Using induced pluripotent stem cell (iPSC) derived microglia to model neuroinflammation in Huntington's disease

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Thesis summary

Huntington's disease (HD) is a neurodegenerative disorder characterised by progressive motor and cognitive dysfunction. It is caused by a single mutation in the gene encoding huntingtin; a protein involved in a multitude of cellular processes throughout development. Currently, there are no treatments available to slow the progression of the disease.

Much research has focussed primarily on the role of neurons in HD and other CNS disorders, however, emerging roles of glial cells including microglia are shifting the focus towards neuron-glia interactions. Neuroinflammation is now recognised as a fundamental component of HD with microglia being the primary innate immune cells orchestrating this process in the CNS. Disease modelling of HD has predominantly relied upon animal models. However, these lack translatability to humans which has consequently led to the failure of compounds in clinical trials. Induced pluripotent stem cell (iPSC) technology has the potential to provide a platform to model disease using human cells without the reliance upon the scarce supply of primary tissue. A number of published protocols are available to generate CNS cell types including neurons, astrocytes, and microglia from iPSC.

In this thesis, inflammatory responses of control and patient microglia in monoculture will be compared along with discussion of the progress towards the development of a platform incorporating neurons, astrocytes, and microglia, to better recapitulate the *in vivo* CNS microenvironment. Interestingly, patient microglia appear significantly less responsive to LPS compared to controls. The methods used to maintain and differentiate iPSC are xenofree, increasing the relevance to the human condition.

Keywords: Huntington's disease, neuroinflammation, iPSC, microglia

COVID impact statement

Due to the COVID pandemic, the scope of this thesis is restricted owing to lab shutdowns for several months and working on a reduced timetable to maintain social distancing upon return to labs. Additionally, due to lengthy differentiation protocols, some of which were further interrupted by supply issues, planned experiments were impacted. Astrocyte work was further limited due to the market withdrawal of a commercial kit and subsequent lead time on the new replacement kit.

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Contents

Abbreviations

AD	Alzheimer's disease
AMPA(R)	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenic protein
BMP2	Bone morphogenic protein 2
BMP4	Bone morphogenic protein 4
BP	BrainPhys neuronal medium
CAA	Cysteine-adenine-adenine
CAG	Cysteine-adenine-guanine
CB-1	Cannabinoid receptor type 1
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
coBP	Co-culture BrainPhys medium
coMM	Co-culture microglia medium
CT-1	Cardiotrophin 1
DAPI	4',6-diamidino-2phenylindole
DARPP32	Dopamine-and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000
DKK1	Dickkopf 1
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HD	Huntington's disease
Hdh	Mouse huntingtin homologue
HTT	Huntingtin
IBA-1	Ionised calcium-binding adaptor molecule 1

IFNγ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-3	Interleukin 3
IL-34	Interleukin 34
IL-4	Interleukin 4
IL-6	Interleukin 6
iPSC	Induced pluripotent stem cell
JAK-STAT	Janus kinase/ signal transducer and activator of transcription
MAP2	Microtubule associated protein 2
M-CSF	Macrophage colony stimulating factor
mHTT	Mutant huntingtin
MM	Microglial medium
MPM	Macrophage precursor medium
MSN	Medium spiny neuron
MS	Multiple sclerosis
NF-κB	Nuclear factor-κB
Ngn 1/2	Neurogenin 1/2
NHP	Non-human primate
NMDA(R)	*N*-methyl-D-aspartate (receptor)
NPC	Neural precursor cell
OCT4	Octamer-binding receptor 4
P2X7	Purinoreceptor 7
P2Y12	Purinoreceptor 12
rs-fMRI	Resting state functional magnetic resonance imaging
ROI	Region of interest
RSN	Resting state network
S100β	S100 calcium binding protein beta
SCF	Stem cell factor
SHH	Sonic hedgehog

TGF β	Transforming growth factor beta
TLR4	Toll-like receptor 4
TMEM119	Transmembrane protein 119
TNF α	Tumour necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
TUJI	Beta-tubulin III
VEGF	Vascular endothelial growth factor
WNT	Wingless-related integrated site
wtHTT	Wild-type huntingtin

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Chapter 1

1: General introduction

1.1. Overview

The central nervous system (CNS) is comprised of extensive networks of neurons supported by an array of other specialised cells including glia. Within the CNS, a unique immunological environment exists whereby responses are tightly regulated to preserve, maintain, and support proper functioning of neural networks. Microglia are the primary first responders of the innate immune system within the CNS (Olson and Miller, 2004). They are a highly dynamic population of cells and continuously monitor the brain microenvironment by extending and retracting their processes (Davalos et al., 2005; Nimmerjahn et al., 2005). Microglial surveillance encompasses more than just traditional immune reactivity, with microglia performing a number of roles in the CNS that may or may not intersect with their immune properties and function, including synaptic pruning (Paolicelli et al., 2011), regulation of neuronal activity, and neurotransmission (Kaindl et al., 2012; Li et al., 2012).

Inflammation within the brain, termed neuroinflammation, is a necessary and important response to challenge. Immune reactivity of microglia in the CNS is observed not only in response to pathogenic material (Mariani and Kielian, 2009) but also during instances of sterile inflammation such as injury (Donat et al., 2017) and disease states (Crotti and Glass, 2015; Hickman et al., 2018).

Neuroinflammation and particularly dysregulated inflammation within the CNS is now recognised as a significant pathological feature of both neurodevelopmental and neurodegenerative disorders (Skaper et al., 2018). Whether this inflammation serves as a cause, or a consequence of disease mechanisms remains unclear. As the primary and most abundant immune cells in the brain, microglia steer neuro-inflammatory responses and as such, are being investigated as a potential target in the treatment of neurological disorders. Despite the traditionally neurocentric focus of much CNS disease research, a number of

neurodegenerative disease-associated genes are in fact found to be enriched on microglia including APOE and TREM2 (Shi and Holtzman, 2018).

Huntington's disease is a monogenic neurodegenerative disorder caused by a mutation in the huntingtin gene (The Huntington's Disease Collaborative Research Group, 1993) in which neuroinflammation, and reactivity of microglia has been identified as a feature, even before clinical onset of symptoms (Tai et al., 2007). HD remains incurable and treatments essentially palliative. In order to develop successful treatments appropriate platforms must be employed. Modelling of brain disorders, including HD, has typically relied on the use of animals and although invaluable to research, it is now apparent that these models significantly lack translatability to humans consequently leading to significant compound attrition in the drug development process (Travessa et al., 2017). Progress in the use of human stem cells including induced pluripotent stem cells (iPSC) has provided an opportunity to more accurately model and investigate mechanisms of human disease (Centeno et al., 2018; de Rus Jacquet et al., 2021).

1.2. Huntington's disease and the role of huntingtin

1.2.1. Huntington's disease (HD)

Huntington's disease (HD) is an autosomal dominantly inherited neurodegenerative disease caused by expansion of the repetitive trinucleotide sequence, CAG, within exon 1 of the gene encoding the protein huntingtin (HTT) (The Huntington's Disease Collaborative Research Group, 1993). The huntingtin gene, also referred to as IT15, is located on chromosome 4 with the disease associated mutation located at position 4p16.3 (Gusella et al., 1983). Huntingtin (HTT) is a large 348 kDa protein, with a sequence of 3, 144 amino acids (Sadou and Humbert, 2016) and is widely expressed, with increased expression in the CNS and testes compared to other tissues (Li et al., 1993).

Typically, in healthy individuals the trinucleotide sequence, CAG, is repeated up to 35 times, with most having between 17-20 CAG repeats (Kremer et al., 1994). Individuals with between 36-39 repeats are at risk of developing HD, a phenomena known as reduced penetrance (Brinkman et al., 1997; Langbehn et al., 2004; Rubinsztein et al., 1996) and those with 40 or above will certainly present with the disease at some point. Although variable in the age of

onset, clinical expression of disease occurs on average around 40 years of age (Ross and Tabrizi, 2011), with an inverse relationship observed between age of onset and CAG repeat length (Andrew et al., 1993; Duyao et al., 1993; Snell et al., 1993). Whilst relatively rare, juvenile cases are estimated to account for between 5-10% of HD diagnoses and are typically associated with larger CAG repeat expansions (Quarrell et al., 2013).

Clinical expression of HD typically involves progressive decline in motor, cognitive and psychological function. Disruptions in cognitive and psychological function are often reported as early indicators of disease, preceding characteristic motor impairments including involuntary movements termed chorea and difficulty regulating voluntary movement (Paoli et al., 2017; Ross et al., 2014). Life expectancy following onset of clinical symptoms is around 18 years. Post-mortem examination of HD patients' brains shows generalised atrophy but with profound volume loss of the striatum including the caudate nucleus and putamen (Halliday et al., 1998; Sharp and Ross, 1996). Although the cortex shows reduced degeneration, striatal atrophy appears to correlate with the degree of severity of atrophy in the cortex (Halliday et al., 1998). Prodromal HD studies report notable changes in the brain many years prior to disease onset (Aylward et al., 2011; Rosas et al., 2008).

Anticipation is also a characteristic of HD, whereby age of clinical onset has a tendency to decrease through consecutive generations as a result of the instability of the CAG repeats and their propensity to expand in length. This is particularly apparent when the mutation is inherited via the male germline (Trottier et al., 1994). Although maternal transmission can generate expansions and result in an increase above the CAG repeat threshold for disease, contractions in CAG length are almost equal in occurrence (Kremer et al., 1994). Individuals with an intermediate number of CAG repeats, between 27-35, are therefore at higher risk of passing on a mutation that results in disease expression.

Although CAG repeat length is the most critical predictor of disease onset, considerable variation exists in the age at which symptoms occur between those with identical CAG expansions, with additional genetic modulation originating at other genomic sites influencing disease expression (Genetic Modifiers of Huntington's Disease (GeM-HD) Consortium, 2015; Gusella et al., 2014; Holbert et al., 2001; Wexler et al., 2004). Genome-wide association studies (GWAS) have identified several gene loci that contain gene modifiers that influence the development of HD and the age of symptomatic disease expression (Genetic Modifiers of

Huntington's disease (GeM-HD) Consortium, 2015). The CAG repeat sequence is unstable in somatic cells as well as during germline transmission, with greatest instability observed in the cerebral cortex and striatum (Wheeler et al., 2007; Swami et al., 2009). This instability particularly as cells age, could result in an increasingly extended polyglutamine tract that has greater toxicity thus altering the pathogenesis of HD and ultimately age of onset (Swami et al., 2009). A significant association is observed between expression of FAN1 and delayed onset and slower progression of the disease, suggesting FAN1 confers a degree of protection in the presence of an expanded CAG repeat (Goold et al., 2019).

1.2.2. The role of huntingtin and its relevance to disease mechanisms in HD

1.2.2.1. The role of huntingtin during normal development

The function of huntingtin is not yet fully understood but it appears to have a multitude of functions throughout the entire lifespan of an organism with essential roles proposed in organelle and vesicle trafficking (Chang et al., 2006; Velier et al., 1998), neurodevelopment (Godin et al., 2010; Zuccato et al., 2003), and transcriptional regulation (Nucifora et al., 2001). The significance of HTT during the early developmental stages of an organism is demonstrated in mouse studies where homozygous knockout of the HTT gene is shown to be lethal around embryonic day 7.5 (E.7.5) (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). In mice, significant depletion of HTT or selective knockdown of HTT in specific neuronal subtypes results in altered neurodevelopment leading to deficits in cognitive and motor function (Godin et al., 2010; Nasir et al., 1995). Reduced expression of wild-type HTT in the CNS of postnatal mice provokes accelerated neurodegeneration (Dragatsis et al., 2000).

HTT contains multiple HEAT (Huntingtin, elongation factor 3, the regulatory A subunit of protein phosphatase 2A and TOR1) repeats and these regions mediate protein-protein interactions, with evidence pointing to HTT functioning as a scaffold protein bringing together molecular binding partners into the correct functional conformations (Andrade and Bork, 1995; Goehler et al., 2004; Takano and Gusella, 2002). Indeed, HTT interacts with a large number of proteins (Harjes and Wanker, 2003; Li and Li, 2004) with the diversity of functions of HTT dependent on the targeted partner protein (s). HTT interacts with HAP-1 (Huntingtin-associated protein-1) which is highly expressed in the brain and was the first interactor

protein of HTT to be identified (Li et al., 1995). HAP-1 is found associated with cell membranes and vesicles suggestive of a role for HAP-1 and its binding partner, HTT, in vesicle trafficking and endocytosis (Martin et al., 1999). HTT has in fact been shown to directly interact with dynein along with HAP-1 and dynactin, forming a complex and facilitating vesicular transport (Caviston et al., 2007). Interactor complexes containing huntingtin also mediate processes such as transcription (Takano and Gusella, 2002) and synaptic function (Sun et al., 2001). PolyQ length has been shown to determine interactions with binding partners in some cases, with increased polyQ expansion altering interactions (Cornett et al., 2005; Modregger et al., 2002).

Normal brain development relies on the correct functioning of HTT, of which the N-terminus of the protein is heavily implicated in developmental processes (Zuccato et al., 2003; Lo Sardo et al., 2012). HTT is required during neural tube formation, controlling cell-cell adhesion between neuroepithelial cells through inhibitory regulation of the interactions of ADAM10 (A Disintegrin and Metalloprotease 10) with N-cadherin (Lo Sardo et al., 2012). Deficits in neural tube formation during HTT knockdown can be rescued by inhibiting ADAM10 activity (Hoettecke et al., 2010). Cortical neurogenesis is dependent on HTT-mediated orientation of mitotic spindle formation (Godin et al., 2010). Progenitor cell migration in the cortex and neural identity specification in the cortex, and also the striatum, is under the influence of HTT (Molero et al., 2009; Tong et al., 2011). Interactions between wild-type HTT and DNA elements leads to the transcriptional regulation of neuronal genes (Zuccato et al., 2003).

An anti-apoptotic function of HTT is also demonstrated including inhibition of components of apoptotic networks including caspase activity, and an upregulation in survival pathways (Rigamonti et al., 2000, 2001; Zhang et al., 2006). Over expression of wild-type HTT in striatal neurons provides protection against cell death in response to adverse stimuli including serum withdrawal and mitochondrial toxin exposure (Rigamonti et al., 2000). This anti-apoptotic effect was observed also in cells expressing only the N-terminal region of the protein, suggesting the N-terminus containing the polyQ tract is responsible for this property (Rigamonti et al., 2000).

1.2.2.2. Disease mechanisms

As the disease-associated region, the poly-Q stretch located at the N-terminus of the protein is the most intensely studied (Sadou and Humbert, 2016). In mouse models, expression of only exon 1 containing the polyQ region is sufficient to induce the characteristic features of HD (Mangiarini et al., 1996).

Huntingtin is expressed throughout the brain; however, a hallmark feature of HD pathology is the selective loss of medium spiny neurons (MSN) in the striatum due to expression of the mutated huntingtin protein, whilst cortical neurons are largely spared as the disease progresses (Graveland et al., 1985; Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). Whilst it still remains unclear why exactly mHTT preferentially affects MSN, a number of mechanisms of pathogenesis leading to degeneration have been proposed including altered transcription (Nucifora et al., 2001), mitochondrial stress (Song et al., 2011), dysregulated protein homeostasis (Hipp et al., 2014; Soares et al., 2019), excitotoxicity (Heng et al., 2009), and synaptic dysfunction (Nithianantharajah and Hannan, 2013).

1.2.2.2.1. Proteolysis, altered protein interactions, and transcriptional dysregulation

Upregulated proteolysis is observed in HD (Hermel et al., 2004; Miller et al., 2010) with inhibition of proteases shown to reduce cellular toxicity (Graham et al., 2006; Miller et al., 2010). This increase in proteolytic activity in HD is an important pathological feature of the disease, as cleavage products of the N-terminal of mHTT containing the poly-glutamine tract in particular, demonstrate enhanced toxicity in comparison to the intact mHTT protein (Barbaro et al., 2015; Gutekunst et al., 1999; Zhou et al.,2003).

