1 # Human iPSC-derived glia models for the study of neuroinflammation

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Abstract

Neuroinflammation is a complex biological process that plays a significant role in various brain disorders. Microglia and astrocytes are the key cell types involved in inflammatory responses in the central nervous system. Neuroinflammation results in increased levels of secreted inflammatory factors, such as cytokines, chemokines, and reactive oxygen species. To model neuroinflammation *in vitro*, various human induced pluripotent stem cell (iPSC)-based models have been utilized, including monocultures, transfer of conditioned media between cell types, co-culturing multiple cell types, neural organoids, and xenotransplantation of cells into the mouse brain. To induce neuroinflammatory responses *in vitro*, several stimuli have been established that can induce responses in either microglia, astrocytes, or both. Here we describe and critically evaluate the different types of iPSC models that can be used to study neuroinflammation and highlight how neuroinflammation has been induced and measured in these cultures.

Keywords: induced pluripotent stem cells, iPSC, neuroinflammation, microglia, astrocytes, monoculture, co-culture, neural organoids, xenotransplantation, cytokines

Background

Defining neuroinflammation

Neuroinflammation is commonly used to describe pathology in multiple central nervous system (CNS) conditions, including Alzheimer's disease (AD), Parkinson's disease (PD), Multiple sclerosis (MS), and Amyotrophic lateral sclerosis (ALS) [1]. Unfortunately, definitions of what constitutes neuroinflammation vary widely across the literature. Within the CNS, the inflammatory response is primarily driven by glia cells, namely microglia and astrocytes [1,2]. These cells mediate the response by releasing cytokines, chemokines, reactive oxygen species (ROS), and secondary messengers. Cytokines can be pro- or anti-inflammatory, thereby either exacerbating or dampening the immune response. For the purpose of this review, we define neuroinflammation as the presence of inflammatory mediators within the CNS [3].

Many researchers have previously classified microglia and astrocytes into dichotomous 'good' versus 'bad' states (e.g. M1 & M2 microglia, A1 & A2 astrocytes) [4,5]. Traditionally, cells representing M1 and A1 states were assumed to serve detrimental roles within diseases, propagating uncontrolled, damaging,

36 inflammatory responses. These M1 and A1 cells have often been referred to as 'activated' (microglia) or
37 'reactive' (astrocytes), thus distinguishing them from the 'resting' cells found in an unstimulated brain. In
38 contrast, M2 and A2 cells were considered 'protective' within diseased brains, acting to limit inflammatory
39 responses and initiate tissue repair processes. Recent research however has proved these rigid, binary
40 definitions to be largely unfounded [6,7]. Rather than adhering to such strict categories, the states of these
41 glia cells are highly dynamic, and they exhibit complex, fluid responses to varied stimuli and environments
42 [6,7].
43 Microglia and astrocytes do not initiate inflammatory responses independently. Instead, they engage in
44 communication with each other as well as with other types of cells in the CNS, including neurons [8]. In
45 general, upon detection of an insult, the secretion of inflammatory factors by microglia (e.g. IL-1α, TNF &
46 C1q) stimulates astrocytes to acquire a more reactive, inflammatory phenotype [9]. In turn, reactive
47 astrocytes likely secrete additional factors that affect microglia-mediated neuroinflammatory behaviours
48 [10]. Depending on the stimuli, distinct activation modes are initiated in these immune cells, although often
49 involving shared signalling pathways and effectors [11]. During CNS injury and disease, microglia and
50 astrocytes can serve both protective and detrimental roles depending on the specific context [12]. Further
51 details about inflammatory signalling pathways and cytokine functions in general, as well as for the
52 pathogenesis of Alzheimer's disease, have already been described elsewhere [13,14].
53

54 ## Microglia

55 Microglia are considered the main immune cells of the CNS. These cells make up between 0.5-16.6% of
56 the total cell population in the human brain, depending on anatomical region, sex, and stage of
57 development, among other variables [15,16]. Microglia possess multiple branched processes, or
58 ramifications, to survey the CNS environment [17]. Despite previous assumptions that microglia were
59 quiescent within the healthy adult brain, they are now known to be crucial players both during
60 development and in maintaining normal brain homeostasis [18]. In brief, the main functions of microglia are
61 to mediate the inflammatory response by secreting cytokines and chemokines, and to phagocytose
62 unwanted material and synapses [19]. Microglia are highly versatile both functionally and morphologically
63 and can rapidly adapt in response to a diverse range of stimuli [20].
64 Upon detection of either pathogen-associated molecular patterns (PAMPs), damage-associated molecular
65 patterns (DAMPs), or neurodegeneration-associated molecular patterns (NAMPs), microglia undergo a
66 rapid phenotypic change [21,22]. NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells), a

67 pleiotropic regulator of many cellular signalling pathways, plays a major role in facilitating the
68 neuroinflammatory response of not only microglia, but also astrocytes, to these stimuli [23]. Activated
69 microglia migrate to the site of damage and injury via a process called chemotaxis, as well as release
70 chemokines (e.g. CCL2 and CXCL1), pro- and anti- inflammatory cytokines (e.g. IL-12, TNF, IL-10, TGF-β),
71 and a variety of other inflammatory mediators [21]. These mediators then further stimulate immune
72 responses in other glia cells [24]. Under certain disease conditions, microglia can also be seen to proliferate
73 and undergo morphological changes (often taking on a more amoeboid appearance). This response,
74 termed microgliosis, likely increases the ability of these cells to survey the brain parenchyma and to
75 migrate more easily towards insults [20]. Transcriptomic studies have revealed the ability of microglia to
76 acquire a vast diversity of transcriptomic states in response to different disease conditions. This includes
77 forming a population of 'disease-associated microglia', which have increased gene expression involved
78 with phagocytosis and lipid metabolism [25]. While there remains difficulty in directly correlating these
79 different transcriptional states with differences in cell function, transcriptomics can provide insight into
80 the heterogeneity of glia in neuroinflammatory conditions.
81

82 ## Astrocytes

83 Astrocytes act as the most prevalent form of glia, making up 17-61% of the total cell numbers in the CNS
84 [26]. These highly heterogeneous cells display a variety of densities, morphologies, gene expression, and
85 proliferation rates depending on many factors including brain region and disease state [27]. Astrocytes have
86 roles in innate and adaptive immunity, neurogenesis, providing metabolic support to neurons,
87 maintaining blood-brain barrier integrity, and are implicated in learning and memory [27,28]. Astrocytes form
88 intimate connections with neurons, thus allowing them to sensitively detect neuronal damage and
89 effectively regulate the subsequent inflammatory response. Astrocytes remove neuronal-secreted
90 glutamate from the synaptic cleft, thus reducing glutamate-induced excitotoxicity and inflammation [29].
91 Furthermore, dying neurons release ATP and potassium, both of which can induce inflammasome
92 activation within astrocytes, which in turn leads to the release of proinflammatory chemokines and
93 cytokines [30,31]. In general, the role of astrocytes following detection of harmful stimuli is to regulate the
94 resulting inflammation and push the brain environment towards a more homeostatic state [32]. However,
95 under certain conditions, astrocytes can also be seen to contribute to both neuroinflammation and tissue
96 damage [33].

97 Within many disease states, astrocytes have been described as taking on a more ‘reactive’ phenotype,
98 characterized by increased expression of the glial fibrillary acid protein (GFAP) [7]. This reactive phenotype
99 involves cell proliferation and hypertrophy (also known as astrogliosis), with astrocytes clustering and
100 integrating with extracellular matrix components to form a ‘glial scar’. The glial scar physically shields the
101 injured region from neighbouring healthy tissue, thus preventing the spread of inflammatory mediators
102 and debris [34]. In addition, ‘reactive’ astrocytes activate NF-κB and produce a variety of chemokines (e.g.
103 CCL2, CXC3L1, CXCL1) that attract other immune cells and cytokines (e.g. IFN-γ, IL-12, TNF, IL-10, and TGF-
104 β), which propagate the immune response by stimulating neighbouring glia [35,36].
105

106 Induced pluripotent stem cells (iPSC)

107 iPSC, initially generated in 2006 by Takahashi and Yamanaka [37], have enormous potential in a wide variety
108 of medical and research applications, as is reviewed extensively elsewhere [38]. These artificial stem cells
109 are formed from somatic cells (e.g. fibroblasts, blood) following the overexpression of transcription
110 factors that stimulate de-differentiation to a state similar to embryonic stem cells. Once formed, these
111 iPSC are capable of infinite self-renewal and can differentiate into all three embryonic germ layers, and
112 more specialized cell types. Protocols have been developed for the differentiation of iPSC into cells of the
113 CNS, including neurons, microglia, and astrocytes, and have been reviewed elsewhere [39–41]. These cells
114 can then be studied in monoculture (single cell type), 2D co-culture (multiple cell types), or in 3D culture
115 (e.g. neural organoids). In addition, iPSC-derived cells can be studied post-injection into a mouse brain
116 (xenotransplantation) [42] (Figure 1).
117 iPSC-derived microglia and astrocytes can produce inflammatory responses that share many similarities
118 with corresponding *in vivo* responses [43,44]. This includes secretion of cytokines, chemokines, ROS, and
119 other inflammatory mediators. The neuroinflammatory response *in vivo* involves numerous complex
120 interactions between multiple brain cell types. For this reason, inflammatory responses are likely to be
121 more physiologically relevant with increasing culture complexity.
122

123 Models for studying neuroinflammation

124 A great number of models to study neuroinflammatory responses are available, ranging from
125 immortalized cell lines (e.g. murine BV2 microglia cells [45] and human HMO6 microglia cells [46]) to primary
126 mouse and human cells, iPSC-derived models, as well as numerous rodent models. All models have both
127 advantages and limitations, but for the purpose of this review we will focus on the reasons for choosing

