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Authors

Warden, Anna S
Han, Claudia
Hansen, Emily
et al.

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Tools for studying human microglia: In Vitro and In Vivo Strategies

Anna S. Warden[3], **Claudia Han**[1], **Emily Hansen**[2,3], **Samantha Trescott**[2,3], **Celina Nguyen**[2], **Roy Kim**[2,3], **Danielle Schafer**[1,2], **Avalon Johnson**[2,3], **Madison Wright**[2,3], **Gabriela Ramirez**[2,3], **Mark Lopez-Sanchez**[2,3], **Nicole G. Coufal**[2,3,*]

[1]Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093, USA

[2]Sanford Consortium for Regenerative Medicine, La Jolla, CA 92037, USA

[3]Department of Pediatrics, University of California, San Diego, La Jolla, CA 92093, US

Abstract

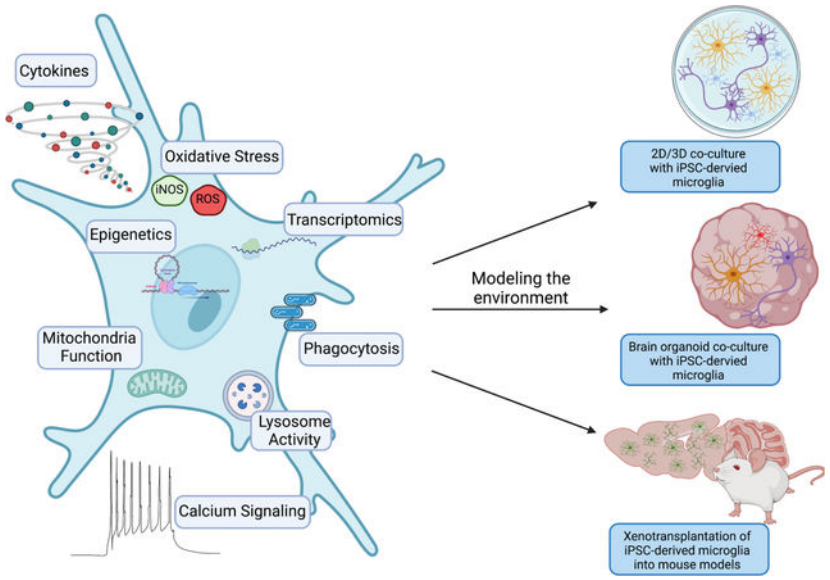
Microglia may only represent 10% of central nervous system (CNS) cells but they perform critical roles in development, homeostasis and neurological disease. Microglia are also environmentally regulated, quickly losing their transcriptomic and epigenetic signature after leaving the CNS. This facet of microglia biology is both fascinating and technically challenging influencing the study of the genetics and function of human microglia in a manner that recapitulates the CNS environment. In this review we provide a comprehensive overview of existing in vitro and in vivo methodology to study human microglia, such as immortalized cells lines, stem cell-derived microglia, cerebral organoids and xenotransplantation. Since there is currently no single method that completely recapitulates all hallmarks of human ex vivo adult homeostatic microglia, we also discuss the advantages and limitations of each existing model as a practical guide for researchers.

Graphical Abstract

*corresponding author.

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.



1. Introduction

Microglia, the resident tissue macrophage of the central nervous system (CNS), are increasingly implicated in neurodevelopmental and neuropathological processes as a therapeutic target of interest (Lewcock et al., 2020). Histological work by Del Rio-Hortega and other early investigators laid the groundwork for our understanding of microglia in animal models, including microglial developmental origin, CNS-specific functions (Salter and Stevens, 2017), morphologic and metabolic phenotypes distinguishing microglia from other tissue resident macrophages (Hoeffel and Ginhoux, 2015; Murabe and Sano, 1982), and environmental dependence (Gosselin et al., 2017).

However, the translational potential of microglia physiology has been limited by species-specific differences. Multiple studies comparing human and murine microglia reported significant differences in the expression of microglia genes, inflammatory response and complement system genes, as well as genes related to neurodegenerative diseases (Galatro et al., 2017; Gosselin et al., 2017). For instance, over half of Alzheimer's disease (AD) risk associated genes share less than 70 % homology between mice and humans (Hasselmann and Blurton-Jones, 2020). Moreover, human and mouse microglia age differently (Galatro et al., 2017), respond to amyloid plaques differently (Zhou et al., 2020), and have distinct sensomes (Abels et al., 2021). Therefore, the development of tools to study human microglia circumvents animal model mitations and enhances the translational capability of microglia research. This review will explore noteworthy techniques in human microglia research and outline their advantages and drawbacks to present a practical toolkit (Table 1).

2. In vitro tools and considerations

In vitro studies allow for in-depth and high-throughput analysis of cellular identity and function. Advantages of in vitro studies include cell number availability, ease of maintenance/manipulation and upon transformation, a high proliferative capacity. However,

microglia undergo substantial alterations once removed from the CNS, including a loss of CNS-specific transcriptional regulation (Gosselin et al., 2017; Vay et al., 2018). Specifically, in vitro microglia differ from ex vivo microglia in gene expression with reduced expression of key microglia genes, such as SALL1, P2RY12, and CX3CR1. Additionally, the in vitro environment leads alterations in differentiation and phagocytosis and increased expression of pro-inflammatory markers (Bohlen et al., 2017; Gosselin et al., 2017; Vay et al., 2018). Molecular techniques, such as co-culturing with other CNS cell types and newly developed in vitro systems, such as induced pluripotent stem cell-derived microglia (iMG), reduce the differences between in vivo and in vitro experiments while allowing for biochemical and molecular assessments that require high cell numbers and high-throughput experimental designs. For a complete listing of in vitro tools see Fig. 1 and Table 2.

2.1. Peripheral macrophage-induced microglia

Human microglia-like cells can be derived from patient PBMCs or monocytes (Ohgidani et al., 2015; Scheiblich et al., 2021; Sellgren et al., 2019; Sellgren et al., 2017) through supplementation of microglia factors including IL-34, CSF1 and GM-CSF in culture. These peripheral monocyte-derived microglia are positive for microglia-specific markers such as TMEM119 and P2RY12 (Sellgren et al 2017; Sellgren et al., 2019). Moreover, these peripheral monocyte-derived microglia engulf synaptosomes, degrade α-synuclein, and have a transcriptomic signature more closely resembling fetal primary microglia rather than monocyte-derived macrophages, suggesting this model produces functional human microglia (Scheiblich et al., 2021; Sellgren et al., 2019; Sellgren et al., 2017). This tool has already been applied to schizophrenia, Nasu-Hakola Disease, and synucleinopathies, demonstrating an ability to model human disease in a monoculture context (Ohgidani et al., 2015; Scheiblich et al., 2021; Sellgren et al., 2019; Sellgren et al., 2017). For instance, schizophrenia patient-derived microglia showed increased synapse elimination in patient-derived synaptosomes, reflecting abnormalities in both microglia-like cells and synaptic structures observed in individuals with schizophrenia (Sellgren et al., 2019).

2.2. Primary culture

Primary human microglia are derived from postmortem samples, perioperative brain resections, or fetal brain tissue (Mizee et al., 2017; Park et al., 2022;Popova et al., 2021). Current popular methods utilize FACS, immuno-panning, MACS, or a percoll gradient for microglial isolation (Agalave et al., 2020; Bohlen et al., 2017; Galatro et al., 2017; Grabert and McColl, 2018). Challenges include significant changes in gene expression within hours after extraction (Bohlen et al., 2017; Gosselin et al., 2017; Popova et al., 2021), limited cell yields, variable culture quality, and a lack of proliferation (Horvath et al., 2008).

In a recent study, Popova and colleagues successfully isolated healthy primary fetal microglia using MACS (Popova et al., 2021). While these isolated primary microglia remained ramified and motile in culture with IL-34 and TGFβ2 supplementation, they had lower homeostatic gene expression, higher immune activation and increased expression of axon tract-associated microglia genes in accordance with previous studies (Gosselin et al., 2017; Hammond et al., 2019; Li et al., 2019; Popova et al., 2021). Interestingly, 3D organoid coculture with primary fetal microglia partially rescued the environment-

dependent homeostatic gene expression signature, but also increased expression of a cytokine-associated signature (Popova et al., 2021).

