Unraveling molecular signaling in neurodegenerative diseases
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CHAPTER 7
Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

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Abstract

The main immune surveillance cell of the brain, microglia, regulate synaptic pruning during development and induce or modulate inflammation during aging or chronic diseases. There is an increasing interest in physiological and pathophysiological functions of microglia, because they are sensitive to brain injury and disease, altering their phenotype and function to adopt a so-called activated state in response to any perceived threat to CNS integrity. They sense environmental changes, migrate towards the injury site, respond by releasing various factors and phagocyte microbes, dead cells, redundant synapses, protein aggregates, and cellular debris. Microglia, as the primary source of pro and anti-inflammatory cytokines or chemokines, are essential mediators of neuro-inflammation, being responsible for a large spectrum of cellular responses. Here, we show an overview of the role of microglia in human neurodegenerative diseases and provide an update on the current model systems to study microglia, including cell lines, iPSC-derived microglia, assembly or integration into 3D brain assembloids. We present various strategies to model and study their role in neurodegeneration, that provide a relevant platform for development of novel and more effective therapies.

Keywords: Microglia, organoids, neurodegenerative diseases

Introduction

Microglia are the most important immune cells of the central nervous system (CNS), being the first line of immune defense. They were first identified by Pio Del Rio Hortega in 1919 and described as cells with phagocytic capacity in brain tissue [1]. Microglia represent 0.5%–16.6% of the total number of cells in the human brain [2], highly depending on the brain region. Their origin during development has been under constant debate and it had been thought that microglia had the same origin as the hematopoietic stem cells (HSC), but nowadays it is widely accepted that the brain’s immune cells derive from the early myeloid progenitors in the embryonic yolk sac (YS) at embryonic day 8.5, and are ontogenetically distinct from peripheral macrophages [3,4]. During embryonic development, these myeloid progenitor cells migrate via neural tube, colonize the entire parenchyma, and become microglia progenitors [5,6], which will mature to microglia cells. After birth, brain microglia already exhibit their definitive local density, following a wave of microglial changes and proliferation. During an organism’s lifetime, the microglial population is long-lived and maintained by a high proliferation rate that accounts for their self-renewal, similar to the behavior of peripheral tissue-resident macrophages, such as lung macrophages. Whether infiltration of peripheral monocytes in the brain during the adulthood contributes to the total number of adult brain microglia is not clearly known. However, recent studies suggest that intrinsic apoptosis and self-renewal by several proliferation cycles maintain a relatively steady number in our brain microglia without any contribution of monocyte infiltration [7]. Microglia function as the brain sentinels by constantly scanning their environment for cell debris, possible infectious agents, and misfolded plaques that reside in the brain of patients suffering of neurodegenerative disorders. The mature cells have a pivotal role in physiological and pathological conditions, mainly in aging and age-related neurodegenerative diseases, but also in other conditions such as brain infections and psychiatric disorders [8–10].

Lessons from microglial ontogeny and its destitution

Most of the cells that comprise the brain parenchyma come from the neuro-ectoderm. However, microglia have a crucially different developmental origin. These developmental differences are intimately related to their function in embryonic and adult life [11]. It is reasonable to think that because microglia are tissue-resident macrophages of the CNS, they share most of their developmental signature with other tissue-resident macrophages [12]. Nevertheless, developmental studies have demonstrated that within the mesoderm-derived myeloid cells, microglia have still a different ontology. In this section, we will comprehensively delineate microglial development.

In mice, tissue-resident macrophages arise from erythromyeloid progenitors (EMP) in two waves of production: an early primitive in the extra-embryonic yolk sac (YS) and a transient definitive, before the establishment of definitive hematopoiesis in the fetal liver and later in
the adult bone marrow. Microglial origins can be traced to the primitive hematopoietic wave of early EMPs at embryonic day 7.5 (E7.5) in the YS. This process is dependent on the transcription factors Spf1 and Irf8 and on colony-stimulating factor 1 (CSF1) signaling. Primitive macrophages from this first wave spread via the embryonic bloodstream and colonize the neuro-epithelium as early as E9.5. Conversely, other tissue-resident macrophages primarily develop from the transient definitive production wave of EMPs from the YS that colonize the fetal liver from E10 onward and mature into tissue macrophages through a monocytic intermediate. Definitive hematopoesis depends on the transcription factor Myb. Genome-wide transcriptome and epigenome studies of mouse microglia showed that microglia cluster very different from other tissue macrophages and other glial cells. However, there is no clear understanding of mechanisms underlying this dichotomy between macrophages and microglia.

The brain is an immune-privileged organ, with a blood-brain-barrier (BBB) which prevents peripheral macrophages and immune cells from infiltrating into brain parenchyma. Self-renewing resident macrophage cell should migrate there in earlier developmental stages, prior to the establishment of the BBB at E10. As the brain develops, microglia must undergo changes in function to support neurogenesis and synaptic pruning. An important transcriptomic and epigenome study demonstrated that microglia cluster in three main groups: early microglia (untill E14), pre-microglia (E14 to few weeks after birth) and adult microglia (few weeks after birth onward). Each of these phases had unique regulatory elements. For instance, disruption of adult-specific transcription factor MAFB led to a disorder of microglial homeostasis. Additionally, germ-free mice and offspring with antecedent of maternal immune activation exhibited dysregulation of adult-specific genes in early microglial stages, impacting microglial function, even demonstrating behavioral features. These findings support that the microglial developmental program is in synchrony with the developing brain and that genetic and environmental perturbations will affect brain homeostasis through microglia.

Most of microglial ontogeny studies have been performed in mice, making room for important debates about interspecies similarity regarding CNS microenvironment, immune system evolutionary divergence, mouse models for neurodegenerative diseases. In human embryos, IBA1 positive microglia are present at gestational week 5.5 in the encephalon, and enter the brain through the ventricles. All in all, the ontogeny of human microglia is a topic that remains to be studied in detail and its understanding is crucial for detection of strengths and limitations of murine findings.

Microglial depletion is a useful approach to study microglial biology in vivo. Absence of microglia in mice has been used to study repopulation capacity of microglia, as well as to interrogate fundamental mechanisms in neurodegeneration. Overall, there are two main approaches for microglial ablation: pharmacological and genetic interventions.