These cleavage products are also prone to aggregation with polyQ expansion length determining the rate at which the protein forms aggregates, with larger expansions correlating with greater aggregation (Scherzinger et al., 1999). These aggregates form inclusion bodies (IB) that are found in the cytoplasm and also at a higher incidence in the nucleus of affected neurons compared to wild-type HTT (Becher et al., 1998; Davies and Scherzinger 1997; DiFiglia et al., 1997; Gutekunst et al., 1999). The function of these inclusion bodies is not well characterised, with evidence even suggesting a neuroprotective role whereby the cell attempts to cope by sequestering the aggregated protein and restricting its

location within the cell. This reduces the levels of mHTT throughout the rest of the neuron and extends cell survival (Arrasate et al., 2004). Interestingly fewer IBs form in the most susceptible brain region in HD, the striatum, compared to the cortex (Gutekunst et al., 1999; Sieradzan et al., 1999) with only 4% of MSNs exhibiting IB formation (Kuemmerle et al., 1999); demonstrating a potentially neuroprotective role of these structures. As well as mHTT, IBs contain components of the proteostasis network including subunits of the proteasome, chaperones, and ubiquitin, which altogether indicates dysregulated proteostasis within the cell due to the presence of misfolded protein (Hipp et al., 2014; Sieradzan et al., 1999).

As previously mentioned HTT interacts with a large number of proteins (Harjes and Wanker, 2003; Li and Li, 2004) of which some interactions appear to be cell-specific, facilitating more pronounced pathology in the striatum during HD (Subramaniam and Snyder, 2011). Ras homologue enriched in striatum (RASD2/Rhes) shows high localisation to the striatum and binds to mHTT with greater affinity compared to wild-type HTT (Subramaniam et al., 2009). This interaction with mHTT mediates sumoylation which in turn reduces aggregation of the protein, and promotes its toxicity (Subramaniam et al., 2009). Rhes gene silencing significantly reduces neurotoxicity (Mealer et al., 2013).

The polyQ region of the protein appears to determine binding to DNA elements, with polyQ expansions associated with increased interactions with DNA elements (Benn et al., 2008). As such transcriptional dysregulation is recognised as a core mechanism of pathology in HD (Luthi-Carter et al., 2000; Malla et al., 2021). Brain-derived neurotrophic factor (BDNF) is a neurotrophin with a critical role in the differentiation, maturation, and survival of neuronal cells throughout development (Huang and Reichardt, 2001). The majority of BDNF present in the striatum is in fact generated in the cortex (Altar et al., 1997) and BDNF production in the cortex is required for proper functioning of the corticostriatal synapse (Jia et al., 2010). HD patient brains show reduced levels of BDNF both in the cerebral cortex and striatum (Ferrer et al., 2000; Zuccato et al., 2001). HTT, with its numerous protein interaction sites regulates the transcription and delivery of BDNF to the striatum (Gauthier et al., 2004; Zuccato et al., 2001, 2003). Therefore, dysfunctional transcription and reduction in striatal BDNF as a result of mHTT may explain to some extent the selective vulnerability of striatal neurons to degenerative processes in HD. Rescue of disease-associated phenotypes is observed in response to overexpression of BDNF in the YAC128 mouse model of HD (Xie et al., 2010).

Dysregulated apoptotic networks are observed in HD, with increased expression of pro-apoptotic proteins including Bax (Vis et al., 2005) and altered function of the anti-apoptotic protein Bcl-2, possibly by increased activity of caspases (Cheng et al., 1997).

1.2.2.2.2. Neurotransmission and Excitotoxicity

Glutamate participates in neurotransmission by activation of metabotropic and ionotropic receptors including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Dingledine et al., 1999). Excitotoxicity refers to the cellular stress and death of neurons triggered by the influx of ions (Na^+/ Cl^-/ Ca^{2+}) and water into the cell as a result of hyperactivity of glutamate receptors (Beck, 2003). Agonism of glutamate receptors in the striatum of animal models induces striatal neuron toxicity (Coyle and Schwarcz, 1976). Some NMDAR complexes contain an NR2B subunit which is upregulated in the striatum (Loftis and Janowsky, 2003). Thus, the presence of this subtype may explain the selective loss of MSN in HD (Arzberger et al., 1997; Zeron et al., 2002). Antagonism of NMDAR demonstrates protection of neurons from excitotoxic insult (Yu et al., 2015).

Striatal MSNs have extensive glutamatergic connections and, therefore, tight regulation of glutamate signalling is required to prevent hyperactivity of networks. Astrocytes have an essential role in clearing extracellular glutamate via transporters including EAAT1 and EAAT2 (Pajarillo et al., 2019). Expression of mHTT in astrocytes impairs their ability to remove glutamate due to reduced expression of the glutamate transporters. Thus, promoting neuronal vulnerability to excitotoxic stress (Shin et al., 2005). The addition of wild-type astrocytes to HD neurons is neuroprotective against excitotoxicity (Shin et al., 2005).

1.2.2.2.3. Huntington's disease as a neurodevelopmental disorder

Although historically described as a neurodegenerative disorder, HD is now also being investigated from a neurodevelopmental perspective. As HTT has such a critical role in brain development it is likely that neurodegeneration is rooted in and a final consequence of abnormal developmental processes (Mehler and Gokhan, 2000; Wiatr et al., 2017). Compared to controls, foetuses of HD carriers' have reduced numbers of actively proliferating progenitor

cells and neuronal specification appears to be accelerated, with increased expression of the transcription factor, TBR2, indicative of cells further committed in the process of neurogenesis (Barnat et al., 2020). In the knock-in mouse model of HD, Hdh Q111, dysregulated neurogenesis within the striatum is observed including disruption in cell cycle progression and abnormal expression patterns of pluripotency markers in the neural stem cells that give rise to medium spiny neurons (MSN) (Molero et al., 2009). These altered early developmental processes are proposed to lead to a vulnerability to cellular stressors later in life, culminating in the symptomatic expression of disease (Arteaga-bracho et al., 2016); and understanding these processes may offer an opportunity to identify potential targets of early intervention before symptoms occur and irreparable damage proceeds.

1.3. Modelling Huntington's disease (HD)

A significant challenge in investigating neurological disorders, including HD, is the limited access to primary human tissue. Tissue that is obtained at post-mortem is the end-point of disease and so development and progression of the pathology cannot be measured. Human brain tissue obtained via surgery for conditions such as epilepsy also presents with issues regarding extrapolation of findings due to the underlying condition. Along with commonly used animal models (Menalled, 2005; Ramaswamy et al., 2007), the development of immortalised CNS cell lines have made it possible to investigate cells that are otherwise difficult to propagate in culture (Furihata et al., 2016; Hu et al., 2018; Hickman et al., 2008). Stem cells and in particular induced pluripotent stem cell (iPSC) technology is providing a patient-specific platform to investigate a wide range of diseases (Takahashi et al., 2007; Park et al., 2008; Vadodaria et al., 2020), and is particularly useful when investigating neurological disorders as human tissue is not readily available.

1.3.1. Animal models

A number of model organisms are used in HD research ranging from invertebrates, including Caenorhabditis Elegans and Drosophila Melanogaster, to rodents and larger animals, such as pigs, sheep, and non-human primates (Howland et al., 2020; Ramaswamy et al., 2007; Rubinsztein, 2002). Early toxin models sought to mimic the catastrophic cell death and atrophy of brain structures in HD (Coyle and Schwarcz, 1976; Beal et al., 1986; Brouillet et al.,

1993). Since the discovery of the HD gene, genetic models have allowed investigation of the disease in an etiologically relevant context (Ferrante, 2009; Howland et al., 2009). Development of genetic animal models of HD typically involves the expression of full-length human mHTT or fragments of the N-terminal disease-causing region, often producing different experimental outcomes (Menalled, 2005; Hersch and Ferrante, 2004). Full-length models provide more genetic accuracy in a system to study HD, but fragment models by comparison, offer the advantage of an accelerated disease phenotype with well-defined behavioural outcomes and neuropathological markers that are easier to identify and score (Hersch and Ferrante, 2004).

1.3.1.1. Toxin models

Prior to the identification of the HD causative gene, neurotoxin models were used to mimic the disease-associated pathology by producing lesions within the striatum, the most affected brain region in HD (Schwarcz et al., 2010; Vonsattel and DiFiglia, 1998). Excitotoxic stress and mitochondrial dysfunction are the common mechanisms by which these toxins induce pathology (Brouillet et al., 2005; Coyle and Schwarcz, 1976; Schwarcz et al., 2010).

The amino acids, quinolinic and kainic acid, have been the most commonly used agents to induce excitotoxic stress, a pathological mechanism associated with HD (DiFiglia, 1990). The glutamate analogue, kainic acid, was used in early excitotoxic studies (Coyle and Schwarcz, 1976; McGeer and McGeer 1976). Targeted injection of kainic acid into the rat striatum results in lesion formation and degeneration of intrinsic GABAergic projection neurons, whilst innervating axons are spared (Coyle and Schwarcz, 1976). These findings reflect the striatal pathology observed in post-mortem HD brains (Vonsattel and DiFiglia, 1998) and supported the excitotoxicity hypothesis of neuronal degeneration in HD via glutamate receptors (DiFiglia, 1990). Administration of kainic acid results in motor abnormalities in both rodents and non-human primates (NHP) (Coyle and Schwarcz, 1976; Kanazawa et al., 1986). The use of kainic acid in modelling HD did, however, present with limitations as it triggers neurodegeneration in brain regions distant from the initial site of injection, due to rapid diffusion of the toxin (Olney and de Gubareff, 1978; Schwarcz and Coyle, 1977; Wuerthele et al., 1978). As a result, ibotenic acid was recognised as an excitotoxin that could produce targeted lesioning when injected into different brain regions without the convulsive effects

observed with kainate administration (Schwarcz et al., 1979; Hantraye et al., 1992; Isacson et al., 1984).

The use of kainic acid to model striatal pathology was also imperfect as it did not capture the selective sparing of somatostatin and neuropeptide Y expressing neurons observed in HD (Beal et al., 1986; Reiner et al., 2011). The excitotoxic compound, quinolinic acid, which acts upon NMDARs, is produced as an intermediate during tryptophan metabolism in the kynurenine pathway, a pathway that is shown to be upregulated in HD leading to elevated levels of quinolinic acid in patients (Campbell et al., 2014; Schwarcz et al., 1988). The use of quinolinic acid, therefore, produced a more refined model that is more reflective of HD neuropathology, with the selective degeneration of GABAergic medium spiny neurons (MSN) while nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase and somatostatin expressing interneurons, and cholinergic aspiny interneurons are relatively spared. (Beal et al., 1986, Bazzett et al., 1994; Burns et al., 1995; Ferrante et al., 1993). Quinolinic acid induced bilateral lesioning of the posterior putamen produces HD-like choreic movement in a non-human primate model (NHP) (Burns et al., 1995).

Mitochondrial dysfunction is proposed as a pathological mechanism in the progression of HD (Alqahtani et al., 2023). Mitochondrial toxin models using 3-nitropropionic acid (3-NP), produce neuroanatomical and behavioural features that, to an extent, resemble those observed in HD (Brouillet et al., 2005). 3-NP elicits striatal toxicity by targeted inhibition of mitochondrial respiratory chain complex II activity which is found to be impaired in the striatum of HD patients (Browne et al., 1997; Gu et al., 1996; Stahl and Swanson, 1974; Tabrizi et al., 1999). Rats treated with 3-NP show preferential degeneration of MSN (Beal et al., 1993; Brouillet et al., 1993) with lesions displaying astrogliosis and abnormalities in MSN morphology, mimicking the histological changes observed in HD patients (Beal et al., 1993). Administration of 3-NP results in progressive degeneration of the striatum with severe motor dysfunction including dystonia and dyskinesia observed in both rats and NHP (Brouillet al., 1998; Palfi et al., 1996).

Interestingly, metabolic inhibitors appear to promote the indirect activation of NMDARs and thus contribute to excitotoxic pathology (Beal 1992). Lesions caused by metabolic inhibitors including 3-NP and malonate resemble those generated by excitotoxic compounds such as

quinolate, and could be inhibited by NMDA receptor antagonism (Brouillet et al., 1999; Greene and Greenamyre, 1996).

1.3.1.2. Genetic models

As polyQ sequences alone are documented to cause pathology in a number of model systems (Mangiarini et al., 1996; Ordway et al., 1997), a method commonly used in modelling polyQ toxicity more generally in associated diseases, is the expression of the polyQ region only along with a fluorescent reporter protein tag (Van Pelt and Truttmann, 2020). Expression of polyQ throughout the C.elegans nervous system using a pan-neuronal promoter (F25B3.3) has been used to investigate how different length polyQ sequences affect different neuronal populations (Brignull et al., 2006). An association between the length of polyQ repeats and their relative toxicity in C.elegans has also been demonstrated in non-neuronal cell types (Morley et al., 2002). Although useful and supporting the phenomena of CAG-length dependent toxicity, these models lack a specific protein and disease context by only expressing the polyQ region.

1.3.1.2.1. Invertebrate models

Invertebrate models including Drosophila and C.elegans are useful model organisms, with their short lifespan and reproductive timeline, low maintenance costs, and genetic amenability, facilitating high throughput testing and screening of potential drug therapies (Ramaswamy et al., 2007). These model systems are also amenable to spatial and temporal modification of gene expression (Gunawardena et al., 2003; Krench and Littleton et al., 2013).

In 1999, a transgenic C.elegans model was first described to investigate huntingtin toxicity, with expression of an N-terminal fragment of the huntingtin protein containing varying CAG repeat lengths from Q2-150, in sensory neurons (Faber et al., 1999). Expression of mHTT with 150 CAG repeats in ASH neurons resulted in progressive neuronal degeneration and protein aggregate formation (Faber et al., 1999). This model of C.elegans expressing 150Q in ASH neurons has been used in chemical screens to identify mutations associated with enhanced neuronal degeneration (Faber et al., 2002) and in pharmacological studies to identify neuroprotectants (Voisine et al., 2007). Ubiquilin, an interactor of polyQ containing proteins (Doi et al., 2004), has been shown to be protective against mHTT-induced toxicity and cell

death in a C.elegans model expressing a GFP labelled genetic HTT construct with 55 CAG repeats (Wang et al., 2006).

More recently a C.elegans HD model (Htt513) was developed expressing Q128 and inclusive of amino acid position 513 (Lee et al., 2017), the location at which the N-terminus of the protein is cleaved by caspase-3 (Wellington et al., 1998). Cleavage at this site results in a fragment containing the polyQ stretch that is thought to promote apoptosis with inhibition of caspase-3 cleavage of mHTT at this position shown to be neuroprotective (Wellington et al., 2000). Inclusion of non-polyQ regions of mHTT into genetic constructs demonstrates the importance of the wider protein context in polyQ-associated toxicity and its downstream effects.

Expression of a fragment of the human HTT gene containing an expanded polyglutamine stretch in the Drosophila eye shows a CAG repeat length-dependent effect on photoreceptor degeneration, with a positive correlation observed between CAG length-dependent levels of nuclear HTT accumulation and neurotoxicity (Jackson et al., 1998). Abnormalities in axonal transport have been observed in Drosophila HD models (Gunawardena et al., 2003; Lee et al., 2004; Sinadinos et al., 2009; Weiss and Littleton, 2016). Expression of amino acids 1-548 and a pathogenic 128 poly Q stretch in the drosophila eye triggers the degeneration and abnormal function of photoreceptors (Lee et al., 2004). Pan-neuronal expression of pathogenic HTT-Q128 results in progressive motor dysfunction, reduced lifespan, and localised huntingtin aggregate formation in the cytoplasm and along neurites compared to Q0 flies that showed diffuse HTT staining throughout the cytoplasm, and with no behavioural abnormalities (Lee et al, 2004). Axonal aggregates were often larger in diameter in HTT Q128 expressing flies suggesting they act as a blockade to axonal transport. The drosophila synaptic vesicle protein, synaptotagmin 1, under normal trafficking conditions in Q0 expressing flies is diffusely distributed along axons and undetectable by immunocytochemistry but is detectable as it becomes concentrated along axons in locations corresponding areas positive for HTT aggregation, implicating dysregulated axonal transport in the pathogenesis of HD (Lee et al., 2004). Deficits in axonal transport in mHTT-expressing drosophila motor neurons are also indicated by Sinadinos et al., 2009. Whilst expression did not result in locomotor abnormalities under normal conditions, exposure to heat stress caused disruption to

movement, indicating a cumulative effect of mHTT expression and a secondary external stimulus in promoting neuronal dysfunction (Sinadinos et al., 2009).

