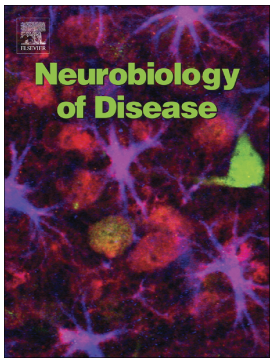

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Brain organoid models for studying the function of iPSC-derived microglia in neurodegeneration and brain tumours

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Abstract

Microglia represent the main resident immune cells of the brain. The interplay between microglia and other cells in the central nervous system, such as neurons or other glial cells, influences the function and ability of microglia to respond to various stimuli. These cellular communications, when disrupted, can affect the structure and function of the brain, initiation and progression of neurodegenerative diseases including Alzheimer's disease and Parkinson's disease, as well as the progression of other brain diseases like glioblastoma. Due to the difficult access to patient brain tissue and the differences reported in the murine models, the available models to study the role of microglia in disease progression are limited. Pluripotent stem cell technology has facilitated the generation of highly complex models, allowing the study of control and patient-derived microglia *in vitro*. Moreover, the ability to generate brain organoids that can mimic the 3D tissue environment and intercellular interactions in the brain provide powerful tools to study cellular pathways under homeostatic conditions and various disease pathologies. In this review, we summarize the most recent developments in modelling degenerative diseases and glioblastoma, with a focus on brain organoids with integrated microglia. We provide an overview of the most relevant research on intercellular interactions of microglia to evaluate their potential to study brain pathologies.

Keywords:
Microglia, organoids, iPSC, neurodegenerative diseases, glioblastoma

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1. Introduction

The interplay between the different cell types in the central nervous system (CNS) has become a focal point of research in the field of neuroscience. Particularly, the role of microglia is increasingly relevant in different contexts of brain development, physiology and pathology. Microglia, the resident immune cells of the CNS, play a multifaceted role in maintaining homeostasis, responding to injury, and influencing brain development. They are able to scavenge dying cells and phagocyte cellular debris and pathogens using pattern recognition receptors that are essential for phagocytosis and endocytosis [1], [2]. Microglia have essential roles: during embryonic development they act as effectors and regulators of synaptic plasticity, synaptic pruning, neurogenesis, angiogenesis and brain homeostasis, while during aging their neuro-immune activity becomes predominant [3], [4], [5], [6].

Microglial interaction with neurons has direct implications in the pathology of many brain diseases. Long-distance indirect communication between microglia and neurons located at the periphery, mainly the gut, have been recently described [7], [8]. In the CNS, neuron-microglia communication involves several processes such as membrane-to-membrane interactions, secreted cytokines, extracellular vesicles, neurotransmitters, peptides, and growth factors. The result of this interaction can affect their microenvironment and other cell types, including neurons, astrocytes, or oligodendrocytes [9], [10], [11], [12]. Moreover, microglial surveillance function involves highly ramified processes that could be transiently and/or repetitively in contact with synapses in different regions of the brain [13], [14]. Similarly, astrocyte-microglia cell interactions substantially rely on the release of soluble molecules. During development and in physiological conditions (homeostasis), astrocytes regulate cholesterol transport and some growth factor, such as IL-34, that are necessary to keep the microglia in a physiological state and maintain their function [15]. Likewise, microglia release many factors required for the physiological function of astrocytes. On the other hand, in disease conditions microglia can produce and secrete cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1-Beta (IL-1β) that could exacerbate inflammation, astrocyte reactivity, and neuronal death [16].

Microglial interaction with other cell types in pathological brain disease contexts such as Alzheimer's disease (AD), Parkinson's Disease (PD), and brain tumours can unravel the underlying affected mechanisms. Due to the neurodegenerative nature of AD and PD, most research has been focused on studying the dysregulation of molecular pathways in neurons. However, there is also strong evidence of the contribution of non-neuronal cells to the pathogenesis of neurodegenerative diseases [17], in which microglia play an important role that involves their neuroinflammatory activity. Glioblastomas (GBMs) represent another example of CNS pathology. They are highly malignant brain tumours known to contain up to 30% of tumour-associated macrophages and microglia, that are recruited to the tumour and can contribute to the pathogenesis of the disease by facilitating tumour proliferation, survival and migration [18]. However, with tumour progression GBM survivors develop progressive neurological deficits associated to tumour growth, side effects of the therapies and brain degeneration [19]. The emergence of brain organoids (three-dimensional *in vitro* models mimicking aspects of human brain physiology) and the embedding of microglia in them provide the opportunity to observe intercellular interactions and to shed light on the complexities of neural networks and their communication with the immune cells.

In this review we gather the most recent advancements in the generation and integration of microglia into brain organoids and their use to model GBM and neurological disorders including AD and PD. We summarise the characteristics and heterogeneity of microglia in health and disease in particular in the context of the aforementioned pathologies.

2. Microglia: from embryo to the bench

2.1. Embryonic origin

Microglial cells are of mesodermal/mesenchymal origin and derive from progenitors, which are generated at the primitive haematopoiesis phase - also known as the first haematopoietic wave - and migrate into the CNS from the periphery [1], [20]. These invading cells migrate from the embryonic yolk sac towards the developing CNS, entering the CNS in several migration steps (e.g., the choroid plexus) [21], [22]. Notably, upon closure of the blood-brain barrier and cessation of monocyte exchange between the CNS and periphery, and also during adulthood, the number of microglia

remains relatively steady based on an intrinsic balance of apoptosis and self-renewal [23], [24].

In mice, primitive macrophages are developed in the yolk sac at embryonic day 8.5 (E8.5) from early erythro-myeloid progenitors. Around E9.5, the primitive macrophages will migrate towards the neural tube where they will give rise to microglia [20]. The migration is a crucial step and depends on the neuronal expression and secretion of interleukin-34 (IL-34) and the colony stimulating factor-1 receptor (Csf1r) expressed by erythromyeloid precursors (EMPs). Both EMPs and developing microglia can sense and respond to IL-34 [25], [26]. At day 17 of gestation, E17, the yolk-sac-derived microglial precursors enter the brain and subsequently mature together with the neurons to become fully functional ramified microglia [25], [27], [28]. In humans, it is assumed that the processes of the ontogeny of microglia follow those reported in mice, although in an extended timeline. The study of microglial ontogeny in humans is limited, but studies on foetuses allowed to determine that at week 4.5 of gestation $Iba1^{+}$ microglia infiltrate the brain via the meninges and begin proliferation and radial migration. In a second wave of migration, microglial cells enter the brain at gestational weeks 12-13 via the vascular route and continue colonising various brain areas [29], [30], [31].

2.2. Microglial heterogeneity and functionality

Microglia are responsible for the brain innate immune response. In the adult mammalian brain, mature differentiated microglia exhibit a small cell soma with little perinuclear cytoplasm and are highly branched with fine ramified processes covered in small fine protrusions [32]. In response to a variety of stimuli, microglia undergo a series of morphological and functional alterations, including changes in morphology, phagocytic activity, motility, gene expression and cytokine/chemokine secretion [2], [33]. Changes can include switching from a highly ramified, motile morphology to an amoeboid morphology and increased phagocytic activity [34]. Upregulation of surface molecules and release of cytokines/chemokines can have a pro- and anti-inflammatory effect indicating a dual effect of microglia depending on the stimuli [35]. Microglial transmembrane transporters, the toll-like receptors (TLRs), are very important in the inflammatory response. Microglia also express multiple other pattern-recognition

receptors (PRRs) next to the TLRs, such as nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), and triggering receptors expressed on myeloid cells (TREMs) [36]. These PRRs are activated by pathogen-associated molecular patterns (PAMPs), which are unique structures present in pathogens, or by damage-associated molecular patterns (DAMPs). When TLRs are activated, several transcription factors are generated, including nuclear factor kappa B subunit 1 (NF-κB). This transcription factor promotes the expression of genes involved in the inflammatory response, such as TNFα, IL-1β and inducible nitric oxide synthase (iNOS) [36]. The aforementioned PAMPs and DAMPs are also present in disease conditions. A particular disease-associated microglial state (DAM) induced via TLR activation and TREM2 signalling, was also characterised by an increased expression of microglial genes, including *Iba1*, *Cst3*, and *Hexb,* and the downregulation of homeostatic genes *P2ry12*, *P2ry13*, *Cx3cr1*, *CD33*, and *Tmem119* [37]*.* Although DAMs were initially identified in AD mouse models, they have also been observed in more models of neurodegenerative diseases, ageing and human post-mortem tissue [37].

In recent years, it has been shown that the dual classification of functional microglia as M1 (activated or pro-inflammatory) and M2 (non-activated or protective) states as well as the context-dependent states such as DAMs are overly simplifying the dynamic nature of microglia as they are constantly receiving signals from the environment and thus are greatly heterogeneous in their morphological, functional, and transcriptional signatures [38]. For instance, a similar expression profile including an upregulation of TREM2, APOE, CD11c, CLEC7A, and LPL and downregulation of TGFβ, CSF1R, P2RY12, and TMEM119 has been observed in both the developing brain and also in aging and neurodegenerative diseases; indicating that one state can be beneficial in one context, although detrimental in another, depending on the context and interaction of the microglia with the environment [38]. Furthermore, even though activated microglia are commonly associated with an ameboid morphology, hyper-ramified microglia have also been detected in response to stress [39], and a variety of microglia morphologies have been reported in stroke conditions [40]. This further shows that microglia morphology cannot be linked directly to function. As such “M1 vs M2” and DAM classification should be avoided as it greatly disregards the heterogeneity of microglial states. As suggested by Paolicelli et at. 2022, a change in nomenclature is

required in which microglia are regarded as highly dynamic, and thus, there is a use of a combination of different markers and as many layers of complexity as possible to place the cells in a specific context and identifying the specific stimuli that microglia react to [38].