2.3. Immortalized cell lines

Immortalized human microglial cell lines have been generated as an alternative to primary cultures, benefiting from ease of maintenance and proliferative advantages (Timmerman et al., 2018). The immortalization method consists primarily of transduction of primary microglia with oncogenes (Blasi et al., 1990) or SV40 large T antigen (Janabi et al., 1995). The validity of immortalized microglial cell lines as an alternative to primary microglia has been an ongoing topic of debate. A study of the SV40, HMC3, and CHME lines revealed these cells are capable of responding to a pattern of chemokines and inflammatory stimuli and regulating the expression of typical activation markers of microglia (Dello Russo et al., 2018). However, there are also significant phenotypic differences between the human microglial cell lines and primary microglia, including significantly decreased expression of microglia-specific genes (i.e., P2RY12, CX3CR1) and notable absence of MHC class II or CD4 expression (Melief et al., 2016). Recently, a new human microglia cell line, SV40 iMhu, was shown to express canonical macrophage markers, such as CD11b and the phagocytic marker CD68, and exhibit morphological and phenotypic immune response profiles (Chiavari et al., 2019). The development and characterization of immortalized lines with improved microglia marker expression and a greater degree of human ex vivo microglia transcriptional overlap, such as the SV40 iMhu line, is essential to the functional dissection of microglia.

2.4. Pluripotent stem cell derived microglia

Human embryonic stem cells (ESCs) and induced-pluripotent stem cells (iPSCs) have recently risen as a technological advancement in the derivation of human CNS cells, including microglia. Numerous strategies have been published on the derivation of microglia from pluripotent stem cells (PSCs) (Hedegaard et al., 2020). Generally, these tools mirror the characteristics of primitive hematopoiesis, including dependence on the transcription factors PU.1 and IRF8 and independence of MYB. Mesoderm/hemogenic endothelium/myeloid differentiation can be achieved using growth factor cocktails (BMP4, VEGF, SCF, IL-3) and then further matured with the addition of microglia maturational/survival factors (IL-34, CSF1, TGFβ1). However, many protocols differ in terms of their final medium composition or monoculture/coculture use to induce microglial maturation (Abud et al., 2017; Banerjee et al., 2020; Chen et al., 2021; Chiavari et al., 2019; Douvaras et al., 2017; Guttikonda et al., 2021; McQuade et al., 2018; Muffat et al., 2016; Reich et al., 2020). A recent study took a novel approach by generating a human iPSC line expressing six transcription factors that enable the generation of microglia-like cells in a rapid and efficient 8-day protocol (Drager et al., 2022). These transcription factors (IRF5, IRF8, CEBPα/β, Pu.1 and MAF) are known to be environment-dependent and responsive to loss of the brain microenvironment (Drager et al., 2022; Gosselin et al., 2017). This rapid protocol for creating iMGs resulted in human microglia capable of phagocytosing synaptosomes, responding to LPS and were cocultured with iPSC-derived neurons (Drager et al., 2022). Taken together, PSC models have several advantages over primary microglia and cell lines by allowing for genetic validation: (1) translational modeling; (2) recapitulating a range

of traditional microglia behaviors; (3) high-throughput experimental designs; (4) reduced maturation times.

A particular strength of this model is the ability to dissect the genetic underpinnings of disease (Nott et al., 2019; Langston et al., 2022; Eitan et al., 2022). For instance, using massively parallel reporter assays to screen 5706 variants identified from genome-wide association studies for both AD and progressive supranuclear palsy, Cooper and colleagues identified 320 functional regulatory variants (Cooper et al., 2022). These variants were validated in iMGs and iPSC-derived neurons using a CRISPRi platform with CROP-seq and then CRISPR directed knockout of five of the regulatory regions to show direction gene regulation (Cooper et al., 2022). These analyses suggest that noncoding genetic risk is driven by common genetic variants through their activity on transcriptional programs (Cooper et al., 2022). By coupling iMG models with screening methods such as CRISPRi/a, these tools permit high-throughput identification of disease-associated variants in multiple disease models.

Although this technology is promising, a significant drawback is the lack of exposure to the complex CNS environment (Timmerman et al., 2018). The culture environment leads to rapid transcriptional and epigenetic differences from in vivo, homeostatic microglia, including a loss of homeostatic markers and increased expression of inflammatory and disease-associated genes (Gosselin et al., 2017). For instance, similar to primary human microglia in vitro, these PSC methods do not fully recapitulate core microglial homeostatic gene signatures such as SALL1 (Gosselin et al., 2017). Moreover, cell-cell interactions between neurons, astrocytes, oligodendrocytes, vasculature and microglia provide key signals and interactions that contribute to microglia phenotypes, identity and their responses to environmental cues (Salter and Stevens, 2017). Without adding complexity to the culture environment via 2D or 3D coculture, iMGs do not fully recapitulate human in vivo microglia. Additional limitations include problems with reproducibility depending on the microglia differentiation protocol and poorly delineated effects of human genetic variation on microglial transcription and functionality (Speicher et al., 2019). For a more detailed discussion of this technique please see (Hasselmann and Blurton-Jones, 2020).

2.5. 2D coculture

2D cocultures can partially rescue the environment-dependent microglia phenotype lost in monoculture. Additionally, they present the opportunity to examine the individual contributions and interactions of microglia with other CNS cell types.

For instance, coculture of hematopoietic progenitors with astrocytes facilitates microglial differentiation based on transcriptomic profiles and marker expression (Pandya et al., 2017). In an AD model, coculture of astrocytes and microglia resulted in fewer intracellular deposits of both α-synuclein and amyloid-β compared with monocultures of either cell type suggesting an anti-inflammatory role of glial crosstalk in AD (Rostami et al., 2021). Additionally, in a TBI model, activated microglia induced a neuroprotective reactive phenotype in astrocytes with enhanced astrocyte migration in vitro and decreased neuronal damage through modulation of ATP signaling (Shinozaki et al., 2017). Taken together, depending on the disease context, astrocyte and microglia interactions can have complex

and diverse consequences, thus underscoring the importance of coculture studies in understanding the relative contributions of each cell type.

Similarly, coculture with neurons facilitates microglial differentiation (Bassil et al., 2021; Guttikonda et al., 2021; Haenseler et al., 2017). iMGs matured in coculture with neurons display canonical macrophage functions such as surveying of the environment, retracting and extending ramifications, and phagocytosis of synaptic material, similar to in vivo microglia (Guttikonda et al., 2021). Neuronal cocultured microglia also exhibit a transcriptional profile more similar to ex vivo primary microglia (Haenseler et al., 2017). Moreover, these cocultured iMGs at baseline resemble primary human microglia rather than monocytes (Chen et al., 2021). A functional assessment demonstrated cocultured iMGs respond to inflammatory responses, exhibit appropriate phagocytic ability and an active “surveying” phenotype involving dynamic crosstalk between microglia and injured neurons (Chen et al., 2021). Interestingly, microglia can also shape neuronal maturation in coculture. A recent study found that microglia secrete factors that enhance dopaminergic differentiation of somatic and NSCs (Schmidt et al., 2021). Together this suggests that coculture of microglia and neurons can enhance cell maturation and permit mechanistic dissection of neuron-microglia crosstalk.

Recently, a tri-culture with neurons and astrocytes was shown to more accurately model in vivo neuroinflammation. Goshi et al., leveraged assays related to mechanical injury response, excitotoxicity, and cytokine profiling (Goshi et al., 2020). In comparison to monoculture and coculture, the tri-culture showed reduced neuronal caspase activity, increased astrocyte migration towards the source of injury, and microglial protection from glutamate-induced excitotoxicity, leading to significantly reduced neuronal loss and astrocyte hypertrophy in the tri-culture (Goshi et al., 2020). Tri-culture may also better reflect AD C3 synaptic loss pathology (Guttikonda et al., 2021; Nishimura and Takata, 2021). Several complement components including C1q, activated C3 and C4 may be produced by glia surrounding plaques in the AD brain (Wu et al., 2019). This overactivation has been hypothesized to contribute to synaptic loss in disease pathology (Wu et al., 2019). Using a tri-culture model, Guttikonda and colleagues found that C3 is secreted from microglia and astrocytes as a feed-forward inducer of astrocytic activation and microglial re-activation that may lead to synaptic loss in AD models (Guttikonda et al., 2021). These findings demonstrate that tri-culture enables the dissection of cellular crosstalk by genetic manipulation and permits a mechanistic study of diseases states in a translational culture model.