A model of HD expressing full-length HTT (HttQ128) in the Drosophila CNS demonstrates an increase in neurotransmitter release and alterations to calcium homeostasis prior to the detection of HTT cleavage and its nuclear translocation (Romero et al., 2008). Flies expressing full-length HttQ128 displayed impaired motor function as a result of age-progressive degeneration of PNS and CNS neurons; and had a shortened lifespan when compared to controls (Romero et al., 2008).

1.3.1.2.2. Rodent models

Mouse models remain the most used animal model to study HD due to their short generation time and the ease at which they can be genetically manipulated (Beal and Ferrante, 2004; Ferrante, 2009). The first and most widely used transgenic mouse model, R6/2, expresses only the N-terminal fragment of the mutated human HTT gene with between 144-150 CAG repeats (Mangiarini et al., 1996). R6/1 mice were developed alongside R6/2 mice, again expressing the N-terminal part of the HTT gene, but with roughly 115 CAG repeats and are less studied than R6/2 (Mangiarini et al., 1996; Li et al., 2005). Both appear to display similar behavioural changes; however, R6/1 mice have a delayed onset, and the progression of symptoms is generally slower and less severe (Li et al., 2005; Naver et al., 2003). R6/2 mice develop a progressive disease-like phenotype such as motor dysfunction including chorea-like movement, weight loss, and a reduced lifespan (Mangiarini et al., 1996; Li et al., 2005; Carter et al., 1999; Stack et al., 2005). They also show neuropathological features, such as, reduced striatal volume and overall brain volume; and neuronal atrophy with the occurrence of astrogliosis (Davies et al., 1997; Mangiarini et al., 1996; Stack et al., 2005). Huntingtin aggregates are also detectable as early as postnatal day 1, which increase in size and number as the animals age (Stack et al., 2005). R6/2 mice show abnormalities in the electrophysiological properties of both MSN and cortico-striatal neurons thus promoting neural circuitry deficits (Kaye et al., 2021). Preceding behavioural symptoms in R6/2 mice, cortical pyramidal glutamatergic projection neurons that synapse with MSN demonstrate hyperexcitability (Burgold et al., 2019; Cummings et al., 2009). Effective glutamate uptake is required to prevent glutamate-mediated excitotoxic insult and neuronal death (Lin et al., 2012). Glutamate transporter 1 (GLT-1) is primarily expressed in astrocytes (Chaudry et al.,

1995; Rothstein et al., 1996). Postmortem HD brains and R6/2 mice show decreased glutamate transporter 1 (GLT-1/EAAT2) expression in both the striatum and cortex (Arzberger et al., 1997; Estrada-Sánchez et al., 2009; Estrada-Sánchez et al., 2019; Hassel et al., 2008) implicating a reduction in GLT-1 expression in attenuated glutamate uptake and thus contributing to glutamate receptor hyperexcitability as a pathological mechanism in HD (Estrada-Sánchez et al., 2019).

N171-82Q is a fragment transgenic mouse model expressing both exons 1 and 2 of the human huntingtin gene, with 82 glutamines, under the control of a mouse prion promoter (Schilling et al., 1999). In this model, expression of the extended polyQ sequence is restricted to only neuronal cells within the CNS. Mice expressing the mutated (82Q) fragment display both nuclear and neurite inclusions when compared to controls (18Q), along with striatal atrophy including moderate MSN degeneration; exhibit altered coordination, tremors, and premature death (Gardian et al., 2005; Schilling et al., 1999; Yu et al., 2003). In comparison to R6/2 mice, disease onset is delayed, and lifespan is longer (Schilling et al., 1999). As determined by resting-state functional magnetic resonance imaging (rs-fMRI), patients with HD show altered functional connectivity of resting-state networks (RSN) associated with impaired cognition and motor function preceding disease onset and in early manifest HD patients (Dumas et al., 2013; Harrington et al., 2015; Wolf et al., 2014). This altered connectivity resulting in motor abnormalities is to some degree replicated in the N171-82Q mouse model (Li et al., 2017).

Transgenic models expressing full-length human HTT have also been developed (Gray et al., 2008; Hodgson et al., 1999; Reddy et al., 1998). YACHD transgenic mice have been produced that express 46, 72, and 128 CAG repeats (Hodgson et al., 1999; Slow et al., 2003). Expanded CAG repeat sequences are inserted into Yeast Artificial Chromosomes (YAC) and cloned (Duff et al., 1994) before microinjection delivery of YAC DNA into FVB/N mouse eggs (Hodgson et al., 1999). YAC mice despite having extensive nuclear huntingtin inclusions display no significant disease-associated phenotype (Slow et al., 2003) suggesting that not all fragments of mHTT promote toxicity (Zhang et al., 2008). BACHD transgenic mice are created by transfection of Bacterial Artificial Chromosome (BAC) DNA that expresses the full human huntingtin locus (Gray et al., 2008). YAC and BAC mice generally exhibit similar behavioural phenotypes and motor deficits (Pouladi et al., 2012). However, the models diverge in their display of certain neuropathological hallmarks (Gray et al., 2008; Slow et al., 2003) which

persist even when background stain is accounted for (Pouladi et al., 2012). Protein expression of human HTT is much higher in both the striatum and cortex in BACHD compared to YAC128 mice. Transcriptional dysregulation and huntingtin aggregation is observed in the striatum of YAC128 mice but not in BACHD mice and thus, YAC128 mice more closely resemble the human condition in terms of striatal pathology (Pouladi et al., 2012). These differences may be attributable to the inserted genetic construct. The human HTT gene contains a close to pure tract of CAG sequences containing a penultimate interrupting CAA sequence (Goldberg et al., 1995). The sequence expressed in YAC128 mice is more closely related to the human HTT polyQ tract with 116 CAG and 9 CAA repeats, compared to BACHD mice containing more CAA interrupting sequences (Pouladi et al., 2012). Such interrupting sequences in the polyQ region appear to delay the age of clinical onset, with patients lacking CAA sequences presenting with symptoms significantly earlier in addition to amplified somatic instability (Ciosi et al., 2019; Wright et al., 2019). Therefore, differences in genomic context may alter expression between different models. Interestingly, both YAC and BACHD mice have higher body weights when compared to controls (van Raamsdonk et al., 2006; Pouladi et al., 2010) with BACHD mice exhibiting a significantly higher body weight (Pouladi et al., 2012). This is in contrast to the human condition whereby patients lose weight as the disease progresses (van der Burg et al., 2017).

Modified BAC mouse models expressing much larger CAG repeat sequences typically associated with juvenile-onset HD, have been developed (Shenoy et al., 2022; Wegrzynowicz et al., 2015). BAC226Q mice express full-length human HTT with 226 CAG repeats (BAC226Q) in a mixed CAG-CAA sequence thus, stabilising the polyQ region (Shenoy et al., 2022). This model recapitulates the development of age-dependent and progressive HD-like phenotypes including early changes in psychiatric and cognitive processing reminiscent of prodromal HD patients (Epping et al., 2016) followed by the development of progressive motor abnormalities around 3-4 months of age. The mice also exhibit gradual weight loss with a significantly reduced lifespan at 11 months. Hallmark HD-like neuropathology including significant striatal and cortical atrophy, with striatal neuron death is also observed (Shenoy et al., 2022).

Knock-in mouse models have an expanded repetitive CAG sequence introduced into the murine huntingtin homologue under the control of the intrinsic murine Hdh promoter

(Menalled, 2005). Despite being more genetically similar due to the genomically relevant location of the insertion, these models display a behavioural and neuropathological phenotype that is mild and prolonged, in comparison to fragment models (Ferrante, 2009). HdhQ knock-in mice have been generated by the replacement of exon 1 of mouse huntingtin with a mutant version of human exon 1 expressing 92 and 111 CAG repeats (Wheeler et al., 1999; Wheeler et al., 2000). These knock-in models demonstrate CAG repeat instability (Wheeler et al, 1999), a phenomenon observed in the human HD population (The Huntington's Disease Collaborative Research Group, 1993).

Other knock-in models expressing varied numbers of CAG repeats have been developed: CAG Q140 (Menalled et al., 2003), Q150 (Lin et al., 2001; Yu et al., 2003), zQ175 (Menalled et al., 2012). Expression of 140 CAG repeats results in nuclear aggregate formation and development of motor abnormalities, with onset of symptoms occurring earlier in these mice compared to those expressing 94 CAG (Menalled et al., 2003). Like other animal models of HD, this CAG length-dependent onset of symptoms is consistent with observations in HD patients (Andrew et al., 1993). Mice expressing 140 CAG also display progressive gliosis and a reduction in the marker of medium spiny neurons, dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP32) (Hickey et al., 2008). HhdQ150 knock-in mouse model has an expanded polyQ sequence with 150 CAG repeats and the mice present with behavioural phenotypes much later than Q140 (Brooks et al., 2012; Franich et al., 2019; Lin et al., 2001; Woodman et al., 2007). The zQ175 line that actually expresses around 188Q emerged through spontaneous expansion of the 140 CAG line (Menalled et al., 2012), and is the first KI mouse model to display robust phenotypes as a heterozygote that resemble HD with an earlier onset than previous KI murine models (Heikkinen et al., 2012). An age-dependent reduction in striatal-associated gene markers (DARPP32, GLT-1, Drd2, Cnr1, PDE10A) is also observed in both homozygous and heterozygous mice (Menalled et al., 2012).

Both homozygous and heterozygous zQ175 mice present with significant motor deficits including body tremors compared to control mice, when assessed at 93 weeks of age, although homozygous mice presented with more severe neurological abnormalities compared to heterozygous animals (Menalled et al., 2012). Further characterisation of zQ175 demonstrates electrophysiological aberrations including age-dependent hyperexcitability of MSN (Heikkinen et al., 2012) and is in agreement with findings in R6/2 mice (Klapstein et al.,

2001). Homozygous and heterozygous zQ175 mice show reductions in striatal, cortical, and whole brain volumes, with reductions most pronounced in the striatum (Heikkinen et al., 2012). To produce a more severe phenotype to facilitate pre-clinical testing zQ175 mice have been backcrossed onto an FVB/N strain background that is more susceptible to neurodegeneration and present with more robust HD-associated phenotypes and earlier onset than parent lines (Southwell et al., 2016). Changes in striatal gene expression, electrophysiological properties of neurons, significant weight loss along with other non-CNS symptoms associated with HD such as testicular degeneration, (van Raamsdonk et al., 2007) are also observed in Q175FDN mice (Southwell et al., 2016).

To investigate how a mouse model may recapitulate HD during development, the zQ175 knock-in mouse model where the animals are heterozygous for the mutated human HTT protein was subjected to longitudinal MRI analysis of striatal development (Zhang et al., 2020). Previous MRI study of children and adolescents with HD indicates altered striatal development in HD compared to control groups with initial hypertrophy in the striatum and globus pallidus followed by a rapid region-specific decline in volume (van der Plas., 2019). Male zQ175 mice recapitulated this same progression from hypertrophy to rapid atrophy in these regions, although, female zQ175 HD mice displayed no significant difference in brain volume through development compared to controls (Zhang et al., 2020).

Fully humanized HD mouse strains have been created whereby only human HTT gene elements are expressed on both alleles and the endogenous mouse homolog is knocked out in BACHD/YAC crossed mice (Southwell et al., 2013; Southwell et al., 2017). Strain Hu97/18 carries one human mHTT gene with 97 CAG repeats and one human wtHTT gene with 18 CAG repeats (Southwell et al., 2013). These mice have a similar HD-like disease profile as BACHD mice including cognitive and behavioural changes compared to controls, although the expression of HD-like behavioural symptoms appear to be marginally later in the Hu97/18 strain (Southwell et al., 2013). As these mice are heterozygous for CAG-tract length, they are also heterozygous at 27 single nucleotide polymorphisms (SNP) identified as therapeutically relevant in the HTT gene. As such this model has been used to test anti-sense oligonucleotides (ASO) as potential silencing therapies targeting SNPs (Southwell et al., 2014), indicating that the Hu97/18 mouse may be more sensitive in the evaluation of SNP discrimination as knockdown of wtHTT is observed in this model compared to zero knockdown in BACHD and

YAC128 mice (Carroll et al., 2011; Southwell et al., 2014). When compared to the human HTT sequence, the BACHD transgene shows variation at 31 sites external to the CAG repeat region and is not suitable for therapeutic targeting of exon 1 DNA and transcripts (Miniarikova et al., 2016; Southwell et al., 2017). Thus, a humanised model expressing a Bac21 transgene was generated by crossing Bac21 and YAC128 mice which have endogenous Hdh knocked down (Hu128/21) (Southwell et al., 2017). Similar to the Hu97/18 line, Hu128/21 mice exhibit progressive motor deficits, and psychiatric disturbances such as depression and anxiety consistent with HD patients and previous models (Duijn et al., 2007; Pouladi et al., 2009; Southwell et al., 2013). Significantly elevated serum levels of IL-6 were also detectable at 12 months compared to control littermates (Southwell et al., 2017).

1.3.1.2.3. Large animal models: Sheep, pigs, and non-human primates (NHP)

Larger animal models including pigs, sheep, and non-human primates (NHP) offer advantages over rodent models due to their larger brains with more anatomical similarity to humans (Howland et al., 2020). Their longer lifespans also facilitate longitudinal study (Ardan et al., 2020; Taghian et al., 202).

In 2010, the transgenic sheep model of HD, OVT73, was created that expresses full-length human HTT with a pure stretch of continuous 69 CAG repeats preceding the penultimate CAACAGCAACAG sequence, and therefore encoding 73Q repeats in total (Jacobsen et al., 2010). These sheep display a HD-like neuropathological phenotype with a marked reduction in DARPP32 and cannabinoid receptor 1 (CB1) immunoreactivity inside the globus pallidus (Jacobsen et al., 2010). Expression of mHTT and the development of associated aggregates and inclusion bodies within the brain is also observed, in a pattern consistent with neuropathological studies of HD patient brains (Reid et al., 2013). However, the model does lack expression of many of the characteristic phenotypes observed in patients, including dystonia and chorea, with no obvious changes in brain anatomy or neurological perturbations. This lack of phenotype may be attributable to the age of the sheep at the time of data collection. Therefore, subsequent studies using this model have collected data at later time points (Taghian et al., 2022; Wild et al., 2015). This model has been used to identify circadian rhythm disturbances (Morton et al., 2014) and metabolic disruption (Handley et al., 2016), as early manifestations of disease prior to any overt clinical expression. MRI scans,

however, revealed no differences in brain structure between HD sheep and controls at 5 years of age (Morton et al., 2014).

Similar to HD patients, mHTT is detectable in CSF collected from OVT73 sheep aged 7-10 years old, however, in contrast to HD patients (Wild et al., 2015) levels of mHTT in CSF were not significantly correlated with neurological scores (Taghian et al., 2022). Although this may be due to the small sample size (Taghian et al., 2022). Significant correlations were, however, observed between MRI findings and a decline in motor function similar to HD patients (Tabrizi et al., 2012; Taghian et al., 2022).