Homeostatic microglia express CSF-1 and survival of microglia depends on CSF-1R signaling. An elegant proof-of-concept experiment in which a CSF-1R inhibitor that crosses the BBB was administered to mice that reported a yellow fluorescent protein (YFP) for microglia demonstrated that CSF-1R blockade effectively clears the microglial population. Moreover, this clearance was harmless for mice. CSF-1R inhibition has been used to investigate microglia-dependent mechanisms in different neurodegenerative disorders. There are contradictory findings about the effects of microglia depletion in mice administered CSF-1R inhibitors, PLX5662 and PLX3397. For instance, PLX5662 exhibited neuroprotection and reduced leukocyte infiltration, whereas PLX3397 presented exacerbated neuro-inflammation. PLX5662, which is more brain-penetrant than PLX3397, successfully depletes microglia; and subsequent repopulation after depletion elicited anti-neuro-inflammatory effects, promoting brain recovery. These findings suggest that after acute microglia depletion and repopulation may have beneficial effects in neurodegeneration. Another CSF-1R inhibitor, GW2850, depleted microglia and macrophages at the same time. It may be that at early stages of development macrophages become sensitive to this drug, or that the treatment is not specific for CSF-1R and suppresses innate immune responses. Further research will interrogate ways to effectively deplete microglia with little side effects on other cell types employing the CSF-1R inhibition.

Hippocampal injections with liposomal clodronate, a bisphosphonate that induces apoptosis in phagocytic cell types, deplete Iba1 positive microglia in vivo. Interestingly, a developmental role for microglia has been strengthened by different effects of acute depletion in adult mice, and in early post-natal life. With the discovery of microglial-specific markers, genetic depletion of microglia has become attainable. However, mice undergo severe developmental defects and rarely survive to adulthood. With the introduction of novel genetic techniques, microglia depletion in adulthood is attainable, more specific and efficient than pharmacological approaches. For instance, expression of the suicide gene herpes simplex virus thymidine kinase (HSVTK) and its mutant version under the CD11b promoter decrease inflammation. HSVTK promotes apoptosis after administration of ganciclovir. Another genetic approach is the administration of diphtheria toxin (DT) to transgene mice that express diphtheria toxin receptor by Cre-mediated recombination driven by the CX3CR1 promoter. Both genetic methods reach up to 90% efficiency and are highly specific for microglia. With these powerful approaches, it remains in the outlook to interrogate the importance of specific microglial subpopulations and developmental stages.

The role of microglia in the CNS
Under healthy physiological conditions, microglia plays an important role during prenatal development when they support neurons and axons to form prenatal connections. During neurogenesis, microglia are able to phagocyte apoptotic neural stem cells. Afterbirth,
Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

Microglia remain as the main responsible for removing not functional or redundant synapses, also called neural pruning [45-49]. On the other hand, they are essential in regulating the synapse strain and plasticity, by releasing different molecular signals, such as reactive oxygen species (ROS), nitric oxide (NO), neurotrophic factors and proinflammatory cytokines [50].

Microglia cells are crucial to CNS homeostasis. They protect neurons against NMDA-induced toxicity, and they are able to communicate with astrocytes to increase the effectiveness and to guarantee the most suitable microenvironment [51-53]. Furthermore, as the macrophages of the CNS, microglia can capture antigens via phagocytic and endocytic receptors, process antigens by the lysosomal machinery, express the major histocompatibility complex class II (MHC class II) and exhibit the peptides, as antigen-presenting cells [54]. Microglial activation leads to morphologic changes, which is one of the possible different ways to classify microglia. The “resting” microglia was described as a state that the microglia cells receive inhibitory signals from the CNS environment but still alert with their highly motile processes [21,55]. The “activated” microglia, has been related to a transformation in morphology in contact with foreign substances, releasing pro-inflammatory mediators as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) [56,57]. The “alternatively activated” microglial phenotype, has been characterized as a phagocytic and an anti-inflammatory morphology, releasing protective and trophic factors [58]. However, recent studies suggest that this classification may be ineffective because due to a wide spectrum of microglial phenotypes [59,60].

Neurodegenerative diseases and microglia
Microglial activation has been associated with several disorders, including neurodegenerative diseases such as Alzheimer Disease (AD), Parkinson Disease (PD), Amyotrophic Lateral Sclerosis (ALS) [61]. These diseases have different protein markers and symptoms, but they have in common a similar physiopathology: accumulation of misfolded proteins that entails intracellular inclusions and followed by neuronal death [62]. As microglia is responsible for CNS homeostasis and phagocytosis, they can become activated in the presence of misfolded proteins, and could initiate molecular pathways detrimental to survival or surrounding cells, such as neurons, by releasing cytotoxic and pro-inflammatory factors [63,64].

Alzheimer disease
Alzheimer Disease (AD) is characterized by progressive neuronal loss in brain regions responsible for learning and memory, being highly associated with dementia. AD represents around 50-75% of dementia patients. 95% of AD cases are sporadic cases, and just 5% is considered familial AD, but in both cases, there is a multifactorial etiology behind [65]. Mutations in ApoE genes are related to late AD development [66], while vascular risk factor is the most related modifiable risk [67]. AD normally occurs in elderly people, and it is very difficult to diagnose at the beginning of symptoms. However, the pathophysiology of AD is well known, and the major markers at the CNS are the beta-amyloid plaques (βA) and neurofibrillary tangles (NFT) [68]. In this scenario, microglial role is crucial at the early stages because they possess the ability to remove the amyloid aggregates. However, with aging, this microglial ability to clear up the debris or apoptotic neurons begins to decrease, in parallel to the inflammatory signals that are increasing. Changes in the cytokine profile and inflammatory markers were detected in brain microglia of post mortem patients, which showed high levels of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β [69,70]. These data were corroborated by observations derived from brain tissue from AD patients, where microglia (positive for Iba-1 marker) lose motility necessary to assist neurons and exhibit high expression of cytokines receptors. Oppositely, other microglial proteins (CD68, MSR-A), the role of which is clearance of damaged cellular material, are positively associated with AD and impaired cognitive function [71].

During aging, microglial capacity to clean up βA decreases, leading to βA accumulation, and inflammation, that in turn facilitates more βA formation and aggregation, leading to a vicious detrimental cycle. Consequently, the AD progression becomes unavoidable, and the sum of it all entails neuronal death, with the activation of caspase-3, 6 and 8 which initiate apoptosis [72]. The initial microglia activation is beneficial up to a limit when it starts to be harmful by releasing cytokines and facilitating further βA aggregation [73].