2.3. Maturation and maintenance

Microglia are self-renewing and proliferate from the resident microglia that originally infiltrated the brain during embryonic development and not from peripheral blood macrophages. Depletion of microglia via targeted diphtheria toxin treatment or CSF1R inhibition was followed by a very fast repopulation of microglial cells. Genetic mouse models showed a minimal contribution of peripheral monocytes to repopulation of microglia, although this has not been determined for humans. Fate mapping experiments showed that residual microglia were responsible for the repopulation after depletion. The underlying mechanisms for the repopulation are yet to be unravelled although some studies have shown a possible role for IL-1, NF-κB and *Mac2*+ microglia [41]. A stable population of microglia is present during the lifespan of an individual [42]. Interestingly, newly repopulated microglia present a downregulation of genes involved in microglia maturation and maintenance of mature phenotypes, such as *TGF*-β, *Mafb*, *P2Y12* and *Tmem119,* as well as an upregulation of cell cycle genes that promote repopulation [41].

After colonisation of the brain during embryonic development, microglia adopt a series of homeostatic markers expressed by adult microglia under physiological conditions, including *Tmem119, Olfml3, P2yr12, Sall1, Hexb, Gpr34, Fcrls,*and *SiglecH* and correlate with an activation of TGF-β signalling [43]. It has been suggested that the activation of TGF-β1 is necessary for the maturation of the mouse microglia in the CNS [44], [45]. Impairment of TGF-β1 signalling results in a lack of microglial maturation characterised by the absence of homeostatic markers [46], [47]. Additionally, the signalling by the microglial CSF1R is essential for the survival of adult microglia as it promotes proliferation and development [48]. In fact, inhibition of CSF1R greatly depleted the microglial population in the mouse brain, highlighting that this pathway is essential for microglial viability [48]. IL-34 is another factor involved in microglial maturation and survival; however, studies have identified that IL-34 inhibition does not deplete microglia as severely as CSF1R inhibition [49]. CSF1R is mainly found in the

white matter, while IL-34 is found in the grey matter indicating differences in functionality. Furthermore, inhibition of IL-34 in prenatal mice did not show a reduction of the microglial population in the brain after birth, indicating that IL-34 is responsible for microglial survival in post-natal stages but not during embryonic development [49].

A comparative transcriptomic analysis of mouse and human microglia revealed that *ex vivo* and *in vitro* cultured microglia present significant changes in gene expression [50], [51]. Genes related to immune function, vascularisation and brain development were downregulated after transfer to the culture environment [50] suggesting the importance of the extracellular context for the development of microglial phenotypes. Furthermore, many of the genes that were downregulated *in vitro* are also the genes involved in development. TGF-β1 treatment of *in vitro* cultured microglia increased the expression of several genes that were downregulated due to *in vitro* cultivation, including *Sall1, Tmem119,* and *P2ry12.* No other *in vitro* treatment was able to better preserve the *ex vivo* phenotype [50]. Loss of TGF-β1 in postnatal mice resulted in a decrease in total microglial numbers, severe motor deficits at an age of 120 days and a reduced survival [45]. These findings reveal the importance of TGF-β signalling for the maintenance of microglial phenotype.

2.4. iPSC technology and its use for microglial generation

iPSC technology brought an excellent opportunity to model different human pathologies by the analysis of patient-specific key cells *in vitro* [52]. Abud, et al. 2017 hypothesized that the *in vivo* conditions of the embryo are crucial for the development and maturation of microglia and therefore iPSCs could be differentiated into human microglia by providing an environment similar to the one of the developing embryo [53].

In recent years, several protocols became available that allowed for the differentiation of human iPSCs into iPSC-derived microglia (iPSC-MG) [25], [53], [54], [55], [56], [57]. While each differentiation protocol uses its own combination of growth factors, there are some common factors that are necessary for a successful differentiation. The most common growth factors are IL-34 and CSF1, both being CSF1R ligands [48]. The pathways related to CSF1 are needed to ensure microglial survival. Another growth

factor that is common across different protocols is bone morphogenic protein 4 (BMP-4), which is mainly applied during the first days of differentiation. The BMP-4 proteins are part of the TGF-β superfamily, which regulates apoptosis, proliferation, and differentiation [58]. More specific functions of the BMP proteins are the inhibition of neurogenesis and the induction of neural stem cell glial differentiation in the subventricular zone of the adult CNS, resulting in a reduction of the stem cell pool [59]. Almost all reported protocols use BMP-4 and CSF1/IL-34, suggesting that these growth factors are essential for microglial differentiation. Another common denominator at the initial steps of each protocol is either the formation of embryonic bodies (EBs) [25], [55] or the differentiation of iPSCs into hematopoietic progenitors [60]. Both methods were shown to result in mature microglia, but different growth factors are needed to ensure a successful differentiation [59]. Lastly, when the microglial progenitors are produced, they need to be matured into microglia. In the current protocols, this has been done either using maturation medium, which is a culture medium containing specific growth factors including TGFβ1, or in a co-culture with astrocytes or cortical neurons [25], [53], [56]. As an example, we differentiated EH1 iPSC into microglial progenitors according to a previously described protocol by Douvaras et al. 2017 [54] (Figure 1a). The differentiation was initiated when iPSC cells reached 80% of confluency (Figure 1b). iPSCs were converted into primitive hemangioblasts induced by bone morphogenetic protein 4 (BMP4). As a result of additional factors (bFGF, SCF, VEGF, IL-3) the primitive hemangioblasts began to develop into microglial progenitors on days 16-25 (Figure 1c), at this stage of differentiation, cells from the supernatant expressed CD45. CX3CR1 was upregulated between days 20 and 25, and more floating cells were observed in the medium (Figure 1d). IL-34 and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of plated microglial progenitors resulted in mature microglia (Figure 1e) with expression of the microglial markers: IBA1 and TMEM119 (Figure 1f,g).

Through a detailed assessment of RNA sequencing of the iPSC-MGs a high similarity was observed between human differentiated microglia from iPSCs with human foetal and adult microglia. First of all, iPSC-MGs showed a similar gene expression profile to foetal or adult microglia, for example *P2RY12, GPR34, C1Q, CABLES1, BHLHE41, TREM2, ITAM PROS1, APOE, SLCO2B1, SLC7A8, PPARD*, and *CRYBB1* genes [53]. The functionality of the mature microglia can also be tested by monitoring the response

of microglia to: LPS, classical Toll-like receptors (TLRs) like TLR4 activation [51], [61], or hallmarks of neurodegenerative pathologies such as amyloid-beta (Aβ) or alpha synuclein (α-Syn) [62], [63].

Indeed, a functional assessment of iPSC-MG activity showed that LPS-induced release of cytokines/chemokines, such as TNFα, CCL2, CCL4, and CXCL10. The Ca^{2+} transients in response to ADP highly resembled that of primary microglial responses to LPS [53], [64] and iPSC-MG could migrate towards Aβ aggregates and perform phagocytosis [53]. Changes in iPSC-MG morphological shape can be visualized by microscopy [47] and phagocytosis assay can be recorded by integration of pHrodo E. coli BioParticles in iPSC-MG (Figure 1h). LPS application to iPSC-MG increased the phagocytosis of these bioparticles (Figure 1i). The morphological changes could be corroborated by real-time cell impedance measurements [65]. Microglial shape during LPS challenge changed from a smaller cell body with fine ramification to a larger amoeboid shape [47]. We recently reported the effect of LPS on microglial cells and on their metabolic changes [47]. Description of the iPSCs-derived microglia protocols can be found in Supportive Supplementary file 1. Based on these findings and the reports of the functionality of iPSC-MG, iPSC technology is an excellent tool for studying the interaction of microglia with their microenvironment in neurological disorders.

3. Microglial function in brain organoids

Until recently, murine brain tissue or cells were largely used to conduct biological experiments. However, the results obtained with pharmaceutical compounds for various brain pathologies were not well translated into human clinical trials due to the intrinsic differences between the human and mouse CNS [51], [66]. These differences include brain size and organization, number, ratio, and type of cells, protein expression and function, fundamental genetic discrepancies, and metabolic reprogramming. Many of the divergent genes are related to signalling pathways and synaptic connectivity. Additionally, human microglia used for research can be obtained from post-mortem tissue, although their availability is limited and does not always recapitulate the brain environment nor the early stages of brain disease progression. The development of iPSC technology has allowed the study of human microglia with the patients' genetic

background while also allowing the introduction of mutations and alterations of the environment. The 2D culture of iPSC-differentiated cells presents several limitations as it does not entirely capture the cellular interactions present *in vivo.*

Brain organoids are *in vitro* 3D models with a collection of stem-cell derived cells (which are mainly composed of progenitors), neurons and astrocytes [59], [67]. Contrary to 2D iPSC models, organoids support cell-cell interactions and provide a 3D organisational structure that better resembles the cellular interactions in the brain. The integration of microglia into organoids allows the study of microglial response to signalling, changes in the environment and cellular interactions, and may provide a more accurate model for brain diseases (Figure 2) [68]. Microglial morphology is dependent on the cues and composition of the microenvironment, as their shape and motility are characteristic for grey and white matter, with distinct features highly dependent on brain anatomical regions. Microglial morphology and movement/migration properties in 3D brain organoids mimic better the human brain microglia. This diversity in shape, motility and regional dependence is not easy to mimic in 2D environments, therefore microglial integration in brain organoids or 3D structures offers opportunities to study microglia in a model with an improved translational value (Figure 2). Prior to the availability of protocols for generating brain organoids, spheroid studies showed that a co-culture of neurons, astrocytes and microglia could contribute to enhance microglial signature compared to mono-cultures of microglia in 2D [55]. Using a similar approach, Song et al., 2019 showed that the integration of microglia on spheroids could improve migration ability, intracellular calcium signaling and the response to proinflammatory stimuli [69] (see Table 1).