The first successful transgenic miniature pig model of HD was generated by pronuclear injection of mutated porcine huntingtin cDNA with 75Q repeats, into fertilised Göttingen miniature pig eggs (Uchida et al., 2001). Founder HD pigs were found to have a varied number of transgene copies, but no HD phenotype of these pigs has been reported (Uchida et al., 2001). In 2010, miniature pig fibroblasts were transfected with a transgene expressing the N-terminus 208 region of human HTT with 105 CAG repeats, followed by somatic cell nuclear transfer (SCNT) to generate N208-105Q pigs (Yang et al., 2010). Appearing normal at birth, the live piglets had multiple transgene loci identified by Southern blotting (Yang et al., 2010). Most of the transgenic HD pigs had died by 53 hours post-birth of which one displayed significant hyperkinesia similar to the chorea observed in HD patients (Novak and Tabrizi et al., 2011; Yang et al., 2010). This early death may be due to toxicity of the N208-105Q fragment or the process of SCNT, possibly due to incomplete reprogramming (Gao et al., 2011). Expression levels of mHTT may be a factor in determining lifespan in transgenic HD pigs as the lowest expression was observed in the pig that was still alive at the time of publishing (4 months), followed by the second lowest mHTT detection in a pig that survived to 25 days (Yang et al., 2010). At post-mortem, both cleaved and uncleaved mHTT was detectable in the brain with increased caspase-3 positive apoptotic neurons (Yang et al., 2010). A larger polyQ expansion appears to be toxic during early development in transgenic pigs, as SCNT of N208-160Q fibroblasts into enucleated pig oocytes failed to produce any offspring (Yang et al., 2010). This is in contrast to mice expressing N208-160Q with only a reduction in lifespan observed (Bradford et al., 2009).

The transgenic minipig model of HD, TgHD, was developed using lentiviral vector delivery of the N-terminal portion of human mHTT (548 amino acids) with 145 CAG repeats along with

the human HTT promoter, into one-cell stage pig embryos (Baxa et al., 2013). However, polyglutamine contraction was observed and as a result the founder pigs only expressed 124Q (N548-124Q). Germline transmission was observed with expression of human mHTT in offspring in all tissues, with highest expression occurring in the brain and testes (Macakova et al., 2016; Vidinská et al., 2018). In contrast to N208-105Q transgenic pigs, the N548-14Q pigs showed no obvious deficits in development or motor function by 40 months of age with no apparent formation of aggregates in the brain by 16 months. Although, DARPP32 immunoreactivity was reduced in neostriatal neurons from the transgenic pigs compared to wild type from 16 months and impaired fertility observed from 12 months (Baxa et al., 2013). Behavioural abnormalities were, however, documented in a subsequent study of N548-124Q pigs at a later stage in development compared to the previous study (Baxa et al., 2013), with a decline in motor function observed at 48 months (Askeland et al., 2018). Due to translational gaps in disease expression, study of this model is essentially ongoing with Ardan et al., 2020 extending the phenotypic characterisation of these pigs with analysis of brain tissue performed later at 4 and 5-5.8 years of age (Ardan et al., 2020). Observations at 60-70 months added additional markers of neurodegeneration in this model including significant cell loss in the caudate nucleus, putamen, and cortex; and marked astrocyte activation (Ardan et al., 2020) indicating the gradual development of more pronounced disease phenotypes. Altogether these findings demonstrate an age-dependent neuropathological signature in TgHD minipigs with a later onset of neurodegeneration.

A knock-in pig model that recapitulates the neurodegenerative pattern observed in HD patient brains has also been developed expressing 140-150Q (Yan et al., 2018). The pigs displayed a reduction in DARPP32 and calbindin-D28k staining indicative of significant neurodegeneration of MSN, with a greater reduction in the caudate nucleus relative to the putamen. The density of interneurons in the striatum, however, were comparable between HD pigs and controls recapitulating the pattern of selective loss and sparing of particular neuronal populations observed in HD (Vonsattel et al., 1985; Yan et al., 2018). Severely altered respiratory function was also documented which has been absent in transgenic rodent models showing evidence of non-CNS symptomology associated with HD progression in patients (Reyes et al., 2014; Yan et al., 2018). This model was later used to demonstrate the safety and efficacy CRISPR-Cas9 mediated removal of mHTT, demonstrating that targeted

replacement of the expanded CAG repeat sequence, was effective in reducing HD-associated neurodegeneration and alleviating neurological abnormalities (Yan et al., 2023).

Non-human primate models of HD have also been described. Transgenic HD rhesus macaques were successfully produced via lentiviral transfection of the mutant HTT gene containing only exon 1 with 84 CAG repeats into mature macaque oocytes along with lentiviruses encoding a green fluorescent protein (GFP) (Yang et al., 2008). Of the transgenic monkeys produced, there were differences in the number of integration sites of the HD transgene with some macaques having two or more sites identified. Variation in the number of expressed CAG repeats was also observed across the group, with one macaque expressing only 29 CAG repeats, below the threshold for HD pathology in humans (Vonsattel and DiFiglia, 1998; Yang et al., 2008). A high copy number of the transgene and variations in CAG length across copy numbers correlated with the severity of phenotype expression (Yang et al., 2008). Of the other macaques, two exhibited respiratory difficulties with accelerated motor abnormalities, culminating in early death. While the two remaining macaques developed motor impairment with one displaying more prominent human HD abnormalities including dystonia and chorea just two days post-birth, and the other displayed sporadic mild involuntary movements from 1 week of age (Yang et al., 2008).

The transgenic macaque, rHD1, expressing one HD transgene copy of exon 1 with 29 CAG repeats, has been subject to longitudinal assessment (Chan et al., 2014; Chan et al., 2015). Rhd1 displays a reduction in both hippocampal and striatal volume associated with decline in cognitive functions compared to controls (Chan et al., 2014). A further longitudinal study comparing the rHD1 monkey to macaques expressing exons 1-10 of the human HTT gene with 67-72Q, demonstrated significant striatal atrophy from 24 months of age in the rHD1 monkey and more acute symptomology reminiscent of juvenile-onset HD including episodic seizures and more aggressive dystonia at observed at a young age (Chan et al., 2015). Thus, the trajectory of decline differs between macaques expressing different genetic constructs including differences in the chosen promoter (Chan et al., 2015).

In addition to the development of progressive motor deficits and cognitive decline, transgenic HD macaques have increased cortisol and pro-inflammatory mediator levels with associated anxiety-like behaviours (Raper et al., 2016), altered gene regulation (Kocerha et al., 2013),

and display a marked reduction in striatal interneurons and reactive astrogliosis (Lallani et al., 2019).

1.3.1.2.4. Considerations of animal models

Early animal models of HD involving the administration of toxins have allowed the investigation of specific pathological features of the disease such as excitotoxicity (Coyle and Schwarcz, 1976). However, they represent an acute and rapid induction of pathology that does not reflect the slow progression of a complex disease caused by a mutated protein. Since the discovery of the gene mutation responsible for the development of HD, a number of genetic animal models have been developed that demonstrate HD-like pathological features with murine models remaining the most commonly used (Ferrante, 2009; Kaye et al., 2021). Given the differences in the aging process, it is largely unsurprising that even when genetically manipulated to do so, animals with short lifespans relative to humans struggle to recapitulate a disease that gains clinical traction over a period of 10-20 years following the first appearance of symptoms (Dawson et al., 2019).

The CAG length boundaries that naturally exist in other species already point to a differential effect of CAG expansion across species. In rhesus macaques the HTT gene carries 10-11 CAG repeats whereas the threshold for HD-associated CAG expansion is greater than 35 repeats (Bates et al., 2015; Putkhao et al., 2013). Rhesus macaques also appear to develop a neurodegenerative associated behavioural and motor phenotype when expressing a CAG expansion sequence below the threshold for HD in humans (Yang et al., 2008). Transgenic rhesus monkeys despite expressing mHTT with a much lower number of CAG repeats at 84Q in comparison to transgenic mouse models expressing 150 repeats, have been shown to be more susceptible to postnatal death (Yang et al., 2008). Thus, different species sensitivities to CAG expansions are apparent with larger animals generally appearing more sensitive to the mutated protein (Yang et al., 2008). Even when genetic construct is accounted for models differ in their phenotypic outcome. Transgenic pigs display increased caspase 3 staining indicative of more apoptotic neurons (caspase-3 staining) in comparison to mice expressing the same mHTT construct, suggesting species and protein context determine apoptosis in the HD pig brain as opposed to protein levels (Yang et al., 2010). Gene modifiers are now known to affect expression of HD in humans (Gusella et al., 2014) and so the genetic background

across a model species or between species is likely to impact the phenotype and these modifiers will be absent in animal models (Menalled and Chesselet 2002).

Fragment models are favoured over full-length models as they produce an accelerated progression of disease (Hersch and Ferrante, 2004). However, the loss of the rest of the protein loses key interaction sites, such as regions containing HEAT repeats that have been shown to be involved in interactions between HTT and several respective binding partners, and regions associated with cleavage of the protein that contribute to pathological features of the disease (Managiarini et al., 1996; Schillin et al., 1999; Tebbenkamp et al., 2011), demonstrating the importance of protein context in disease expression.

Genetic manipulation tools and transgene effects are likely to contribute to the different observations made within species and between species. Transgenic models are influenced by the promoter which varies across models, the size of the huntingtin sequence inserted into the genome, and the number and location of transgene loci (Mangiarini et al., 1996; Menalled, 2005; Yang et al., 2008). The background strain of transgenic mice has also been identified as a cause of variation across mouse models due to the differences in modifier genes affecting the expression of the HD gene between mouse strains (Menalled et al., 2002).

Growing ethical concerns over the use of animals in research and the lack of disease-modifying treatments for HD and other neurodegenerative diseases is bringing into question the ability of animal models to accurately reflect disease burden in humans that translates into beneficial clinical outcomes (Dawson et al., 2018). As such there is a significant need for more human relevant models.

1.3.2. Human cell platforms for investigating Huntington's disease (HD)

1.3.1. Human stem cells

In 1998, human embryonic stem cells (hESC) were first isolated from donated human blastocysts and propagated in culture (Thomson et al., 1998). This provided a basis for modelling genetic diseases by providing, in theory, an unlimited source of stem cells to then differentiate into desired cell types (Thomson et al., 1998; Verlinsky et al., 2005). A number of HD hESC lines have been produced from HD-affected embryos and from genetic correction of normal hESC (Bradley et al., 2011; Lu et al., 2013; Niclis et al., 2009; Ruzo et al., 2018; Verlinsky et al., 2005). Formation of mHTT aggregates has only been noted in neurons derived

from hESC modified to express mHTT (Lu et al., 2013; Ruzo et al., 2018), although this discrepancy may be due to the CAG repeat lengths, with the modified hESC expressing much longer repeat sequences and as such may display pathology earlier than their HD-affected counterparts. The embryonic origin of these cells is, however, the subject of much ethical controversy.

Induced pluripotent stem cell (iPSC) technology involves the vector delivery of defined factors to reprogram somatic cells back to an early pluripotent stage of development and allows the cells to be differentiated towards a target cell of choice (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2008). This technology offers a platform to model development and disease using human cells. The relevance and importance of iPSC technology is perhaps more significant in the study of CNS disorders as the supply of primary human tissue is severely limited. Indeed, multiple protocols have been developed to differentiate iPSC into multiple CNS cell types including a number of neuronal subtypes (Chambers et al., 2009; Shi et al., 2012) along with astrocytes (Krencik and Zhang, 2010; Emdad et al., 2012) and microglia (Haenseler et al., 2017; McQuade et al., 2016; Muffat et al., 2016; Pandya et al., 2017). Oligodendrocytes have also been successfully generated from iPSC (Wang et al., 2013).

iPSC lines were first generated from HD patient fibroblasts in 2008 by retroviral delivery of the pluripotency factors, Oct4, Sox2, Klf4, c-Myc (Park et al., 2008) and there are now numerous lines available (Monk and Connor, 2021). HD iPSC have successfully been differentiated into neural stem cells and DARPP32 positive neurons (Mattis et al., 2015; Ring et al., 2015; Zhang et al., 2010; Mehta et al., 2018). Glial cells including astrocytes and microglia have now also been generated from HD patient iPSC (Garcia et al., 2019; O'Regan et al., 2021). Co-cultures of HD patient neurons and astrocytes demonstrate a reduced ability of HD astrocytes to support neuronal function including prevention of excitotoxicity (Garcia et al., 2019).

iPSCs are embryonic-like stem cells and during reprogramming lose epigenetic changes and thus age-related disease phenotypes (Mollica et al., 2018). As such direct reprogramming cell technology is an attractive platform to model human disease with much shorter differentiation timelines and reduced labour (Martens et al., 2015). Patient-derived somatic cells are directly reprogrammed to desired cell types without first reprogramming them back to an early pluripotent stem cell state, and in doing so avoids the epigenetic reset associated

with iPSC generation (Pang et al., 2011; Pfisterer et al., 2011). Non-viral delivery of neural transcription factors including *Sox2* and *Pax6* directly reprograms donor fibroblasts to induced neural precursor (iNP) cells omitting a pluripotent cell intermediate in generating target cell types. These iNP demonstrated further differentiation capacity to generate neurons and astrocytes (Maucksch et al., 2012). Transfection of DNA-free chemically modified mRNA (cmRNA) (Connor et al., 2018) has been used to generate HD iNP from patient fibroblasts with CAG repeat lengths of 41Q-57Q, followed by subsequent differentiation to DARPP32-positive striatal neurons (Monk et al., 2021). Generated neurons exhibit ubiquitinated mHTT aggregates (Monk et al., 2021).

Aggregate formation is a key neuropathological hallmark of HD (Difiglia et al., 1997) and is reported in other induced neuron (iN) models of HD (Liu et al., 2014; Victor et al., 2018) but not iPSC-derived neuronal models unless precursors were cultured for an extended amount of time or engrafted into neonatal rodent brains (Jeon et al., 2012). Increased apoptotic gene expression and impaired neuronal maturation is also observed in patient cells compared to controls (Monk et al., 2021). Despite these benefits, they do not progress through the successive time-dependent stages of development and, therefore, are limited in their application for modelling all stages of development (Wapinski et al., 2013). The ability to model earlier stages of disease is useful in developing therapeutic interventions particularly for diseases characterised by substantial cell loss, including HD, to prevent progression to cell death and irreversible damage. Gene editing technology including CRISPR/ Cas9, has allowed the genetic correction of disease cell lines (An et al., 2014) to generate isogenic controls allowing the investigation of a particular mutation in the same wider genomic context (Heman-Ackah et al., 2016; Xu et al., 2017).

1.4. Investigating Huntington's disease (HD) pathology: A focus on neuroinflammation and the role of microglia

1.4.1. Microglia

1.4.1.1. Origin and development

Microglia are the immune sentinels of the CNS, constituting between 5-15% of cells within in the human brain (Lyck et al., 2009; Pelvig et al., 2008). Microglia are a highly dynamic population of cells, and continuously extend and retract their ramified processes, surveying

the brain microenvironment (Nimmerjahn et al., 2005). In response to stimuli microglial cells retract their processes and present with an amoeboid morphology such as when phagocytosing pathogenic material and cell debris, as well as synapses (Gomez-Nicola and Perry, 2014; Hanisch and Kettenmann, 2007).

Microglial origin and mechanisms of development had long been contested up until relatively recently, however, it has since been determined that they are of mesodermal origin and arise from precursors present in the yolk-sac from where they migrate to and differentiate in the embryonic brain prior to blood brain barrier (BBB) formation (Ginhoux et al., 2010; Kierdorf et al., 2013). These specialised cells represent a population of tissue resident macrophages within the CNS, distinct from macrophages present in non-CNS tissue (Ginhoux *et al.*, 2010; Gomez-Perdiguero *et al.*, 2015; Gosselin *et al.*, 2014; Hoeffel *et al.*, 2015; Sheng *et al.*, 2015). Microglial differentiation is dependent upon the myeloid master regulator transcription factor (PU.1) and interferon regulatory factor (IRF8), similar to other myeloid cells, but does not require the transcription factor MYB (Kierdorf et al., 2013; Schulz et al., 2012). Differentiation of microglia is dependent on a number of signals generated from the CNS such as TGF-β (Matcovitch-Natan et al., 2016). Engagement of the colony stimulating factor 1 receptor (CSF-1R) located on microglia is essential for the development, survival, and maintenance of the microglial pool (Elmore et al., 2014; Ma et al., 2012; Wang et al., 2012). As they mature upon colonisation of the brain, microglia transition from an early ameboid-shape with little to no processes, into cells with an increasingly ramified morphology (Kettenmann et al., 2011; Rezaie and Male, 1999).