Another novel study recently showed iMGs can be cultured with hippocampal mouse slices, permitting the study of human microglia in a more brain-like environment (Ogaki et al., 2022). Researchers found that cocultured iMGs with hippocampal slices were responsive to neuronal death and phagocytosed cell debris. Thus, this study provides a method to transplant iMGs into the mouse hippocampal slice cultures with a high replacement rate (Ogaki et al., 2022). Future studies should include electrophysiological observations in combination with genetic manipulations of cocultured iMGs to parse the effects of human microglia on neuronal signaling.

2.6. 3D cocultures

While these 2D coculture systems allow for further understanding of the interactions between different cell types in the brain, these systems do not fully recapitulate the complex 3D brain environment. The primary 3D coculture approaches are cerebral organoids, which are designed to mimic early brain development and organization, and assembloids/spheroids, which are 3D combinations of multiple brain cell lineages and lack cortical layering but more closely control cell type fate and cell ratios.

2.6. a. Cerebral organoids—Organoids are self-organizing in vitro structures that contain some organ-specific cell types and structural organization thereby mimicking a portion of organ-specific functions (Chiaradia and Lancaster, 2020; Di Lullo and Kriegstein, 2017) (Table 3). Although brain organoids are not able to recapitulate the full structure of the human brain, these organoids are able to model cortical development and yield insights into cellular interactions in development. The first protocol to generate 3D structures containing cortical organization arose from the seminal application of extracellular matrix (Matrigel) to neuro-ectoderm embryoid bodies (EBs) to increase layer formation and neural budding (Lancaster et al., 2013), mimicking human development (Di Lullo and Kriegstein, 2017; Lancaster et al., 2013). Numerous novel protocols have furthered the development of brain region specificity, such as the usage of dual SMAD inhibition in the generation of the telencephalon/forebrain (Chambers et al., 2009; Kadoshima et al., 2013; Qian et al., 2016). These brain region specific organoids contain neurons at different stages of maturity, neuroepithelial progenitors, astrocytes, and cells expressing retina-specific genes (Di Lullo and Kriegstein, 2017; Marton and Paşca, 2020; Quadrato et al., 2017). Despite the organoid cellular diversity, there are some crucial cell types that are lacking, specifically endothelial cells, mature oligodendrocytes, and microglia (Di Lullo and Kriegstein, 2017). It has been reported that innate development of microglia can occur in an unpatterned organoid system without SMAD inhibition (Ormel et al., 2018). Unpatterned neural organoid strategies allow for differentiation to occur spontaneously, without the addition of specific patterning factors. As a result, these unpatterned organoids are heterogenous and vary from batch-to-batch in the cell types they produce (Lancaster and Knoblich, 2014). Importantly, any organoid protocols that include SMAD inhibition results in inhibition of mesoderm formation and an absence of microglia (Chambers et al., 2009). Recently it has been shown, that overexpression of the transcription factor PU.1 results in microglia-like cells in cerebral organoids, suggesting this may be a powerful tool for 3D coculture systems (Cakir et al., 2022). Therefore, coculture systems of microglia with brain organoids should be considered for robust characterization of microglia interactions with other cells of the brain. See Tables 3–4 for a list of current 3D coculture applications.

An example of how these models can be leveraged to determine the underlying mechanisms of neurological disorders has been recently demonstrated by Jin and colleagues (Jin et al., 2022). A common feature of Down Syndrome is altered synaptic development, induced by changes in microglia synaptic pruning. Cerebral organoids from NPCs and Down Syndrome iPSC-derived PMPs replicated enhanced synaptic pruning in observed in Down Syndrome and a reactive microglia morphological phenotype (Jin et al., 2022). Future studies should

pair these types of 3D in vitro models with xenotransplantation to further dissect human microglia function in the brain.

2.6.b Assembloids & Spheroids—While brain organoid technologies are the closest replication of cortical structure and development in vitro, there are also more simplified spheroid/assembloid structures derived from NPCs and other 3D culture models that can be used to study interactions between CNS cells (Table 4) (Bodnar et al., 2021; Brüll et al., 2020; Miura et al., 2022; Pamies et al., 2017; Papaspyropoulos et al., 2020; Song et al., 2019). Cell type or brain-region specific spheroids can be generated to understand specific brain regions in a 3D environment with less variability in cell type composition than organoids. These region-specific spheroids can then be utilized to further understand interactions across brain regions through the creation of assembloids. Multi-region assembloids are produced by fusing two region-specific spheroids together and multilineage assembloids are generated by inclusion of other CNS cell types such as microglia or pericytes (Makrygianni and Chrousos, 2021). Multiple studies have reported 3D coculture of microglia with brain spheroids or neurons to create multilineage assembloids (Abreu et al., 2018; Brüll et al., 2020; Muffat et al., 2016; Park et al., 2018; Song et al., 2019). As these models are more homogenous than other brain organoid models higher throughput assays, such as drug screening, can be applied. Additionally, the decrease in variability allows detection of interactions between specific neuronal populations and microglia with higher sensitivity (Brüll et al., 2020). However, these cocultures are limited by the lack of brain tissue architecture displayed in the 3D organoid coculture models. Therefore, researchers have focused on tools to better replicate this environment. For instance, the enhanced release of neuronal soluble factors has been shown to better replicate the brain environment (Victor et al., 2022; Umpierre et al.,2020).

A major consideration for both organoids and spheroids/assembloids is the maturity of each individual cell type and the degree to which these methods can appropriately model cellular maturation and senescence. A current study found that prolonged culture (10–13 weeks) led to an increase in senescence-associated β-galactosidase (Shaker et al., 2021). However, it should be noted that organoids more closely resemble fetal brain and do not fully recapitulate the ex vivo microglia signature (Hasselmann and Blurton-Jones, 2020). Further studies are needed to validate if prolonged culture is sufficient for modeling human in vivo aging phenotypes, and should be taken into account in interpreting the application of these models to human aging.

2.7. Functional outputs for in vitro human microglia models

The diverse role of microglia in both immune response and homeostasis leads to a broad range of both core macrophage functions and brain-specific roles (Salter and Stevens, 2017). These functional outputs can be studied by leveraging high-throughput in vitro systems such as PSC-derived microglia and immortalized cell lines.

2.7.a Phagocytosis—Phagocytosis of a wide range of pathogens, debris, and neurodegenerative, and apoptotic stimuli is a core macrophage function, and can be quantified through the addition of pHrodo-labeled bioparticles (E.coli, Aβ1–42, apoptotic

cells, neural precursors, etc.) to microglia culture paired with: time lapse imaging via fluorescence microscopy, imaging in live-cell imaging systems such as the Incucyte Live Cell Analysis System (Satorius, Germany) or by flow cytometry. This assay is useful for measuring microglia phagocytosis phenotypes of expected components (debris, bacteria, etc.) or atypical responses (synapse loss, plaque formation, etc.) in disease states. Seminal reference: (Abud et al., 2017).

2.7.b Migration—Migration towards injury or in response to chemokines is assessed by live cell imaging systems or via time-lapse imaging in scratch wound assays (Justus et al., 2014; Taylor et al., 2018). Scratch wound assays involve using a pipette tip or 96-well or 384-well pin tool such as the WoundMaker™ (Essen BioScience, USA) to create a linear scratch in the center of a monolayer followed by imaging over time to determine how quickly microglia migrate back in to fill the vacated space (Justus et al., 2014; Taylor et al., 2018). Boyden chamber assays, also commercially available as Transwell invasion assays, can also be used to assess migration in response to a particular chemoattractant or stimulus (Justus et al., 2014; McQuade et al., 2020). Seminal reference: (McQuade et al., 2020).