The majority of CNS cells are neuroectoderm-derived, while microglia are mesodermal-derived, therefore generated neural organoids from iPSCs lack non-neuroectodermal cells such as microglia [66], [68], [70]. Ormel et al., 2018 developed a CNS organoid in which the differentiation of brain organoids was performed without inhibitors of mesoderm lineage or associated mesoderm lineage modulators. This specific protocol showed an innate presence of microglia in organoids [71], although variability in the proportion of microglia has been described. The innate generation of microglia within brain organoids has been considered challenging and not easily reproducible. Instead, several studies have focused on alternative methods to integrate

microglia into brain organoids which involve the separate generation of differentiated iPSC-MG followed by their integration into mature cerebral organoids [69], [71]. The origin of the microglia may differ: (1) co-culture of brain organoids with mature microglia either from human primary microglia, human microglial cell lines, or microglia from iPSC or human embryonic stem cells (hESCs), (2) co-culture with human iPSC-derived microglial progenitors, or (3) co-culture of human induced microglial progenitors with neural progenitor cells (NPCs) both derived from either iPSCs or hESCs [72]. It was shown that for a successful integration the analysis of microglial morphology within the organoid is an important factor. As an example, our group generated organoids according to Pasca et al., 2015 [73], Sloan et al., 2018 [74], and Yoon et al., 2019 [75] (Figure 3a) and evaluated the integration of iPSC-MG in non-disease cortical brain organoids (Figure 3b-e). As previously mentioned, homeostatic microglia show a ramified morphology. These ramified microglia survey the environment and their phenotypic morphology is changed upon various insults or alterations in microenvironment [76], changing their morphology to more ameboid microglia [77]. Likewise, in brain organoids with integrated microglial cells, microglia appear ramified resembling their surveillant state in both control and pathological settings [53], [71]. Furthermore, integrated microglia in midbrain organoids are also functionally active, as they secrete cytokines (IL-7, IL-12p17, IL-3, TNFα, IL-1α, IL-1β, IL-6, IFNα, IL-10, and IFNγ) and chemokines (CXCL8, CCL2, CXCL2, CCL3, CXCL1, CCL4, CX3CL1, and MIP-3β) [78], [79]. Microglia integrated into brain organoids respond to various stimuli, display an increased phagocytic capacity, and increase the expression of genes involved in oxidative stress and immune response [78], [79]. Microglial integration in the brain organoids facilitates communication with other brain cells and could promote neurogenesis and synaptic remodelling [78], [79]. Furthermore, if damage occurred to the organoid such as piercing with a needle [53], the integrated microglia responded by migrating to the site of injury adopting an ameboid morphology [53], as observed in Figure 3f-h. Abud et al. 2017 and Fagerlund, et al. 2021 showed spontaneous migration of iPSC-MG in the brain organoid towards the injury site following an injury via a needle prick (Table 1) [80], as also shown in Figure 3h. More recently, Park et al, 2023 demonstrated that microglia incorporation can promote organoid maturation by limiting NPC proliferation and promoting axonogenesis in a mechanism mediated by cholesterol transfer from microglia to NPCs [81]. Besides, addition of macrophage precursors to midbrain organoids or cerebral organoids, have been used to study

Parkinson Disease (PD) or the pathology of Zika virus infection, respectively (Table 1). In the case of PD, inclusion of microglia increased neuronal maturation and excitability favouring synaptic remodelling [78]. Xu et al., 2022 used cerebral organoids and added microglia observing the response of incorporated microglia to Zika virus infection [82]. These findings support the successful integration of functional microglia – observed by morphology, secretion of cytokines and migration – into brain organoids, allowing the study of microglial role in the CNS pathology.

Table 1. Addition of iPSC-derived microglia to organoids or spheroids

Study purpose	***Addition to***	***Remarks***	***Reference***
Testing microglia progenitors	- Spheroids from NPCs that generate neurons and microglia	- Neuronal co-cultures enhance the microglial signature	Muffat et al., 2016 [55]
Testing functionality of microglia	- 3D Cortical organoids (12 weeks)	- After injury with 25Gs needle, iPSC-MG clustered close to the injury site, responding similar to brain microglia	Abud et al., 2017 [53]
Modelling microglia in organoids	- Cortical organoids (Day 30, 66, 120, 213)	- Incorporation of erythromyeloid progenitors (EMPs - Day 8) promote a microglial phenotype in the organoids and also neuronal bursting and network activity	Fagerlund et al., 2021 [80]

3.1. Response of microglia to radiation damage

Upon damage to a brain organoid, microglia can respond by migrating to the site of injury or changing their morphology. This is consistent with the findings of Schafer et al., 2023 that presented data on how stem cell-derived microglia integrated into human brain organoids. When microglia were xenotransplanted into mouse brains they migrated towards the site of injury following a laser lesion [83]. Since radiotherapy is a common treatment for many brain cancers, including glioblastoma, it is essential to understand the effect of radiation, not only on tumour cells but also on the surrounding normal tissue which is unavoidably irradiated as well. This leads to progressive radiotherapy-induced neurocognitive decline in over half of all patients [84]. Radiotherapy induces DNA damage, which induces cell death and can profoundly impact the brain environment through immunomodulating effects [84], [85]. These immunomodulatory effects include the radiation-induced cytosolic release of damaged nuclear DNA, mitochondrial DNA and double-stranded RNA, which in turn mediate

immune cell activation, inflammation and secretion of pro-inflammatory factors by activation of the cGAS-STING pathway, the ZBP1 (DNA-dependent activator of IFN-regulatory factors (DAI) and DLM-1) pathogen sensor, the AIM2 (Absent In Melanoma 2) and NLRP3 (nucleotide-binding and leucine-rich repeat receptor (NLR) family pyrin domain containing 3) inflammasomes [86].

In both human and mouse models, irradiation of microglia induces a response characterised by the migration of microglia to the site of injury and increased phagocytic activity as well as increased ROS and inflammatory cytokines and chemokines [87], [88]. Microglia in the mouse brains respond to irradiation with a shift in morphology from ramified to round and amoeboid, increased phagocytic activity, and increased pro-inflammatory markers, including IL-1β, IL-6, IL-18, TNF-α and COX-2, [89], [90]. This is concordant with the response of *in vitro* mouse microglia cell line BV-2 after irradiation [91]. Irradiated microglia also increase the production of ROS observed as an increase in DCF fluorescence with increasing radiation dosage [92] or decreased activity of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD), as observed in the brains of rats after irradiation [93]. GPx4 is a main regulator of ferroptotic cell death and inhibition of GPx4 leads to an increase in ROS, lipid peroxidation and cell death by ferroptosis [94], [95], [96], [97]. Therefore, a decrease in antioxidant enzymes after irradiation could suggest a ferroptotic fate in irradiated microglia. Interestingly, the peak of ROS production precedes pro-inflammatory factors TNF-α and MCP-1 in irradiated mouse brains, indicating that microglia respond to irradiation via ROS which in turn induces the release of pro-inflammatory factors [98]. ROS and the pro-inflammatory factors released by microglia after irradiation lead to adverse effects on the surrounding cells, such as neurons and astrocytes, leading to a vicious cycle of brain damage and further microglial-mediated inflammatory processes. For instance, irradiation of microglia increased the expression of IL-1β, IL-6, IL-18, TNF-α and COX-2 (all commonly associated with a pro-inflammatory phenotype), which leads to astrocyte gliosis [99], neuronal apoptosis and diminished brain function [89], [100]. Microglia have been shown to be neurotoxic in several radiation models. Exposure to X-rays was associated with depression-like behaviour in mice and neuronal apoptosis induced by microglial activation of the NLRP3/ASC/Caspase-1 pathway [101]. The neurotoxic effect of radiation is mediated by microglia, as temporary decrease of microglia activity

by CSF1R inhibition ameliorated cognitive function in mice [102] and reduced the expression of the pro-inflammatory genes TLR9, SYK (spleen tyrosine kinase), CCL6 (Chemokine (C-C motif) ligand 6), CD14, CLECL5a (C-type lectin domain family 5 member A), TSLP (Thymic stromal lymphopoietin), and TNFRSF13b (Tumour necrosis factor receptor superfamily member 13B) [103]. Modulation of cellular pathways in microglia, such as those induced by inhibition of P2X7R (P2X purinoceptor 7), Kv1.3 channel, CXCR1 (C-X-C chemokine receptor type 1) or PPAR (peroxisome proliferator-activated receptors) rescued the brain damage induced by irradiation, thereby shedding light on the underlying mechanisms involved in radiation-mediated microglial activation and subsequent brain damage [87]. Recently, brain irradiation has been shown to lead to persistent innate immune reprogramming of rat microglia into a primed state, enhancing the response to subsequent inflammatory stimuli [104], [105]. The extent of microglial priming was shown to be dependent on age and the radiation dose, and still be present after dose fractionation as delivered clinically. Importantly, evidence of radiation-induced microglial priming was also found in human post-mortem normal-appearing brain samples of patients with GBM [104], [106].

In recent years, brain organoids have increasingly been used to study the effect of radiation on the normal brain, where microglia localization change after irradiation, and exhibit an amoeboid morphology (Figure 3i) similar to the microglial response to irradiation damage in *in vivo* and *in vitro* murine models [89], [90], [91]. The increased localization of microglia at the edge of the organoids might be due to either the fact that microglia react to damage on a “first-come, first-served” basis or that astrocytes, largely located at the edges of the cortical organoids, interact with microglia mediating their local activation.