128 iPSC-derived models. Mouse models, although valuable, are unable to faithfully replicate human
129 neuroinflammatory responses due to species-specific differences in immune system architecture and
130 signalling pathways [47]. For instance, it has been demonstrated that mice exhibit different immune
131 responses to humans following injection of one of the most commonly used experimental
132 neuroinflammatory activators, LPS [48]. Amongst other species-specific differences, mouse astrocytes, but
133 not human astrocytes respond to LPS [49]. Primary human *ex-vivo* microglia and astrocytes, which are most
134 often derived from postmortem samples, are difficult to obtain and manipulate, thus limiting their
135 widespread use in research. Immortalized (human) cell lines, although easy to maintain and abundantly
136 available due to their unrestricted proliferative capacity, have genetic and functional abnormalities, thus
137 do not closely recapitulate microglia and astrocyte behaviour [50,51]. The utilization of iPSC-derived cells
138 overcomes these limitations by providing a direct and consistent source of human microglia and
139 astrocytes, enabling investigation of human-specific responses [43,52]. Moreover, iPSC-derived microglia and
140 astrocytes can be generated from patient-specific iPSC, offering a personalized approach to understanding
141 disease pathology. Their ability to model genetic variability and susceptibility enhances the translational
142 relevance of findings and holds promise for drug screening and precision medicine approaches [53].
143 Disadvantages of iPSC models however include their resembling of a 'foetal' rather than an 'adult' state
144 [52], considerable genetic variability between different iPSC lines [54], and the relatively high cost of iPSC
145 generation and maintenance [55].
146 In this review, we summarise and access how neuroinflammation has been studied in human iPSC-
147 microglia and iPSC-astrocyte cultures of increasing culture complexity. We highlight and critically evaluate
148 which stimuli have been used to initiate an inflammatory response and mention the commonly used
149 methods of detection, thereby providing a comprehensive guide on how best to model
150 neuroinflammation in human iPSC models.
151

152 Neuroinflammation studied in monoculture models

153 iPSC-microglia monocultures

154 Multiple protocols are available for the differentiation of iPSC-microglia [56–62]. These protocols mimic
155 microglia ontogeny by replicating *in vivo* pathways. Lineage-tracing studies in mice revealed that microglia
156 progenitors are produced from mesodermal yolk sac macrophages, which migrate into the early brain
157 before blood-brain barrier formation. Once inside the brain, these progenitors continue differentiating to
158 a microglia phenotype [63]. Differentiation protocols attempt to recapitulate these events with a

combination of growth factors and physical conditions that directs cells towards a mesodermal lineage and initiates early haematopoiesis. This results in the production of embryonic macrophage precursors which can be harvested and directed towards a specialized microglia phenotype. Microglia differentiation methods have been reviewed in more detail elsewhere [39], and some commonly used protocols are listed in Figure 1A.

Modelling neuroinflammation in microglia monocultures

Neuroinflammation is most commonly induced following the addition of a stimulant to the culture. Multiple stimulants are reported in the literature with the most common for iPSC-microglia being lipopolysaccharide (LPS). LPS is a component of gram-negative bacteria cells walls and stimulates microglia through a series of interactions with several proteins, including the LPS binding protein, CD14, MD-2 and toll-like receptor 4 (TLR4) [64]. LPS addition results in a potent inflammatory response, with short stimulation paradigms of only 3-4 hours shown to be sufficient in increasing gene transcription and secretion of multiple cytokines, such as IL-1β, IL-6, and IL-10, measured by RT-qPCR and enzyme-linked immunosorbent assay (ELISA) [65,66]. However, much longer time courses are more commonly used ranging from 8-24 hours, and prolonged LPS stimulation leads to an increase in further cytokines and chemokines [52,57,59,67–72]. In addition to stimulation time, the concentration of LPS is important to consider. LPS concentrations are used in the range of 10 ng/mL to 1 µg/mL, with 100 ng/mL used most often. A brief comparison of 10 ng/mL and 100 ng/mL LPS stimulation for 6 hours to unstimulated iPSC-microglia revealed that 10 ng/mL is sufficient to cause a significant increase in IL-1β and IL-6 gene transcription, as well as IL-6 protein secretion. In this study, stimulation with 100 ng/mL LPS showed only IL-1β gene transcription to be significantly increased compared to unstimulated iPSC-microglia, as well as IL-6, IL-1β and TNF protein secretion [65]. This indicates that LPS concentrations as low as 10 ng/mL can initiate a neuroinflammatory reaction in iPSC-microglia. The variability in significantly increased cytokines between studies shows that duration and concentration of the stimulant can have a huge effect on measuring neuroinflammatory findings, and it might be useful to include multiple stimulation timepoints into the study design. The importance of this has previously been demonstrated in alternative microglia models including primary rat microglia and the rodent microglia cell line BV2. In one study a range from 5 ng/mL to 5 µg/mL was reported after either 24 hours or 48 hours of stimulation. Comparison of key cytokines TNF, IL-1β and IL-6 was performed using an ELISA. This revealed stimulating with 5 µg/ml LPS for 24 hours to produce the most profound production of cytokines for both cell line. However, the magnitude varied

190 drastically between the two cell lines with primary microglia secreting more of all cytokines than BV2 cells.
191 Indeed, no significant increase of IL1B was reported from BV2 cells at any concentration or time point.
192 They also showed that stimulation with LPS as low as 5 ng/mL to still elicit a response showing significant
193 increases of TNF secretion at 24 hours in both cells but failed to show significant increases of IL-1β or IL-6
194 at either time points in either cell lines. Although 5 µg/mL was found to be an acceptable concentration
195 in this study others have reported LPS concentrations of up to 10 µg/ml as being cytotoxic in 24-hour
196 assays with primary microglia cells. Therefore, it is advisable to not go beyond this upper limit. Highlighting
197 variations in cytokine responses between primary microglia and BV2 cells underscores a significant
198 challenge encountered while endeavouring to characterize inflammatory responses using this
199 immortalized cell line. [73]

200 A final point to be aware of when selecting LPS as a neuroinflammatory stimulant is the variation between
201 LPS-species produced by different bacterial strains. LPS-species variation has shown to produce different
202 biological activity. LPS is consists of three parts; an inner lipid A core, the core oligosaccharide and antigen-
203 O which is a chain of repeating oligosaccharides exposed to the outside of the structure. The number of
204 O-antigen repeats is diverse amongst LPS strains, with over 160 O-antigen structures being identified for
205 different strains of E. *coli*. Comparison of two LPS strains, K-235 and 0055B5, was performed on primary
206 rat microglia. ELISA data for TNF and IL-10 production showed addition of 10 ng/mL of both LPS strains
207 for 24 hours significantly increased the production of both cytokines, however IL-10 showed a significantly
208 higher difference in secretion compared to controls with 005B5 stimulation compared to addition of K-
209 235 however no significant differences between this secretion between strains was shown. A Griess assay
210 was used to measure production of NO showing both strains significantly increased production from
211 unstimulated controls with a significantly increased amount of production shown following 005B5
212 stimulation compared to after K-235 stimulation. Despite this comparison of different phagocytosis
213 receptors at each stimulation time point showed transcript of receptors MARCO and MHCII to have
214 significantly higher expression after K-235 stimulation than 005B5 stimulation indicating that this strain is
215 more consistent with innate activation of iPSC-microglia. [74]

216 As LPS is considered not to be very physiologically relevant for many disease applications, cytokines, such
217 as IFN-γ or IL-1β, can be alternatively used to induce neuroinflammation in iPSC-microglia either on their
218 own [52,59,71], or in combination with LPS [59,61,70,71,75,76]. The dual stimulation paradigm allows for the cytokine
219 to prime the microglia, resulting in a heightened inflammatory response. This priming response also
220 triggers canonical activation of the inflammasome, which results in increased caspase 1 production, which

221 then cleaves cytokines IL-1β and IL-18 to their active forms, the latter of which stimulates IFN-γ production
222 [77]. Abud and colleagues compared a 24-hour stimulation of iPSC-microglia with 20 ng/mL IFN-γ, 20 ng/mL
223 IL-1β, or 100 ng/mL LPS. Multiplex ELISA data for 10 key cytokines and chemokines revealed that LPS had
224 the largest effect on protein production with all but CCL3 showing large significant increases. Stimulation
225 with IFN-γ and IL-1β showed increases in 7-8 cytokines with no significant changes in the secretion of IL-6
226 and IL-10 with either stimulant, and no change in CXCL10 with IFN-γ stimulation. When used in
227 combination, a stimulation of 100 ng/mL LPS and 20 ng/mL IFN-γ is often selected. The combined stimulus
228 induced an increase in multiple cytokines and chemokines when compared to unstimulated iPSC-
229 microglia, including TNF, IL-6, IL-8, IL-1β, CXCL1 and CXCL10, measured by RT-qPCR or protein arrays
230 [59,61,70,75]. Additional stimuli have been investigated in other microglia models and it would be valuable to
231 test the response of iPSC-microglia to these in future studies For instance, one group compared 24 hour
232 stimulation on rat primary microglia with 10 ng/mL LPS alone and a dual cytokine cocktail of 20 ng/mL
233 IFN-γ and 50 ng/mL TNF. Both TNF and IFN-γ are high expressed cytokines, which have been implicated in
234 many disease pathways, including AD. Nanostring was used to compare transcriptomic differences of
235 proinflammatory markers after each stimulation. Although both stimulation paradigms induced increased
236 expression of pro-inflammatory markers, LPS resulted in a higher and wider range of cytokine production
237 in key markers including IL-6 and TNF. Additionally, LPS affected some genes which the dual cytokine
238 cocktail did not, such as increases in IL-1β and IFN-γ gene expression and reductions in C5Ar1 and CCL3
239 gene expression. [78]

240 Additional stimuli have also been reported in a recent transcriptomics paper which utilised iPSC-microglia.
241 Here, CONC ATPγS, CONC PGE2 or LPS and IFN-γ combined treatment were added for 24 hours and
242 differential gene expression changes compared. All challenges evoked an inflammatory response with a
243 strong correlation being observed following stimulation with LPS & IFN-γ and ATPγS. A later analysis then
244 added primary mouse microglia to the stimulation model and projected the subsequent data, from both
245 microglia cell models, onto reported transcriptional chances seen in activated microglia of various AD
246 mouse models. This analysis indicated that despite the similarities in ATPγS and LPS + IFN-γ response it is
247 LPS alone that best promotes transcriptional shift towards a state more similar to the activated microglia
248 from mouse AD models. This indicates that although LPS is not physiologically present within the brain
249 environment it still offers a robust and valuable stimuli for measuring neuroinflammatory changes in
250 microglia. [79]

