hickman et al., 2018). Badanjak et al (Badanjak et al., 2021). demonstrated increased inflammation in idiopathic PD (IPD) microglia, as evidenced by upregulation of the pro-inflammatory cytokines *IL-10* and *IL-1B* as well as the inflammasome protein NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) (Badanjak et al., 2021; Swanson et al., 2019). Furthermore, IPD microglia exhibited increased phagocytosis, indicating functional alterations under pathological conditions. This study highlighted the potential of iPSC-derived microglia in exploring PD mechanisms and suggested that inflammation and dysfunctional microglia might contribute to the pathogenesis of IPD.

One distinctive characteristic of microglia is that they are known to adopt a spectrum of cell "states" *in vivo (*Salter and Stevens, 2017; McQuade et al., 2020; Dolan et al., 2023), leading to heterogeneity within a cell population. Two recent studies have found that human microglia derived *in vitro* also exhibit this property. Following the protocol of Abud et al (Abud et al., 2017)., Dolan et al (Dolan et al., 2023). treated iMGLs with CNS substrates such as synaptosomes, synthetic Aβ fibrils, apoptotic neurons, and myelin debris, and analyzed the transcriptional states of treated iMGLs through single-cell RNA sequencing. They identified 11 distinct clusters, with the proportion of iMGLs in each cluster changing in response to CNS substrate treatment. These findings suggested alterations in cell states upon substrate stimulation. Comparisons with human biopsy microglia single nuclei RNA-seq data indicated that iMGLs adopt analogous transcriptional states like *in vivo* human microglia. Such similarity validates hESCs-derived microglia as a reliable model system. Disease-associated microglia (DAM)-like clusters identified in iMGLs share transcriptional signatures with both human and mouse DAMs. This microglial state is triggered specifically by CNS substrates in a TREM2-dependent manner, similar to the findings observed using TREM2-KO microglia by McQuade et al. (2020). Through ATAC-seq and gene regulatory network analysis, the transcription factor microphthalmia-associated transcription factor (MITF) was identified as a regulator of the DAM state. Overexpression of MITF resulted in differential expression of pathways upregulated in DAM transcriptional signature, and comparison of MITF-induced genes with gene expression signatures in each iMGL state revealed a larger overlap with DAM-like iMGL clusters, suggesting a role for MITF in promoting transition into DAM state. Functionally, MITF overexpression led to increased phagocytic activity, as reported in neurodegeneration models. Through the identification of key transcription factors such as MITF that can control the activation of DAM states, microglia could be manipulated to adopt DAM-like states to study the role of this cell subpopulation in diseases. Finally, the iMGL transcriptional cell states were also observed in microglia derived from three other independent iPSC lines, demonstrating the replicability of microglia cell states *in vitro*.

In a second study, Drager et al. (2022). introduced a novel microglial differentiation protocol using six transcription factors over eight days, as mentioned above (Drager et al., 2022). The researchers integrated inducible CRISPRi/a machinery into the cell lines to allow sgRNA transduction at the iPSC stage, as differentiated microglia become resistant to lentiviral transduction. The study involved three types of pooled screens on the druggable genome to identify genetic modifiers of microglia survival, inflammatory activation, and phagocytosis. The screens demonstrated the utility of the platform for high-throughput studies of microglia-specific biological mechanisms. For example, phagocytosis CRISPRi/a screens revealed that PFN1, a gene implicated in ALS, exhibited opposite phenotypes: silencing of PFN1 slightly increased phagocytosis, while its overexpression inhibited phagocytosis. RNA-seq of PFN1-OE microglia revealed alterations in immune-related genes and AD risk genes, including HLA-DRA and HLA-DQA1. Further studies are needed to delve deeper into the potential disease mechanisms involved. In addition, a CRISPR droplet sequencing (CROP-seq) screen using scRNA-seq as a readout revealed nine distinct microglial states, including a proliferation cluster characterized by MKI67 and TOP2A expression that was also identified in the study by Dolan et al. (2023). Both studies acknowledged the inherent heterogeneity of transcriptional cell states in their respective *in vitro* microglia models. However, Drager et al. did not compare the transcriptional signatures of each cluster to the identified microglial states in human or mouse models, leaving the generalizability of these microglial states unknown.

Table 2
Published protocols for the generation of MGLs through overexpression of transcription factors.