3.2. Microglia-containing brain organoids as cellular models for Alzheimer’s Disease

AD is characterised by accumulation of amyloid beta (Aβ) plaques and neurofibrillary tangles in the brain that are associated with alterations in mitochondrial function [107] and an inflammatory response mediated by microglia [63], eventually leading to neurodegeneration [108]. In homeostatic conditions, microglia are able to sense the soluble Aβ peptides and clear them from the brain by a process called macropinocytosis, or through phagocytosis in the case of fibrillar Aβ peptides. Even

though microglia can take up these fibrillar peptides, their degradation is very difficult [109]. Paresce, et al (1997) showed that once these peptides have been internalized by microglia, they can remain undegraded for more than 72 hours [110]. Another study showed that microglia could secrete the Aβ peptides into the medium following their internalization [111]. Next to the microglia being unable to remove the Aβ peptides properly, there is also an inflammatory response that can affect the surrounding cells, including neurons and astrocytes. Microglia and Aβ interact via activation of the microglial PRRs, leading to production of ROS, RNS and pro-inflammatory cytokines [108]. The pro-inflammatory cytokine production induced by Aβ contributes to the inability of microglia to clear the Aβ plaques, thus further promoting their accumulation leading to a detrimental vicious cycle [59]. The same process essentially happens with tau-protein and its interaction with microglia: the microglia can sense and clear hyperphosphorylated tau to protect the brain against tau toxicity. However, when these microglia are activated in the context of AD, they adopt a pro-inflammatory phenotype thereby increasing tau phosphorylation. To summarise, the microglial dysfunction in AD leads to a dysregulation in the defence against toxicity, and thus results in a vicious cycle, finally contributing to neuronal damage and loss [108].

Many different cell types and models have been used to study AD, but no animal or cellular model fully represents the physiology of the human brain and the pathology caused by neurodegenerative diseases [112]. Due to the complexity of the disease, organoids provide a great tool to study the pathogenesis and intercellular interaction including microglial involvement [113]. There have been many studies on brain organoids in the context of AD [114]. These models are derived from patients with familial or sporadic AD and therefore carrying AD-related or high-risk mutations such as APP (amyloid precursor protein), PSEN1 (presenilin 1), PSEN2 (presenilin 2) (familial AD) or APOE4 (apolipoprotein E4) (idiopathic/sporadic AD). Furthermore, comparing cells derived from healthy subjects and AD patients can provide insightful details about the relative genetic differences and the progression of the pathology [115]. The generated organoids recapitulate characteristics of AD pathology including Aβ deposition, tau hyperphosphorylation, neuronal loss, impaired neuronal activity, and endosomal dysfunction [113], [114], [116] or even ferroptotic pathway alterations (PSEN1Δ9 brain organoids) [117]. However, not all organoid protocols develop non-neuronal cells therefore, considering the relevance of these cells to the

pathophysiology of AD, new protocols for the incorporation of astrocytes, microglia and cerebrovasculature have been developed [118], [119], [120], [121], [122].

Lin et al. 2018 generated brain organoids derived from patients carrying the AD risk factor APOE4 or APOE3 allele variants using a protocol that generates astrocytes in later stages of the organoid formation. APOE4 organoids contained more Aβ plaques than those with low-risk APOE3 variant. In the same study, APOE4 iPSC-derived microglia exhibited an increase in inflammatory gene expression and deficits in Aβ clearance, which could explain the accumulation of Aβ. However, microglia have not yet been integrated in this AD brain organoid model [123]. Another approach to study the influence of microglia on AD pathology made use of a 3D triculture chip model where the inner chamber contained a 3D co-culture of neurons and astrocytes, and the outer chamber contained the microglia. There was an increased accumulation of $A\beta_{40}$ in the AD triculture compared to an AD co-culture without microglia or a WT triculture [124]. Furthermore, it was observed that microglia migrated to the AD triculture and increased the expression of chemokines (CCL2, CCL3/4, CCL5, CXCL1 and CXCL10) and soluble factors (IL6, IL8, G-CSF, GM-CSF, MIF, PAI1 and TNFα) in comparison to the AD co-culture without microglia or the WT triculture. Furthermore, the presence of microglia in the 3D model induced damage to neurons and astrocytes in an INFγ- and TLR4-dependent manner [124]. A recent study generated human cortical organoids (hCOs) containing microglia-like cells (mhCO) and treated them with $A\beta_{1\text{-}42}$ oligos to recapitulate AD pathology [125]. Phagocytic microglia-like cells (MG-L) were observed to colocalize with the Aβ oligos in the mhCOs. Furthermore, upon Aβ treatment mhCOs presented an increase of the expression of several genes, including *Aif1, C1qc, Csf1r, Lcp1, Ptprc,* and *Ctss* often associated with microglial activation. In addition to these genes, several other genes involved in neuronal differentiation, maturation, and prevention of cell death via apoptosis and ferroptosis were changed compared to brain organoids without integrated microglia. Overall, the treatment with Aβ oligos induced an AD-associated profile in organoids, which was rescued by the presence of MG-L [125]. Another recent study showed that in spontaneously generated microglia in familial AD organoids carrying APP mutations, the generated organoids presented an increased expression of genes associated to activated microglia and staining with TMEM119 revealed a ramified morphology of microglia [126]. The generated AD model successfully presented characteristics of AD pathology, such as

Aβ accumulation, altered neuronal excitability and a dysregulated gene profile similar to that found in AD brains [126]. Although these studies present an important role of microglia in AD pathology that could be mimicked in brain organoids containing microglia, further research is necessary to further delve into the mechanisms on how microglia contribute to AD pathology. These findings, which are summarised in Table 1, show promising results of integrated functional microglia into brain organoids as a novel tool to study their involvement in AD pathology.

Table 2. Microglia containing brain organoids in AD research.

AD model	*Organoid protocol & microglia integration*	*Organoid Features*	*Reference*
APP mutation	Human tri-culture model: AD neurons and astrocytes differentiated from NPCs in the inner chamber Adult microglia cells (human microglia-SV40 cell line) were added to the outer chamber.	Microglia migration to AD triculture Aβ aggregation and p-tau formation in AD triculture Increased chemokines/cytokines CCL2, CCL3/4, CCL5, CXCL1 and CXCL10, and soluble factors IL6, IL8, G-CSF, GM-CSF, MIF, PAI1 and TNFα. 3D AD triculture leads to neuron and astrocyte damage.	Park et al. 2018 *[124]*
$A\beta_{1-42}$ oligo treatment	Microglia-containing human cortical organoids (mhCOs) generated from PU.1 doxycycline-inducible human embryonic stem cells (hESCs). Neural induction medium: SB431542, LDN-193189, XAV-939, Y27632. Organoid medium: 1:1 DMEM-F12 and neurobasal media (minus vitamin A). Organoid medium with vitamin A, BDNF and ascorbic acid. Doxycycline for microglia-like cell generation (MG-L).	MG-L migrate towards Aβ and engulf Aβ oligos. Reduced apoptotic areas in mhCOs Upregulation of *Aif1, C1qc, Csf1r, Lcp1, Ptprc*, and *Ctss* in mhCOs in response to Aβ. Aβ treatment downregulates genes involved in synapse and dendrite development, and neuronal differentiation and maturation in hCOs but not in mhCOs. Reduced expression of ferroptosis genes in mhCOs after Aβ treatment. MG-L rescue AD-associated profile after Aβ treatment in hCOs	Cakir et al. 2022 *[125]*
APP mutation	iPSCs → EBs. Neural induction medium Neural progenitor medium (StemCell Technologies). 3D structures generated by embedding NPCs in silk-collagen scaffolds. Neural progenitor media BrainPhys media (StemCell Technologies) supplemented with SM1 and N2.	Spontaneous generation of $TMEM119^+$ microglia Increased expression of activated microglia genes Ramified microglia Increased Aβ accumulation in AD organoids Increased neuronal excitability in AD AD organoids recapitulate human AD gene signature	Lomoio et al. 2022 *[126]*

3.3. Microglia-containing brain organoids as cellular models for Parkinson's Disease

Parkinson's disease (PD) is a late-onset neurodegenerative disease associated with misfolded α-synuclein and loss of dopaminergic neurons in the substantia nigra. Not only does α-synuclein induce neuronal toxicity, but it also triggers microglial activation and a pro-inflammatory response [127]. Studies identified an increase in microglia in the substantia nigra of PD post-mortem brain tissues with an amoeboid morphology, and a pro-inflammatory profile characterised by increased levels of IL-1β, GPNMB (Transmembrane glycoprotein NMB), and HSP90AA1 (heat shock protein 90 alpha family class A member 1) [128]. These so-called activated microglia can produce large amounts of superoxide radicals, which are a source of the oxidative stress possibly responsible for the dopaminergic cell death in PD [129]. Other inflammatory processes amongst which, lipid peroxidation, are also increased in the substantia nigra. Alterations in the LOXs (lipoxygenases), COXs (cyclooxygenases) and EPOX (epoxygenases) pathways may be associated with the pathogenesis of PD and increase the inflammatory response [130], [131]. Another mechanism through which microglia participates in PD is internalization and degradation of α-synuclein. A dysregulation in the process of clearing α-synuclein can lead to an accumulation of α-synuclein, which in turn causes microglia to migrate near the aggregation sites. Through interaction via PRRs, just like Aβ can do in AD, α-synuclein will alter the function of microglia to adopt pro-inflammatory characteristics [127]. Microglial activation is promoted by α-synuclein via activation of TLR2 [36]. α-synuclein directly engages with TLR2 receptor at the cell membrane, resulting in an increased production of pro-inflammatory cytokines TNF-α and IL-1β [132]. Another study showed that α-synuclein needs to be phagocytosed in order to activate the microglia, and that blocking the internalization of α-synuclein could prevent the production of superoxide and iROS, mediated by α-synuclein [133].