Parkinson disease
Parkinson Disease (PD) is a neurodegenerative disorder that affects 1-3% of the world population above 60 years [74]. There are two different types to develop PD: genetic hereditary or familial and sporadic disease [75,76]. Therefore, multiple mechanisms lead to the same pathophysiology in the PD brain: the loss of dopaminergic neurons as an essential feature, together with α-synuclein aggregation [77].

In healthy physiological conditions, genes, such as protein declycase DJ-1, Phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1), Parkin, and Leucine Rich repeat kinase 2 (LRRK2), regulate microglia function - inflammation, surveillance and phagocytosis, and those genes are the most affected in PD [78-79]. These changes lead to an increase in the markers of inflammation and an increase in ROS associated with a loss of DJ-1 and PINK1 function and gain of Parkin and LRRK2 function [80-82]. There is no doubt about the crucial role of microglia in PD pathophysiology, by releasing more proinflammatory substances, which accelerate neuronal cell death. The understanding of PD pathology is hampered due to the disease complexity.

Amyotrophic lateral sclerosis
Amyotrophic Lateral Sclerosis (ALS), a motoneuron degeneration of the CNS, is a multifactorial disease, with 5-20% of cases having a hereditary component while the majority are sporadic cases [83]. The ALS physiopathology involves changes in various physiological pathways,
including increased oxidative stress, reduction of neurotrophic support, failure in protein homeostasis and RNA processing. In ALS patients, motoneuron degeneration has been associated with stimulation of excitotoxic pathways, glial inflammation [84], and microglial activation. Infiltrating lymphocytes at sites of motoneuron injury are highly correlated with the disease severity [85,86]. These processes contribute to the release of pro-inflammatory cytokines and chemokines, a decrease of neurotrophic factor expression and release, and an increase in the secretion of neurotoxic factors, that ultimately contribute to motoneuron cell death and neuronal network degeneration [87].

Huntington disease
Huntington's disease (HD), an autosomal dominant neurodegenerative disease is characterized by progressive motor dysfunction, cognitive impairment, and is accompanied by neuropsychiatric symptoms characterized by progressive motor dysfunction, cognitive impairment, and is accompanied by Huntington's disease (HD), an autosomal dominant neurodegenerative disease. The mutant protein in HD, huntingtin (mHTT) results from expanded CAG repeats and contributes to the formation of a polyglutamine strand of variable length at the N-terminus [88]. Although the pathogenic mHTT is ubiquitously expressed in the CNS and also different types of neuronal cells, it causes a preferential damage and cell loss in the striatum, particularly affecting medium spiny neurons. As HD progresses, the atrophy of caudate and putamen expand to surrounding brain areas, reaching the cerebral cortex [89]. At the cellular level, mHTT proteins promote neuronal dysfunction and cell death through a number of molecular mechanisms, including disruption of cellular proteostasis, transcription and mitochondrial structural and functional alterations [89]. Marked astrogliosis and microgliosis were detected in post-mortem brains of HD patients, while in healthy brains these processes were absent [90]. It was reported that microglial activation and its associated structural alterations were present in all grades of HD patients’ brains, and the structural alterations correlated to the degree of neuronal dysfunction [91]. Increased immune activation in the CNS and peripheral immune system in HD has been described by identifying increased IL-6, IL-8 and chemokines such as eotaxin-3, MIP-1β, eotaxin, MCP-1 and MCP-4 in plasma [92,93]. Monocytes isolated from HD gene carriers, which highly express mHTT, are pathologically hyperreactive in response to various stimuli, including lipopolysaccharide (LPS) stimulation [94]. Therefore, a hyperreactive immune system, together with microglial activation, have been recognized as an important feature of HD.

Other neurodegenerative diseases such as multiple sclerosis, and frontotemporal dementia, highlight the key role of microglia in both cellular homeostasis and neurological disorders that affect millions of people worldwide (Figure 1) [95,96]. In conclusion, better understanding of physiological and pathophysiological mechanisms in microglial biology is fundamental to elucidate ways to tackle progressive neurodegeneration.

Figure 1. Microglia in neurodegenerative diseases. Microglia serve numerous functions in the brain, including synaptic pruning, phagocytosis, secretion of growth factors to maintain homeostasis, immune surveillance, shaping axonal projections, among others to control the homeostasis. Usually, microglia is activated in response to different stimuli, such as protein aggregates, myelin debris, apoptotic cells, etc. Its answer is described as reparative or toxic depending of the anti-inflammatory or proinflammatory factors secreted, respectively. AD: Alzheimer disease; ALS: Amyotrophic lateral sclerosis; CTE: Chronic traumatic encephalopathy; ECM = extracellular matrix; FTD: frontotemporal dementia; FTLD: frontotemporal lobar degeneration; HD: Huntington disease; HTT: huntingtin; PD: Parkinson disease; mSOD: superoxide dismutase 1; ROS = reactive oxygen species; TLR = toll-like receptor.

Models to study microglia function in the CNS

In vitro studies on microglial cells provide a good platform to understand fundamental questions on microglial biology under healthy and conditions modeling various brain pathologies. Currently, there are five main ways to culture microglia and perform experiments to learn more about the balance between the beneficial and detrimental role of microglia: cell lines (murine or human), primary microglia (mainly murine), stem cell-derived microglia (murine and human), organotypic brain slices of transgenic animals and brain spheroids/organoids.

Cell lines
Cell lines are immortalized cells, collected and generated by treatment with oncogenes, that induce them to become immortalized, although cells might undergo spontaneous immortalization as well. However, immortalized cells can be very sensitive to differentiation,
which represent an inconvenience. There are various cell lines generated from rat, mouse, macaque, and human. From rat, the most studied microglial-like cell line is HAPI - highly aggressively proliferating immortalized - and it is considered the first cell line generated by unprompted immortalization, although the exact mutation that enables immortalization is not yet known. The most numerous protocols for microglial cell lines are generated from mouse, with the cell line BV2 being the most widely used. BV2 cell line was generated via v-raf/v-myc oncogenes. These cells respond to LPS, are able to phagocyte and increase pro-inflammatory gene in response to LPS or various stimuli, mediate increases in ROS levels after exposure to βA fibrils and α-synuclein [101–103]. Meanwhile, HMO6 cells are derived from embryonic human primary microglia with a v-myc oncogene carrying PASK 1.2 retroviral vector.