251 Neuroinflammation can also be modelled by the introduction of disease-related aggregates, which has
252 been done to model the neurodegenerative disorders AD (using amyloid-β) and PD (using α-synuclein).
253 Addition of 3 µM oligomeric amyloid beta (AβO) to iPSC-microglia for 24 hours resulted in a significant
254 increase in IL-1β, TNF, and IL-6 gene expression, measured by RT-qPCR. The authors compared AβO
255 stimulation to the stimulation with 100 ng/mL LPS and showed that the LPS challenge increased all three
256 cytokines to a much larger extent. However, both compounds triggered morphological changes in the
257 iPSC-microglia towards an amoeboid phenotype [80]. Ihnatovych and colleagues found that a 24-hour
258 stimulation with 1 µM of the peptide $Aβ_{1-42}$ increased iPSC-microglial gene expression of TNF, IL-6, IL-1β
259 and the NF-KB subunit P65 (encoded by the gene *RELA*) [72]. Stimulation of 5 µM $Aβ_{1-42}$ peptides for 6 hours
260 on primary human microglia and microglia cell line HMO6 also revealed significant increases in TNF and
261 IL-18 as measured by ELISA. However, significant increases in IL-1β and MIP only measured in primary
262 human microglia not in HMO6 cells, further demonstrating the limitations of immortalised cell line
263 microglia models. Rostami and colleagues found dissimilar results when stimulating iPSC-microglia with
264 0.2 µM amyloid-beta fibrils (Aβ-F). They stimulated iPSC-microglia for 24 hours, then[81]A cytokine array
265 measuring 36 human chemokines & cytokines revealed no significant differences after Aβ-F stimulation
266 compared to unstimulated iPSC-microglia [81]. This might be due to the rather lowthe different species of
267 amyloid-β used. The authors also chose to 6 days. An earlier timepoint or the absence of the washing step
268 might have led to the detection of a cytokine increase.

269 Rostami and colleagues also used the PD related peptide α-synuclein to study a neuroinflammatory
270 phenotype in iPSC-microglia. Stimulation with 0.5 µM α-synuclein fibrils (αSYN-F), with a similar washing
271 and conditioning procedure as mentioned above, led to no significant changes in 36 chemokines and
272 cytokines measured via a cytokine array [81]. Trudler and colleagues, however, found that both α-synuclein
273 monomers and oligomers can induce an inflammatory phenotype in iPSC-microglia, measured by ELISA
274 after 6 hours. Stimulation with 700 nM of α-synuclein monomers induced a significant increase in IL-6 and
275 TNF. The same concentration of α-synuclein oligomers induced a significant increase in IL-6, TNF, IL-1β,
276 and caspase-1 [65]. These findings indicate that neuroinflammation triggered by neurodegeneration-related
277 peptides can be studied in iPSC-microglia cultures, although more research is needed to better understand
278 the variable results.

279 When modelling neuroinflammation in iPSC-microglia, the choice of stimulation paradigm is not the only
280 important factor to consider. Autocrine and paracrine feedback mechanisms regulate cytokine release,
281 therefore, the plating density of cells can significantly impact the final output measure. Although no data

282 has been published for the effect of plating density on cytokine release in iPSC-microglia, it has been
283 shown to have a large effect on iPSC-macrophages [82]. Stimulation was performed with either 100 ng/mL
284 LPS alone or in combination with 10 ng/mL IFN-γ for 24 hours. The results reveal density-associated effects
285 with both stimulation paradigms, where densely plated cells secreted significantly less TNF, measured by
286 ELISA. Transcriptomic analysis of unstimulated iPSC-macrophages indicated that the plating density had a
287 significant effect on cytokine transcription. The most significant differences were seen in CCL2, CCL4,
288 CCL5, CXCL1, CXCL11, IL-1β, IL-13, IL-17E, IL-32A and TNF. Significant differences were also shown
289 following stimulation with 100 ng/mL LPS and 20 ng/mL IFN-γ for 24 hours, with significant reductions of
290 CCL3, CXCL10, GM-CSF, IL-12p70, IL-18 and IL-32A transcription in the iPSC-macrophages plated at higher
291 density [82]. This demonstrates the importance of keeping consistent plating densities when investigating
292 neuroinflammation *in vitro.*

293 iPSC-microglia are an advantageous model for the study of microglia inflammatory responses that elicit a
294 strong response following challenge of multiple stimulants. As microglia are highly reactive cells, this
295 model offers an advantage over those that require extraction and purification of the cells, such as primary
296 microglia. The removal of these cells from their original environment likely causes a reactive response,
297 which may skew any neuroinflammatory data later collected. For instance, one study stimulated primary
298 human microglia with 100 ng/mL LPS alone or with 10 ng/mL IFN-γ for 18 hours. RT-qPCR analysis revealed
299 increase of key cytokines including TNF and CCR7 but to a much lower than expected magnitude, and
300 much lower to monocyte derived macrophages. Characterization of primary microglia revealed virtual
301 absence of CD14, which binds LPS with high affinity and, alongside TLR4, facilitates transmembrane
302 signalling. This is consistent with others who have shown the absence of CD14 mRNA and protein in resting
303 primary microglia in situ and ex vivo. Extended culturing of primary microglia results in steady increase of
304 CD14 expression which coincides with increased responsiveness to LPS. Furthermore, others have
305 reported absence of IFN-γ receptors in situ human microglia. Although increasing the culturing of primary
306 microglia has shown to improve the responsiveness to LPS, bulk RNA sequencing study has shown that in
307 vitro cultured microglia to represent an overactivated phenotype at baseline, which may make it difficult
308 to distinguish changes due to stimulation in these models. A possible reason for this overactivation could
309 also be due to removal of cell-cell interactions which may act to maintain homeostatic state in microglia.
310 For instance, TGF-β secreted from local astrocytes and neurons has shown to be essential in maintaining
311 homeostatic microglia. The lack of cell-cell interaction also exists for monocultures iPSC-microglia,
312 however, this issue is discussed in greater detail in section X of this review.

313 Although iPSC offer a unique model to study human microglia *in vitro,* multiple unknown still exist around
314 the model. The large range of protocols offers a diverse number of range which are likely to vary in their
315 response to stimulation. Many protocols utilize key growth factor TGF-β in the media, which
316 aforementioned would skew cells towards a more homeostatic phenotype compared to those that do
317 not. Furthermore, iPS microglia represent a young population with little information being available for
318 the aging of these cells. This is a particular problem in these study of neurodegenerative disorders, which
319 occur mostly in aged patients. Moving towards refining iPSC models and increasing their complexity is
320 essential in undercovering key process of microglia neuroinflammatory functions.

321

322 iPSC-astrocyte monocultures

323 Many protocols have been developed in recent years for generating iPSC-astrocytes [83–88], see Figure 1A.
324 Unlike microglia, astrocytes have a neuroectodermal origin and are derived from radial glia *in vivo* [41].
325 Astrogenesis, which broadly follows neurogenesis in humans, is initiated following activation of the JAK-
326 STAT canonical pathway. To differentiate iPSC-astrocytes *in vitro*, iPSC are differentiated into neural
327 progenitor cells, followed by addition of a mixture of growth factors such as CNTF and FGF2, which aids
328 the development of mature astrocytes [41].

329

330 Modelling neuroinflammation in astrocyte monocultures

331 The most commonly used stimulus for triggering an inflammatory response in astrocytes *in vitro* is TNF.
332 TNF, known to be secreted by microglia, was shown to play a key role in astrocyte activation in both
333 human and murine models [9,89]. However, it is clear that several important species differences exist
334 following TNF stimulation, with human astrocytes displaying an elevated and more divergent cytokine
335 response when compared to mouse cells [89]. For the stimulation of iPSC-astrocytes, TNF has been used at
336 concentrations of 10 ng/mL to 100 ng/mL and stimulation times vary widely between studies. Stimulation
337 with 50 ng/mL TNF for just 1.5 hours was sufficient to induce NF-κB nuclear translocation, measured by
338 immunostaining [90], and increased IL-6 and IL-8 were detected after 5 hours of TNF stimulation using flow
339 cytometry [91]. However, most studies to date used longer TNF stimulation times of 24 hours up to 7 days
340 [90,92–96]. In order to measure neuroinflammation in iPSC-astrocytes, cytokine and chemokine gene
341 transcription and protein secretion have been studied most often. Two studies performed bulk RNA
342 sequencing comparing unstimulated iPSC-astrocytes to those stimulated with 10 ng/mL TNF for 5 days [94]

343 or 100 ng/mL for 7 days [92]. The results are largely overlapping, indicating that numerous cytokines and
344 chemokines are upregulated after TNF stimulation. In addition, GFAP was used as a marker of astrocyte
345 activation. GFAP intensity, measured via immunostaining, was shown to be increased after 5 days of
346 stimulation with 10 ng/mL TNF [90]. However, in a second study, 7 days of stimulation with 50 ng/mL TNF
347 did not affect GFAP, measured by western blot [95]. Furthermore, it was shown that TNF stimulation induced
348 NF-κB nuclear translocation and phosphorylation [90,92]. In summary, these data indicate that stimulating
349 iPSC-astrocyte monocultures *in vitro* with TNF appears to induce a robust and reproducible inflammatory
350 phenotype. However, more research is necessary to reconcile different observations in the literature
351 regarding effects of TNF stimulation on GFAP levels. Moreover, when interpreting outcomes, it's essential
352 to factor in published reservations regarding the accuracy of GFAP as an indicator of astrocyte activation,
353 rather than just indicating the presence of astrocytes [10].