Similar to most models of chronic disorders, animal models for brain disorders do not always recapitulate the human characteristics of the disease. Human samples often derived from post-mortem tissue do not allow for the study of early stages of the disease or the progression of the disease. Several studies have been carried out using iPSC-derived dopaminergic neurons from patients with familiar and sporadic forms of PD providing insight into the underlying molecular mechanisms [134]. However, these

2D models cannot adequately simulate the cell-cell interactions between different cell types in the brain. Therefore, 3D models, such as organoids could provide a better understanding of PD pathology. Jo et al 2016 [135] developed a protocol to generate midbrain organoids that later were used for LRRK2-G2019S (leucine rich-repeats kinase 2) mutation inserted via CRISPR/Cas9 technology. LRRK2-G2019S is one of the most common genetic cause of familial PD and is also found in sporadic PD. The generated organoids presented decreased neurite length of dopaminergic neurons, increased cell death and abnormal localization of α-synuclein [136]. Using the same protocol, another study generated patient-derived organoids from iPSC cells obtained from patients with the sporadic form of PD. They successfully developed mature midbrain organoids expressing late neural markers such as TUBB3 (beta tubulin protein family), NURR1 (nuclear receptor subfamily 4 group A member 2) and TH (tyrosine hydroxylase). However, the mature organoids derived from PD patients also expressed early neuronal markers, suggesting dysregulated neural development. PD organoids showed reduced levels of TH concomitant with the loss of dopaminergic neurons observed in PD patients [137]. Smits et al. 2019 developed a different protocol to generate patient-derived midbrain organoids carrying the LRRK2-G2019S mutation to study familial PD. These organoids contained functional dopaminergic neurons producing dopamine. The LRRK2-G2019S organoids displayed a reduced number of dopaminergic neurons when compared to the control organoids [138].

These studies show the relevance of organoid models for the study of PD; however, the generated organoids lack microglia. In the past years there has been progress in the incorporation of non-neuronal cells into midbrain organoids: Nickels et al 2020 developed an improved midbrain organoid protocol based on Smits et al 2019 by starting the differentiation steps earlier in the protocol, and this improved method showed the presence of different functional neuronal subtypes as well as GFAP-positive glial cells [139], and Sabate-Soler et al 2022 developed midbrain organoids with integrated and functional iPSC-derived microglia [78]. These results shed light on the progress made to mimic and understand the brain environment however, to the best of our knowledge, no research has been carried out yet on organoids with integrated microglia mimicking PD pathogenesis, which could more closely resemble the CNS and provide insights into the cell-cell interactions in the development of the disease.

3.4. Glioblastoma and their interaction with microglia

Gliomas are a collective name for brain tumours that originate from glial cells. GBM is the most aggressive type of glioma, characterised by rapid tumour growth, resistance to therapy and diffuse invasiveness [140], [141], [142]. GBM is made up of neoplastic and non-neoplastic cells, which are both immune and non-immune cells and they have been described due to the studies in GBM from patients (Figure 3j). Between 30-50% of the tumours consist of microglia, together with infiltrating monocytes and macrophages [143]. GBM cells can interact with the non-neoplastic cells for instance they recruit microglia by secreting chemokines, growth factors, cytokines, ECM constituents, angiogenic molecules and inducers of vascular permeability (Figure 4) [144]. Once microglia accumulate around and in the GBM tumour region, they exhibit an amoeboid morphology [18]. The most important recruiting factor is the monocyte chemoattractant protein-1 (MCP-1), also called CCL2, which is secreted by GBM cells [145]. It has been demonstrated that GBM cell-derived MCP-1 increases the infiltration of microglia into the tumour. GBM cells also secretes stroma-derived factor-1 (SDF-1), which promotes accumulation of microglia in normoxic tumour regions, and M-CSF, which promotes microglial cell motility and the phenotype shift towards an anti-inflammatory profile [146], [147]. Two other factors are GM-CSF, which facilitates the invasion of microglia, and epidermal growth factor (EGF) that directs microglia to the lesion site [148]. Microglia also produce factors that alter tumour development and thus promoting tumour cell invasion (Figure 4) [149]. One of these factors is TGF-β2, which induces the expression of MMP-2 [150]. This is an enzyme that degrades the extracellular matrix and thereby facilitates the GBM cell invasion. The co-chaperone stress inducible protein 1 (STI-1) is a chemoattractant derived from microglia, which facilitates GBM cell proliferation and migration. It has also been shown that expression of STI-1 by microglia increases with the tumour severity and remains unchanged in monocytes that circulate in the blood [151]. Not only is there chemoattraction between microglia and GBM cells, but microglia can also facilitate the vascularization of the tumour [152]. They secrete pro-angiogenic factors at high levels like vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6), both of which facilitate the vascularization and thus promote tumour growth [153]. The microglia can ensure the presence of an immunosuppressive environment inside and around the tumour [144]. To be able to create this immunosuppressive environment, microglia secrete high

amounts of cytokines (e.g. TFG-β, IL-6, IL-10) [154]. TGF-β signalling contributes to a reduced phagocytic activity due to induction of the downregulation of MHCII- and co-stimulatory molecules (e.g. CD80 and CD86) [155]. IL-10 also contributes to creating an immunosuppressive environment by inhibiting antigen-presenting cells and T-cell proliferation. The transcription of this cytokine is mainly dependent on STAT3 signalling, which is enhanced in tumour-derived microglia compared to normal microglia [156]. Blockage of STAT-3 has been shown to lead to a decrease in secretion of IL-10 and other inflammatory molecules (e.g. IL-4, IL-6, IL-11 and IL-23) and a decrease in a variety of growth factors, such as FGF, EGF, PDGF and HGF. These factors may also contribute to the resulting immunosuppressive environment [157].

Patients suffering from GBM present neurological symptoms. These have been shown to cause brain compression as a result of tumor growth, and synaptic interactions between GBM and neuronal cells, as well as from the side effects of radiotherapy and chemotherapy [106], [158]. GBM cells extend a network of microtubes through which mRNA, proteins and organelles can be exchanged affecting cell functioning. These microtubes surround neurons and take up growth factors which eventually lead to neuronal cell death [19]. Since the emergence of organoid protocols, this model has also been used to study GBM. There are several strategies to develop GBM models such as inserting oncogenic factors into brain organoids [159], [160] or co-culturing patient-derived GBM models with healthy brain organoids to generate a hybrid GBM organoid [161], [162], [163], [164]. An iPSC-derived cerebral organoid co-cultured with patient-derived GBM cells was able to successfully recapitulate the invasive characteristics of GBM observed *in vivo*. Furthermore, GBM cells and neurons were observed to interact through enrichment of ligand-receptor pairs involved in tumour invasion [165]. Recently Jacob et al. 2020 developed a novel protocol to develop patient-derived GBM organoids [159], where fresh GBM tumour tissue was dissected into small pieces and cultured in organoid medium. These GBM organoids recapitulate the characteristics of the original tumour and environment, including the presence of microglia, observed through Iba1 staining, and a characteristic microglial gene signature, showing great potential for modelling GBM [159]. Nevertheless, the interaction of microglia with neurons has not yet been studied in this model. Zhang et al. (2020) cultured microglia on a collagen matrix followed by embedding of GBM tumoroid and measured the outgrowth of the tumoroid to determine tumour

invasiveness. They observed that the presence of microglia increased tumoroid outgrowth and similarly, the exposure of GBM organoids to microglia-conditioned media also facilitated tumour invasiveness [166]. Additionally, Leite et al. 2020 developed a 3D GBM model with a microglia co-culture. They observed that microglia grew in proximity to GBM cells and resulted in a moderate increase in GBM cell proliferation. Furthermore, the presence of microglia conferred resistance to drugs and it has been showed the integration of microglia in the GBM after different time points (Figure 3j) [167]. Together these results show the relevance of microglia presence in GBM models to recapitulate the cell-cell interactions and their modulation on tumour progression.

4. Perspectives on brain organoid research

4.1. Integration of various types of immune cells

Beyond microglia, there are other resident macrophages in the CNS called border-associated macrophages (BAMs). BAMs present mixed origins as they derive from both yolk sac and liver progenitors and are found in the barriers between the blood and brain and CSF (cerebrospinal fluid) and in the meninges [168]. They present much greater heterogeneity compared to microglia regarding their origin, morphology and motility [169], however they share some common markers with microglia including CD45, CX3CR1 and IBA1 making it difficult to discriminate between different populations [169], [170]. Similar to microglia, BAMs seem to play a dual role in the brain as they are involved in a variety of homeostatic functions, including regulating brain development, clearance of debris and resolution of inflammation, but on the other hand they also could contribute to immune-related pathologies [171]. Subpopulations of BAMs have been identified in AD, PD and gliomas, among other CNS diseases and are linked to the pathogenesis and progression of the disease [169], [172], [173]. In AD, an increase in BAMs has been linked to Aβ overload and ROS [174]. Furthermore, BAMs can contribute to microglia function in particular synapse pruning, as decrease in BAMs resulted in a decrease in synapse loss in an AD mouse model [175]. Similarly, in PD, BAMs specifically play an important role in α-synuclein inflammation by acting

as antigen presenting cells [176]. In brain cancer, like gliomas there is a large population of tumour associated macrophages (TAMs) which include microglia and BAMs among other immune cells. TAMs create an immunosuppressive environment which facilitates tumour growth, invasion, metastasis, angiogenesis, immune evasion and resistance to treatment [18]. Together these findings show that other immune cells are also relevant for CNS pathology. BAMs have only recently been discovered therefore, there is still limited research carried out. Thus, the integration of BAMs to the brain organoids will add another layer of complexity to model the CNS, and if the organoids are placed in the context of neurodegenerative diseases or gliomas, we can further understand the role of the immune cells in brain-related diseases.