2.7.c Cytokine release—Cytokine release by microglia, in response to stimuli, can be pro- or anti-inflammatory and quantified using ELISA, flow cytometry-based panels or dot-blot based immunoassays. The percentage of the microglia population involved in cytokine production can be determined by treatment with protein transport inhibitors followed by activation, permeabilization, staining with antibodies for cytokines of interest, followed by flow cytometric quantitation. This assay is useful for determining how disease states influence the inflammatory responses of activated microglia. Seminal reference: (Haenseler et al., 2017).

2.7.d NO and ROS production—NO and ROS production are also released by microglia in response to proinflammatory stimuli. There are several commercially available dyes that react with NO, ROS, or both simultaneously to produce fluorescence which can then be quantified with plate readers, flow cytometry, or imaging (Adamiak et al., 2017; Habibalahi et al., 2020). This assay is useful for determining how disease states respond to proinflammatory environments (i.e., shift to an activated microglia phenotype). Seminal reference: (Pandya et al., 2017).

2.7.e Mitochondrial function—Mitochondrial function is determined by monitoring oxygen consumption and mitochondrial respiration and measuring fluctuation in cellular oxygen consumption rate upon the sequential introduction of compounds that inhibit the electron transport chain. Additionally, potential mitochondrial superoxides can be detected via fluorescent probes that are commercially available (Table 5), such as MitoSOX (ThermoFisher, USA). When coupled alongside a Mitotracker probe for active mitochondria (ThermoFisher, USA), this assay permits for normalized data to compare mitochondrial superoxide levels. Seminal reference: (Piers et al., 2020).

2.7.f Lysosomal activity—Lysosomal activity can be evaluated by using by combining a fluorogenic probe to tag lysosomes and live cell imaging (Dolman et al., 2013). Commercially available LysoTracker (ThermoFisher, USA) probes consist of a fluorophore

linked to a weak base that is only partially protonated at neutral pH. This allows LysoTracker probes to freely permeate cell membranes. LysoTracker probes are highly selective for acidic organelles, and unlike dinitrophenyl and can be combined with other live imaging dyes. Seminal reference: (Ihnatovych et al., 2020).

2.7.g Calcium imaging—Calcium imaging measures the orchestrated calcium response in microglia that is driven by the purinergic receptors to signal cytokine synthesis. Calcium imaging is performed by coupling live imaging and commercially available fluorogenic calcium sensors such as the NeuroBurst Orange Reagent (a genetically-encoded fluorescent calcium sensor from AXOL, USA). Radiometric calcium imaging has also been successfully performed in iMGs using Fluo-4 and Fura-Red (Dolman et al., 2013) to investigate the role of CXCR4 signaling in AD (McQuade et al., 2020). Some labs are also beginning to apply CRISPR/Cas9 editing in iPSC lines to create stable GcAMP6 reporters (Jiang et al., 2018) and Salsa6f reporters (Jairaman et al. 2022). Seminal reference: (Victor et al., 2022).

2.10. Phenotyping microglia using cellular markers

Additionally, analyzing microglia marker expression can be a sensitive tool to assess the effect of the CNS environment on both in vitro and in vivo models (Jurga et al., 2020; Gosselin et al., 2017; Schwabenland et al., 2021). Appropriate markers for general microglia include: IBA1, CD11b, CD14, CD45(med), CD80, CD115, CX3CR1, FCER1G and the transcription factor PU.1. Many if not most of these markers will also mark peripheral macrophages so coupling these markers with microglia-specific markers is necessary. These relatively microglia-specific markers include: TMEM119, P2RY12; and the transcription factor SALL1 (Jurga et al., 2020; Schwabenland et al., 2021).

General markers of an activated microglia phenotype include: increased pro-inflammatory factors (TNFα, IFNγ, IL-6, IL-12, IL-17, IL-18), receptor upregulation (CD14, CD16, CD32, CD40, CD68), cytokines and chemokines (CCL2, CCL5, CXCL10) and MHC class II cell surface (HLA-DR, HLA-DRA).

However, microglia phenotype is influenced by several factors including: (1) brain region heterogeneity, (2) species-specific expression; (3) morphological heterogeneity, (4) environment-dependent signaling; and (5) microglia density (Jurga et al., 2020; Gosselin et al., 2017; Schwabenland et al., 2021). Shifts in microglia phenotype are disease-dependent but carry certain general characteristics such as an ameboid morphology, loss of homeostatic markers and enhanced inflammatory signaling (Jurga et al. 2020). For instance, P2RY12 is an excellent marker for the homeostatic phenotype of microglia and is rapidly lost in many pathologies (Schwabenland et al., 2021). Whereas homeostatic microglia do not express CD9 or CD163 but microglia upregulate these markers in AD and MS respectively (Keren-Shaul et. al., 2017; Schwabenland et al., 2021). Therefore, researchers must account for which markers, species, regions and environments are critical when assessing microglia phenotype both in vitro and in vivo.

3. In vivo tools and considerations

As discussed above there are significant changes in gene expression and function when microglia are removed from the brain and placed in a culture environment (Gosselin et al., 2017). Therefore, studying human microglia in their environment is critical to understand the brain microenvironment's contribution to microglia identity and function. The advantages and limitations are listed in Table 1.

3.1. Xenotransplantation models

As a method to model human microglia in the in vivo environment, researchers have turned to engraftment of iMGs or progenitors into murine systems to restore the ex vivo microglia transcriptional signature. The protocols for this type of engraftment have been recently reviewed (Fattorelli et al., 2021; Hasselmann and Blurton-Jones, 2020). Therefore, instead of a full methods review, we will focus on the findings and their applicability to disease states, tools to couple with xenotransplantation and the future usage of this technique (see Fig. 2).

Briefly, the unique developmental ontogeny of microglia can be recapitulated such that engraftment can be accomplished with several PSC-derived cell types including iMGs (Mancuso et al., 2019; Svoboda et al., 2019), hematopoietic progenitors (Hasselmann et al., 2019) or PMPs (Xu et al., 2020). Immunodeficient mice are not sufficient for successful engraftment, expression of the human version of either CSF1 or IL34 are necessary for transplanted human microglia survival (Fattorelli et al., 2021; Hasselmann and Blurton-Jones, 2020; Hasselmann et al., 2019; Mancuso et al., 2019; Xu et al., 2020). This finding is highly consistent with prior studies that delineate the importance of CSF1 signaling for microglia development and survival in vitro and in vivo (Elmore et al., 2014; Erblich et al., 2011; Abud et al., 2017).

Xenotransplanted microglia (xMG) result in strong expression of microglia markers TMEM119, P2RY12 (Hasselmann et al., 2019; Mancuso et al., 2019; Xu et al., 2020). Even though in culture, iMGs share many of the same genes as cultured primary human microglia, once engrafted, xMGs share a similar gene expression signature to human ex vivo microglia, reinforcing that the environment dictates a more complete shift to the microglia identity in brain (Hasselmann et al., 2019; Mancuso et al., 2019; Xu et al., 2020). However, some researchers have reported an "immature" microglia phenotype (Hasselmann and Blurton-Jones, 2020; Hasselmann et al., 2019), potentially reflecting younger, postnatal engrafted microglia at homeostasis. xMGs also expresses key microglia-associated transcription factors such as SALL1, KLF4, and EGR1, suggesting a return to the human microglia transcriptional program (Hasselmann and Blurton-Jones, 2020; Hasselmann et al., 2019). Furthermore, xMGs retain microglia-specific functions in the murine brain, with demonstrated ability to engulf synapses, respond to immune stimulation, phagocytose neural precursors, and migrate towards wounds (Fattorelli et al., 2021; Hasselmann and Blurton-Jones, 2020; Hasselmann et al., 2019; Mancuso et al., 2019; Xu et al., 2020).