354 The second most commonly used stimulus when attempting to activate iPSC-astrocytes *in vitro* is IL-1β,
355 which is known to be secreted by activated microglia [97]. IL-1β has been used at a concentration of 10
356 ng/mL or 100 ng/mL for 5 hours up to 7 days to stimulate iPSC-astrocytes. Using a short incubation time
357 of 5 hours led to an increase in IL-6 and IL-8, measured by flow cytometry [91]. Longer incubation times of
358 24 hours to 7 days could show an increase in IL-8, IL-6, GM-CSF, TNF, IL-23, IFN-β, IFN-α, CCL5 and CXCL8,
359 as well as increased GFAP [93,95,96]. No activation of NF-κB was observed in IL-1β treated astrocytes, which
360 is dissimilar to the downstream effects of TNF stimulation [92]. Two studies performed bulk RNA sequencing
361 on IL-1β-stimulated iPSC-astrocytes. Santos and colleagues used a stimulation of 10 ng/mL for 5 hours and
362 reported an enrichment for inflammation-related GO terms such as inflammatory response, immune
363 response, chemokine activity, and cytokine activity in the IL-1β-stimulated iPSC-astrocytes [91]. Zhou and
364 colleagues compared the effects of 7 days of stimulation with either 100 ng/mL TNF or 100 ng/mL IL-1β
365 to unstimulated iPSC-astrocytes. They found that both upregulate pro-inflammatory genes, but by widely
366 different magnitudes. Cross-comparison of genes significantly upregulated by IL-1β and TNF treatments
367 revealed a large overlap, however treatment with TNF led to a much greater number of differentially
368 expressed genes [92]. Contradicting this finding, two studies found IL-1β to be a more potent activator of
369 iPSC-astrocytes than TNF [91,96]. After 5 hours of stimulation with either 50 ng/mL TNF or 10 ng/mL IL-1β,
370 Santos and colleagues found the response to IL-1β stimulation to be higher, measured via flow cytometry
371 of IL-6 and IL-8 [91]. Using a multiplex bead-based immunoassay, Perriot and colleagues showed that
372 stimulation with 10 ng/mL IL-1β for 5 days increased IL-6, GM-CSF, TNF, IL-23, IFN-β and IFN-α in iPSC-
373 astrocytes, compared to only GM-CSF and IL-1β in iPSC-astrocytes stimulated with 10 ng/mL TNF for 5
374 days [96]. Overall, these findings indicate that both TNF and IL-1β can individually induce a

375 neuroinflammatory phenotype in iPSC-astrocytes but suggest that the degree of activation is highly
376 dependent on the experiment set-up. Contradictory findings between studies when comparing iPSC-
377 astrocyte responses to IL-1β and TNF demonstrate how further research must be undertaken before firm
378 conclusions can be drawn.

379 Several studies to date have investigated the effect of the co-stimulation with both 10 ng/mL TNF and 10
380 ng/mL IL-1β on iPSC-astrocytes. Hyvärinen and colleagues detected NF-κB nuclear translocation, increased
381 IL-6 secretion, as well as increased transcription of CCL5, CXCL8, C3 and LCN2 after co-stimulation, but
382 also a decrease in GFAP gene expression [98]. Perriot and colleagues report that co-stimulation with TNF
383 and IL-1β resulted in a huge synergistic effect, enhancing the production of both pro- and anti-
384 inflammatory mediators. Compared with IL-1β stimulation only, co-stimulation with IL-1β and TNF
385 induced a massive increase in the secretion of GM-CSF, IL-6, IL-10, IL-12, IL-23, IFN-β, and IFN-α [96].

386 A seminal study by Liddelow and colleagues demonstrated how activated primary mouse microglia
387 secrete IL-1α, TNF, and C1q, which in turn leads to astrocyte activation, and in turn neuronal death [9]. This
388 finding has important implications regarding the role of astrocytes in neurodegenerative diseases. Since
389 its publication, several groups have attempted to replicate this finding using human iPSC-astrocytes. One
390 such study, by Soubannier and colleagues, found increased TNF and IL-1β following 24-hour treatment
391 with this cytokine cocktail [99]. However, this group also found decreased GFAP expression following
392 treatment, contrasting with findings of Liddelow and colleagues. Moreover, when Barbar and colleagues
393 performed the same triple-stimulation as Soubannier and colleagues but compared the resulting cytokine
394 releases with stimulations with TNF and IL-1β, they observed no or incredibly minor differences in the
395 cytokine release profiles between the two groups, and no change in GFAP protein levels [100]. These data
396 suggest that TNF stimulation, which was shared between the two conditions, may be more important
397 regarding human iPSC-astrocyte activation than IL-1α and C1q.

398 To date, neuroinflammation in the context of neurodegenerative diseases has been mostly studied in
399 iPSC-microglia. Rostami and colleagues did not only investigate the effect of Aβ-F and αSYN-F on iPSC-
400 microglia, but also iPSC-astrocytes. However, just like for the iPSC-microglia, stimulation of iPSC-
401 astrocytes with Aβ-F and αSYN-F did not result in changes to any of the 36 measured chemokines and
402 cytokines [81].

403 More studies are needed to draw conclusions on the neuroinflammatory responses of iPSC-astrocytes to
404 disease-related stimuli. This would include comprehensively testing all cytokines thought to be important

405 for astrocyte activation both in combination and individually. Moreover, differences in the responses of
406 mouse and human cells when stimulating with certain immune stimuli [49,89] highlights the importance of
407 human models when studying inflammatory responses.

408 To conclude this section on monoculture models, thus far neuroinflammation has been studied more
409 often in iPSC-microglia than iPSC-astrocytes, however both cell types have been demonstrated to respond
410 to inflammatory stimuli *in vitro*. Different stimuli were used to activate glia cells, with LPS being the most
411 commonly used for iPSC-microglia, and TNF and IL-1β for iPSC-astrocytes. Disease-relevant stimuli such
412 as Aβ and α-synuclein can be used, however, so far they showed inconsistent inflammatory reactions
413 when added to iPSC-microglia cultures and no effect on iPSC-astrocytes, indicating that more studies are
414 needed to fully understand the neuroinflammatory response to disease-associated peptides. Following
415 the addition of inflammatory stimuli, monocultured glia release a range of inflammatory mediators, that
416 can be measured using several well-established techniques. Factors to take into consideration when
417 attempting to measure neuroinflammatory responses in monocultured glia include choice of stimuli,
418 concentration, treatment duration, and cell plating density.

419

420 Complex 2D culture models

421 Introduction to complex models

422 The establishment of iPSC-derived monoculture systems in the past 15 years has led to advances in the
423 study of neuroinflammatory processes. Analysing iPSC-derived microglia and astrocytes in monoculture
424 allow for a higher level of detail in their characterization, which can be challenging to attain with more
425 complex models. However, microglia, astrocytes, and neuronal cells interact with each other in the brain
426 environment, both via physical contact and secreted factors [101]. This constant communication between
427 brain cells is of paramount importance as it results in reciprocal changes to gene expression and cellular
428 function. Excluding cell-cell communication from neuroinflammatory models may therefore lead to
429 oversimplified or inappropriate conclusions [101]. Methods for generating iPSC-cultures with a higher degree
430 of complexity have been established to simulate a more physiological CNS environment. 2D co-culture
431 systems can be generally divided into two categories: (1) Culturing one population of cells using the
432 conditioned medium derived from another population or, culturing the two populations of cells in the
433 same vessel separated by a porous membrane. In both instances, the main aim is to allow secreted factors

434 to be shared without physical contact. (2) Culturing more than one cell type in a dish with physical contact
435 to each other.

436

437 Modelling neuroinflammation using iPSC-derived conditioned medium

438 Microglia and neurons cultured with astrocyte-conditioned medium (ACM)

439 Two decades ago, scientists speculated that astrocytes might play an important regulatory role in
440 microglial differentiation. Using primary mouse microglia, Schilling and colleagues demonstrated that
441 microglia ramification is controlled by the astrocytic factors TGF-β, M-CSF, and GM-CSF. Cultured primary
442 microglia were treated for one day with primary mouse ACM, which induced significant microglia
443 ramification compared to non-conditioned medium. Moreover, microglia ramification was inhibited by
444 neutralizing antibodies against TGF-β, M-CSF, and GM-CSF [102]. Zhang and colleagues compared the
445 morphology of primary mouse microglia that were either treated with primary mouse ACM, cultured with
446 primary mouse astrocytes but separated by a transwell, or cultured with direct contact to astrocytes.
447 Microglia showed a more complex morphology when treated with ACM and cultured in transwells with
448 astrocytes, compared to control medium. In co-culture these microglia however showed an even more
449 complex morphology, indicating that astrocytes regulate microglia ramification through contact-
450 dependent and -independent mechanisms [103]. Nowadays, many iPSC-microglia differentiation protocols
451 use TGF-β, M-CSF, GM-CSF and further growth factors (including SCF and IL-34) to differentiate iPSC to a
452 microglia phenotype [39]. However, to our knowledge no research has been published presenting a
453 comparison between standard microglia media and the use of iPSC-derived ACM for iPSC-microglia
454 culture. Further studies are needed to explore the potential beneficial effects of ACM on microglia
455 cultures, as well as to investigate the effects of ACM from pro-inflammatory stimulated astrocytes on
456 microglia phenotypes.

457 Hyvarinen and colleagues compared human embryonic stem cells (ESC)-derived neurons cultured in the
458 presence of ACM from either control or reactive iPSC-astrocytes (treated with 10 ng/mL IL-1β and 10
459 ng/mL TNF for 7 days, then washed out and collected 48 hours later). No increase in apoptosis or
460 cytotoxicity was observed in ESC-derived neurons cultured with the reactive ACM. ESC-derived neurons
461 were functionally supported when treated with both control and reactive ACM, which led the authors to
462 speculate that the treatment of astrocytes with IL-1β/TNF might represent a neurosupportive rather than
463 a neurotoxic stimulus [98]. However, these results are in contradiction to previous observations by Liddelow

464 and colleagues, who found that reactive ACM from primary rodent astrocytes was highly neurotoxic and
465 caused cell death of ESC-derived neurons within 24 hours of culture. In this study, rodent astrocytes were
466 stimulated with IL-1α (3 ng/mL), TNF (30 ng/mL) and C1q (400 ng/mL) [9]. The striking differences observed
467 in the two studies could be explained by the different stimuli and concentrations used to trigger astrocyte
468 reactivity, as well as differences in cell biology between mature rodent astrocytes and human iPSC-
469 astrocytes [89].