Publication	Transcription factors (TFs) required	Time required for MGL generation.	Overview of protocol
Chen et al., 2021	PU.1 and CEBPα	9 days after induction	iPSCs infected with FUW-rtTA and pTetO-CEBPA-T2A-SPI1-T2A-Puro were induced with doxycycline on day 0, in a medium containing BMP-4, FGF2, and Activin A. On day 1, cells were selected with puromycin in a medium supplemented with VEGF, SCF, and FGF2, and then replaced with a medium containing IL-34, M-CSF, and TGFβ-1 on day 2. After day 4, half of the medium was replaced with IL-34, M-CSF, GM-CSF, and TGFβ-1 and the cells were ready for analysis on day 9.
Speicher et al., 2022	PU.1 and CEBPβ	Approximately 16 days after induction	iPSC lines expressing PU.1 and CEBPβ were cultured in a medium supplemented with Activin A, BMP4, FGF, VEGF-A, LY, CHIR, and doxycycline. From days 2–4, the medium was enriched with SCF, IL-3, IL-6, M-CSF, FGF and doxycycline. On days 6 and 8, only CSF1 and doxycycline remained in the medium. By day 10, detached cells were collected, replated, and maintained in a medium containing IL-34, CSF1, and TGFβ-1, without doxycycline.
Sonn et al., 2022	PU.1	Approximately 30 days after induction	An inducible SPI1 expression plasmid, along with plasmids encoding for rtTA and piggyback transposase, were introduced into the iPSC line using a non-viral method. Following successful selection with hygromycin and puromycin, clones overexpressing PU.1 were cultured in BMP-4 and doxycycline-containing media for the initial 2 days. VEGF and FGF2 were introduced on day 2, BMP4 was removed on day 4, and the culture was maintained under hypoxic conditions until day 6. Subsequently, VEGF, FGF2, IL-3, IL-6, and IWR-1 endo were added between days 6 and 12. Differentiated HPCs were cultured in an HPC differentiation medium containing FGF2, SCF, IL-3, and IL-6 until day 18. Successfully generated MGLs were replated and maintained in a microglia differentiation medium enriched with M-CSF, CD200, IL-34, CX3CL1, and TGFβ-1.
Drager et al., 2022	PU.1, MAFB, CEBPα, CEBPβ, IRF5 and IRF8	8 days after induction	The iPSC line expressing six different TFs was induced on day 0. On days 2 and 4, it was supplemented with a culture medium containing GM-CSF, IL-34, M-CSF, and TGF-β which aimed to promote the generation of mature MGLs.
Liu et al., 2022	PU.1, CEBPα, CEBPβ, IRF8, FLI1 and MEF2C	4 days after induction	iPSCs harbouring the 6 different TFs were cultured in mTESR Plus medium with the addition of doxycycline and Y-27632 ROCK inhibitor for 24 hours. Subsequently, a single medium change was performed using mTESR Plus and doxycycline. Notably, the generation of MGLs did not necessitate the use of small molecules or growth factors in this protocol.

The advent of hPSC-derived culture offers a unique opportunity to study neurological disorders in the human context. This is critical because microglial biology has been shown to be species specific when comparing humans and rodents (Dragunow, 2020). Moreover, in contrast to the scarcity and rarity of primary human neural cells, the self-renewal property of hPSCs promises a non-exhaustive source for deriving desired cell types for research (Garcia-Leon et al., 2020). This scalability, combined with protocols that generate high-quality and physiologically relevant microglia, holds promise for establishing high-throughput drug screening platforms.

In addition, the use of patient-derived microglia in drug screening allows drug development in a disease-specific context, offering the potential for personalized drug screening. However, drug screening using hPSC-derived microglia has not been reported to date, possibly due to the relatively recent development of this model. Nevertheless, microglial drug screens have been performed using both primary human or murine microglia and murine microglial cell lines (Rustenhoven et al., 2018; Figuera-Losada et al., 2017; Zhou et al., 2016; Oliveira et al., 2018), often targeting microglial activation status as a screening phenotype using readouts such as proinflammatory cytokine secretion, metabolite generation, or gene expression. With the refinement of protocols that improve the reliability of derived microglia to reflect *in vivo* physiological or pathological responses, and the establishment of robust disease-relevant phenotypes suitable for high-throughput screening, it is anticipated that drug screens identifying potential new therapeutics using hPSCs-derived microglia could be reported within the next few years. Tables 1, 2.

5. Conclusions

In conclusion, hPSC-derived human microglia are emerging as promising models for both *in vitro* and *in vivo* studies of neurological disorders. Their advantages include retention of human-specific biology and scalability for high-throughput functional screens. These models have demonstrated the ability to recapitulate key findings observed in human tissues and mouse disease models, and thus could serve as valuable tools for elucidating the role of microglia in both physiological and pathological settings. It is worth noting that the functionality and identity of these hPSC-derived human microglia need to be validated by a comprehensive approach including immunostaining of various microglial markers, phagocytic assay, calcium activity analysis, and assessment of inflammatory responses.

Ethics approval Statement

This study did not involve human subjects

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

F.T., C.K., M.J.T., and H.S.J. contributed to the review, writing, and editing of the study; F.T. and H.S.J. contributed to the preparation of the tables.

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