The additional advantage of xenotransplantation is that xMGs can be evaluated to cross validate how accurately mouse models reflect changes observed in human microglia. Recent studies validated that xMGs respond to cuprizone-induced demyelination resulting in upregulation of CD74 and SPP1, similar to what is observed in human MS patients (Masuda et al., 2019; Xu et al., 2020). Similarly, in AD models, xMGs near Aβ plaques expressed both the mouse disease-associated microglia (DAM) signature and the human microglia neurodegenerative (MgnD) signature associated with AD (Claes et al., 2021; Hasselmann et al., 2019; Yuan et al., 2016). This suggests that xMG recapitulate disease-associated changes in vivo and are a powerful tool to study disease-associated phenotypes and genetic risk factors.

To date only a handful of studies have applied xMGs to understand genetic risk factors associated with diseases, although many more are sure to follow. For instance, Hasselmann et al., demonstrated that the R47H-TREM2 xMGs have impaired the association between microglia and plaques in an AD mouse model, similar to what is observed in human AD patients (Andreone et al., 2020; Hasselmann et al., 2019). Claes and colleagues also found TREM2-R47H xMGs have reduced reactivity to plaques, including diminished plaque-proximity, reduced CD9 expression, and lower secretion of plaque-associated APOE (Claes et al., 2021). Similarly, TREM2 knockout xMGs do not become DAMs in vivo, further supporting the hypothesis TREM2 is critical in plaque formation and inflammatory response in human AD models (McQuade et al., 2020). A study from Jin et al., leveraged Down syndrome (DS) and control iPSCs and engrafted them into chimeric mice to model DS synaptic pathology in development. Researchers found that DS xMGs phagocytose more synapses during development than control xMGs and display a more ameboid morphology (Jin et al., 2022). This increased phagocytosis led to impaired synaptic transmission in CA1 neurons surrounded by DS xMGs, which was rescued by IFN inhibition (Jin et al., 2022). Xenotransplantation in conjunction with genetically edited cell lines appears promising and will most likely pave the way for mechanistic dissection of the complex interplay between the brain microenvironment and microglia function in disease models.

In addition to genetically edited cell lines, researchers can leverage crossed disease mouse models with xenotransplantation to further dissect disease states. Kiani Shabestari and colleagues recently leveraged an AD engraftable mouse that genetically lacks microglia to show that microglia-deficient mice shift amyloid plaques from the parenchyma to a cerebral amyloid angiopathy. This shift resulted in increased DAM transcriptional signature as well as increased brain calcifications, hemorrhages and premature lethality (Kiani Shabestari et al., 2022). Furthermore, iMGs were leveraged to demonstrate that microglia phagocytose calcium crystals in a TREM2-dependent manner (Kiani Shabestari et al., 2022). This study demonstrates the ability of this model to provide a tool for dissecting the complex interplay between the brain microenvironment and microglia function through the combination of cell type genotype and mouse genotype in neurological disorders.

3.2. Xenotransplantation applications

3.2a Mouse genotype.—Several different mouse lines have been applied to create xenotransplantation models (Claes et al., 2021; Fattorelli et al., 2021; Mancuso et al.,

2019; McQuade et al., 2020; Xu et al., 2020). The first xenotransplantation was performed in MITRG mice (M− CSFh/h IL-3/GM-CSFh/h SIRPah/h TPOh/h Rag2− / − IL2rγ−/−; The Jackson Laboratory, no. 017711) (Abud et al., 2017). Later studies refined the genotype required for successful engraftment, requiring only humanized CSF1 (RIM:Rag2−/−;Il2rg−/−;CSF1h/h (The Jackson Laboratory no. 017708). Some groups also report that depletion of the mouse microglial niche with a CSF1 inhibitor prior to engraftment is necessary (Mancuso et al., 2019). It should be noted that CSF1 inhibition with BLZ945 or PLX5622 are not microglia-specific and also transiently deplete peripheral macrophage populations simultaneously (Lei et al., 2020) An alternative to pharmacological depletion of the microglial niche, recently researchers showed removal of tissue resident macrophage populations in mice through deletion of a super-enhancer, the fms-intronic regulatory element (FIRE), within the CSF1R gene locus (Rojo et al., 2019). $Csf1r^{\Delta FIRE/\Delta FIRE}$ mice developmentally lack microglia, and surprisingly exhibit no reported developmental or neurological abnormalities; differentiating them from Csf1r−/− mice (Rojo et al., 2019). Recently, researchers have been able to model neurological diseases by crossing $Csf1r^{\Delta FIRE/\Delta FIRE}$ mice with the 5xFAD humanized amyloid mutation murine model to generate an engraftable AD murine model (Kiani Shabestari et al., 2022).

3.2b Behavior.—One potential application of xenotransplantation models is the degree to which microglial dysfunction contributes to behavioral abnormalities. This question is inherently dependent on the degree to which microglial dysfunction contributes to disease initiation and progression. An example of this is within AD, where sustained microglia depletion in 5xFAD mice results in a further reduction in anxiety-like behaviors compared to 5xFAD controls and wild-type animals (Spangenberg et al., 2019). For instance, researchers could leverage PSC-derived microglia from patient lines such as from Coriell, Jackson Laboratories (iPSC Neurodegenerative Disease Initiative, the Tau Consortium (http://neuralsci.org/tau) or other NINDS repository. Microglia derived from these patient lines can be engrafted to determine if patient pathology alone can reproduce disease-specific behavioral deficits. In Alzheimer's disease, could patient lines replicate learning and memory deficits in aged mice? Or in Autism Spectrum Disorder, could patient lines replicate the social behaviors observed in animal models?

A second approach would be to leverage the genetic editing capabilities of PSC-derived microglia and target specific SNPs, genes and enhancer regions implicated in disease such as the R47H variant in Alzheimer's Disease; followed by engraftment of these CRISPR/Cas9 edited lines into xenotransplantation mouse models for disease-specific behavioral assays. It should be noted that these behavioral assays are not limited to neurodegenerative diseases but can and should be applied in neurodevelopmental and neuropsychiatric disorders as well.

3.2c Calcium imaging.—Multiple calcium indicators have been employed in mice to study calcium dynamics in microglia (Tvrdik and Kalani, 2017). A promising candidate for PSCs is GCaMP6. GcaMP6 belongs to a family of sensitive fluorescent calcium sensors, CaM and CaM-interacting M13 peptide (Chen et al., 2013). Several GCaMP variants have been developed and applied in previous studies in PSCs (Tvrdik and Kalani, 2017). However, researchers should use caution when using lentiviral transduction approaches

as they lead to the random genomic GCaMP insertion. CRISPR/Cas9 technology can be leveraged to ensure that GCaMP is safe harbor site targeted in PSCs. As a proof of concept, a recent study successfully knocked in a GCaMP6 reporter in PSCs (Jiang et al., 2018), suggesting that similar approaches can be used in PSC lines prior to microglia differentiation and xenotransplantation.

3.2d In vivo imaging.—Microglia are highly dynamic in the brain. Therefore, real-time imaging is a useful tool to examine how microglial behavior is regulated and how it affects the surrounding environment across time. In vivo two-photon imaging in the xenotransplantation mice coupled with engraftment of a GFP-tagged PSC line would permit long term imaging of microglia dynamics. A non-invasive approach such as PET or SPECT would also permit longitudinal, three-dimensional, imaging of transplanted microglia in vivo. TSPO 18kDA, a mitochondrial molecule upregulated in activated microglia, is a common marker for assessing neuroinflammation and microglial activation in PET studies (Beaino et al., 2021). These well-established neuroscience techniques could be leveraged to assess microglia processes dynamics, migration after injury, phagocytosis and even interaction with other cell types in vivo (Andoh and Koyama, 2021).

3.2e Epigenetics.—To date all xMG studies have relied solely on transcriptomics. For instance, our group demonstrated that the transcriptomic signature is similar between xMGs and human ex vivo microglia (Hasselmann et al., 2019). However, it remains unclear if the epigenetic landscape is conserved between xMGs and ex vivo human microglia. Future studies leveraging open chromatin and poised and active histone marks will be useful in determining how the CNS microenvironment regulates the xMG epigenetic landscape.