470 **Microglia cultured with neuronal (precursor)-conditioned medium (N(P)CM)**

471 Banerjee and colleagues derived iPSC-microglia by supplementing media with GM-CSF and IL-34 or by
472 exchanging that medium with an increasing gradient of neuronal precursor cell conditioned medium of
473 up to 50%. The comparison between the two media conditions revealed increased gene expression of
474 common microglia markers, including TMEM119, CX3CR1, and SALL1, in the NPCM-treated microglia,
475 measured by RT-qPCR [68]. In line with these findings, Muffat and colleagues reported that iPSC-derived
476 microglia exposed to stem cell-derived mature neuronal secreted factors via a transwell setup showed a
477 gene expression signature closer to foetal human microglia, when compared with iPSC-microglia cultured
478 in defined media, measured by RNA sequencing [61]. This indicates that N(P)CM can contribute to the
479 differentiation of iPSC towards a microglia phenotype by providing factors normally present in the CNS.
480 To our knowledge, no study has yet focused on investigating the effects of N(P)CM of injured/dying
481 neurons on potential iPSC-microglia inflammatory responses.

482 **Neurons cultured with microglia-conditioned medium (MCM)**

483 Recently the effect of microglia-conditioned medium on neuronal cultures has been explored in the
484 context of the neurodegenerative disorder Huntington's disease. O'Regan and colleagues stimulated ESC-
485 microglia, derived from control or mutant *HTT* gene carrying human ESC, with 1 μg/mL of LPS and 10
486 ng/mL of IFN-γ. The stimulated MCM was added to the culture of isogenic ESC-derived striatal neurons
487 for 5 days. Surprisingly, no differences in neuronal cell identity, cell viability, or DNA damage markers were
488 observed after treatment with activated MCM, compared to control MCM [104]. Further research on the
489 effect of MCM from microglia stimulated with commonly used pro-inflammatory activators, such as LPS,
490 on iPSC-neurons is needed to better understand the effect of neuroinflammatory secreted factors on
491 neuronal identity and viability.

492 In conclusion, various groups have started to use conditioned medium to explore the complex
493 communication of CNS cells using iPSC cultures, however the use of conditioned medium remains

494 uncommon. A huge disadvantage of using conditioned medium is that the medium composition is
495 undefined and can vary between batches, as well as that it is unclear which factors contribute to the
496 observed findings. However, the use of conditioned medium could potentially answer questions of non-
497 physical cell-cell communication in a simple iPSC-derived monoculture model.

498

499 Modelling neuroinflammation in iPSC co-culture models

500 A more recently developed and complex way to study interactions between brain cell populations *in vitro*
501 involves using a culture system where cells are in physical contact. Over recent years, an increasing
502 number of groups have established novel iPSC-derived CNS co-culture models.

503 **iPSC-microglia and neuron co-culture**

504 The first iPSC-derived microglia-neuron co-culture protocols were established in 2017 [44,52]. In the healthy
505 brain, microglia are proposed to be maintained in a homeostatic state by a crosstalk of the neuronal
506 CD200 glycoprotein and microglial receptor CD200R, as well as interaction between microglial receptor
507 CX3CR1 with the neuronal transmembrane chemokine fractalkine (CX3CL1) [105]. Abud and colleagues
508 compared gene expression of iPSC-microglia in co-culture with rat hippocampal neurons to iPSC-microglia
509 monocultures supplemented with the neuronal factors CD200 and CX3CL1 via RNA sequencing. They
510 demonstrated that the direct contact of microglia with neurons suppressed pro-inflammatory signalling
511 and maintained a microglia homeostatic transcriptional state, indicating that CD200 and CX3CL1 are not
512 the only interactions important for microglia-neuron crosstalk, especially in the context of microglia
513 homeostasis [52]. Haenseler and colleagues described a protocol to co-culture iPSC-cortical neurons with
514 iPSC-microglia. Unstimulated iPSC-microglia in co-culture with neurons displayed resting 'surveillance'
515 motility similar to *in vivo* microglia behaviour. When stimulated with 100 ng/mL of LPS, 5 hours post-
516 stimulation microglia showed reduced ramifications and an increased area-to-perimeter ratio, indicative
517 of an amoeboid, activated morphology. Cytokine response upon activation with 100 ng/mL of LPS and 100
518 ng/mL of IFN-γ was compared between monocultured and co-cultured iPSC-microglia measured with a
519 Luminex multiplex bead array assay. Co-cultured iPSC-microglia exhibited lower pro-inflammatory
520 cytokine secretion in both basal and stimulated conditions, and in fact, their stimulated cytokine profile
521 was broadly anti-inflammatory [44], indicating that co-culture with neurons induces a more 'homeostatic'
522 state in iPSC-microglia, which might be more representative of *in vivo* microglia. More recently, Vahsen
523 and colleagues described a co-culture of iPSC-microglia with iPSC motor neurons. Transcriptomic analysis

524 demonstrated a microglial signature for the co-cultured iPSC-microglia, and they also showed increased
525 ramifications, indicative of a homeostatic microglial identity. When stimulated with 100 ng/mL of LPS and
526 100 ng/mL of IFN-γ, after 18 hours co-cultured iPSC-microglia revealed an amoeboid morphology and
527 clustered together. Cytokine and chemokine secretion after the stimulation was measured using a
528 membrane-based supernatant proteome array. In comparison with microglia monocultures, co-cultures
529 showed a moderately attenuated secretion profile, with the downregulation of CHI3L1 and serpin E1 [106].
530 The results from both studies support the hypothesis that microglia acquire a less activated status when
531 in direct contact with neurons [107].

532 **iPSC-astrocytes and neuron co-culture**

533 Multiple iPSC-astrocyte and neuron co-culture protocols have been established in recent years, however
534 with little focus on neuroinflammation. Santos and colleagues explored the consequences of co-culture
535 of neurons with activated astrocytes on the viability and dendritic length of the neurons. iPSC-derived
536 astrocytes were treated with either IL-1β (10 ng/mL) or control vehicle for 24 hours and co-cultured with
537 neurons for 48 hours after the removal of the cytokine. They observed a large decrease in the survival
538 rate and reduction in dendritic length of the neurons when co-cultured with activated astrocytes, in
539 comparison to astrocytes treated with vehicle, demonstrating that IL-1β-stimulated astrocytes have a
540 negative impact on the maturation and survival of neurons [91]. A similar study, using a microfluidic platform
541 to culture iPSC-astrocytes and iPSC-neurons, gave contradictory results. The microfluidic device contained
542 separate chambers for neurons and astrocytes allowing cell-to-cell interactions within microtunnels,
543 where astrocyte processes connected with neuronal axons. Hyvärinen and colleagues explored the effect
544 of reactive astrocytes using IL-1β (10 ng/mL) and TNF (10 ng/mL) on number and density of neuronal
545 axons. Co-culture of reactive astrocytes with neurons led to increased axonal density compared to co-
546 culture with control astrocytes. The authors, therefore, hypothesised that reactive astrocytes might
547 display a neurosupportive function towards axonal growth [98]. The discrepancy between the studies might
548 be explained by a difference in the activation strategy and suggests the need for further studies on
549 neuroinflammatory responses to different cues in co-culture models.

550 **Triple cultures of microglia, neurons, and astrocytes**

551 iPSC-derived triple-culture systems have recently been developed to investigate complex cell-cell
552 interactions *in vitro.* Ryan and colleagues established an iPSC-derived triple culture of microglia,
553 astrocytes, and neurons for the study of cognitive disorders associated with infection from human

554 immunodeficiency virus (HIV). They independently generated iPSC-neurons, iPSC-astrocytes, and iPSC-
555 microglia, combined the three into a common culture, and performed experiments 14 days later. In this
556 study, the authors compared the tri-culture system in the presence or absence of HIV infection and
557 assessed the outcome of common antiviral therapies. Single-cell RNA-sequencing performed on the tri-
558 culture confirmed several findings previously documented using *in vivo* models, including enhanced
559 inflammatory responses by all three cell types upon HIV infection. Microglia appeared to be the main
560 culprit in driving the initial inflammatory response to HIV infection, as examination of inflammatory genes
561 revealed the largest change in this cell type compared to astrocytes and neurons [108]. HIV is a virus that
562 infects only human cells, limiting the use of animal models for studying this infection, and therefore the
563 iPSC-derived triple culture system offers a great tool for the study of HIV infection in the brain. Guttikonda
564 and colleagues developed an iPSC-derived triple culture primarily for the study of secreted complement
565 C3, as C3 has been reported to be increased under inflammatory conditions and to be implicated in
566 neurodegenerative disorders such as Alzheimer's disease [109]. Upon stimulation with 1 µg/mL LPS for 72
567 hours, increased C3 levels, measured by ELISA, were observed in co-cultures of iPSC-microglia and iPSC-
568 neurons, and to an even greater extend in triple cultures of iPSC-microglia, iPSC-neurons, and iPSC-
569 astrocytes, suggesting a potentiation of C3 secretion via cellular crosstalk of microglia and astrocytes.
570 Furthermore, LPS stimulation increased secretion of the cytokines IL-6, TNF, IL-1β, IFN-γ, GM-CSF, and IL-
571 10, measured by ELISA, in microglia-neuron co-cultures and tri-cultures, but not in astrocyte-neuron co-
572 cultures and neuronal monocultures [58]. Bassil and colleagues developed a high-throughput iPSC-derived
573 triple culture to model Alzheimer's disease pathology by adding $A\beta_{1-42}$ oligomers to the culture. In
574 comparison to a neuron-astrocyte co-culture, the presence of microglia in a triple culture resulted in
575 decreased neuronal death and increased Aβ plaque formation, suggesting that the Aβ plaque formation
576 may be neuroprotective. When a pro-inflammatory stimulus (50 ng/mL IFN-γ, 100 ng/mL IL-1β, 50 ng/mL
577 LPS) and $A\beta_{1-42}$ oligomers were added to the triple culture system, microglial-plaque association was
578 increased, but the neuroprotective effect of microglia was lost. The authors hypothesize that microglial
579 activation in response to Aβ may be beneficial in plaque compaction and neural protection, but over-
580 activation could counteract these benefits through toxic microglial activities such as cytokine secretion
581 [110].

582 In summary, over recent years, several groups have established iPSC-derived co-culture systems, either
583 with two or three cell types, in order to achieve more physiological culture conditions, as well as to model
584 complex cell-cell interactions. These human-derived cultures have the potential to validate evidence
585 previously generated by monocultures or animal models. Compared to more complex neural organoids,

586 co-cultures have the advantage of being easily accessible for analysis and variability is considerable low
587 due to defined cell numbers. However, even the co-culture models of neurons, microglia and astrocytes
588 lack further cell types, as well as vasculature, which are normally present in the brain. In this model, direct
589 cell-cell contact is limited to the 2-dimensional space, a disadvantage compared to 3D neuronal organoids.
590 Overall, iPSC co-culture models are a versatile, yet not too complex, system for the study of
591 neuroinflammation. Future studies will show how this model contributes to understanding cell-cell
592 communication between the major cell types of the CNS.