These cell lines show distinctive microglial/macrophage cell markers and similar behavior (cytokine release, migration, and phagocytosis) in the presence of the gram-negative bacteria lipopolysaccharide (LPS). However, it has been described that IL-1β release and NO production in BV2 and HMO6 cells differs from primary microglia. Likewise, Nagai and collaborators demonstrated important differences in protein profile and mRNA expression after βA induction between primary microglia and human cell lines. Protein profile and mRNA expression after LPS or βA induction were distinct from human microglia responsiveness.

Butovsky et al. showed that the treatment of N9 and BV2 cells with MCSF macrophage colony-stimulating factor and TGF-β 1 did not induce the expression of microglial molecular pattern as the adult microglia. These studies underline the limitation of microglial cell lines in terms of molecular expression, morphology, proliferation, and adhesion.

**Primary microglia**

Primary microglia cultures can be extracted from non-human primate, rodent, or human brains. All three cultures have positive and negative features in relation to microglial function. Cells from rodents are easier to obtain because the animal's centers are accustomed to raising them. However, many unanswered questions remain about the use of rodents, concerning developmental, genetic, and physiological differences among human and mice. Studies have shown many divergences in mice and humans' embryonic development, particularly during gastrulation and organogenesis. An extensive comparison in microglial gene expression of human and mice resulted in critical differences, principally in aging-related genes. Moreover, in uncommon cases, the same genotype could trigger divergent phenotypes in both rodents and humans.

On the other hand, in most studies, human brain tissue was derived from neonatal donors and humans, especially in the context of elderly, increase the need for new and relevant models to study neurodegenerative disorders and also microglial biology related to healthy aging.

Although non-human primates are evolutionarily closer to humans, and they are in specialized centers what allows control postmortem conditions of brain tissue, they are difficult to raise, and just few centers are able to handle them, therefore, primary microglia from them is not widely used. On the other hand, primary microglia from humans started to be more commonly used and these microglia are considered one of the best option to study and understand the human microglial biology. However, there are two principal technical features that hamper their study in age-related neurodegenerative diseases: i) the difficulty in obtaining healthy samples, since one can only obtain brain tissue from abortion, and this young microglia is quite distant and different from old, more mature microglia, or ii) from autopsies, where it is impossible to control antemortem conditions; and the existing microglia phenotype, which can be altered by the delay in withdrawing the brain tissue. Considering these impediments and limitations, microglia extracted from autopsies of patients suffering from different neurodegenerative disorders could provide answers on their implication on the molecular pathways that link disease pathogenesis and the degree of brain degeneration.

**Organotypic brain slices**

The study of microglial function in neurodegenerative conditions presents various technical complications. First, the most accepted in vivo and in vitro models of neurodegenerative disease recapitulate key molecular phenotypes, but they do not accurately replicate disease progression and/or associated pathology (i.e. hyper-phosphorylated tau without neurofibrillary tangles, APP isoform influence AD progression, over-expression of tau impairs motor function and interferes with cognitive assessment). Second, efforts towards ex vivo approaches, such as primary microglia obtention, present technical culprits regarding the physiological state after dissociation of brain tissue and culture. Third, the co-culture system of glial cells and neurons, which aims to mimic the complex brain microenvironment, does not accurately replicate it. Efforts to study microglial function without acutely affecting microglial microenvironment and interactions have risen, in particular with the use of organotypic brain slices.

An important feature of the organotypic brain slice culture system is that it preserves the cytarchitecture of the brain, which is close to an in vivo-like situation. Moreover, the maintenance of the tridimensional structure and architecture of the brain preserves critical cellular interactions, making possible to perform dynamic profiling and long-term cellular tracking. Working with brain slices reduce, refine and replace animals used and increase the capacity to perform medium-to-high throughput drug screenings. Furthermore, brain slices can be obtained from embryonic, neonatal and adult specimens, highly valuable for neurodevelopmental stages and neurodegenerative conditions.
Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

slices are a valuable model for microglial research, while there are some considerations when employing brain slices: although they may be a good in vivo replacement, behavioral testing and correlation cannot be performed. Brain slices are axotomized, and there is an acute inflammatory response to cutting which is driven by astrocytes [121]. Microglia undergo morphological changes in the first days in vitro (DIV), but after 10 DIV, microglia return to an in vitro-like situation [122]. Lastly, they lack blood flow, limiting their viability. In spite of this, efforts to extend viability have led to up to 28 DIV [122] of life-time. All in all, brain slices may provide acceptable replacement for in vivo studies to elucidate microglial physiology.

In homeostasis, microglia support neurons by secreting neurotrophic factors, and clearing debris in a house-keeping manner [125,127], in neurodegenerative disease, microglial function is profoundly impaired [128] perpetuating chronic neuroinflammation. These important interactions are not entirely recapitulated in cell culture systems. For instance, functional studies of isolated microglia are confounded by the disruption of the microenvironment [126]. The slice culture system is a well-established model [129–131] to study microglial function as close to as in situ as possible. A valuable tool to study microglial function is their depletion [130] in brain slices. Jung et al have successfully depleted microglia in brain of microglia/macrophage reporter mice with the toxin clodronate [133], paving the way for microglial depletion in brain slices [134–137]. Hellwig and colleagues have demonstrated for the first time that microglia prevent amyloid burden in brain slices from wild-type mice by depleting them, strengthening their phagocytic role [136]. Following this approach, a phagocytic and chemotactic role for microglia in AD has been proposed by Daria and colleagues. In their study, they employed AD mice brain slices that were treated ex vivo with clodronate to deplete the endogenously expressed microglia. By replenishing the brain slices with either young or old microglia, the amyloid burden present in the brain slices was decreased and correlated with microglial recruitment to the plaque. Moreover, exposing old microglia to secreted factors of young microglia or supplementing the culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) could elicit functional recovery of old microglia and even reduce amyloid plaque size [124]. Overall, these findings indicate the critical role of microglia in clearing protein aggregates and propose potential therapeutic approaches aimed to reinforce microglial phagocytosis to revert neurodegenerative disease pathology.