4.2. Integration of the brain vasculature

The CNS consists of other non-neuroectodermal cells, namely the components of the blood brain barrier (BBB) and the vasculature are essential for the correct development and function of the brain. The neurovascular system includes neuronal and glial cells, as well as the components of the vasculature such as endothelial cells, pericytes, smooth muscle cells and a basal lamina of extracellular matrix [177]. It is not only required for oxygen and nutrient supply, but also regulates neurogenesis and brain functions. Dysfunction of the neurovascular unit has been observed in many neurodegenerative diseases [178] as well as in brain tumours [179]. Interestingly, different components of the neurovascular unit were observed to be altered in AD post-mortem brain samples [180]. The lack of vasculature is a major disadvantage of brain organoid models. Sun Ju et al. recently developed a vascularised brain organoid (VBO) model through the formation of an assembloid of vascular organoids fused to cerebral organoids. The resulting VBOs contained not only cells characteristic of the neurovascular unit, such as endothelial cells and astrocytes, but also functional microglia capable of responding to LPS stimulation [181]. This is one step further towards a more comprehensive CNS model that contains multiple cell types, allowing the study of multiple cell-cell interactions. However, there is still no blood flow in the generated vasculature. To overcome this limitation, vascularised brain organoids have been transplanted into mice resulting in successful integration, maturation of neuronal cells and infiltration of microglia as well as extension of vessels [182]. Despite this research, the field of vascularised organoids still requires further advancements.

4.3. Interaction of the CNS organoids with other peripheral tissues

Brain organoids can also be fused with other types of organoids generating assembloids to study the interaction between various tissues [183], [184]. For instance, assembloids comprised of cortical-spinal-skeletal organoids showed that stimulation of brain neurons resulted in contraction in the muscle area of the assembloid [185]. A similar construction could be used in a model of PD to study motor dysfunction. Assembloids represent an advanced *in vitro* model to study not only interaction between cells of different lineages but also between tissues and organs providing a broader view of the physiological or pathological conditions.

4.4. Inclusion of iPSC-derived microglia in a more complex environment: xenotransplantation

While brain organoids with integrated microglia offer valuable insights, they still lack the complexity and structural maturity of an *in vivo* environment. In this context, xenotransplantation provides a promising alternative, allowing for the transplantation of human iPSC-derived microglia into the mature, structurally developed brain of a mouse. Table 3 summarizes key studies chronologically conducted in this field. Many studies validate microglial functionality *in vivo*, where transplanted microglia exhibit similar behaviours to human foetal microglia, including migration, process extension, and homeostatic regulation in response to various stimuli [53], [60]. Additional protocols have been developed for introducing iPSC-derived microglia into mouse pups and adult mice, with transplanted cells displaying gene signatures and behaviours consistent with human primary microglia [186], [187], [188], [189]. Notably, alternative delivery methods, such as intranasal administration, have also been explored [190]. Beyond the brain, other organs like the retina have been used as transplantation sites, where iPSC-derived microglia maintained a stable, homeostatic profile over time [191].

Similarly, xenotransplantation of human iPSC-derived microglia into mouse brains has been important in studying neurodegenerative diseases such as Alzheimer's disease. This approach has provided insights into microglial function and their distinct transcriptomic profiles in response to AD pathology [53], [192], [193]. In the context of multiple sclerosis (MS), Xu et al. (2020) [194] transplanted iPSC-derived microglia into immunodeficient mice, where the cells matured successfully and exhibited functional

responses to cuprizone-induced demyelination. This work highlights the potential of transplanted microglia to model MS pathology and study microglial dynamics in demyelinating conditions. More recently, research on Down syndrome (DS) has shown that iPSC-derived microglia from DS patients transplanted into mouse brains display enhanced synaptic pruning activity, which in turn disrupts neuronal synaptic function [195]. Despite these advancements, there is much more to be explored. For instance, the use of microglia in xenotransplantation models to study glioblastoma has yet to be investigated, highlighting an important area for future research. These findings illustrate the versatility of xenotransplantation of microglia across various neurodegenerative and neurodevelopmental disorders.

Table 3. Xenotransplantation of iPSC-derived microglia

Purpose	***Injected to***	***Remarks***	***Reference***
Disease context: Alzheimer Disease	MITRG mice: Cortex Rag-5xfAD: APPSw FlLon, PSEN1* M146L* L286V)	iPSC-MG behave similarly to human foetal microglia: migration, extension of processes and phagocytosis βA	Abud et al., 2017 [53]
Testing microglia progenitors	MITRG mice - Hippocampus & Cortex	Engraftment of iPSC-MG - Homeostatic microglia	McQuade et al., 2018 [60]
Disease context: Alzheimer Disease	Hippocampus & Cortex - Pups (Day 4) $Rag2^{-/-}$ $IL2r\gamma^{-/-}$ hCSF1KI	Successful engraftment of embryonic stem cells ESC-derived microglia mimic primary human cells at the transcriptome level. - Divergent response of mouse and human microglia to oligomeric βA	Mancuso et al., 2019 [192]

5. Conclusions

This review summarizes the importance of cell-cell interactions in brain diseases like AD, PD and GBM, in which microglial interactions play a key role. Their great heterogeneity allows them to carry out a dual function in the brain by maintaining homeostasis and protecting the brain, but also favouring disease progression upon

activation by damage or secreted factors. The complexity of the brain and the interplay between different cell types call for new models that can recapitulate the intricate network of cellular interactions and mimic the context of different diseases. Brain organoids prove to be a potential candidate as they have been successfully developed for the study of AD, PD and GBM, and a lot of effort has been put into the integration of other cell types including microglia and their characterisation in the context of healthy brain organoids. However, there is still limited research performed on the role of microglia in the progression of neurodegenerative diseases, especially in the context of PD where there is no evidence yet on the incorporation of microglia into PD-organoids. In the context of brain tumours, such as GBM, microglial interaction with the tumour seems to favour invasion but this still remains to be proven in 3D GBM models. Brain organoids are unarguably a valuable model system to recapitulate the brain environment and the addition of non-neuronal cell types, like microglia, can further mimic the environment found in the brain since it is clear that microglia play a crucial role in the progression of different CNS diseases. Additional research into the specific role that microglia play in these diseases is necessary, and elucidating how the modulation of microglial behaviour and response to different stimuli can aid in concomitantly modulating the disease pathophysiology and progression and thus finding potential disease-modifying treatments for neurodegenerative diseases, such as AD and PD, and also brain tumours, like GBM.

However, there is still room to closely mimic the full environment of the CNS by including other CNS immune cells like BAMs and components of the brain vasculature. Additionally, assembloids can provide insights into the connectivity between different organs and tissues. Until this field is further developed, microglia-containing organoids present one step further in mimicking the complexity of layers in the CNS and serve as a functional and versatile model to study a range of CNS diseases including neurodegenerative diseases and glioblastoma.

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Figure 1. iPSC Differentiation to human microglia and its functional activity. iPSC differentiated to microglia through myeloid progenitors according to Douvaras et al., 2017 [54]. (a) Diagram depicting the major steps of the microglial differentiation through myeloid progenitors according to Douvaras et al., 2017 protocol. (b-e) Representative pictures of the main steps in the differentiation protocol until day 45, when microglial cells were collected from the supernatant. (f) immunofluorescent labelling with TMEM119 of mature microglia and (g) Iba-1, a marker for microglia. Cell imaging by an Incucyte system displaying phagocytosis without (h) and with LPS (i) [47]. Figure 1a was created with BioRender.

Figure 2. Advantages of microglia in 3D environments. Isolated microglia post-mortem and microglia derived from hESC/iPSC that are cultivated in a 2D format have some limitations in structure and function. Microglia that have been cultivated in either Matrigel or organoids showed enhanced bona fide microglial markers and highly branched morphology that can improve their immune surveillance function, phagocytic and injury response capacity.

Figure 3. Integration of microglia in brain organoids. (a) Diagram depicting the major steps of generation of brain organoids according to Pasca et a., 2015, Sloan et al., 2018, and Yoon et al., 2019 [73], [74], [75] (b). Scheme of Integration of the iPSC-MG: Microglial cells were transfected with an integration-free (episomal) expression of EGFP vector for constitutive expression of EGFP [Addgene, Cat #27082]. Four days after transfection microglia cells were integrated into the organoids. Microglia stained with PKH67 green fluorescent dye (Sigma Aldrich, Cat #:PKH67GL) were added to the brain organoids. (c) To observe the response to injury stimulation of integrated microglia into organoid, control organoids (d) were exposed to two different types of damage, local and global injury. (e) Local damage was induced by a needle at the surface of the organoid [49]. Pictures were taken after 12 hours with and without any damage. (f) Scheme showing a global injury can be induced by irradiation of organoids. (g) Scheme showing the generation of oncospheres in suspension from the GSC of GBM patients [141] and cultivated in 96 well plate where microglia could be added. (h) Scheme showing microglia morphological changes Figure 3a, b, c, f, g and h were created with BioRender.