593

594 Complex 3D iPSC culture models

595 Neural organoid models

596 Neural organoids offer the investigation of neuroinflammation in an organ-like structure containing
597 several cell types and extracellular matrix, while being accessible for functional analysis and drug
598 interventions. Due to the increase in complexity, culture time and cost, to date very few research groups
599 have used organoid models to validate findings from iPSC monocultures. Recently, guidelines for the
600 nomenclature of three-dimensional cellular models of the CNS have been defined, recommending that
601 these are termed 'neural organoids' [111]. Neural organoids recapitulate (parts of) the developmental
602 process of the brain that leads to the generation of the brain's unique 3D arrangement, establishing
603 specific and unique substructures of the brain [112]. Two different types of methodologies are used to
604 generate neural organoids: unguided and guided methods. Unguided methods rely on spontaneous
605 morphogenesis of stem cell aggregates, whereas guided organoid methods require external patterning
606 factors for the differentiation towards desired lineages. Further details about methodologies are reviewed
607 elsewhere [113].

608 Most neural organoids recapitulate the diversity of cells originating from a neuroectodermal lineage,
609 including radial glia, interneurons, astrocytes, and oligodendrocyte precursor cells [114]. Astrocytes appear
610 after radial glia and neuronal cell types and mature over time. In order to study mature astrocyte functions
611 in neural organoids, prolonged cultures well over 100 days are needed [115,116]. Most protocols lack cells of
612 non-neuronal origin, including mesodermal-derived vascular cells and microglia [117]. Incorporating
613 microglia is recommended, as microglia play crucial roles in neural development and diseases [19].

614 Distinct techniques are used to include microglia in neural organoids. Firstly, microglia can be separately
615 differentiated from iPSC and subsequently added to the culture medium, where they adhere to the

616 surface of neural organoids and migrate into the interior [52,61,118,119]. This approach allows the use of
617 defined numbers of microglia, however it also means that neural organoid and microglia differentiations
618 have to be performed in parallel. Secondly, neuronal progenitor cells have the capacity to self-assemble
619 into 3D neural organoids, and neuronal progenitor cells and microglia precursor cells can be combined to
620 generate uniform and cell-type ratio-controlled neural organoids [120]. Finally, it has been demonstrated
621 that microglia can arise alongside neural cell types in minimally patterned organoids, which avoid directing
622 differentiation towards a single germ layer [121–124]. No microglia precursors need to be added in these
623 protocols, however the timing of appearance of microglia varies between protocols and microglia
624 numbers are heterogenous between individual organoids. This induces high variability into an already
625 complex system and potentially leads to variable results between similar samples, for instance when
626 measuring cytokine concentrations in the supernatant. A summary of organoid models can be found in
627 Figure 1D. A major focus in recent years has been to improve culture conditions, for example by improving
628 nutrient supply into the inner core of the organoids, as well as the development of region-specific neural
629 organoid models. These approaches can help to improve reproducibility and robustness of organoid
630 models for future studies. Compared to iPSC-monoculture, as well as co-culture, where it is easy to control
631 for cell numbers and ratios, studying microglia and astrocytes in organoids can lead to high variability
632 within groups, so that high sample sizes should be considered.

633

634 Modelling neuroinflammation using neural organoids

635 Neuroinflammation has been studied in cerebral organoids stimulated with 100 ng/mL LPS for 24 hours
636 and 72 hours. Ormel and colleagues used both ELISA and RT-qPCR to demonstrate increased levels of the
637 pro-inflammatory cytokines IL-6 and TNF, but not the anti-inflammatory cytokine IL-10, after LPS
638 exposure. Both astrocytes and microglia were present in the studied organoids, and the increased
639 cytokine levels are likely to be induced by the interplay between both cell types, however this has not
640 been dissected [121]. When treated with a combined stimulus of 100 ng/mL LPS for 24 hours, followed by 2
641 mM ATP for 30 minutes, microglia incorporated into 'tubular organoids', which also contained astrocytes,
642 changed towards an amoeboid-like morphology, suggestive of activation. These tubular organoids
643 secreted higher levels of IL-1β, IL-18, and TNF, measured by ELISA, and showed NLRP3 inflammasome
644 activation, quantified by colocalization of NLRP3 and ASC via proximity ligation assay. The authors
645 repeated the experiments with a second stimulus, 100 nM DAMGO, a synthetic μ-opioid receptor agonist,
646 for 24 hours. In rat experiments, where 1 μM DAMGO was delivered to the nucleus accumbens core via

647 microdialysis, a significant increase in IL-1α, IL-1β and IL-6 was measured using flow cytometry [125]. Here,
648 DAMGO induced a similar inflammatory phenotype to LPS/ATP stimulation in the tubular organoids,
649 indicated by a change in microglia morphology, increased secretion of IL-1β, IL-18, and TNF, and NLRP3
650 inflammasome activation. Interestingly, the authors found that TNF levels were increased after LPS/ATP
651 stimulation in tubular organoids but not in 2D iPSC-derived microglia monocultures, which could be
652 explained by crosstalk of the different cell types (including astrocytes) present in the tubular organoids
653 model [126].

654 Neuronal damage has been modelled in neural organoids using needles and focal laser injury. When
655 pierced with a 25-gauge needle, microglia near the injury side were found to adopt a more amoeboid
656 morphology, suggestive of activation [52]. After focal laser injury, proximal microglia were observed to react
657 within minutes by extending a single long process towards the injury centre, contacting the damaged
658 zone. They then rapidly migrated their cell bodies to surround the damaged area, while microglia distant
659 from the injury site remained immobile [61]. No study to date has investigated neuroinflammatory
660 mediators after neural damage in neural organoids.

661 Neural organoids have also been used to investigate neuroinflammation in the context of disease. Zika
662 virus (ZIKV) infection was modelled in microglia-containing neural organoids. After virus exposure,
663 microglia adapted an ameboid morphology, suggestive of activation. Expression of IL-6, IL-1β, and TNF
664 was examined by qRT-PCR and was significantly increased in the ZIKV-exposed organoids. Furthermore,
665 the virus exposure had an effect on astrocytes as the authors found a significant increase in GFAP+ cells
666 in the ZIKV-exposed organoids [120]. Alzheimer's disease-related neuroinflammation was modelled by Song
667 and colleagues in microglia-containing brain region-specific organoids. Stimulation of microglia-containing
668 dorsal cortical organoids with $A\beta_{1-42}$ oligomers for 72 hours led to a significant increase in TNF, but not IL-
669 6 gene expression measured by RT-qPCR. Furthermore, $A\beta_{1-42}$ oligomer treatment induced an increase in
670 reactive oxygen species production in microglia-containing ventral cortical organoids [127].

671 Until recently, specifically astrocyte inflammatory responses have not been investigated in neural
672 organoids. A recent study utilized bioengineered neural organoids that included a method of selectively
673 initiating astrocyte reactivity through a genetically encoded chemogenetic tool. This chemogenetic
674 astrocyte activation elicited a dynamic inflammatory reaction, which was detected by RNA sequencing. A
675 Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses identified TNF signalling, IL-17 signalling,
676 MAPK signalling, Kaposi sarcoma-associated herpesvirus infection, and NF-κB signalling as the top five

677 significantly upregulated pathways. This tool allowed for the direct activation of astrocytes in neural
678 organoids and offers an opportunity for further studies of astrocyte reactivity in a 3D culture system [128].

679 To summarize, recently researchers started to use 3D neural organoids to study neuroinflammation,
680 however, to date there has only been a few studies, which mostly focussed on microglia changes after
681 stimulation. Just like in 2D cultures, LPS is a commonly used stimulus. As mentioned above, human
682 astrocytes have been reported to not respond to LPS stimulation [49], however within neural organoids,
683 microglia stimulated by LPS can release factors, such as TNF and IL-1β, that can activate astrocytes.
684 Therefore, the recorded inflammation in neural organoids is likely due to the communication of microglia
685 and astrocytes, which resembles a model of the human brain cell-cell interaction. More detailed studies
686 are needed to break down the communication between microglia and astrocytes in neuroinflammation,
687 and organoid models could hold a useful tool to do that. Additionally, in these complex systems the effect
688 of neuronal damage on inflammatory responses can be exploited, thereby providing a more physiological
689 induction of inflammation than the addition of external factors to the culture media. Before deciding to
690 use neural organoids for a research study, it should be considered to start investigating the research
691 question using iPSC-derived monoculture or co-culture models. Due to the cost and time of neural
692 organoids, these can be a great tool to validate findings from less complex models, as well as interrogate
693 cell-cell interactions.

694

695 # Xenotransplantation

696 ## Microglia and astrocyte xenotransplantation models

697 *In vitro,* iPSC models have a limited variety of brain cell types and extracellular matrix, which are important
698 for regulating microglia and astrocyte functions. Microglia are particularly plastic cells, and at a
699 transcriptional level their gene expression changes dramatically when isolated from the brain and cultured
700 *in vitro* [129]. This has led researchers to develop a method to culture iPSC-microglia *in vivo*, where iPSC-
701 microglia precursors are transplanted into brains of live postnatal or adult mice, with or without prior
702 chemical depletion of the mouse microglia and are allowed to establish and mature over a period of 2-4
703 months [52,130–133]. The resulting xenotransplant iPSC-microglia, or ‘xenoMG’, morphologically and
704 transcriptionally resemble freshly isolated adult human microglia [131,132]. The success of these initial
705 methods required immunodeficient mouse strains to avoid massive immune activation and astrocyte
706 proliferation resulting from invasive brain surgery [52,130,132]. These mice lack B, T, and NK adaptive immune

707 cells [134]. T cells are critical for microglia maturation during mouse brain development [135], and infiltrate the
708 brain during chronic neurodegeneration where they are likely to interact with microglia [136], so this should
709 be considered as a limitation of the model. More recently a new xenotransplantation model with non-
710 immunodeficient mice was developed, which relies on trans-nasal injection of the iPSC-microglia
711 precursors to minimize disruption of the blood-brain barrier and avoid glial or immune activation [137]. A
712 further innovation in microglia xenotransplantation models has been to integrate iPSC-microglia into iPSC-
713 derived forebrain organoids, and transplant these into the retro-splenial cortex of immunocompromised
714 NOD/SCID mice [138]. This method has the advantage of allowing human microglia interactions with human
715 neurons to be studied and manipulated in an authentic brain environment, as the iPSC-microglia were
716 shown to remain within the organoid graft. However, it is not clear how faithfully the organoid
717 recapitulates brain tissue organization, thus more detailed characterization of the model would be
718 helpful.