Microglia are the first glial cell population present in the brain developing concurrently alongside neurons (Pont-Lezica et al., 2011), and throughout development are closely associated with neurons and other glial cells including astrocytes and oligodendrocytes, and also neural progenitor populations (Frost and Schafer, 2016; Schafer et al., 2012). This close contact with neural networks allows microglia to not only react in response to immune challenge (Mariani and Kielian, 2009; Olson and Miller, 2004), but also permits microglia to participate in and modulate processes such as neurogenesis (Cunningham et al., 2013; Sierra et al., 2010, Vukovic et al., 2012), synaptic pruning (Stevens et al., 2007; Schafer et al., 2012) and synaptogenesis (Miyamoto et al., 2016), neuronal activity (Badimon et al., 2020) and neurotransmission (Kaindl et al., 2012). Microglia demonstrate a broad distribution

throughout the brain and with vast heterogeneity with regards to their number, size, proliferation capacity, morphology, and receptor expression across brain regions (Tan et al., 2020).

1.4.1.2. Functions within the CNS: surveillance, immune responses, and neural circuit modulation

Microglia have a plethora of roles during the development and maturation of the CNS. As the primary macrophages of the CNS, microglia are commonly investigated for their roles in immune defence (Olson and Miller 2004). However, they are now recognised as essential mediators in processes such as synaptic pruning (Paolicelli et al., 2011) and synaptogenesis (Miyamoto et al., 2016), neurogenesis (Aarum et al., 2003; Kreisel et al., 2018) and neurotransmission (Kaindl et al., 2012; Liu et al., 2012); contributing to overall CNS maintenance and homeostasis (Li and Barres, 2017).

1.4.1.2.1. Microglia as active sensors and mediators of immune responses in the CNS

Immune responses are determined by a basic principle of pattern recognition by specific receptors. As immune cells, microglia express key receptors and ligands to support both innate and adaptive immune responses. Toll-like receptors (TLRs) are a key group of pattern recognition receptors (PRR) expressed by macrophages, including microglia, allowing them to respond to disturbances in the CNS (Olson and Miller, 2004). Activation of TLRs 3 and 4 expressed in microglia are responsible for much of their proinflammatory cytokine release in response to stimuli (Jack et al., 2005).

Similar to other macrophage cells, M1/M2 phenotypic descriptions are typically used to describe the opposing pro- and anti-inflammatory processes observed in microglia. M1 is characterised by an upregulation in the expression of proinflammatory cytokines including IL-6, TNF α, and IL-1β, whereas M2 microglia are characterised by their production of anti-inflammatory mediators such as IL-10, TGFβ, CD206 and Arg1 (Franco and Fernandez-Suarez, 2015). This discrimination of phenotypes is now viewed as rather simplistic with microglia existing on an increasingly nuanced scale between these two highly polar states, and is dependent on cellular context (Ransohoff, 2016).

1.4.1.2.2. Homeostatic functions and regulation of microglial activity in the healthy CNS

Although in the healthy CNS microglia exhibit a suppressed phenotype, they are in fact actively participating in processes that support maturation of the CNS and maintain homeostasis.

Under normal conditions microglial activity is attenuated by regulatory signals received from the brain microenvironment. Two of the key regulatory mechanisms of microglial responses: the CD200-CD200R and CX3CL1-CX3CR1 pathways, involve bidirectional signalling between microglia and neurons (Gomez-Nicola and Perry, 2015). Engagement of the CD200 receptor (CD200R) expressed on microglia with the neuronal membrane-bound glycoprotein, CD200, is essential to maintaining microglia in a surveillant, neuroprotective state in the absence of activating stimuli (Hernangómez et al., 2012). Reduced expression of either CD200/ CD200R or both, have been reported in neurodegenerative disorders such as Alzheimer's disease (Walker et al., 2009) suggesting progression of a number of neurological disorders may involve a breakdown in the mechanisms aimed at regulating microglial activity and maintaining them in a neuroprotective state. The chemokine, fractalkine (CX3CL1), is expressed by neurons and engages with its receptor (CX3CR1) expressed on microglia, conserving the homeostatic functions of microglia and protecting neurons (Cardona et al., 2006; Liang et al., 2009).

Engulfment of synapses by microglia refines network dynamics and occurs in an activity-manner, supporting the development and maturation of the CNS (Badimon et al., 2020). Immune signalling molecules of the innate complement system are involved in regulating synaptic pruning by microglia. The complement component, C1q is transformed to C3, the cognate ligand for complement C3 receptor, which is microglial associated. Deficiency in any of these components in mice has been shown to stunt the ability of microglia to effectively engulf synapses, contributing to synaptic dysfunction (Schafer et al., 2012; Stevens et al., 2007). In contrast, control mechanisms prevent the overzealous phagocytic activity of microglia. The receptor SIRPα (signal-regulatory protein α) expressed on microglia binds to the neuronally expressed ligand, CD47, with engagement preventing microglia-mediated phagocytosis (Ding et al., 2021; Lehrman et al., 2018). CD47 has been shown to be localised at active synaptic regions preventing their loss (Lehrman et al., 2018).

Microglia provide trophic support for the brain microenvironment by secreting factors that regulate the activity of multiple cell types (Cunningham et al., 2013). Like other neural cells, microglia release BDNF under normal conditions and in response to adverse stimuli to promote repair mechanisms and maintain homeostasis (Gomes et al., 2013; Nakajima et al., 2001). Oligodendrocyte myelinogenesis is modulated by the microglia-specific secretion of insulin-like growth factor 1, IGF-1, with knockdown of IGF-1 leading to defective myelination and ablation of the microglial pool resulting in a decreased number of oligodendrocytes and their respective progenitor population (Wlodarczyk et al., 2017).

Microglia act as sensors and regulators of local cellular activity. The purinergic receptor, P2Y12, is highly expressed in microglia when compared to their peripheral immune cell counterparts (Butovsky et al., 2014; Hickman, 2013). This receptor is an important metabolite sensor, responding to adenosine nucleotides including ATP (Haynes et al., 2006). Activation of microglial P2Y12 leads to the activation of the enzyme, CD39, expressed at the microglial cell surface which is then responsible for catalysing the conversion of ATP to adenosine and initiates purinergic signalling in neurons and suppresses neuronal activity in a microglial-dependent manner (Badimon et al., 2020).

1.4.2. Modelling neuroinflammation in Huntington's disease (HD)

Under normal conditions microglia are monitoring and responding to the CNS microenvironment, secreting neurotrophic factors, and supporting the functionality of neural networks (Hickman et al., 2018). Inflammation is a necessary and protective mechanism in order to remove stimuli that disrupt the local cellular environment and promotes repair. However, dysregulated immune responses lead to persistent, chronic inflammation that promotes long-term damage (DiSabato et al., 2016). Neuroinflammation is now recognised as a significant pathological feature of HD and attention is now turning to the role of microglia in disease progression (Crotti and Glass, 2015; Jia et al., 2022). Accumulation of microglia is observed in HD patient brains and this correlates with disease severity and neuronal loss (Pavese et al., 2006; Sapp et al., 2001). Microglial activation is in fact observed in mHTT gene carriers prior to clinical symptom onset (Politis et al., 2015; Tai et al., 2007). Presymptomatic microglial accumulation and activation in the striatum is also corroborated in animal models of HD, including the R6/2 mouse expressing exon 1 of human mHTT with 150 CAG repeats (Simmons et al., 2007). The role of neuroinflammation in the pathology of HD, however, is

not well understood, and whether microglia serve a neuroprotective role in HD or are in fact detrimental to disease progression remains unclear.

Morphologically microglia adopt a more amoeboid shape, with increased soma size and fewer primary processes suggestive of a reactive phenotype and this appears to become more pronounced as the disease progresses (Sapp et al., 2001; Savage et al., 2020; Simmons et al., 2007). Impaired migration of immune cells has also been observed in mouse models of HD and patients (Kwan et al., 2012). Comparison of primary microglia isolated from YAC128 and BACHD mice expressing mHTT with HD patient monocytes, demonstrates impaired migration in response to chemotactic cues including adeonise triphophophate (ATP), monocyte-chemotractant protein-1 (MCP-1), and complement component 5a (C5a), although respective receptor expression remained the same (Kwan et al., 2012). Microglia are typically highly dynamic cells (Davalos et al., 2005; Nimmerjahn et al., 2005) but demonstrate reduced baseline process extension and retraction when expressing mHTT (Kwan et al., 2012).

Both patients and HD mouse models confirm an upregulation in markers associated with an M1 polarisation state in microglia including significantly elevated levels of pro-inflammatory cytokines. Increased levels of pro-inflammatory cytokines are detected in post-mortem HD brain tissue, including IL-6 and IL-8 (Sillvestroni et al., 2009). In HD patients, inflammatory cytokines are detected early in the progression of the disease prior to the onset of symptoms, with IL-1β, IL-6, IL-8, and TNFα appearing elevated not only in the striatum and cerebrospinal fluid (CSF) but also in the periphery, as measured in plasma samples (Björkqvist et al., 2008; Chang et al., 2015; Rodrigues et al., 2016). Determining the origin of such increases in inflammatory mediators can be a challenge due to the presence of other contaminating cells, however, transcripts for IL-6 have been found to be elevated in primary microglia isolated from zQ175 mice (Crotti et al., 2014). Early symptomatic R6/2 mice show an upregulation in peripheral cytokine mRNA transcripts but no changes in the striatum (Pido-Lopez et al., 2018). Despite gene upregulation in the periphery, cytokine release remained undetected but an increase in the number of recently activated macrophages was observed (Pido-Lopez et al., 2018). Elevated levels of inflammatory cytokines including IL-1β, TNF α, IL-6, and IL-10, were only observed in late symptomatic stage R6/2 mice (Pido-Lopez et al., 2018). Thus, peripheral immune activation appears to be an early pathological feature that precedes CNS immune activity in R6/2 mice, and is age-progressive (Pido-Lopez et al., 2018). Late-stage symptomatic

mice show elevated levels of TNF α in both the brain and periphery, despite no evidence of increased TNF α gene expression in the periphery, suggestive of cross-talk and migration between the CNS immunological mileu and that of the periphery (Pido-Lopez et al., 2018).

As well as dysregulated pro-inflammatory signalling, an impairment of anti-inflammatory networks has also been described in the literature with low levels of the anti-inflammatory cytokine, IL-10, recorded in peripheral blood samples taken from R6/1 mice; with sex differences observed in the elevated levels of pro-inflammatory cytokines including IL-6, TNFα, and IL-1β (Podlacha et al., 2022). Similarly, alterations to receptor signalling pathways that regulate microglial activity under normal physiological conditions promote aberrations in microglial behaviour, contributing to disease pathology (Chamera et al., 2019; Kim et al., 2020). R6/1 mice display increased synaptic pruning as a result of downregulated fractalkine/ CX3CR1 signalling and this precedes the onset of symptoms and any morphological changes associated with inflammatory microglia (Kim et al., 2020).

mHTT expressing neurons activate microglia initiating an inflammatory response localised to dysfunctional neurites (Kraft et al., 2012). Microglia not only respond to mHTT as a result of exogenous signals, but also through cell-autonomous mechanisms with mHTT expression in microglia promoting expression of genes associated with inflammation without the presence of an extrinsic pro-inflammatory stimulus (Crotti et al., 2014). This response relates to upregulated expression of PU.1 and CCAAT-enhancer binding proteins (C/EBPs), which determine macrophage/ microglia lineages and shape their function (Kierdorf et al., 2013; Heinz et al., 2010). These factors responsible for determining myeloid identity then select enhancers and promoters, which are modulated in a signal-dependent manner by proinflammatory transcription factors including NFκB (Heinz et al., 2010). This increased activity of PU.1 and C/EBPs means microglia expressing mHTT display a reactive profile that is in turn toxic to non-HD neurons compared to wild-type microglia (Crotti et al., 2014), indicating cell-autonomous mechanisms play a role in mediating neurotoxic outputs of microglia in HD. Reducing mHTT levels by siRNA knockdown is shown to ameliorate NF-κB transcriptional dysregulation, and subsequently downregulate expression of pro-inflammatory mediators in HD (Träger et al., 2014).

The addition of wild-type primary microglia to neurons expressing mHTT promotes neuronal survival with the effects proportional to microglia numbers (Kraft et al., 2012). This same amelioration of disease phenotype is observed *in vivo* where supplementation with healthy human glia to the transgenic HD mouse model, R6/2, conferred neuronal protection (Benraiss et al., 2016). Supplementation with corrected microglia and subsequent improved neuronal survival indicates that expression of mHTT in microglia is to some extent, important to neuronal health in HD and that neurons expressing mHTT can be rescued by healthy microglia. Therefore, a balance between intrinsic and extrinsic cell signalling appears to determine the reactivity of microglia in the context of neuroinflammation in HD.

Similar to microglia, the number of reactive astrocytes in the brains of HD patients correlates with disease severity (Vonsattel et al., 1985). Primary cultures of R6/2 mouse astrocytes demonstrate increased NF-κB signalling, a pathway involved in inflammatory processes, leading to increased release of pro-inflammatory cytokines (Hsaio et al., 2013). Interestingly, this pathway appears to be aberrantly activated in mHTT expressing astrocytes under basal conditions, suggesting a role for astrocytes in early neuroinflammatory processes in HD (Hsaio et al., 2013). The expression of mHTT in astrocytes also renders them unable to provide effective neuroprotection (Bradford et al., 2010) including reduced glutamate uptake, contributing to excitotoxic mechanisms (Shin et al., 2005) and reduced antioxidant release (Rebec, 2013). The role of astrocytes in neuroinflammatory mechanisms in HD, however, is not well understood.

In summary, much of the literature indicates a pro-inflammatory environment in HD in both the CNS and periphery that propagates disease phenotypes (Björkqvist et al., 2008; Pido-Lopez et al., 2018; Sillvestroni et al., 2009). However, there is contradictory evidence in mice models. The application of IL-6 neutralising antibodies is shown to ameliorate disease expression in R6/2 mice with partial rescue of motor deficits (Bouchard et al., 2012), however, mice lacking IL-6 displayed exacerbated behavioural phenotypes through altered regulation of genes associated with HD pathology including those involved in synaptic function (Wertz et al., 2020). The relationship between astrocytes and microglia, and how they act in concert in the pathology of HD is largely unknown. However, amelioration of disease phenotypes when either is targeted demonstrates their potential as therapeutic targets in the treatment

of HD and the need to more thoroughly explore glial cell interactions (Siew et al., 2019; Wood et al., 2019).

1.5. Aims and objectives of this thesis

To date there are no treatments available to successfully modify the progression of Huntington's disease (HD). Animal models have provided significant insight into the mechanisms of disease pathology but are failing to accurately predict efficacy in patients leading to significant attrition of compounds during the drug discovery process. As such, models of increasing human relevance are needed to bridge this gap. Firstly, induced pluripotent stem cell (iPSC) technology has allowed access to patient derived cells that can then be differentiated into multiple CNS cell types by addition of developmental factors. This technology has provided access to otherwise unattainable cell populations of the CNS under normal conditions and the ability to investigate a range of genetic backgrounds in disease states. Neuroinflammation is now increasingly recognised as a modifier of disease progression and the potential to modulate microglial activity is an attractive target for therapeutic intervention. The overall aim of this thesis, therefore, is to develop a model of neuroinflammatory processes in HD with a focus on the role of microglia before considering their activity in the context of other CNS cell types with the aim of developing a platform containing neurons, astrocytes, and microglia. To achieve this the following objectives will be considered:

1) To successfully differentiate control and HD patient iPSCs into microglia, astrocytes, and neurons, and characterise them by specific marker expression.
2) To assess the inflammatory function of iPSC-derived microglia in monoculture and determine if HD patient microglia demonstrate increased pro-inflammatory activity compared to control cells.
3) To build upon previous methods developed in house for co-culture of CNS cell types and assess whether the media conditions are suitable for microglial survival and function.
4) To co-culture microglia with astrocytes in two different media compositions and assess the inflammatory responses when treated with activating stimuli and determine if co-culture of astrocytes with microglia produces a synergistic elevated pro-inflammatory response; and if this is further enhanced by the presence of HD patient cells.