3.2f Spatial Transcriptomics.—Emerging technologies for genome-wide spatial transcriptomics offer significant potential for providing detailed molecular maps of microglia within the CNS environment (Burgess, 2019; Rodriques et al., 2019). Currently, scaling and overcrowding of probes presents a particular challenge for spatial transcriptomic analysis. Yet in theory, this approach could capture gene expression within the architecture of intact tissue and permit researchers to investigate how crosstalk between microglia and the CNS affects microglia function and brain homeostasis (Burgess, 2019; Rodriques et al., 2019).

3.2g Electrophysiological recordings.—To date no studies have recorded from xMGs themselves, although it is of great interest to see how human microglia signal in the brain microenvironment and whether there is regional heterogeneity in membrane potentials as seen in mouse microglia (De Biase et al., 2017). However, researchers have recently shown that xMGs modulate the synaptic transmission of surrounding neurons depending on their genotype (Jin et al., 2022). Therefore, future studies examining xMG-adjacent neurons and their membrane properties will be useful in determining how microglia regulate and maintain synaptic transmission.

3.2h Microglia heterogeneity.—Given challenges with heterogeneity in microglial engraftment, regional specific contribution of engrafted human microglia has not been reported (Tan et al. 2020). It will be of great interest the degree to which xMGs recapitulate

both normal microglial heterogeneity, such as cerebellar microglia phenotypes, as well as disease specific microglial heterogeneity, such as within the substantia nigra in Parkinson's models.

4. Conclusions and perspective on the future of microglia research

The advent of iPSC approaches to model human microglial biology is rapidly furthering our collective understanding of how microglia contribute to brain development and aging and the impact of microglial pathology on neurological disorders. However, significant gaps in knowledge and techniques remain. One such area is the crucial interaction of human microglia with the blood brain barrier, which can be elucidated using a combination of organoids or assembloids and the xenotransplantation model. A second area of future growth and importance is microglial heterogeneity during development and neurological disease and how to accurately and adequately models recapitulate microglial heterogeneity in vitro, in cocultures, and in the xenotransplantation model. Future areas of interest in the field will include the modeling of microglial heterogeneity using regional organoids and the xenotransplantation approach. Secondly, the ability of these models to incorporate microglial maturation and cellular senescence has not been fully investigated. In neurons it is well described that direct differentiations maintain senescence phenotypes compared to stem cell derived neurons (Mertens et al., 2021), but both direct differentiation and comparable studies in microglia are lacking in the field. Lastly, the application of these technologies to drug screening and to CRISPR screens is in its infancy compared to other iPSC derived cell types, foreshadowing future insights into pathophysiology and targeted drug development. Moving forward, assays for microglial activity and the establishment of benchmarks for modeling will greatly expand our understanding of human microglial function.

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Data availability

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

Abbreviations

5xFAD	5 familial AD mutations
Aβ	Amyloid-beta
AD	Alzheimer's Disease
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
BMP4	Bone morphogenetic protein 4

C1q	Complement component 1q
C3	Complement component 3
C4	Complement component 4
CAM	Calcium-binding protein calmodulin
Cas9	Caspase 9
CCL/CCR	C-C Motif Chemokine Ligand/Receptor
CD	Cluster of differentiation receptors
CEBP	CCAAT/enhancer-binding protein
CNS	Central nervous system
CO	Cerebral organoid
CRISPR/	Clustered regularly interspaced short palindromic repeats/
CRISPRi/a	CRISPR interference/activation
CSF1/CSF1R	Colony stimulating factor 1/receptor
CX3CR1	C-X3-C motif chemokine receptor 1
CXCL/CXCR	C-X-C motif chemokine ligand/receptor
DAM	Disease-associated microglia
EGR1	Early growth response 1
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FCER1G	High-affinity immunoglobulin epsilon receptor subunit gamma
FIRE	Fms-intronic regulatory element
FTD	Frontal temporal dementia
GCAMP	Genetically encoded calcium indicator
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IBA1	Ionized calcium-binding adapter molecule 1

IFN	Interferon
IL	Interleukin
iMG	Induced microglia
iNOS	Inducible nitric oxide synthase
iPSC	Induced-pluripotent stem cell
IRF	Interferon Regulatory Factor
KLF4	Kruppel like factor 4
LPS	Lipopolysaccharide
oMG	Organoid microglia
NINDS	National Institute of Neurological Disorders and Stroke
NO	Nitric oxide
NPC	Neural progenitor cells
NSC	iPSC-derived human neural stem cells
MACS	Magnetic-activated cell sorting
MAF	MAF BZIP Transcription Factor
MHC	Major histocompatibility complex
MS	Multiple sclerosis
P2RY12	Purinergic receptor P2Y12
PET	Positron emission tomography
PMP	Primitive macrophage progenitor
PSC	Pluripotent stem cell
Rag2	Recombination Activating 2
ROS	Reactive oxygen species
SALL1	Spalt like transcription factor 1
SCF	Stem cell factor
SIRP	Signal-regulatory-protein
SMAD	Suppressor of mothers against decapentaplegic
SPECT	Single positron emission computed tomography
SPP1	Secreted phosphoprotein 1

TBI	Traumatic brain injury
TGF	Tumor growth factor
TMEM119	Transmembrane protein 119
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TREM2	Triggering receptor expressed On myeloid cells 2
TSPO	Translocator protein
ULA	Ultra low attachment
VEGF	Vascular endothelial growth factor
xMG	Xenotransplanted microglia

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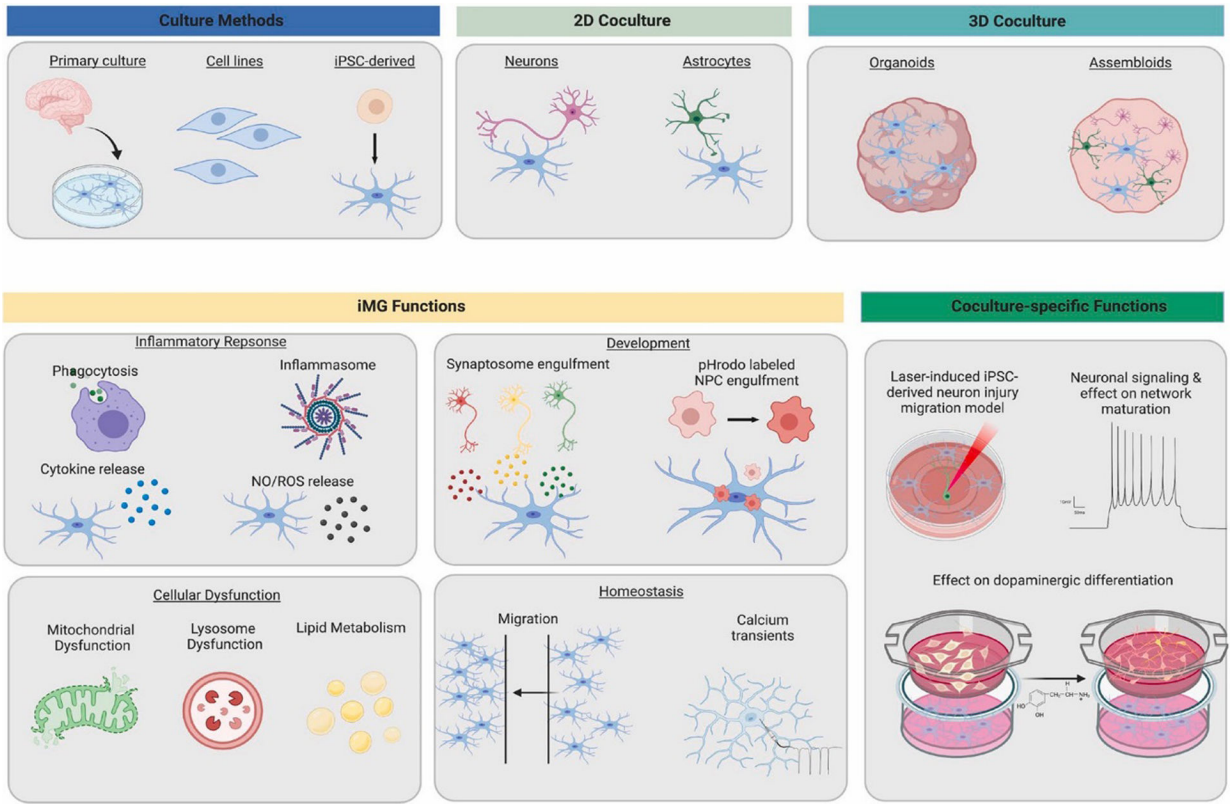

Fig. 1.
Human Microglia In Vitro Toolbox.