Induced Pluripotent Stem Cells (iPSC)

Stem cell biology is evolving every day. Microglia can be generated from two different types of stem cells: embryonic stem cells (ESC), derived from a blastocyst, and induced pluripotent stem cells (iPSC). Although ESC can be reprogrammed in any cell type, currently the majority of the protocols of human microglial generation are not employing ESC as a primary cell type. On the contrary, iPSC technology is widely used to differentiate and study microglial biology. The iPSC can be produced from adult human cells, which have undergone a reprogramming via overexpression of immature state-specific transcription factors (OCT4, SOX2, KLF4, and c-MYC) knowns as Yamanaka reprogramming factors [130]. With the help of these factors, the nullipotent, mature cell is able to convert its fate backwards into a pluripotent cell, embryonic-like state. iPSC technology was particularly innovative, as it allowed it for the first time to evaluate the effects of a particular gene on familial monoallelic diseases as well as complex non-familial idiopathic diseases. The latter studies are performed using iPSC patient-derived cells, that are differentiated into a plethora of brain cells, including neurons, astrocytes, oligodendrocytes and recently microglia, systems that were previously not easily available for experimental investigation [139,140]. As a consequence, primary microglial cultures, organotypic brain tissue culture systems of transgenic animals and emerging iPSC-based microglia represent valuable experimental systems to study human familial neurodegenerative diseases and age-related non-familial neurodegenerative diseases [141,142].

Recent studies have shown that human aging can be modeled across direct cell type conversion in any kind of cell and transcriptomic signatures of their donors age do not disappear after reprogramming protocols, indicating the importance of iPSC tools to study age-related diseases [143,144]. Nonetheless, iPSC technology has its own limitations. It was suggested that residual epigenetic features from donors might sometimes persist in iPSC [145–147] and most cases of degenerative diseases have multifactorial risks, which is hard to mimic in vitro [144,148].

To reverse this scenario, new techniques are emerging to induce and even accelerate aging. iPSC-neurons were submitted to general stressors such as hydrogen peroxide, MG-132 and concanamycin [149,150], showing promising results; however more tests are necessary to secure their reproducibility, since cells showed differential vulnerability to various stressors. Other experiments performed for inducing an accelerated aging phenotype, a progerin-a protein involved in Hutchinson-Gilford progeria syndrome, was overexpressed that initiated age-related markers and cell death pathways in neurons [151,152].

Despite all this, iPSC-derived neurons and glial cells have been used to answer various questions related to their relevance to model human diseases and also their experimental practicality. Disease modeling and drug testing seem to be the mainstream end goal on using iPSC technologies, in addition to 3D models to study the CNS cell interactions that are generally difficult to mimic in a 2D in vitro monoculture [141,153].

Microglia differentiation protocols

Since the discovery of the exact origin of microglia, few protocols have been described to generate microglia from iPSC. Differentiation of iPSC to microglia is really new, and the first protocol appeared in 2016. The first author to publish was Mussati et al. 2016 [154], followed by Abud et al. 2017 [155], Douvras et al. 2017 [156], Haenseler et al. 2017 [157], Pandya et al. 2017 [158], Brownjohn et al. 2018 [159], Garcia et al. 2018 [160] and McCuade et al. 2018 [161] as illustrated in Figure 2. These reported protocols share similar patterns in the microglial differentiation steps, however the most common denominator is represented by colony-stimulating factor 1 (CSF1) receptor ligands. CSF1-related pathways are required for macrophage proliferation, differentiation and even its own survival. Bone morphogenic protein 4 (BMP4) is also commonly applied during the first days of iPSC differentiation. BMP proteins are known to inhibit neurogenesis and induce neural stem cell (NSCs) glial differentiation in the adult CNS, particularly in subventricular zone, that results in the reduction of the stem cell pool [162,163].
BMP4 proteins belong to transforming growth factor β (TGF-β) superfamily, and beside their function in bone formation, they regulate proliferation and differentiation, cell-fate determination, and apoptosis. Some protocols employ embryonic bodies (EB) as an early step in the microglial generation and CSF1/IL-34 or IL-3/M-CSF as differentiation factors. To overcome potential variability in EB formation, and related batch-to-batch variability, Abud and colleagues directly differentiated iPSCs into hematopoietic progenitors by using FGF-2 and BMP4. In addition, they have used secreted proteins by neurons/astrocytes/endothelial cells to mimic the natural microglial environment, by adding factors like TGF-β. All protocols validated differentiated microglial-like cells, by assessing their capacity to migrate, secrete cytokine/chemokine and also phagocyte, common functions mediated by human brain microglia.

Muffat et al. 2016, elaborated a protocol to create microglia from iPSC and ESC. First, human embryonic stem (hES) and induced pluripotent stem (iPS) cells have been cultivated in hES medium. Cystic and neutralized embryonic bodies (EB) were produced in a serum-free medium containing IL-34 and colony-stimulating factor 1 (CSF1). After 14 days, early yolk-sac myelogenesis markers became detectable, including VE-cadherin, c-kit CD41, CD235a, and, mainly PU.1, which is crucial for microglia maturation and viability. Subsequently, EBs positive for yolk-sac markers were passed to polystyrene plates, where, after 30 days, semi-adherent cells exhibited a highly motile morphology and stained positive for markers such as PU.1, CD11b, and allograft inflammatory factor 1, which are well-defined microglia markers in several species. The protocol expands over 56-60 days and the reported microglial yield was 1–8 x 10⁶ pMGLs from 2 x 10⁶ hPS.
To test the effectiveness of this protocol, Muffat et al., analyzed chemokines and cytokines under stimuluated and stimulated conditions with interferon y (IFN-γ) and LPS. Before stimulation, the generated microglia, termed pluripotent stem cell-derived microglia-like cells (pMGLs) released various types of cytokines and chemokines, including IL-8, C-X-C motif chemokine ligand 1 (CXCL1) and C-C motif ligand 2 (CCL2). Under stimulation, the cells released these substances, but above baseline, in particular, CXCL10, CCL3 (or MIP1A), IL-6 and TNF-α - those last two were highly released and also expressed, as detected at transcriptional levels. Moreover, pMGLs appeared as a vastly ramified structure with slim end filaments, which resemble the phenotype of primary microglia. Transcriptomic data shown that pMGLs clustered with fetal microglia showing a unique signature. Besides, functional assays, as phagocytosis and migration were demonstrated in these differentiated pMGLs [154].

Abud et al. [155] reported that human induced microglial-like cells (iMGLs) could be generated after five weeks from iPSCs. First, iPSCs were differentiated into hematopoietic progenitors (iHSC) CD43+/CD235a+/CD41+. After ten days, iHSC CD43+ were cultivated in a serum-free differentiation medium containing CSF-1, IL-34, and transforming growth factor-β1 (TGFβ1). After more 14 days, the cells were positive for PU.1 and receptor expressed on myeloid cells 2 (TREM2), and were grouped in CD45+/CX3CR1- and CD45+/CX3CR1+, which occurs equally in vivo. Following 35-38 days of differentiation, iMGL resemble human microglia and their gene profile started to diverge from macrophages and monocytes gene profile. They express several proteins such as MERTK, ITGB5, CX3CR1, TGFβR,1 and PROS1, which are microglial-enriched proteins, and purinergic receptors as P2RY12 and TREM2 [166]. Differentiated mature microglia was generated over a period of 38-40 days and the reported yield was 3–4 × 10⁶ iMGLs from 1 × 10⁶ hPSCs.