Chapter 2

2: Materials and Methods

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

2.1. Cell maintenance

2.1.1. General procedure for plate preparation

Cell culture surfaces were coated with cell specific coatings. Depending on the end-point assay cells were plated onto either coated plastic or glass coverslips (immunocytochemistry; live cell imaging). Glass coverslips (13 mm; VWR) were pre-sterilised in 70% ethanol and allowed to dry in the laminar flow before addition of coating reagent.

Cell culture surfaces were prepared as follows:

Vitronectin

A final solution of vitronectin at 5 µg/mL or 10 µg/mL (cell line and differentiation dependent) was prepared by dilution of vitronectin human recombinant protein (Gibco) in Dulbecco's phosphate buffered saline (D-PBS). Coated culture vessels were then incubated for 1 hour 30 minutes at room temperature. The solution was simply removed before addition of culture medium without the need for wash steps.

Poly-L-ornithine (pORN)/ laminin-511

A 20 µg/mL poly-L-ornithine (pORN) solution was prepared by dilution in tissue culture grade water and added to the culture surface at 200 µL/cm^2 followed by incubation overnight at 37^{o}C/ 5% CO_2. The pORN solution was then removed, and the culture surface washed twice with water, followed by a single wash with D-PBS before addition of 2 µg/mL recombinant human laminin BG iMatrix-511 (Biogems by Peprotech) in D-PBS at 200 µL/cm^2 and incubation at 37^{o}C/ 5% CO_2 overnight.

Poly-D-lysine (PDL)

Poly-D-lysine (PDL; Gibco) was diluted in tissue culture grade water to a concentration of 50 µg/mL. The solution was added to tissue culture plates and incubated at 37°C/ 5% CO_2 overnight. Following incubation, the PDL solution was removed, and the plates washed twice with water followed by a single D-PBS wash.

2.1.2. Induced pluripotent stem cells (iPSC)

Control (HPSI1113i-podx_1; Human Induced Pluripotent Stem Cell Initiative – HipSci) and HD 109 (ND50036; NINDS) iPSCs were maintained on 5 µg/ml and 10 µg/ml vitronectin human recombinant protein respectively, in complete Essential 8 Medium (E8; Gibco). Full medium exchanges were performed daily, and cells passaged at around 80% confluency. To passage the cells, media was removed, and the cells rinsed with D-PBS without calcium and magnesium before the addition of 0.5 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen). The cells were then incubated at 37 °C/ 5% CO_2 for 2-3 minutes before EDTA removal and addition of fresh complete E8 Medium to the culture surface. Cells were washed from the surface by gently pipetting up and down using a 5 ml pipette to retain the cells in small clusters and plated into vitronectin coated culture vessels preconditioned with fresh complete E8 Medium. Cells were typically passaged every 4-5 days at a ratio of 1:5.

2.1.3. Neural precursor cells (NPC)

Neural precursor cells (NPC) were maintained on pORN (20 µg/mL) and recombinant human laminin BG iMatrix-511 (2 µg/mL; Biogems by Peprotech) in Neural Maintenance Medium (NMM). NMM was composed of a 1:1 mix of Advanced DMEM/ F-12 (Gibco) with 1X N-2 supplement (Gibco), 1X GlutaMAX (Gibco) and 50 µM 2-mercaptoethanol (Gibco); and Neurobasal Medium (Gibco) with 1X B-27 supplement (Gibco), 1X GlutaMAX (Gibco) and 50 µM 2-mercaptoethanol (Gibco). Full medium exchanges were carried out every 2-3 days and cells typically passaged every 4-6 days when the cells reached 90-95% confluency using Stem Pro™ Accutase (Gibco). For cell passage, spent medium was removed and the cells washed once with D-PBS before incubation with Accutase for 3-5 minutes at 37°C/ 5% CO_2. Following incubation, Accutase was neutralised by addition of DMEM at a ratio of 3:1 DMEM to Accutase. Cells were gently rinsed from the culture surface, collected, and centrifuged at 200 x g for 5 minutes. Cells were then resuspended in NMM containing 10 µM Y-27632 (Hello Bio).

24 hours after cell passaging, medium containing Y-27632 was removed and replaced with NMM only.

2.1.4. Neurons

iPSC-derived neurons were maintained on pORN LAM-511 in BrainPhys™ SM1™ Neuronal Medium (STEMCELL Technologies) supplemented with 20 ng/mL BDNF (STEMCELL Technologies) and 20 ng/mL GDNF (STEMCELL Technologies). Half medium exchanges were carried out every 2-3 days.

2.1.5. Astrocytes

iPSC-derived astrocytes were maintained on pORN LAM-511 in Astrocyte Medium (AM) composed of Advanced DMEM/ F-12 supplemented with 1X GlutaMAX, 1X N-2 supplement and 50 µM 2-mercaptoethanol. Complete medium exchanges were carried out every 2-3 days and astrocytes passaged weekly using Accutase as previously described (Section 2.1.3) with the exception of centrifugation at 400 x g for 5 minutes.

2.1.6. Macrophage precursors

Macrophage precursors were maintained in cell culture treated flasks with X-VIVO™ 10 Haematopoietic Medium supplemented with 1X GlutaMAX, 50 µM 2-mercaptoethanol, 25 ng/mL Interleukin-3 (IL-3; Peprotech), and 100 ng/mL Macrophage Colony Stimulating Factor (M-CSF; Peprotech). Half medium exchanges were carried out every 7 days.

2.1.7. Microglia

iPSC-derived microglial cells were maintained on PDL (50 µg/mL) in Microglial Medium (MM) composed of Advanced DMEM F-12 medium supplemented with 1X GlutaMAX, 1X N-2 supplement, 50 µM 2-mercaptoethanol, and 100 ng/mL Interleukin-34 (IL-34; Peprotech). Full medium exchanges were carried out every 2-3 days.

Cell type	Media composition
iPSC	Essential 8 Medium (Gibco)
NPC	Neural maintenance medium (NMM): 1:1 mix of Advanced DMEM/ F-12 (Gibco) with 1X N-2 supplement (Gibco), 1X GlutaMAX (Gibco), and 50 µM 2-mercaptoethanol (Gibco); and Neurobasal Medium (Gibco) with 1X B-27 supplement (Gibco), 1X GlutaMAX (Gibco), and 50 µM 2-mercaptoethanol (Gibco)
Neurons	BrainPhys™ SM1™ Neuronal Medium (STEMCELL Technologies) supplemented with 20 ng/mL of BDNF (STEMCELL Technologies) and GDNF (STEMCELL Technologies)
Astrocytes	Astrocyte Medium (AM): Advanced DMEM/ F-12 (Gibco) supplemented with 1X GlutaMAX (Gibco), 1X N-2 supplement (Gibco), and 50 µM 2-mercaptoethanol (Gibco)
Macrophage/ microglial progenitors	Macrophage precursor medium (MPM): X-VIVO™ 10 Haematopoietic Medium supplemented with 1X GlutaMAX (Gibco), 1X N-2 supplement (Gibco), 50 µM 2-mercaptoethanol (Gibco), 25 ng/mL IL-3 (Peprotech), and 100 ng/mL M-CSF (Peprotech)
Microglia	Microglial Medium (MM): Advanced DMEM/ F-12 (Gibco) supplemented with 1X GlutaMAX (Gibco), 1X N-2 supplement (Gibco), 50 µM 2-mercaptoethanol (Gibco), and 100 ng/mL IL-34 (Peprotech)

Table 1. Cell culture medium compositions.

2.1.8. Cryopreservation

iPSCs, NPCs and astrocytes were frozen in their respective maintenance medias containing 10% DMSO.

2.2. Cell differentiation

2.2.1. Neural induction: Generation of neural precursor cells (NPCs)

iPSCs were maintained as previously described (Section 2.1.2). At around 80% confluency, iPSCs were passaged at a 1:5 ratio using Accutase. Briefly, cells were washed once with D-PBS before addition of Accutase for 2 minutes at 37 ^{o}C/ 5% CO_2. DMEM was then added to the cells to neutralise the Accutase at a ratio of 1:3 parts DMEM and cells gently removed from the surface using a cell lifter to keep the cells in small sheets before centrifugation at 200 x g for 5 minutes. The supernatant was removed and the cell pellet gently resuspended in complete E8 Medium supplemented with 10 µM Y-27632 (Hello Bio). The cell suspension was then added to tissue culture plates coated with 10 µg/mL vitronectin and the cells incubated at 37^{o}C/ 5% CO_2. The following day a full medium exchange with Neural Induction Medium (NIM) (Axol Bioscience, Cambridge UK) was carried out. Full medium exchanges with NIM were then carried out daily.

On day 11, cells were passaged at a 1:1 ratio using Accutase and plated into pre-coated pORN-LAM 511 tissue culture plates with NIM supplemented with 10 µM Y-27632. Full medium exchanges with NIM were carried out daily for a further 2 days. On day 14, a full medium exchange with Neural Maintenance Medium (NMM) was carried out. Full medium exchanges with NMM were then carried out every 2-3 days and NPCs passaged using Accutase every 3-5 days. Cells were plated with NMM supplemented with 10 µM Y-27632 at the time of passaging and the medium exchanged after 24 hours.

2.2.2. Neuronal differentiation

Neural precursor cells (NPCs) were passaged as previously mentioned (Section 2.1.3) and plated onto pORN-laminin 511 at a density of 2 x 10 ^{5} cells/ cm^{2} in Neural Maintenance Medium (NMM) supplemented with 10 µM Y-27632. After 24 hrs the medium was removed and replaced with fresh NMM for a further 24 hrs before initiating neuronal differentiation with the gamma secretase inhibitor, Compound E. After 48 hours post initial plating a full

medium exchange with BrainPhys™ SM1™ Neuronal Medium (STEMCELL Technologies) supplemented with 20 ng/mL of BDNF and GDNF (STEMCELLTechnologies), and 2 µM Compound E (Hello Bio) was carried out. Half medium exchanges with this same medium supplemented with 2 µM Compound E were carried out every 72 hours thereafter. On day 9, a half medium exchange was carried out with complete BrainPhys™ SM1™ with BDNF and GDNF. Neuronal cultures were then subsequently maintained with half medium exchanges carried out every 2 days.

2.2.3. Astrocyte differentiation

Neural precursor cells (NPC) were passaged using Accutase as previously described (Section 2.1.3) and resuspended in Neural Maintenance Medium (NMM) supplemented with 10 µM Y-27632. Cells were plated at a density of 2 x 10^5 cells/ cm^2 onto pORN-laminin 511 coated culture plates and incubated at 37 °C/ 5% CO_2. After 24 hours, a full medium exchange with NMM was carried out. After a further 24 hours, NMM was exchanged with Astrocyte Differentiation Medium (ADM; STEMCELL Technologies) and full medium exchanges with ADM carried out daily for 6 days. On day 7, astrocyte precursors were passaged 1:2 using Accutase and cells resuspended in ADM. Complete medium exchanges were then carried out on alternate days instead of daily. Cells were passaged again 1:2 at 14 days and resuspended in ADM with full medium exchanges continuing every other day for a further 7 days. On day 21, cells were passaged 1:2 and plated in Astrocyte Maturation Medium (AMM; STEMCELL Technologies) with complete medium exchanges carried out every 48 hours and cells passaged every 7-9 days. On day 46, AMM was replaced with serum-free Astrocyte Medium (AM) composed of Advanced DMEM/ F-12 medium supplemented with 1X N-2 supplement, 1X GlutaMAX, and 50 µM 2-mercaptoethanol.

2.2.4. Microglia differentiation

Microglia differentiation from iPSC was carried out according to a published method (Haenseler et al., 2017). Differentiation to microglia comprises two stages: macrophage induction to produce macrophage precursors followed by terminal differentiation to microglia. At around 80% confluency iPSCs were passaged for the first stage. Firstly, cell medium was removed, and the cells washed with D-PBS without calcium or magnesium before incubation with Accutase at 37 °C/ CO_2 for 2-3 minutes. DMEM was then added to the

Accutase at a ratio of 3:1 respectively before gently dislodging iPSC colonies. Cells were collected and centrifuged at 200 x g for 5 minutes. Following centrifugation, the cells were resuspended in embryoid body (EB) medium composed of complete mTeSR™ 1 medium (STEMCELL Technologies) supplemented with 50 ng/mL of bone morphogenic protein-4 (BMP4; Peprotech) and vascular endothelial growth factor (VEGF; Peprotech), 20 ng/mL stem cell factor (SCF; Peprotech), and 10 µM Y-27632. Cells were plated at a concentration of 2 x 10^6 cells/mL into Aggrewell 800™ (STEMCELL Technologies) microwell plates pre-treated with Aggrewell Rinsing Solution™ (STEMCELL Technologies). The microplate containing cells was then centrifuged for 3 minutes at 100 g/ 37°C and the cells returned to the incubator for embryoid body (EB) formation. 2/3 medium exchanges with fresh EB medium were then carried out daily for 5 days. On day 5, the cell suspension containing EBs was carefully collected and passed through a 40 µm cell strainer to capture the EBs. The EBs were then resuspended in Macrophage Precursor Medium (MPM) composed of X-VIVO™ 10 Haematopoietic Cell Medium (Lonza) medium supplemented with 1X Glutamax, 50 µM 2-mercaptoethanol, 100 ng/mL macrophage colony stimulating factor (M-CSF; Peprotech), and 25 ng/mL Interleukin-3 (IL-3; Peprotech). EBs were incubated at 37 °C / 5% CO_2 and left undisturbed for 7 days to allow attachment. Half-medium exchanges were then carried out weekly. Macrophage precursors were visible in suspension around 4 weeks in MPM.

For final differentiation to generate microglia, floating macrophage precursors were collected at week 7 in MPM and first passed through a 40 µm cell strainer before centrifugation at 400 x g for 7 minutes. Following centrifugation, the cells were resuspended in Microglial Medium (MM) composed of Advanced DMEM/ F-12 medium supplemented with 1X GlutaMAX, 1X N-2 supplement, 50 µM 2-mercaptoethanol, and 100 ng/mL IL-34 (Peprotech). Cells were differentiated to microglia for a further 2 weeks.

2.3. Experimental procedures

2.3.1. Glial cocultures: iPSC-derived astrocytes and microglia

iPSC-derived astrocytes and microglia were cocultured at a ratio of 1:2 respectively. First astrocytes were plated onto pORN LAM-511 in AM at a concentration of 1.5 x 10^4 cells/cm^2. The following day, medium was removed and replaced with AM containing 2 µM cytosine arabinoside (Ara-C). After 24 hours treatment with Ara-C, the medium was replaced with AM

only for a further 24 hours before addition of microglial progenitors at a concentration of 3.0 x 10^4 cells/cm^2. Progenitors were added to the astrocyte cultures in MM and full medium exchanges carried out every 2-3 days. Cultures were assessed for expression of key markers by immunocytochemistry (ICC) and responses to inflammatory stimuli.

2.3.4. Medium compatibility assessment for microglia in coculture and triculture

Macrophage precursors were plated as previously described (Section 2.2.4) at 5.0 x 10^4 cells/cm^2 and initially plated in MM for 2 hours to allow cells to attach. Following attachment, cells were treated with either MM or BrainPhys™ SM1™ Neuronal medium supplemented with 20 ng/mL BDNF, 20 ng/mL GDNF, and 100 ng/mL IL-34 for the remainder of differentiation. Differentiation of the progenitors into microglia was assessed by immunocytochemistry of key markers.