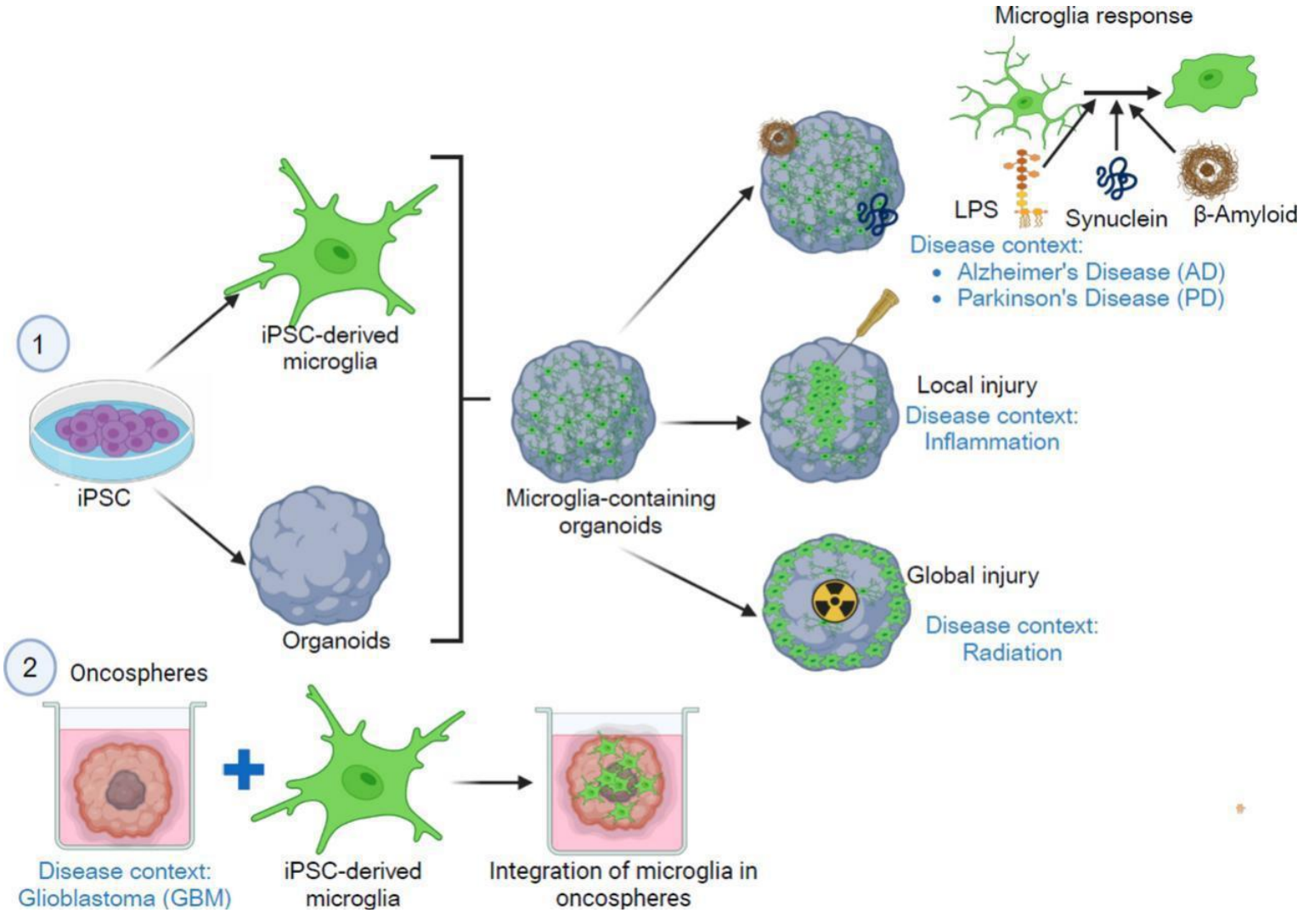
Figure 4. 2-way communication between GBM and microglia. GBM creates a tumour-favourable environment by secreting factors that recruit microglia to the tumour. These ensure an immunosuppressive environment that allows tumour survival. Microglia in turn also secrete factors that facilitates tumour growth and invasiveness thus enabling tumour progression. Figure 4 was created with BioRender.

Authors declare no conflict of interest.

Graphical abstract

Highlights

- Modelling microglial function is a growing field with technical culprits to overcome
- Microglia integrated into 3D models allow studies on microglial interactions with CNS-resident cells
- Integrated microglia can respond to local and global organoid damage
- iPSC-derived microglia-containing organoids can shed light on microglial implications in disease pathology



Graphics Abstract

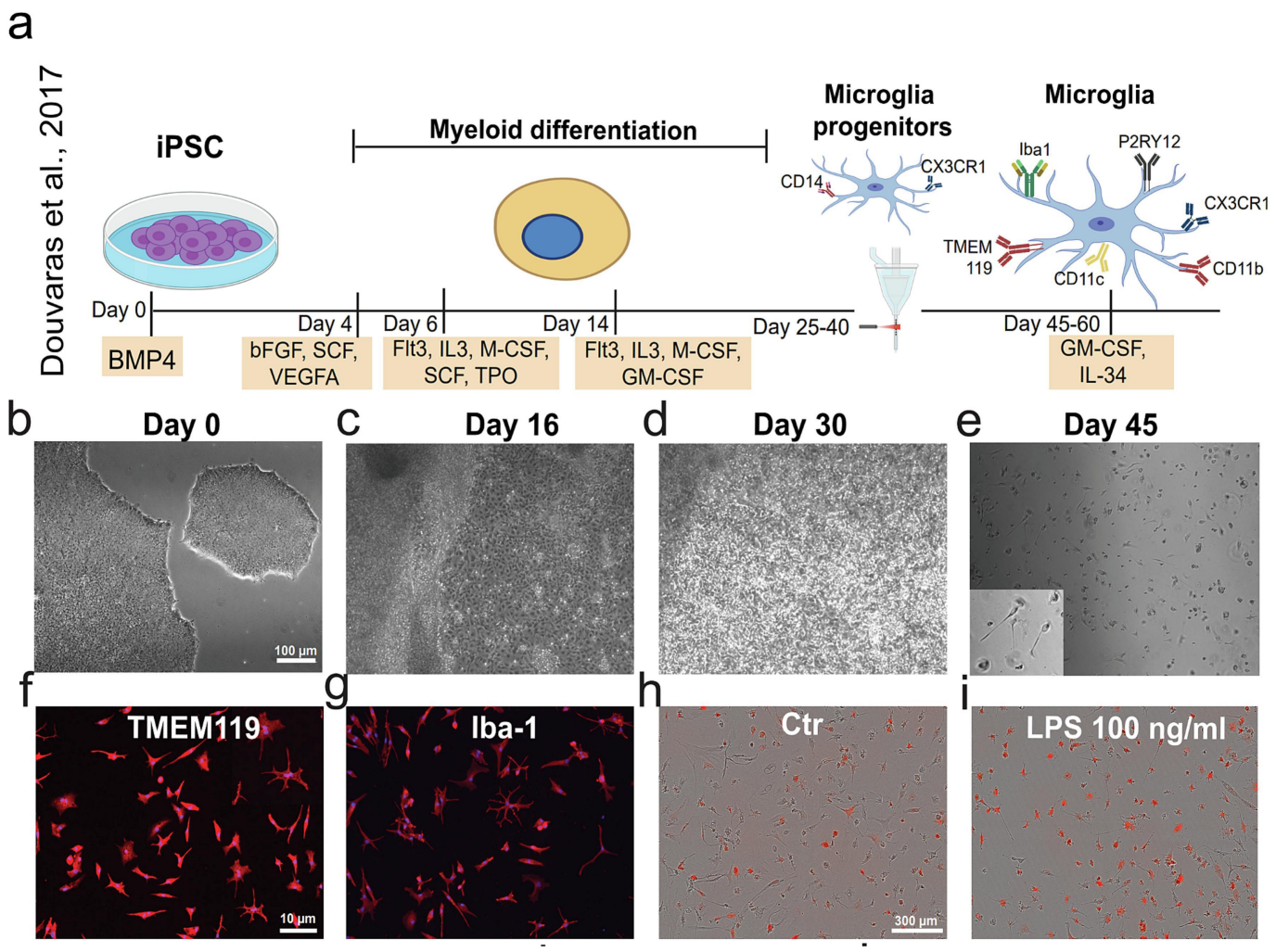

a
Douvaras et al., 2017
iPSC
Myeloid differentiation
Microglia progenitors
Microglia
CD14
CX3CR1
Iba1
P2RY12
CX3CR1
TMEM 119
CD11c
CD11b
Day 0
BMP4
Day 4
bFGF, SCF, VEGFA
Day 6
Day 14
Flt3, IL3, M-CSF, SCF, TPO
Flt3, IL3, M-CSF, GM-CSF
Day 25-40
Day 45-60
GM-CSF, IL-34
b
Day 0
100 µm
c
Day 16
d
Day 30
e
Day 45
f
TMEM119
10 µm
g
Iba-1
h
Ctr
300 µm
i
LPS 100 ng/ml