Culturing iPSC-derived microglia with factors that are normally produced and released by the surrounding healthy brain cells enabled them to exhibit a transcriptomic profile similar to human fetal and adult microglia. Remarkably, this transcriptomic profile is distinct from monocytes or blood dendritic cells. These factors include CXCL1, CD200 and TGFβ that highly mimic the surrounding microglial environment in the CNS, and provide a functional and relevant model to study microglial functions.

On transcriptomic level, iMGL clustered with human adult and fetal microglia [167,168]. Moreover, analyzing the cytokine/chemokine secretion and phagocytosis capacity the authors showed that iMGLs respond to their surface’s receptors stimuli, resembling primary microglia activity. Their resemblance with primary microglia function was also demonstrated by the capacity of iMGLs to phagocyte human synaptosomes. Besides, iMGL responded well to ADP stimuli and were able to phagocyte fluorescently-labeled fibrillar AB and pHodo-labeled brain-derived tau oligomers, indicating that this iMGL might represent a relevant model system to study AD pathology [169,170]. Moreover, iMGLs were co-cultured with rat hippocampal neurons, which increased the gene expression of neuroprotective function and decreased pro-inflammatory genes. Additionally, they included iMGLs in the brain cortex of mice and demonstrated the cells ability to engrave and survive into real CNS environment.

Douvaras et al. 2017 [171] developed a protocol in which myeloid progenitors generated microglia-like cells. First, the human PS cells were grown in feeder-free media with bone morphogenetic-protein 4 (BMP4) for four days, to generate primitive hemangioblasts. Then, medium containing basic fibroblast growth factor (BFGF), stem cell factor (SCF), and vascular endothelial growth factor A (VEGF) was added for additional two days. During the next eight days (6-14), the factors in the medium were replaced by interleukin-3 (IL-3), thrombopoietin (TPO), SCF, macrophage colony-stimulating factor (M-CSF) and FMS-like tyrosine kinase 3 (FLT3). From day 14 to 25 medium was supplemented with M-CSF, FLT3, and granulocyte macrophage colony-stimulating factor (GM-CSF). Microglia progenitors CD14+/CX3CR1+ were isolated by FACS and were placed in contact with medium with IL-34 and GM-CSF for one to two weeks to generate microglia. Differentiated mature microglia was generated over a period of 35-60 days and the reported yield was 2–3 × 10⁶ iPSC-MG from 1 × 10⁶ hPSCs. To evaluate iPSC-microglia, Douvaras et al., 2017 clustered iPSC-microglia with human fetal microglia (hMG), and they confirmed the expression of six-genes specific to human microglia [167,171]. In addition to the gene characterization, the cytokine/chemokine profile and the phagocytosis assay validated the functionality of mature microglia. P2RY12, a gene that encodes a G1 protein was capable of inducing intracellular Ca²⁺ transients in response to ADP in iPSC-microglia resembling the activity of primary microglia [172,173].

Haenseler et al. 2017 [157] described a protocol based on the study of van Wilgenburg et al. 2013 [174] generating microglia starting from embryonic-like myeloblastosis (MYB)-independent macrophage precursors. Following one month of differentiation, macrophage precursors were harvested in the supernatant, and the cells were collected and co-cultured with iPSC-derived cortical neurons in a medium enriched with IL-34 and M-CSF. Two weeks following co-culturing, macrophage precursors had a similar phenotype as primary microglia, with ramified branches (co-pMG). Differentiated mature microglia was generated over a period of 30 days and the reported yield was 1–4 × 10⁷ Macpre from 1 × 10⁶ hPSCs. Transcriptomic analysis corroborated than iPSC microglia was clustered with fetal microglia and showed 6 specific genes for microglia; also, protein expression for Iba-1, P2RY12, TMEM119, and MERTK. Besides, gene ontology assays demonstrated a downregulation of genes involved in viral, bacterial and yeast recognition response and upregulation of genes responsible for survival as differentiation, chemotaxis, regulation cell-cell adhesion, and metal ion response was evidenced [175]. iPSC microglia were able to respond to LPS/IFN-γ stimulation and to promote phagocytosis.

Pandya et al., 2017 [174] developed a protocol based on a co-culture with astrocytes where iPSC were cultured in medium with VEGF, BMP4, SCF, and ActivinA for four days. Afterwards, new growth factors were added to the medium (FIt3, IL-3, IL-6, F-CSF) for more ten days. On day 15, cells expressed myeloid progenitors’ markers: CD34 and CD43. These cells were co-cultured with human astrocytes in a medium containing GM-CSF, M-CSF, and IL-3 for additional two weeks. At this stage, the differentiated cells were positive for microglial markers, such as CD11b and Iba1. The protocol expands over 30-60 days and the reported microglial yield was 1–3 × 10⁶ IPS-MG from 1 × 10⁶ hPSC. Gene expression signature of IPS-MG was evaluated and it displayed clusters with human fetal microglia and also with
Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

Chapter 7

Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

Dendritic cells and macrophages, resembling their myeloid lineage. Phagocytic activity and ROS production were assessed using pHrodo E. coli Bio Particles and phorbol myristate acetate (PMA), respectively and demonstrated the capacity of iPSC-MG to respond to various stimuli, including LPS and TNF-α cytokine.

Brownjohn et al., 2018 and Garcia et al., 2018 developed similar protocols to generate microglia from iPSC trying to understand TREM2 mutations in neurodegenerative diseases [159,160]. They divided their protocol into two phases: the differentiation of iPSC in primitive macrophages precursor (PMP) and then in iPSC-microglia. First, embryoid bodies were developed in ultra-low attachment plates with medium containing BMP-4, SCF, and VEGF-121 for four days. EBs were exposed to IL-3 and M-CSF for additional 3-4 weeks. After this, PMP were cultured in enriched medium with GM-CSF and IL-34 for 6-10 days in Brownjohn protocol. In Garcia protocol a 40-micron filter was used to separate cells and the induction of cell maturation was performed in medium containing only M-CSF. Functional studies such as phagocytic assays, injury responses, and physiological responses complemented transcriptomic studies and probed the resembling to primary microglia.