2.3.2. Glial monocultures and cocultures: Treatments

2.3.2.1 Assessment of inflammatory responses in iPSC-derived microglia, astrocytes, and co-cultures

Macrophage precursors were plated at a density of 1.0 x 10^4 cells/ cm^2 and terminally differentiated to microglia as described above (Section 2.2.4). Complete medium exchanges with microglial medium (MM) were carried out every 2-3 days. At day 15 from initial plating, microglial cells were ready for experimental treatments. Culture medium was removed, and cells washed once with D-PBS before addition of MM supplemented with either 70 ng/mL lipopolysaccharide (LPS) or 20 ng/mL IL-4 (Peprotech) for a total of 24 hours. In a further condition, cells were treated with 70 ng/mL LPS for 4 hours before removal, washing with MM, and addition of 20 ng/mL IL-4 for the remainder of the experiment. At 24 hours, conditioned medium samples were taken for cytokine release assays, and the cells lysed for total protein quantification. Monocultures of astrocytes or microglia, as well as cocultures, were subject to treatment with 1 µM amyloid and high dose LPS (1 µg/mL). All treatment incubation periods were 24 hours.

2.3.3. Phagocytosis assay

To assess the ability of the iPSC-derived microglia to phagocytose material, microglia were plated at a cell density of 7 x 10^4 cells/ cm^2 on PDL coated glass coverslips. After 2 weeks differentiation cell medium was removed, and the cells washed once with D-PBS before addition of MM containing 10 µg/mL pHrodo™ E.coli BioParticles™ (Invitrogen). Cells were then imaged with an Incucyte S3 (Sartorius) live imaging platform. Two images per well were taken every 15 minutes for a total of 10 hours at X10 objective.

2.4. Immunocytochemistry (ICC) and fluorescence microscopy

2.4.1. Cell fixation

Cell culture medium was removed, and cells rinsed twice with cold D-PBS without calcium and magnesium before addition of 4% paraformaldehyde (PFA) in D-PBS to the cells and incubation at room temperature for 20 minutes. The PFA was removed, and the cells washed twice again with cold D-PBS. 0.1 % sodium azide made up in D-PBS was added to the cells for storage at 4°C until antibody staining was performed.

2.4.2. Antibody staining procedure

Following fixation, PBS was removed, and cells treated with permeabilization buffer containing 0.2% Triton X-100 in PBS. Three five-minute washes were carried out in permeabilization buffer before incubating the cells in blocking buffer containing 2 % BSA/ 0.2 % Triton X-100 in PBS for 3 hours at room temperature. After removal of blocking buffer, primary antibodies diluted in blocking buffer were then added to the cells and incubated at 4 °C overnight. Primary antibodies were then removed, and the cells washed in blocking buffer for three five-minute washes before addition of secondary antibodies diluted in blocking buffer. Cells were incubated with secondary antibodies for 2 hours at room temperature. Following incubation, secondary antibodies were removed as per previous wash step. 4',6-Diamidino-2-phenylindole dihydrochloride, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) diluted in D-PBS (1 in 1000) was then added to the cells for 20 minutes followed by two D-PBS washes before mounting coverslips onto glass slides using ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen).

Antibody	Target cell	Species	Dilution
Anti-OCT4	iPSC	Rabbit	1 in 400
Anti-SOX2	NPC/ iPSC	Mouse	1 in 100
Anti-PAX6	NPC	Rabbit	1 in 300
Anti-NESTIN	NPC	Mouse	1 in 500
Anti-Ki67	NPC	Rabbit	1 in 500
Anti-MAP2	Neurons	Chicken	1 in 1000
Anti-TUJI	Neurons	Mouse	1 in 500
Anti-GFAP	Astrocytes	Mouse	1 in 200
Anti-S100β	Astrocytes	Rabbit	1 in 100
Anti-IBA1	Microglia	Goat	1 in 200
Anti-TMEM119	Microglia	Rabbit	1 in 200
Anti-HTT	All	Mouse	1 in 100

***Table 2*. Primary antibodies used for immunocytochemistry (ICC).**

2.4.3. Fluorescence microscopy

Imaging of fluorescently labelled cells was carried out using a Zeiss Axiovert 200M epifluorescence microscope with Leica Application Suite Advanced Fluorescence (LASAF) Software (Leica Microsystems, Milton Keynes, UK). Suitable gain and exposure settings for each stain were determined and kept constant for each experiment. Cells were imaged at X20 with 3 regions of interest (ROI) taken per coverslip.

2.4.4. Image analysis and quantification

Images were first adjusted in Leica Application Suite Advanced (LASAF) Lite Software and exported to Image J (Fiji, NIH) for analysis. To assess colocalization of cell-specific markers with DAPI, stained nuclei images were first split into individual channels and subject to thresholding. The colocalization plugin was selected and each channel corresponding to a particular marker was compared to the DAPI channel creating a 8-bit image of overlapping

regions. The number of positively stained cells was then determined using the Analyse Particles function and the number of marker positive cells represented as % of DAPI.

2.5. Cell lysis and protein quantification: BCA assay

2.5.1. Cell lysis

Cell lysis buffer was prepared by addition of 1X Halt™ Protease Inhibitor Cocktail EDTA-free (Thermo Scientific) to Pierce™ RIPA Buffer (Thermo Scientific). All reagents and cells were kept on ice during the cell lysis process.

Following experimental treatments cells were lysed by first removing the cell culture medium and washing the cells twice with ice cold D-PBS without calcium and magnesium. Ice cold RIPA buffer complete with protease inhibitors was added to the cells at 15 µL/ cm^2 for 20 minutes on ice, with occasional swirling of the culture plate to ensure coverage of the cells. Lysates were then collected by scraping the culture surface with a pipette tip/ cell scraper and transferring the cell suspension to a microcentrifuge tube. Samples were then centrifuged at 13,000 x g/ 4°C for 20 minutes to collect the cell debris. Following centrifugation, supernatants were transferred to new tubes and stored at -80°C until further analysis.

2.5.2. BCA assay

The Pierce™ BCA Protein Assay kit (Thermo Scientific) was used to determine total protein of cell samples.

Protein standards (0-2000 µg/mL) were prepared in the same buffer as the samples tested. Working reagent was made up by mixing 50:1 reagent A and reagent B. Complete working reagent was then added to the standards and samples at a ratio of 20:1. Plates were then agitated at 500 rpm for 60 seconds using a Stuart SSL3 gyro-rocker (Cole-Parmer, Staffordshire, UK) before incubation at room temperature for 2.5 hours. Following incubation plates were read at 562 nm using a FLUOstar Omega (BMG Labtech) plate reader.

2.6. Cytokine release and quantification: Enzyme-linked immunosorbent assay (ELISA)

All ELISA kits listed in table 3 were purchased from R&D Systems and the procedure carried out as per manufacturer instructions, with all steps carried out at room temperature.

Briefly, Nunc MaxiSorp immunoassay plates were coated with capture antibody diluted in PBS and incubated overnight at room temperature. The following day, the capture antibody solution was removed, and plates washed three times with 0.05% Tween 20 in PBS and plates blocked with blocking buffer (1% BSA in PBS) for 2 hours. Once the blocking buffer was removed, samples and standards were then loaded into corresponding wells and incubated for 2 hours at room temperature. Samples were removed and the plate washed again as previously described with 0.05% Tween 20 in PBS. Detection antibody diluted in block buffer was then added to the wells and incubated for a further 2 hours. Following removal of the detection antibody and wash steps repeated as before, streptavidin-HRP diluted in block buffer was added to the wells for 20 minutes before washing again, and addition of colour reagent for 20 minutes. 2N sulfuric acid (H_2SO4) was then added to the wells and mixed thoroughly by pipetting the contents of each well several times.

Plates were read at 450 nm and 570 nm using a FLUOstar Omega (BMG Labtech) plate reader. Values at 570 nm were deducted from those taken at 450 nm followed by blank correction.

Cytokine	Detection range
IL-6	9.4 – 600 pg/mL
IL-1β	3.9 – 250 pg/mL
TNF α	15.6 – 1,000 pg/mL
IL-10	31.2 – 2,0000 pg/mL

Table 3. List of cytokines analysed, and respective detection ranges of ELISA kits used.

Chapter 3

3: Generation and functional assessment of microglial cells derived from iPSC with comparisons to HD cells

3.1. Introduction

Microglia are the primary immune cells of the central nervous system (CNS) and the first to detect and respond to disturbances within the CNS microenvironment to eliminate toxic stimuli, promote repair, and maintain homeostasis (Kettenmann et al., 2011; Li and Barres, 2017; Michell-Robinson et al., 2015). As the primary mediators of neuroinflammation, microglia are becoming increasingly recognised for their role in various brain pathologies and as a potential therapeutic target (Fatoba et al., 2020). The role of microglia in neuroinflammatory disorders, and their relative contribution to both neuroprotective and neurodegenerative processes is an area of contentious debate, particularly due to the contrasting findings that different models both *in vivo* and *in vitro*, produce (Carson et al., 2008; Timmerman et al., 2018).

Microglia pose a number of challenges when attempting to investigate their function. Primary human microglia are difficult to obtain due to the limited availability of CNS tissue and typically, the tissue is either of foetal origin, resected tumours, epileptic foci, or post-mortem tissue (Jana et al., 2007; Rustenhoven et al., 2016). The methods used to isolate the tissue/ cells and the fact damage may exist previous to the isolation process can have a significant impact on the quality and relevance of any data acquired. Animal models are commonly used to provide ease of access to brain tissue but are lacking in key areas of translatability (Smith and Dragunow, 2014). Microglia have also been shown to rapidly lose transcriptional signatures characteristic of their physiological state when removed from their natural microenvironment and cultured in artificial conditions (Bohlen et al., 2017; Butovsky et al., 2014; Gosselin et al., 2017).

Rodents are the most common animals of choice for investigating and modelling microglia function both *in vivo* and *in vitro* (Nimmerjahn et al., 2005; Paolicelli et al., 2011; Stevens et al., 2007). Brain slice cultures provide 3D sections containing all the CNS cell types and their connections, meaning the cellular context of the microglia is more physiologically relevant. However, the slicing process causes mechanical tissue trauma as well as destabilising environmental changes, disrupting normal cellular interactions and inducing processes such as micro- and astrogliosis (Aitken et al., 1995; Kettenmann et al., 2011; Takano et al., 2014). The potential impact of this damage and activation is demonstrated in cultures that show amplified neuronal responses due to the presence of activated microglia and astrocytes (Bernardino et al., 2008), meaning data should be cautiously interpreted.

Primary microglia have been isolated from rodent CNS tissue using well established methods (Cardona et al., 2006; Giulian and Baker 1986). This process is technically demanding and time consuming and so, to overcome these disadvantages, immortalised microglia cell lines have been developed, the most commonly used being the murine cell lines, BV-2 (Blasi et al., 1990; Tahara et al., 2006) and N9 (Righi et al., 1989; Hickman et al., 2008). These lines are advantageous in that they provide high numbers of microglia for study but due to their genetic modification exhibit increased proliferation rates not typical of *in vivo* microglia and differential responses to stimulation exist between cell lines and when compared to primary microglia (Horvath et al., 2008). Advances in the labelling of genetic markers and imaging techniques has also enabled live *in vivo* imaging of mouse microglia, demonstrating the highly dynamic nature of microglia under physiological conditions (Davalos et al., 2005; Nimmerjahn et al., 2005) and how they interact with other cells and structures within the brain.

Rodent and human microglia share highly conserved gene expression profiles (Galatro et al., 2017; Gosselin et al., 2017) but do show important differences in expression of genes, particularly in those associated with aging (Galatro et al., 2017; Grabert et al., 2016). Divergent roles for receptors such as CD33, in regulating phagocytosis (Bhattacherjee et al., 2019), are also observed between them. Such species differences pose significant challenges for biological research as they often confer disparities in experimental outcomes particularly in relation to pharmacological manipulation (Gibbons et al., 2011; Smith et al., 2010).

In an attempt to improve the translatability of animal models, ‘’humanised’’ models of microglia function have been developed. Transgenic mice models are now commonplace,

where genes related to human disease are overexpressed (Croft et al., 2017; Lee et al., 2010). Human-rodent chimeras in which human cells are transplanted into an immunodeficient animal host have demonstrated that human induced pluripotent stem cell (iPSC) generated haematopoietic progenitors transplanted into the mouse brain fully differentiate into human microglia as demonstrated by their marker expression and highly ramified morphology (Hasselmann et al., 2019).

Like rodent microglia, immortalised human microglia cell lines have been generated including the cell line HMO6 (Nagai et al., 2001). The microglia were isolated from embryonic telencephalon tissue and transformed with a PASK 1.2 retroviral vector carrying a v-myc oncogene (Nagai et al., 2001). Although demonstrated to be similar to primary human microglia, HMO6 cells appear stunted in their ability to produce TNF α and MIP-1α after long-term exposure to LPS compared to their primary human microglia counterparts (Nagai et al., 2001), further demonstrating the challenges associated with current microglial cell lines.

The ability to produce CNS cells including microglia from human stem cells offers a unique opportunity to generate cellular models of the brain in health and disease that encompass multiple CNS cell types without reliance upon obtaining primary human tissue and the use of animal models which often lack translatability to human disease (Smith and Dragunow, 2014; Galatro et al., 2017). It also provides patient specific disease models with the potential to cover a more significant range of genetic backgrounds and phenotypes of a particular disease.

There are a number of published methods for generating microglia from both embryonic stem cells (ESC) (Muffat et al., 2016; Douvaras et al., 2017) and iPSCs (Abud et al., 2017; Douvaras et al., 2017; Haenseler et al., 2017; McQuade et al., 2018; Muffat et al., 2016; Pandya et al., 2017). Each protocol involves the addition of factors identified as essential to the development and maintenance of microglia (Butovsky et al., 2014; Wang et al., 2012). Normal development and maturation of microglia is dependent on the presence of other brain cell types, and this is demonstrated in data that shows the loss of expression of specific transcription factor groups in adult microglia (Gosselin et al., 2017). As such co-cultures with neurons (Haenseler et al., 2017) or astrocytes (Pandya et al., 2017) have been implemented and more recently, triculture systems including neurons, astrocytes, and microglia (Guttikonda et al., 2021) to better recapitulate the physiological environment of microglia.

Haenseler et al., 2017 is the published method of choice used to generate microglia in this thesis. In this chapter, the generated microglia will be considered in the context of monocultures with comparisons to HD cells where indicated.

The aims of this chapter are to:

1) Differentiate control (HipSci) and patient (HD 109) iPSC and characterise microglia-like cells by immunocytochemistry (ICC).
2) Assess the function of iPSC-derived microglia in monoculture including responses to inflammatory stimuli (e.g. LPS and amyloid) and their phagocytic competency.
3) Determine if HD 109 microglia produce a heightened inflammatory response when stimulated with pro-inflammatory stimuli including LPS and amyloid (Aβ 1-42).

3.2. Results

3.2.1. HipSci and HD CAG 109 iPSC express the pluripotency markers, *Oct4 and Sox2*

Both HipSci and HD 109 iPSCs display typical iPSC morphology as described in the literature (Nagasaka et al., 2017; Wakao et al., 2012). They cluster into rounded and compact colonies with bright, smooth edges when examined by light microscopy, with individual cells exhibiting a large nucleus with reduced cytoplasmic area (Wakao et al., 2012). *Oct4* and *Sox2* are commonly used markers to assess pluripotency (Rizzino, 2013). HipSci and HD 109 iPSC both express *Oct4* and *Sox2* (Figure 1). Immunofluorescent staining was carried out across a range of iPSC passages which were used for further differentiation to generate microglia and other CNS cell types used in this thesis. For quantification of markers across passages used for subsequent differentiations, two coverslips were stained with appropriate antibodies and three regions of interest (ROI) were selected per coverslip and the number of positively stained cells were counted as a percentage of DAPI.