719 Astrocyte maturation at least partly requires neuron synaptic activity and a 3D environment [139,140],
720 therefore iPSC-astrocytes are likely to achieve limited maturity in monoculture. Stem cell-derived
721 astrocytes have also been successfully transplanted into the brains of mice. The first protocol for astrocyte
722 xenotransplantation used human ESC- or iPSC-derived glial progenitor cells, which developed into both
723 astrocytes and oligodendrocytes in the mouse brain [141,142]. These were used to investigate the non-
724 inflammatory phenotypes of astrocytes with schizophrenia- or Huntington's disease-linked genetic
725 mutations. More recently, pure iPSC-astrocyte progenitors injected into immunodeficient NOD-SCID mice
726 were used to characterize the morphologies of human astrocytes in the presence of Alzheimer's disease
727 pathology [143]. Neuroinflammation was not explored in these astrocyte xenotransplantation studies,
728 therefore there is large scope for future research with this iPSC model. A summary of xenotransplantation
729 models for iPSC-microglia and iPSC-astrocytes can be found in Figure 1E.

730

731 Modelling microglial neuroinflammation using xenotransplantation

732 Neuroinflammation has been studied in xenoMG models using systemic LPS challenge, with 2-5 mg/kg
733 injected intraperitoneally. This resulted in xenoMG adopting 'amoeboid' morphology, suggestive of
734 activation, downregulation of the homeostatic marker P2RY12 and upregulation of the activation marker
735 CD45, measured by immunostaining [131,132]. In one xenoMG study, the transcriptomic signature of systemic
736 LPS challenge (2 mg/kg LPS given as three intraperitoneal injections spaced 24 hours apart) was directly
737 compared to *in vitro* LPS treatment of iPSC-microglia (100 ng/mL for 24 hours) and observed to have very

738 limited overlap and fewer differentially expressed genes than *in vitro* LPS challenge of iPSC-microglia
739 monoculture [132]. The difference between microglia responses to *in vivo* and *in vitro* LPS challenge may
740 arise from the peripheral immune system acting as the signal intermediary *in vivo*, with very little LPS
741 crossing the blood-brain barrier and directly activating microglia [144]. However, the different timescales
742 used (84 hours *in vivo* versus 24 hours *in vitro*) could also have had a confounding effect. Brain injury has
743 also been modelled in xenoMG models using focal laser damage, and 2-photon time-lapse imaging of
744 microglia at the site of injury showed that xenoMG rapidly extend their processes toward the lesion, at
745 the same speed as mouse microglia [132].

746 Additionally, neuroinflammatory responses to specific neurodegenerative disease-associated pathologies
747 have been modelled by injecting protein oligomers into the brains of xenotransplanted mice [65,130,145].
748 Synthetic amyloid-β oligomers caused the majority of the xenoMG to adopt a 'cytokine response'
749 transcriptional signature, with upregulation of IL-1β, IL-6, CCL2, and CCL4, whereas treatment with a
750 'scrambled' peptide used as a control resulted in a homeostatic transcriptional signature for most cells,
751 measured by single-cell RNA sequencing [130]. Alzheimer's disease brain tissue-derived soluble tau fractions
752 caused xenoMG to accumulate phosphorylated tau protein, likely due to endocytic uptake [145]. Single-cell
753 RNA sequencing revealed an inflammatory transcriptional signature, when compared with xenoMG from
754 mice injected with healthy control brain extracts. Significantly upregulated genes were associated with
755 immune response, the type-I interferon response pathway, and antigen processing and presentation [145].
756 Finally, synthetic α-synuclein oligomers, a stimulus associated with Lewy Body diseases caused induction
757 of inflammasome activity in xenoMG, measured by ICC for caspase-1 [65]. The direct acute injection of
758 disease-associated protein oligomers into xenotransplantation models allows the response of iPSC-
759 microglia to these oligomers to be dissected, however this is not useful as a model of neurodegenerative
760 disease, since neurodegenerative diseases are chronic and multiregional in nature.

761 To summarise, microglia and astrocyte xenotransplantation models are currently the most complex iPSC
762 models with the most authentic brain-like environment. For research questions where
763 microglia/astrocyte 'maturity', and interactions with other brain cell types, extracellular matrix, or the
764 peripheral immune system are critical, these models will prove invaluable. However, time, expense,
765 difficulty of manipulation, and ethical considerations will most likely restrain use of these models. Recent
766 research has shown that it is possible to model neuroinflammation *in vivo* using xenotransplanted iPSC-
767 microglia, although this should be done with caution and bearing in mind the potential impact of species
768 differences on immune responses, and the use of immunodeficient animals that have no adaptive immune

769 system. It is likely that xenotransplanted iPSC-astrocytes will be similarly exploited for the study of
770 neuroinflammation in future.

771

772 ## Conclusion

773 Neuroinflammation plays a significant role in various brain disorders, including neurodegenerative
774 conditions, and methods to study iPSC-derived microglia/astrocyte inflammatory responses are of great
775 importance. In order to induce neuroinflammation in microglia, most studies to date used LPS at a
776 concentration ranging between 10 ng/mL and 1 µg/mL, and measured changes between 3-24 hours after
777 stimulation. In astrocytes, TNF (ranging between 10 ng/mL and 100 ng/mL, measured between 1.5 hours
778 and 7 days after stimulation) and IL-1β (10 ng/mL, measured between 5 hours and 5 days) were most
779 often used to induce a neuroinflammatory phenotype. Even though these stimuli are known to induce a
780 pro-inflammatory phenotype and are by now quite well characterized, the cellular responses might not
781 be relevant for every disease. Therefore, more research is needed to improve and validate stimulations
782 with disease-relevant stimuli. Complex cultures, including co-culture models, neural organoids and
783 xenotransplantation have the advantage that advanced methods, such as neuronal damage, can be used
784 to induce a more physiological pro-inflammatory environment. Future studies will help to re-evaluate
785 which stimuli to choose for neuroinflammatory research.

786 In order to measure inflammatory changes, most studies used cytokine secretion or gene transcription as
787 an indicator of activation. As a best practise, measuring both, gene expression changes, but also the
788 release of cytokines into the extracellular space, is recommended and will highlight different aspects of
789 the neuroinflammatory cascade. Furthermore, as immune responses to stimuli evolve over time,
790 measuring responses over a series of timepoints will often provide the most comprehensive picture of
791 neuroinflammation [73,146].

792 Here we discussed inflammatory findings in a range of iPSC models with increasing complexity:
793 monoculture, the use of secreted factors, 2D co-culture with other iPSC-derived brain cell types, 3D neural
794 organoids and xenotransplantation. There are some disadvantages to increasing cellular complexity. The
795 cost of setting up and maintaining cultures increases, and the ease of manipulation, throughput, and
796 reproducibility decreases. The main advantage is that improved cellular complexity is anticipated to
797 improve the maturity and 'authenticity' of microglia/astrocyte phenotypes, in addition to allowing specific
798 interactions between cell types to be modelled, including microglia-neuron and astrocyte-neuron

799 interactions. However, cell-type specific questions are difficult to address in models where the recorded
800 neuroinflammatory response is likely due to an interplay of multiple cell types and their interactions.
801 Research questions could first be investigated in iPSC monoculture models, and then further validated in
802 increasingly more complex cultures. Evidence is limited for how the different iPSC-derived models affect
803 microglia and astrocyte phenotypes, as direct comparisons are rare. However, Xu and colleagues
804 performed a meta-analysis of single-cell transcriptomic data from multiple studies using iPSC-microglia
805 monoculture, iPSC-microglia developed within cerebral organoids, and two xenoMG studies including
806 their own, and combined these with datasets of primary human foetal and adult microglia. Clustering by
807 principal component analysis showed that the foetal and adult primary microglia separated by the first
808 principal component, implying that this represents differences in relative age or maturity. Interestingly,
809 the monoculture and organoid-culture iPSC-microglia clustered closely with primary foetal microglia,
810 whereas the xenoMG were closer to adult primary microglia [133]. This suggests that xenoMG are either
811 more mature/aged or perhaps just more quiescent and 'homeostatic' in phenotype than other iPSC-
812 microglia models. The underlying biological differences warrant further characterization.

813 Future studies comparing complex cell culture models of microglia and astrocytes would be highly
814 beneficial to the field, particularly with regards to neuroinflammatory responses. Moving towards higher
815 levels of iPSC model complexity is assumed to improve the 'brain-like' authenticity of inflammatory
816 responses, however there is currently little evidence to support this assumption, given the scarcity of
817 cross-model comparisons. More studies comparing the different iPSC-model systems are needed to aid
818 future researchers in the design and interpretation of their iPSC experiments.