One of the most recent protocol was presented by McQuade et al., 2018. After seeding, cells were exposed to medium A (Supplement A), after three days, the medium was changed to medium B (Supplement B). On day 10-12, non-adherent cells were positive for CD43 and termed Hematopoietic progenitor cells (HPC). Their medium was supplemented with IL-34, TGfβ1, and M-CSF and after additional 4-6 weeks medium was changed with new growth factors (IL-34, TGFβ1, M-CSF, CD200, and CX3CL1) to further ensure the microglial maturation and cellular homeostasis. To evaluate the functional activity of iPSC-microglia, phagocytic activity was demonstrated by exposure of microglia to different stimuli. Transcriptomic analysis of generated iPSC-microglia presented an exclusive profile with genes resembling primary microglia and distinct from hematopoietic progenitor cells, monocytes, and dendritic cells.

The first protocols of microglial differentiation showed maturation and fully functional microglia following 60-75 days in culture. More recent protocols demonstrated that microglia can be differentiated from iPSCs in only 24 days according to Konttinen et al., 2019 [170]. The protocol they developed involved specific oxygen concentrations in the first stages of differentiation. 48 hours after seeding, mesodermal cells were treated with bFGF, VEGF, and insulin to induce hemogenic differentiation. Then, cells were subjected to MCSF1 and IL-34 to induce microglial differentiation. To mature the cells, they were cultured on poly-D-lysine (PDL)-coated plates until D24, when they expressed IBA1. On their study of iPSC-derived microglia, APOE4 mutation exhibited profound impact on fundamental aspects of microglial function such as phagocytosis, migration and metabolism, supporting the hypothesis of impairment of microglial function by APOE4 [177]. Interestingly, APPSw, and Psen1 mutations had minor effects. Moreover, the authors acknowledge that these microglia, may represent relatively young microglia based on the expression of P2RY12, a marker of mature microglia.

Despite encouraging results, caution must be taken to interpret these data, as microglia in this study were differentiated in a fetal bovine serum (FBS)-containing media. FBS is not a well-defined supplement and may promote microglial priming, which can mask differences between groups. All in all, this recent study proposed a short differentiation protocol for microglia.

Transcriptomic analysis of microglia obtained from differentiation protocols.

Efforts to standardize protocols to differentiate microglia from patient-derived iPSCs have led to identification of transcriptomic signatures that support the use of these microglia-like cells as an alternative to human primary microglia. For instance, the transcriptomic clustering approach has been utilized to highlight similarities and differences between microglia-like cells, other CNS-cells, myeloid cells, iPSCs, fetal and adult microglia [134,155,157,160,176].

Regarding their similarity to human microglia, Muffat et al found that their pluripotent stem cell-derived microglia-like cells (pMGLs) did not differ in any of the canonical myeloid ontology terms with human fetal microglia [116]. As the first study to propose the use of iPSC-derived cells as surrogates for human microglia, it paved the way for further transcriptomic and functional characterization of pMGLs. However, this study did not assess the similarity to adult microglia. Later, Abud and colleagues compared their human microglial-like cells (iMGLs) with adult microglia finding important similarities, especially in the expression of CD11b, ITGB2, CSF1R, CD45, IBA1, LGMN [155].

By correlation and principal component analysis (PCA) of whole transcriptome, Abud et al. have shown that their iMGLs do not cluster with other myeloid cells, such as CD14+ CD16− monocytes and blood dendritic cells (DCs) [155]. Additionally, García et al., Douvaras et al. and Haenseler et al. focused on genes proposed by Butovsky et al., 2014 to be preferentially expressed in microglia, such as TREM2, C1QA, TMEM119, GPR34, PROS1 but not in monocytes [116,137,140,178]. These genes showed to be expressed in microglia but not in peripheral blood monocytes and primary macrophages.

More recent protocols employed in studies by McQuade and colleagues focused in the comparison of the so-called iPSC-microglia 2.0 with the previously published iPSC-microglia from Douvaras and colleagues [184]. This study concluded that iPSC-microglia 2.0, which result from a less complex protocol, are virtually identical to iPSC-microglia regarding to their transcriptomic profile. Similarly, they study of Konttinen and colleagues compared their iMGL transcriptome data with Abud’s, concluding that they clustered with human microglia and published iMGLs [117]. All in all, transcriptomic analysis of iPSC-derived microglia-like cells is a valuable tool to understanding function and evaluate expected responses to overcome the culprits and technical difficulties of primary microglia harvesting and culture.
Microglia in brain organoids

Limitations of 2-dimensional (2D) culture systems to replicate and evaluate the human brain pathologies, have led to the generation of 3D culture systems or brain organoids/spheroids/assembloids from human iPSCs by direct replicating the neurological development.

At the moment, there are a variety of strategies available to develop brain organoids/spheroids/assembloids as for other types of tissues or organs, such as retina, intestine, thyroid, liver, inner ear, pituitary gland, and kidney [170-172]. Cerebral assembloids are stem-cell derived models in a three dimensional in vitro culture systems that aim to recapitulate the developmental processes and structural brain organization of the developing or adult human brain [3]. Current 3D assembloids are able to accurately summarize defects in early brain development as nicely demonstrated for microcephaly by Lancaster et al. 2013 [181] and for Zika virus infection by Qian et al. 2016 [182].

To create a 3D assembloid similar to the human brain is complex, as it must contain different neural populations, astrocytes, oligodendrocytes, microglia and the blood brain barrier cell population. Since microglia has another embryonic origin than neuronal or astrocytic cells, the generation of assembloids that are spontaneously populated with microglial cell population. Since microglia has another embryonic origin than neuronal or astrocytic cells, has been proven to be a difficult task [182,184]. However, recent studies proposed novel strategies to create an assembloid with cells of all germinal layers, including microglia. This combination enables a better understanding of a healthy brain and also the pathophysiology of neurodegenerative disorders, since interactions between microgila and macrogia/neurons are crucial during brain development, and aging [185,186].