Figure 1

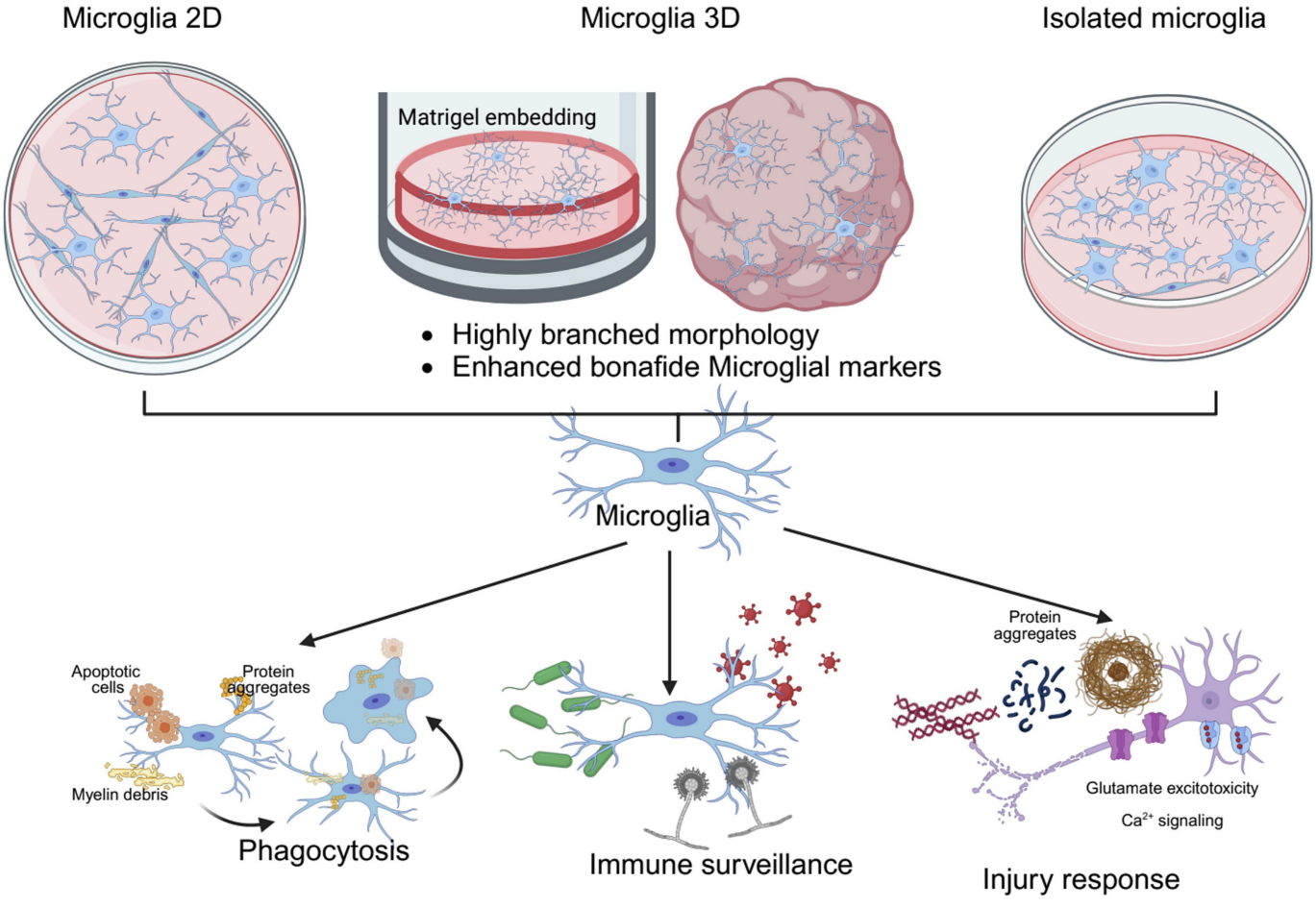

Microglia 2D
Microglia 3D
Isolated microglia
Matrigel embedding
• Highly branched morphology
• Enhanced bonafide Microglial markers
Microglia
Apoptotic cells
Protein aggregates
Myelin debris
Phagocytosis
Immune surveillance
Protein aggregates
Glutamate excitotoxicity
Ca2+ signaling
Injury response

Figure 2

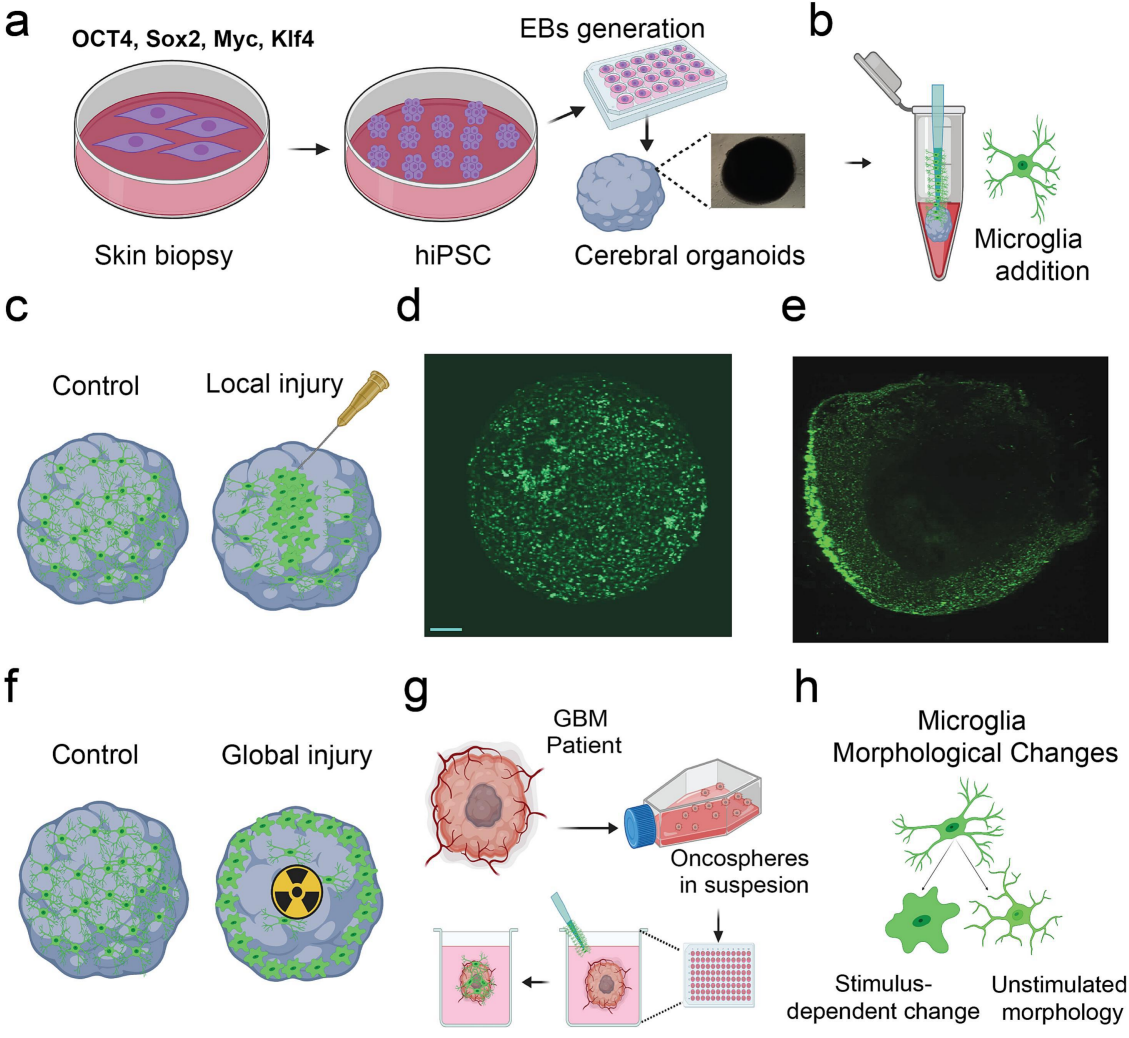

a
OCT4, Sox2, Myc, Klf4
EBs generation
Skin biopsy
hiPSC
Cerebral organoids
b
Microglia addition
c
Control
Local injury
d
e
f
Control
Global injury
g
GBM Patient
Oncospheres in suspesion
h
Microglia Morphological Changes
Stimulus-dependent change
Unstimulated morphology

Figure 3

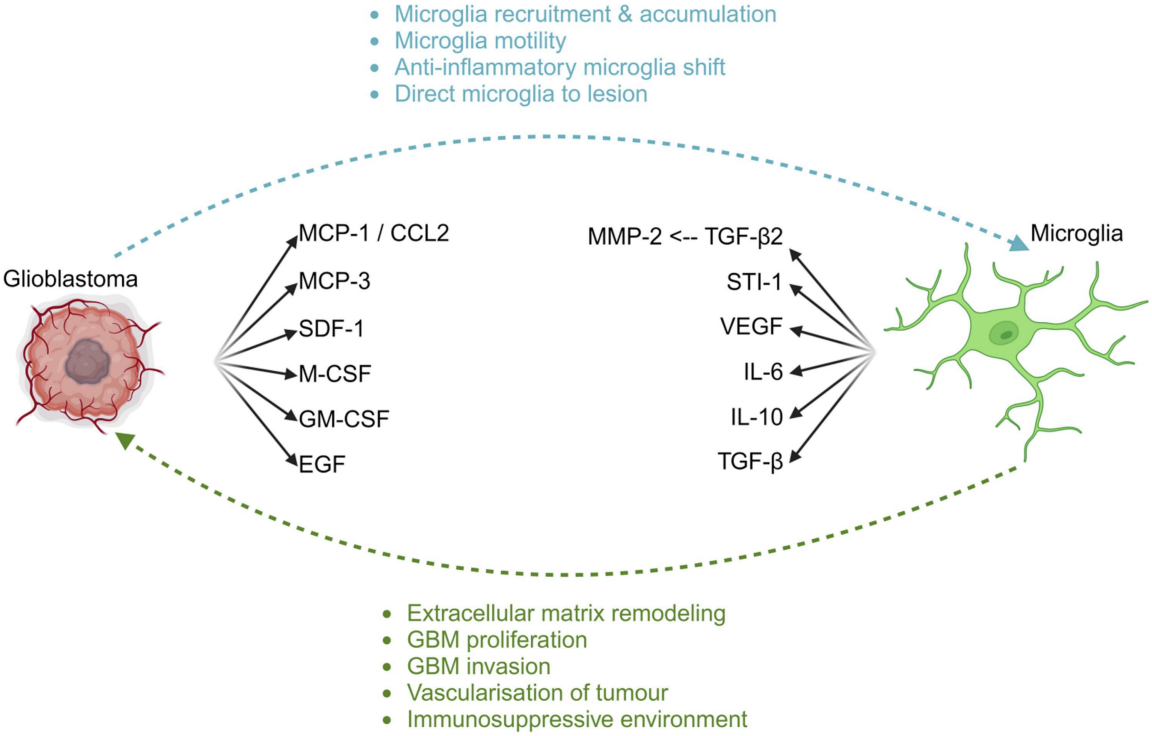

• Microglia recruitment & accumulation
• Microglia motility
• Anti-inflammatory microglia shift
• Direct microglia to lesion
Glioblastoma
MCP-1 / CCL2
MCP-3
SDF-1
M-CSF
GM-CSF
EGF
MMP-2 <-- TGF-β2
STI-1
VEGF
IL-6
IL-10
TGF-β
Microglia
• Extracellular matrix remodeling
• GBM proliferation
• GBM invasion
• Vascularisation of tumour
• Immunosuppressive environment

Figure 4