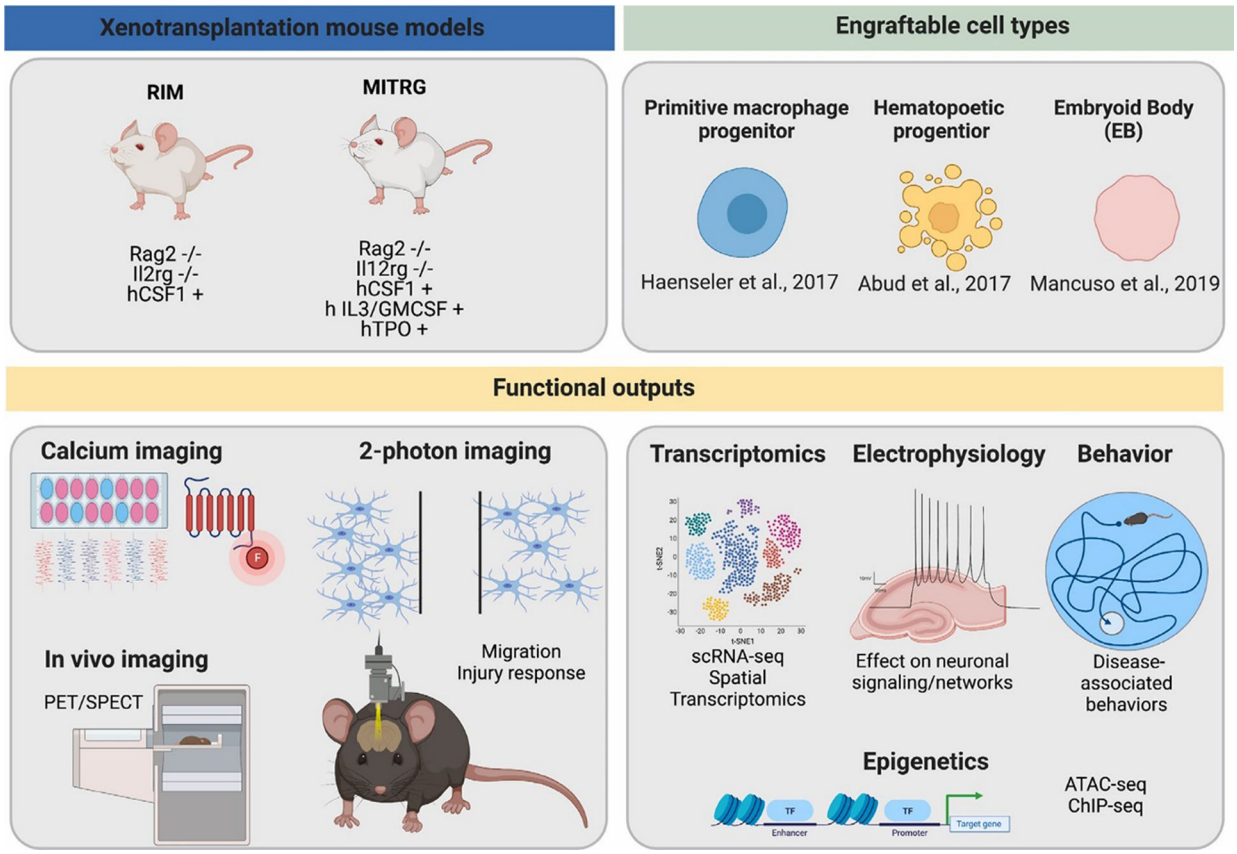

Fig. 2.
Human Microglia In Vivo Toolbox

Table 1

Advantages and limitations of human microglia tools

	Monoculture	2D Coculture with Microglia	3D Coculture with Microglia	Xeno-transplantation
Advantages	High throughput; scalable for screening	Transcriptome more closely resembles in vivo microglia	Authentic cell interactions to model interactions with multiple CNS cell types	xMGs mature within an intact brain microenvironment
	Ability to easily genetically modify	Microglia interactions with other CNS cell types are observable	Transcriptome similar to in vivo microglia	Transcriptome similar to adult in vivo microglia
	Ability to use patient derived lines to model disease	Ability for live imaging and 2D quantification of the results of CNS cell interactions	Robust and defined protocols for triculture systems	Can be transplanted into a variety of murine disease models, intact BBB
	Simple cell lysis for protein and RNA isolation	Ability to independently modulate individual cell type genetics to measure interactions	Ability to independently modulate individual cell type genetics to measure interactions	Ability to independently manipulate microglial genetics
Limitations	Loss of in vivo transcriptome/epigenome in culture	Transcriptome may be more closely related to fetal microglia rather than adult microglia	Long maturation times for organoids	Long maturation times after engraftment
	Many microglia functions require cell–cell interactions and therefore cannot be observed in monoculture	Technically challenging protocols for RNA and protein isolation (i.e., MACS, FACS)	Low throughput	Low throughput
	Not all microglia differentiation protocols are robustly reproducible	Additional cell types are involved in neurological disease that are not modeled	Difficulty reproducing phenotypes due to heterogenous cell composition and differentiation in organoids	Genetic background differs between host and xMGs, variable engraftment efficiency
			Technically challenging protocols for RNA and protein isolation (i.e. MACS, FACS)	Prescence of host microglia unless ablated pharmacologically or genetically
			Substantial batch variability	No peripheral immune cell interactions due to mouse genotype
Functional Outputs & Applicable Tools	Cytokine release, NO/ROS, mitochondrial function, calcium signaling, immuno-fluorescence, morphology, phagocytosis, lysosome dysfunction, lipid accumulation, migration assays, transcriptomic/epigenomic sequencing, ease of application to mass spectrometry	Immuno-fluorescence, morphology, migration assays, differentiation/maturation, phagocytosis, effect on neuronal firing (e.g. Multielectrode array recording), transcriptomic/epigenomic sequencing	Immuno-fluorescence, morphology, migration assays, differentiation, phagocytosis, transcriptomic/epigenomic sequencing, effect on neuronal firing, neuronal maturation	Immuno-fluorescence, morphology, calcium imaging, behavioral assays, transcriptomic/epigenomic sequencing, regional microglial heterogeneity and specification, interaction in complex and local networks, blood brain barrier contributions

Table 2:

In vitro toolbox references

Tool	Technique	Reference
Culture Methods	Induced from peripheral macrophages	(Ohgidani et al., 2015; Scheiblich et al., 2021; Sellgren et al., 2019; Sellgren et al., 2017)
	Primary culture	(Gosselin et al., 2017; Horvath et al., 2008; Popova et al., 2021)
	Cell Lines	(Blasi et al., 1990; Janabi et al., 1995)
	PSC-derived	(Abud et al., 2017; Chen et al., 2021; Douvaras et al., 2017; Guttikonda et al., 2021; Haenseler et al., 2017; McQuade et al., 2018; Muffat et al., 2016; Pandya et al., 2017)
Coculture	2D coculture	(Akiyama et al., 2020; Bassil et al., 2021; Chen et al., 2021; Guttikonda et al., 2021; Haenseler et al., 2017; Pandya et al., 2017; Reich et al., 2020; Schmidt et al., 2021; Takata et al., 2017)
	3D coculture	(Abud et al., 2017; Brownjohn et al., 2018; Guttikonda et al., 2021; Lin et al., 2018; Popova et al., 2021; Ryan et al., 2020; Schmunk et al., 2020; Takata et al., 2017)
Functional Outputs for iMGs	Phagocytosis	(Abud et al., 2017; Douvaras et al., 2017; Healy et al., 2017; Konttinen et al., 2019; Lin et al., 2018; Marschallinger et al., 2020; McQuade et al., 2018; McQuade et al., 2020; Muffat et al., 2018; Pandya et al., 2017) Garcia-Reitboeck et al., 2018)
	Calcium transients	(Abud et al., 2017; Chen et al., 2021; Douvaras et al., 2017; McQuade et al., 2020; Reich et al., 2020)
	Migration	(Justus et al., 2014; Panagiotakopoulou et al., 2020; Reich et al., 2020; Taylor et al., 2018; Yarrow et al., 2004)
	Cytokine release/ NLRP3 activation	(Cosker et al., 2021; Marschallinger et al., 2020; Schmidt et al., 2021; Trudler et al., 2021)
	NO/ROS release	(Abud et al., 2017; Brownjohn et al., 2018; Lin et al., 2018; Wißfeld et al., 2021; Zhang et al., 2020)
	Synaptic/NPC engulfment	(Justus et al., 2014; Liang et al., 2007; Marschallinger et al., 2020; Sellgren et al., 2019; Sellgren et al., 2017; Yarrow et al., 2004)
	Cellular dysfunction	(Andreone et al., 2020; Logan et al., 2020; Piers et al., 2020; Reich et al., 2020)
Functional Outputs for 2D/3D co-culture	Laser-induced injury/ Migration	(Abud et al., 2017; Chen et al., 2021; Muffat et al.)
	Effect on cell type differentiation	(Schmidt et al., 2021)
	Neuronal signaling/ Network maturation	(Fagerlund et al., 2021; Popova et al., 2021; Victor et al., 2022)
	Lipid accumulation	(Victor et al., 2022)
	Inflammatory response/ Response to viral infection	(Abreu et al., 2018; Guttikonda et al., 2021; Muffat et al., 2018; Park et al., 2018; Xu et al., 2021)
	Phagocytosis/Synaptic Pruning	(Cakir et al., 2022; Jin et al., 2022; Popova et al., 2021; Xu et al., 2021)
	Calcium signaling	(Victor et al., 2022; Song et al., 2019)

Table 3.

3D Organoid Systems References

Study	Method of coculture	Microglia Protocol References	Organoid Protocol References	Application	Disease model
(Abud et al., 2017)	iMGs added to 12-week-old brain organoid for 7 days of coculture	(Abud et al., 2017)	(Abud et al., 2017; Lancaster et al., 2013)	Functional characterization of iMGs and response to injury	CNS injury (needle poke)
(Brownjohn et al., 2018)	iMGs added to Day 102 COs, supplemented with iMG growth factors. Cocultured for an excess of 100 days	(van Wilgenburg et al., 2013)	(Brownjohn et al., 2018; Qian et al., 2016)	Functional characterization of TREM2 mutation in iMGs and COs	Studying FTD missense TREM2 mutations and Nasu-Hakola disease
(Lin et al., 2018)	iMGs added to 2-month-old organoid for 1 month of coculture	(Muffat et al., 2018)	(Muffat et al., 2018; Raja et al., 2016)	Aβ clearance by microglia in organoids	Role of APOE4 variant in Alzheimer's Disease
(Ormel et al., 2018)	Innate development in organoids "organoid-grown microglia" for 60 + days	Differentiate in organoids	(Lancaster and Knoblich, 2014)	Functional characterization of oMGs	Proposed as a 3D-model to explore role of microglia in disease
(Popova et al., 2021)	MACS-purified microglia added to week 5 organoids for coculture for 14–35 days	(Abud et al., 2017)	(Popova et al., 2021)	Study the impact of microglia on developing cortical networks in organoids, single cell transcriptome of "neuroimmune organoids"	N/A
(Bodnar et al., 2021)	Innate development in organoids of organoid-grown microglia for 20 + days	Differentiate in organoids	(Ormel et al., 2018)	Create a higher throughput method for microglia-containing COs	N/A
(Xu et al., 2021)	7,000 NPCs plus 3,000 PMPs were co-cultured for up to 35 days to create COs	(Haenseler et al., 2017;	(Xu et al., 2021)	Create a model where the number of human microglia can be controlled to study viral infections in COs	Zika virus
(Fagerlund et al. 2021)	Day 8 microglial progenitors added to day 30 organoids and cocultured for up to 180 days	(Konttinen et al., 2019)	(Lancaster et al., 2013)	Determine how microglia-like cells within the organoids promote neuronal and network maturation	N/A
(Cakir et al., 2022)	Mixed 10 % of PU.1-infected ESCs with 90 % parental ESCs to generate COs. COs matured for up to 90 days.	Differentiate in organoid	(Cakir et al., 2022)	Study if PU.1 expression can generate COs with microglia-like cells. Also, examined COs in context of Alzheimer's Disease.	Alzheimer's Disease
(Kenkhuis et al., 2022)	iMGs added to day 43 organoids and cocultured for 7 days	(Haenseler et al., 2017)	(Abud et al., 2017; Konttinen et al.,2019)	Study role of iron accumulation in neurodegenerative disorders	Alzheimer's Disease

Table 4.

3D Spheroids, Assembloids, Coculture Systems References

Study	Method of coculture	Microglia Protocol Reference	Other cell types Protocol Reference	Application	Disease model
(Haenseler et al., 2017)	iMGs cocultured with iPSC-derived neurons for 14 days	(van Wilgenburg et al., 2013)	(Shi et al., 2012)	Microglia-neuron coculture model	N/A
(Muffat et al., 2018)	Zika-infected iMGs added 24 h after infection to a single 14 day assembloid for 7 days of coculture	(Muffat et al., 2016)	(Muffat et al., 2016)	Microglia spreading Zika virus to NPCs and assembloids	Zika and Dengue virus
(Park et al., 2018)	Triculture model using neurons, astrocytes, and microglia in a 3D microfluidic platform –SV40 immortalized microglia injected into microfluidic chamber	SV40 microglia line	(Choi et. al., 2014)	3D human Alzheimer's Disease tri-culture model	Alzheimer's disease
(Abreu et al., 2018)	SV40 immortalized microglia added to the suspension and maintained in coculture for 1 week	SV40 microglia line	(Pamies et al., 2017)	Microglia altering response to viral infection in assembloids/ spheroids	Zika and Dengue virus
(Song et al., 2019)	iMGs and neurons added to spheroids and cocultured for 30 days	(Song et al., 2019)	(Paşşa et al., 2015)	Investigated the neural-microglia interactions for dorsal and ventral spheroids	N/A
(Brüll et al., 2020)	3D dopaminergic neuron aggregates with iMGs. plated for 14 day coculture	(van Wilgenburg et al., 2013)	LUHMES cell line	3D cultured human dopaminergic neurons interactions with glia for neurotoxicity studies	N/A
(Guttikonda et al., 2021)	Coculture iMGs with neurons, astrocytes and triculture aggregates for up to 14 days	(Wang, et al., 2012)	(Qi et. al., 2017; Tchieu et. al., 2019)	Tri-culture model investigating C3 potentiation in Alzheimer's Disease	Alzheimer's Disease
(Victor et al., 2022)	Combinatorial experiments mixing forebrain spheroids and iMGs derived from CRISPR-edited isogenic iPSC lines harboring either APOE3 or APOE4	(Abud et al., 2017)	(Sloan et. al., 2018)	Spheroid coculture with genetically edited iMGs investigating role of APOE genotypes on disease progression	Alzheimer's Disease

Table 5.

Commercially available in vitro human microglia tools.

Product	Function	Company	Catalog #
Mitotracker	Cellular Dysfunction	ThermoFisher	M7514
MitoSOX	Cellular Dysfunction	ThermoFisher	M36008
iNOS	iNOS release	Abcam	Ab211085
Lysotracker	Lysosomal Activity	ThermoFisher	L7528
CellROX	Inflammation	ThermoFisher	C10443
Fluo-4 AM	Calcium imaging	Molecular Probes	F14201
Fura-Red AM	Calcium imaging	Molecular Probes	F3020
phRodo labeling	Phagocytosis	ThermoFisher	P36600
Cytokine Assay	Immune response	*Meso* Scale Diagnostics Biolegend R&D	K15345D 740399 ARY005B
Incucyte	High-throughput imaging for phagocytosis, migration, and other fluorescent assays	Satorius	4763
Seahorse MitoStress test	Mitochondrial respiration	Agilent	103015