819 ## List of Abbreviations

820 **3D:** Three-dimensional

821 **Aβ-F:** Amyloid-beta fibrils

822 **AβO:** Oligomeric amyloid beta

823 **αSYN-F:** α-synuclein fibrils

824 **ACM:** Astrocyte-conditioned medium

825 **AD:** Alzheimer's disease

826 **ALS:** Amyotrophic lateral sclerosis

827 **CNS:** Central nervous system

828 **DAMPs:** Damage-associated molecular patterns

829 **ELISA:** Enzyme-linked immunosorbent assay

830 **iPSC:** Induced pluripotent stem cells

831 **LPS:** Lipopolysaccharide

832 **MCM:** Microglia-conditioned medium

833 **MS:** Multiple sclerosis

834 **NAMPs:** Neurodegeneration-associated molecular patterns

835 **NF-κB:** Nuclear factor κ-light-chain-enhancer of activated B cells

836 **NPCM:** Neuronal precursor-conditioned medium

837 **PAMPs:** Pathogen-associated molecular patterns

838 **PD:** Parkinson's disease

839 **ROS:** Reactive oxygen species

840 **TLR:** Toll-like receptor

841 **xenoMG:** Xenotransplant iPSC-microglia

842 **ZIKV:** Zika virus

843 **FIGURE 1: Overview of culture models for iPSC-microglia and iPSC-astrocytes.** (A) In recent years,
844 numerous protocols have been developed to differentiate iPSC to microglia and astrocytes, of which a
845 selected number is mentioned here. (B) PSC-derived cell conditioned media has been used to investigate
846 its effect on other CNS cell types. Here we list all the studies mentioned in the review. (C) iPSC-derived
847 CNS cells can also be studied in co-cultures of two or three different cell types. (D) Several strategies for
848 the generation of 3D neural organoids have been established and a number of representative protocols
849 are highlighted here. (E) Recently, methods to transplant human pluripotent stem cell-derived microglia
850 and astrocytes into rodent brains have been established. The lists of protocols mentioned in this figure
851 are not exhaustive.

852 *Table 1: Stimulation procedures and outcomes for microglia monocultures*

Stimulus	Stimulus concentration	Stimulus time	Method	Measure of inflammation	Reference
LPS	10 ng/mL	4 hours	RT-qPCR	Increased *IL1B, IL6* Decreased *CX3CR1*, *TREM2*, *CD33*, *CSF1R*	65
			ELISA	Increased IL-6	65
	50 ng/mL	24 hours	RT-qPCR	Increased *IL1B*, *CCL2*, *TNFAIP3*	67
	100 ng/mL	3 hours	RT-qPCR	Increased *IL1B, IL10, NLRP3*	66
			Western Blot	Increased NLRP3	66
		4 hours	RT-qPCR	Increased *IL1B* Decreased *CX3CR1, TREM2, CD33, CSF1R*	65
			ELISA	Increased IL-6, IL1B	65
		8 hours	RT-qPCR	Increased *IL1B*	68
		18 hours	RNA sequencing	*Increased IL1B*, *TNF*, *IL6*	69
			RT-qPCR	*Increased IL1B*, *IL6*	69
		24 hours	Multiplexed ELISA	Increased TNF, IL-6, IL-8, IL-10, IL-1α, CCL2, CCL4, CXCL10, CCL17	52
			Multiplexed ELISA	Increased IL-1β, IL-6	70
			Profiler human cytokine kit	Increased TNF, Serpin E1, IL-8, IL-6, IL-1Rα, IL-1β, ICAM-1, CXCL10, CXCL1, CCL5, CCL2, CCL1	57
			Human magnetic Luminex assay	Increased IL-6, TNF, IL-10, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2	71
			Cytometric bead array	Increased IL-6, MCP-1, IL-8, RANTES, GM-CSF, TNF	59
	1 µg/mL	24 hours	RT-qPCR	Increased *IL6*	72
			ELISA	Increased IL-6	72
IFN-γ	10 ng/mL	24 hours	Human magnetic Luminex assay	Measured 10 cytokines, no change detected	71
	20 ng/mL	24 hours	Multiplexed ELISA	Increased TNF, IL-8, CCL2, CCL3, CCL4, CCL17	52
			Cytometric bead array	Increased IL-6, MCP-1, IL-8	59
LPS & IFN-γ	100 ng/mL LPS and 10 ng/mL IFN-γ	24 hours	Human magnetic Luminex assay	Increased TNF, CCL2, CCL3, CCL4, RANTES	71
	100 ng/mL LPS and 20 ng/mL IFN-γ	24 hours	RT-qPCR	Increased *TNF* and *IL6*	61
			RT-qPCR	Increased *TNF, IL6, IL1B*	75
			Cytokine antibody panel membrane	Increased MIPα/β, TNF, IL-6, IFN-γ, CXCL1, CXCL10	61
			Human cytokine array kit	Increased CCL2, MIPα, IL-6, IL-8, PA1-1, CXCL1, CXCL10	75
			Multiplexed ELISA	Increased IL-1β, TNF, IL-6	70
			Cytometric bead array	Increased IL-6, TNF, MCP-1, IL-8, RANTES, GM-CSF	59

		48 hours	Cytometric bead array	Increased IL12p40, IL12p70, IL-6, IL-4, IL-10, IL-1RA, TARC, TNF, IFN-γ, IL-23	76
IL-1β	20 ng/mL	24 hours	Multiplexed ELISA	Increased TNF, IL-8, CCL3, CCL4, CXCL10, CCL17	52
Amyloid beta ($A\beta_{1-42}$)	1 µM	24 hours	RT-qPCR	Increased *TNF*, *IL6*, *IL1B*, *RELA*	72
Amyloid-beta fibrils (Aβ-F)	0.2 µM	24 hours	Cytokine array	Measured 36 human chemokines & cytokines, no change detected	81
Amyloid beta oligomers (AβO)	3 µM	24 hours	RT-qPCR	Increased *TNF*, *IL6*, *IL1B*	80
α-synuclein monomers	750 nM	6 hours	ELISA	Increased IL-6, TNF	65
α-synuclein oligomers	750 nM	6 hours	ELISA	Increased IL-1β, caspase-1, IL-6, TNF	65
α-synuclein fibrils (αSYN-F)	0.5 µM	24 hours	Cytokine array	Measured 36 human chemokines & cytokines, no change detected	81

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862 *Table 2: Stimulation procedures and outcomes for astrocyte monocultures*

Stimulus	Stimulus concentration	Stimulus time	Method	Measure of inflammation	Reference
TNF	10 ng/mL	24 hours	RT-qPCR	Increased *IL8, IL1B, IFNG, TNF, IL2, IL4, IL6, IL10*	[90]
			Cytometric bead array	Increased IL-8, IL-1β, IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10	[93]
		48 hours	Cytometric bead array	Increased IL-8	[93]
		5 days	Bulk RNA sequencing	Increased *IL6*, *C3*, *CXCL10, CXCL11* amongst others	[94]
			Immunostaining	Increased GFAP intensity	[90]
			Multiplex bead-based immunoassays	Increased GM-CSF, IL-1β	[96]
	30 ng/mL	7 days	Western Blot	Increased NF-κB phosphorylation	[92]
	50 ng/mL	1.5 hours	Immunostaining	Increased NF-κB nuclear translocation	[90]
		5 hours	Flow cytometry	Increased IL-8 & IL-6	[91]
		24 hours	Cytometric bead array	Increased IL-8	[93]
		48 hours	Cytometric bead array	Increased IL-8	[93]
		48 hours	RT-qPCR	Increased *CCL5*, *CXCL8*	[95]
		7 days	Western blot	No change in GFAP expression	[95]
			ELISA	Increased IL-6	[95]
	100 ng/mL	7 days	Bulk RNA sequencing	Increased *IL8*, *C3, CXCL10* and *CXCL11* amongst others	[92]
			Western Blot	Increased NF-κB phosphorylation	[92]
IL-1β	10 ng/mL	5 hours	Bulk RNA sequencing	Enrichment for inflammation-related GO terms	[91]
		5 hours	Flow cytometry	Increased IL-6 and IL-8	[91]
		24 hours	Cytometric bead array	Increased IL-8	[93]
		48 hours	Cytometric bead array	Increased IL-8	[93]
		48 hours	RT-qPCR	Increased *CCL5*, *CXCL8*	[95]
		5 days	Multiplex bead-based immunoassays	Increased IL-6, GM-CSF, TNF, IL-23, IFN-β, IFN-α	[96]
		7 days	Western Blot	Increased GFAP expression	[95]
			ELISA	Increased IL-6	[95]
	100 ng/mL	7 days	Bulk RNA sequencing	Increased *IL8*, *C3, CXCL10* and *CXCL11* amongst others	[92]

			Western Blot	No change in NF-κB phosphorylation	92
TNF & IL-1β	10 ng/mL TNF and 10 ng/mL IL-1β	1 hour	RT-qPCR	Decreased *GFAP*	98
			Western blot	Decreased GFAP	98
			Immunostaining	Increased NF-κB nuclear translocation	98
		5 days	Multiplex bead-based immunoassays	Increased GM-CSF, TNF, IL-1α, IL-6, IL-10, IL-12, IL-23, IFN-β, IFN-α	96
		7 days	RT-qPCR	Increased *CCL5, CXCL8, C3, LCN2*	98
			ELISA	Increased IL-6	98
		24 hours	Multiplex bead-based immunoassays	Increased C3a, MCP-3, I-TAC, GRO-a, sICAM-1, GM-CSF, IL-1RA, MIG, RANTES, IL-6, MCP-2, MIP-1a	100
IL-1α, TNF, & C1q	3 ng/mL IL-1α, 30 ng/mL TNF, & 400 ng/mL C1q	24 hours	Multiplex bead-based immunoassays	Increased C3a, MCP-3, I-TAC, GRO-a, sICAM-1, GM-CSF, MIG, RANTES, IL-6, MCP-2, MIP-1a	100
			Western blot	No change in GFAP	100
			RT-qPCR	Increased *TNF* & *IL-1β*, decreased *GFAP*	99
			Whole proteome analysis	Enrichment of GO-terms related to immune responses, cytokine-associated signalling, and recruitment of peripheral immune cells following treatment	147
Amyloid-beta fibrils (Aβ-F)	0.2 µM	24 hours	Cytokine array	Measured 36 human chemokines & cytokines, no change detected	81
α-synuclein fibrils (αSYN-F)	0.5 µM	24 hours	Cytokine array	Measured 36 human chemokines & cytokines, no change detected	81

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Declarations

Ethical Approval: not applicable.

Competing interests: The authors declare no competing interests.

Author contributions: Conceptualization, N.S., E.S., and H.H.R.; Writing - Original Draft, N.S., E.M., E.S., B.S., and H.H.R.; Writing - Review & Editing, N.S., E.M., E.S, B.S and H.H.R.; Visualization, N.S., E.M., and B.S.

Funding: NS, EM, BS and HRR were funded by the UK-DRI (Project reference UK DRI-DSI2020-IPMAR). ES was funded by the GW4 BioMed MRC Doctoral Training Partnership. The figure was created with BioRender.com.

Availability of data and materials: not applicable.

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