Brain assembloids have a certain degree of resemblances to in vivo conditions, although some features and cell interactions still cannot be reproduced. Interactions between microglia and brain-blood barrier (BBB) can be relevant in the context of neuroinflammation, considering that microglia could be activated by changes in the BBB permeability, that is often compromised in age-related neurodegenerative diseases [187-189]. Further, Erny and colleagues described the importance of host microbiota to microglia maturation, morphology, and function [190]. More studies are necessary to understand the relationship between microglia and neural populations in neurodegenerative diseases. Moreover, to model neutromiumological interactions in human brain and investigate the consequence of these interactions on brain pathology, researchers combined brain neuronal assembloids with immune cells, such as microglia-like cells [190,191].

Hitherto, only a limited number of studies have showed the interaction of neurons and microglia in a 3D setting, by incorporation of differentiated microglia in brain organoids/assembloids, as illustrated in Figure 3. Muffat and colleagues added the differentiated pMGLs in a 3D brain structure of a three months old spheroid. NPCs were used to generate the 3D brain assembloid (oMG) model and exhibit their 3D characteristic ramified morphology. The

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Figure 3. A scheme for iPSC modeling and microglia in organoids. (a) Generation of organoids using patient-derived iPSCs mainly includes the following steps. First, iPSCs are generated from patients by reprogramming somatic cells using 4 Yamanaka factors, OCT4, SOX2, KLF4, and MYC. Human-induced pluripotent stem cells will create embryonic bodies (EBs) and consequently, cerebral organoids with different growth factors, mediums, and reagents. (b) Graphic representation of direct re-aggregation of microglia, astrocytes and neurons in spheroids or 3D stacks in transwells, according to Muffat et al., 2016 [154]. (c) Abud et al., 2017 [155] showed how ameboid microgila was localized close to damaged area (pierce with 25G needle) in cerebral organoids. (d) Microglia migrate into preformed cortical organoids and assume a pronounced ramified morphology, which is demonstrated by Brownjohn et al., 2018 [159]. (e) Ormel et al., 2018 [192] developed a protocol where the organoid by itself shows microgila after two months.
Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

Chapter 7

Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

oMG was IBA-1 positive following 2 months of culturing in the cerebral organoid. Extensively transcriptomic assays were assessed comparing oMG with adult and fetal primary human microglia, 2D iPSC microglia, iPSC, and fibroblasts. Interestingly, oMG clustered with adult primary microglia, whereas 2D iPSC microglia clustered with fetal primary microglia [190].

Besides, stimulation with pro- or anti-inflammatory triggers - LPS and dexamethasone, respectively – led to an increased cytokine release (IL-6 and IL-1β) in oMG and a comparable response for dexamethasone (CD163 and MRC1) with adult primary microglia.

To understand how microglia affect βA clearance in AD, Lin and colleagues cultured human differentiated microglia with two-month-old familial AD-derived forebrain organoids that have an increased expression of amyloid precursor proteins (APP) [191]. Microglia was generated from iPSC derived from AD patients carrying either a low-risk gene variant (APOE3) or a high-risk gene variant (APOE4). One month of co-culture rendered comparable numbers of microglia integrated into organoids regardless of APOE genotype. However, the morphology and function of microglia-like cells derived from a high susceptibility background, the APOE4 variant was different than the APOE3 microglia-like cells, with longer processes and reduced capacity to phagocyte βA. As a result, organoids populated by APOE3 microglia-like cells contained fewer βA aggregates compared to organoids with APOE4 microglia. Morphological alterations in APOE4 microglia correlated well with the capacity of βA uptake, that potentially restricts the ability of microglia to clear extracellular βA plaques from AD brains [191].

Most of microglial derivations lack specificity regarding region-specific microenvironment. For instance, forebrain microglia depend on IL-34 for maintenance, while cerebellar does not. Work from Song and colleagues was the first to address this issue by generating dorsal and ventral brain organoids and co-culturing them with microglia [192]. Interestingly, dorsal organoids showed higher anti-inflammatory cytokine secretion, while ventral organoids exhibited higher TNF-α expression. Transcriptomic analysis exhibited microglia-specific genes that were differentially expressed in both groups. Regarding disease modeling, findings from this study add a layer of complexity to more accurately resembling diseased region-specific microenvironments.

A more sophisticated cell model system was used by Park and colleagues to study brain cell interactions in a microfluidic-based system. The microfluidic chambers contained neurons and astrocytes differentiated from NPCs cultured together with a human immortalized SV40 microglia-like cell line. The culturing was realized in a microfluidic-based system where neuroimmunologic interactions related to AD pathology could be easily modeled and tested.

Neuronal cells and astrocytes were differentiated from ReNcell VM cells (immortalized hiPSC-derived hNPCs) expressing multiple familial AD mutations, including APP mutations (mAPP). Microglial morphology and activity were altered in the presence of mAPP neurons/astrocytes, with microglia migrating faster towards βA aggregates and causing cell death to mAPP neurons/astrocytes. Using this model system, Park and colleagues tested different pathways targeting microglia-neuron interaction, including anti-CCL2 neutralizing antibodies or knockdown of TLR4 in microglia. These strategies reduced microglial migration and also neuronal toxicity of mAPP neurons/astrocytes, providing insights into the cytokine signaling pathways activated and potentially druggable in AD [194].

Taken together these studies, underline the importance of microglia in cerebral organoids as a tool to study the effects of cell interactions on CNS during development, maturation, inflammation, and neurodegenerative diseases.

Conclusions

Microglia are the resident immune surveillance cells within the CNS and are involved in a plethora of physiological as well as pathophysiological functions. Microglial role in neurodegenerative diseases, such as PD, AD, ALS is complex and there is an urgent need to understand better the pathways that regulate their proinflammatory response to injury. Recently, microglia in vitro models have been established with respective advantages and disadvantages as summarized previously. Lately, ESC and iPSC-derived 2D and 3D models, in combination with exposure to CNS microenvironmental cues, form a strong basis to pursue studies of microglial biology in health and disease. Besides, it brings the opportunity to study stem cell-derived 3D human brain organoids/assemblyoids where one can recapitulate features of the human brain with greater complexity than the classical Petri-dish 2D models. Considering that neuroinflammation is involved in neurological diseases, generation of a brain organoid/assembly with all neural population will form an environment where human microglia interacts with other brain populations offering a relevant model to study brain function and pathologies. A better comprehension of inflammatory pathways and novel reliable, easily reproducible and relevant human stem cell-based models will represent an important step in our understanding of the pathogenesis of neurodegenerative disease, hence finding efficient therapies.
Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms

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Chapter 7
Microglia alterations in neurodegenerative diseases and their modeling with human induced pluripotent stem cell and other platforms
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Chapter 7
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