HipSci iPSC expressed *Oct4* with an average of 99.79% (±0.18%) of DAPI-positive cells with slightly lower average expression of *Sox2* at 99.47% (±0.15%). HD 109 iPSC demonstrated a similarly high expression of both *Oct4* and *Sox2*, averaging 99.50% (±0.74%) *Oct4* positive cells but with slightly higher *Sox2* expression at 99.64 (±0.52%) compared to *Oct4*. Morphologically abnormal cells identified by disruption to the smooth edges of iPSC colonies and cobblestone like appearance (Yamamoto et al., 2022) were minimal as indicated by the smooth colony edges and high expression of *Oct4* and *Sox2*. This suggests both HipSci and HD 109 iPSC maintain pluripotency to a similar degree in culture with minimal spontaneous differentiation between passages.

For differentiations ‘’n’’ refers to an individual differentiation from a particular passage of an iPSC. Thus, differentiations were carried out three times unless otherwise indicated. Within each differentiation, experimental procedures such as labelling for ICC and cell treatments were carried out in duplicate.

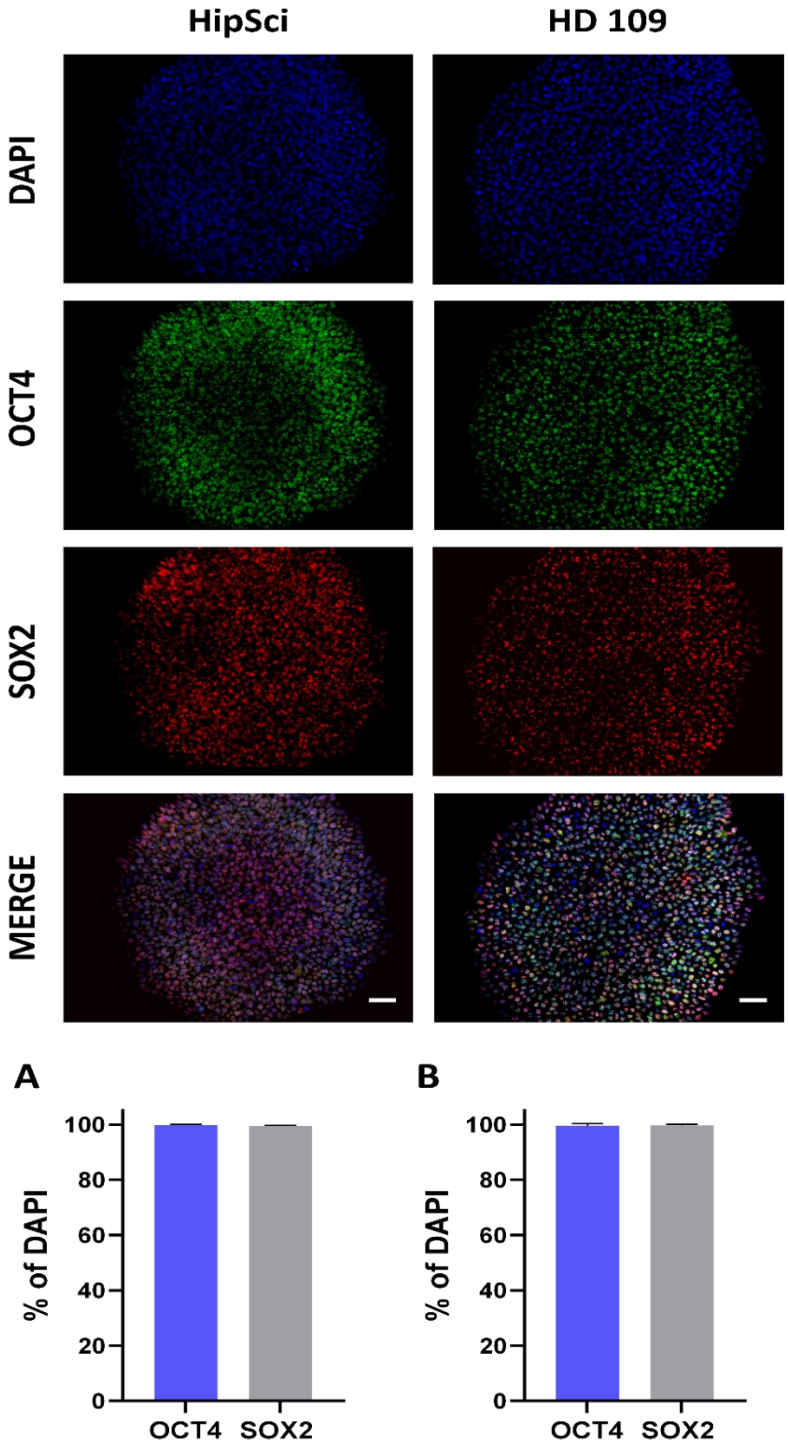

Figure 1. Immunocytochemistry (ICC) of control and patient iPSC*. HipSci (control) and HD 109 (patient) iPSC were plated onto vitronectin (5/10 µg/mL respectively) coated glass coverslips and maintained in E8 medium before fixing and staining with DAPI (blue), Oct4 (green), and Sox2 (red), with respective merge images displayed. Images were taken at X20 magnification and are representative. Scale bar: 50 µm. Graphs (A) HipSci, (B) HD 109, display the quantification of Oct4 and Sox2 per line (n=3) and are represented as % of DAPI-positive stained cells ± standard deviation.*

3.2.2. Induced pluripotent stem cells (iPSC) successfully differentiate into microglia displaying typical morphology and expression of the microglial markers, *TMEM119* and *IBA-1*

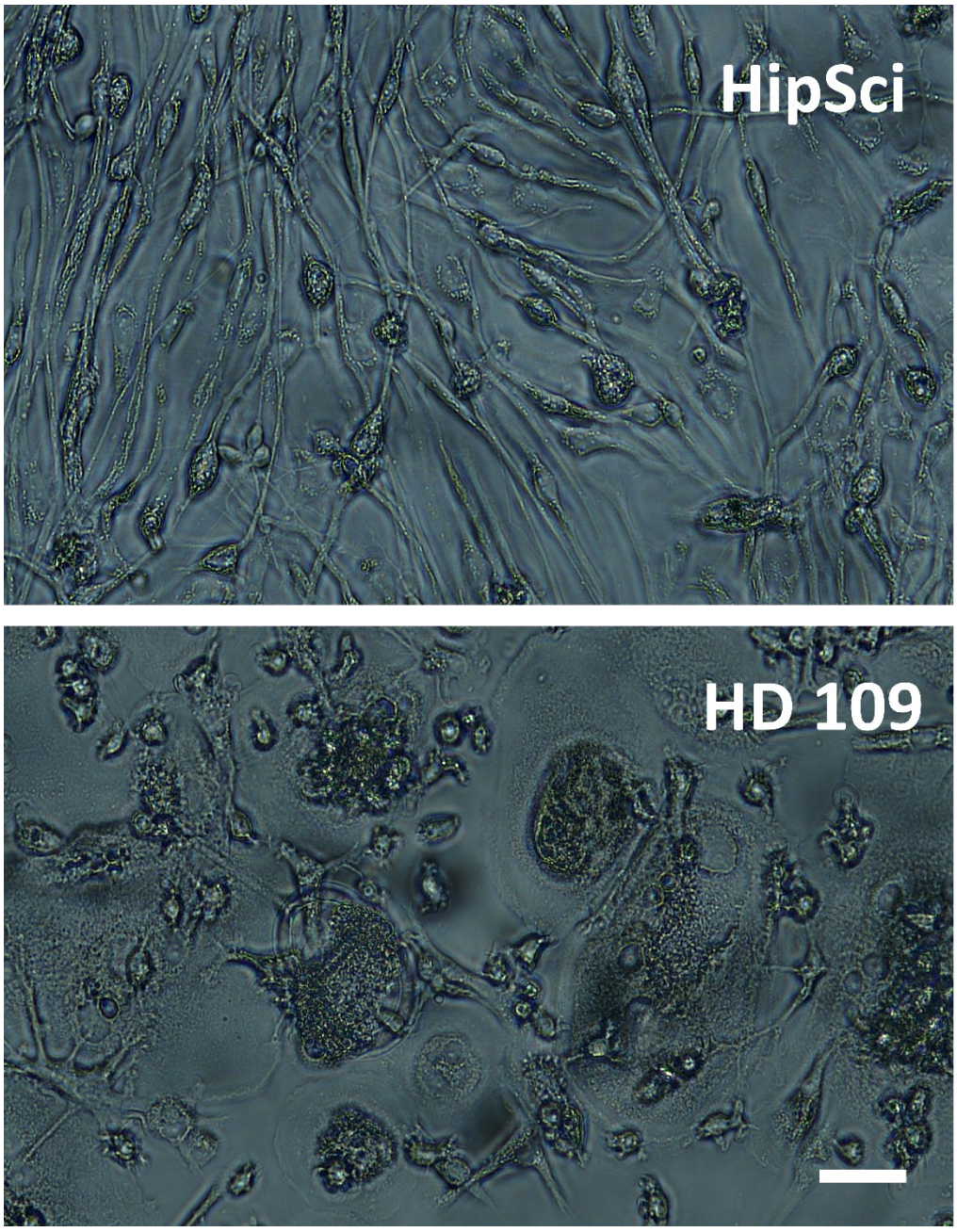

Figure 2. Phase images of iPSC-derived control (HipSci) and patient (HD 109) microglia. *iPSC were first exposed to macrophage precursor medium (MPM) before collection at 7 weeks and subsequent terminal differentiation to microglia in microglial medium (MM). Images were taken at day 15 in MM at X20 objective. Scale bar 50 µm. (n=3).*

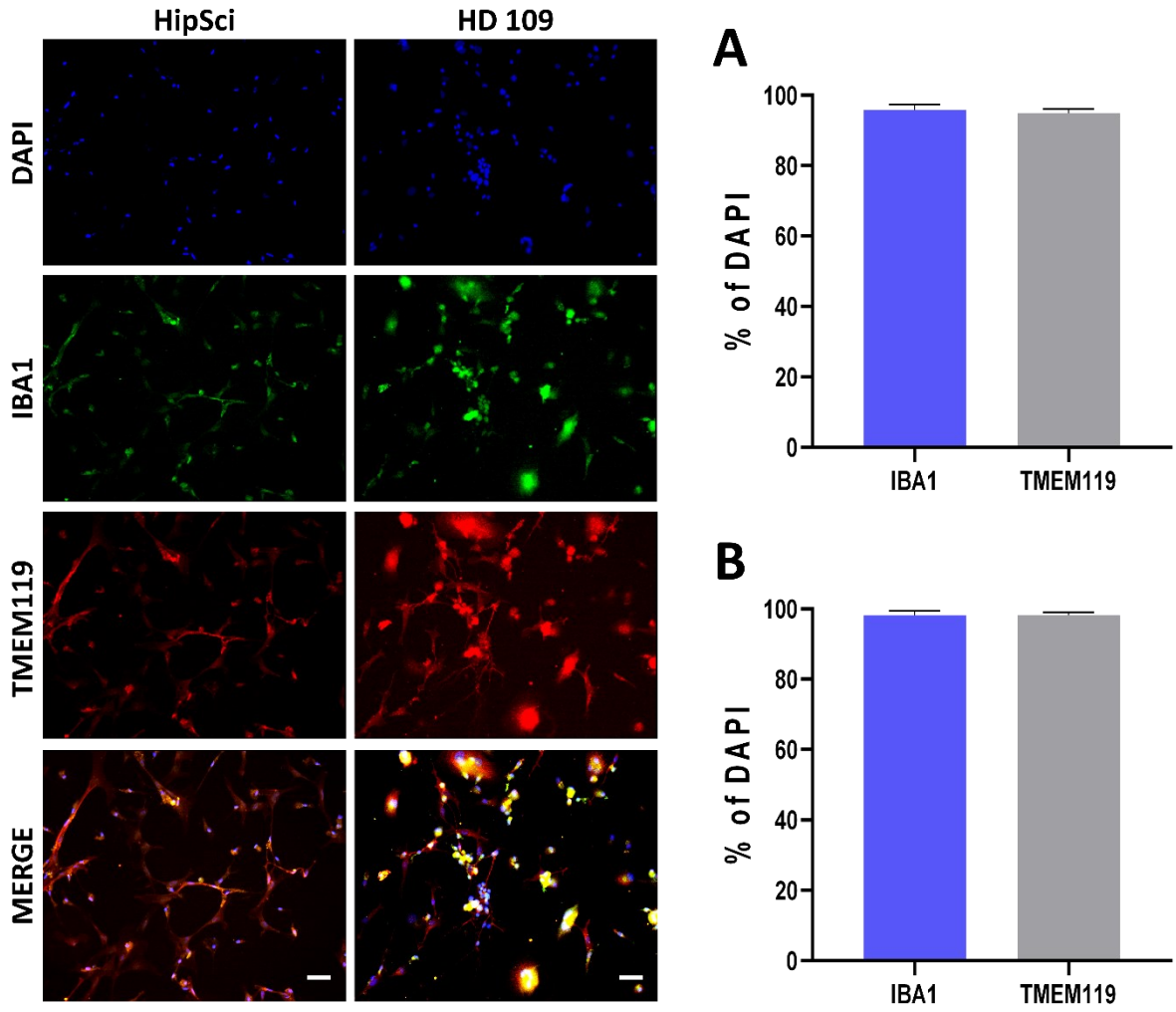

Figure 3. Generation of microglia from control and patient iPSC*. HipSci (control) and HD 109 (patient) week 7 macrophage precursors were plated onto PDL (50 µg/mL) coated glass coverslips and terminally differentiated into microglia in Microglial Medium (MM) for 15 days. Cells were then fixed and stained for DAPI (blue), Iba1 (green), and Tmem119 (red), with respective merge images displayed. Images were taken at X20 magnification and are representative. Scale bar 50 µm. Graphs (A) HipSci, (B) HD 109, display the quantification of Iba1 and Tmem119 per line (n=3) and are represented as % of DAPI-positive stained cells ± standard deviation.*

Both HipSci and HD 109 iPSC differentiate successfully into microglia expressing the microglial markers *Iba1* and *Tmem119* (Butovsky et al., 2014; Jurga et al., 2020). Both cell lines express high levels of *Iba1* and *Tmem119* as determined by immunocytochemistry (Figure 3). On average, HipSci cells are 95.90% (±1.540%) positive for *Iba1* and 95.01% (±1.092%) positive

for *Tmem119*. HD 109 cells are 98.14% (±1.426%) and 98.27% (±0.7967%) *Iba-1* and *Tmem119* positive, respectively. All nuclei is associated with either *Iba1* or *Tmem119* indicating a relatively pure population of cells.

Of note, HipSci and HD 109 microglia appear to demonstrate striking differences in morphology, with HD cells displaying a more amoeboid shape with fewer processes and appear to demonstrate increased clustering compared to their HipSci counterparts (Figure 2, 3). This altered morphology is described in the literature in both mouse models of HD and patients (Franciosi et al., 2012; Sapp et al., 2001). This difference in morphology indicates an early expression of dysfunction and importantly occurs in the absence of other CNS cells suggesting the involvement of cell autonomous mechanisms at least in terms of morphological characteristics (Crotti et al., 2014).

Interestingly, HD 109 microglia display an aberrant nuclear morphology with irregular nuclear shape and in some instances multiple nuclei are observed (Figure 4). Fibroblast cultures from R6/2 HD mice and juvenile HD patient fibroblasts also demonstrate this same multinucleate property in cells (Sathasivam et al., 2001). Binucleation is observed in the CNS under pathological conditions including neurodegenerative disorders such as Alzheimer's disease (AD) (Zhu et al., 2008) and multiple sclerosis (MS) (Kemp et al., 2012) whereby binucleation is observed in neurons. Microglia can also form binucleate cells, the accumulation of which is associated with inflammation, aging, and certain brain pathologies (Hart et al., 2012; Hornik et al., 2014). As this appears to be occurring even in control conditions and in the absence of other cell types, it is suggestive of an intrinsic mechanism related to the presence of mHTT that is causing this phenomenon.

HD microglia also exhibit large vacuole like structures (Figure 2). These structures have also been observed in HD astrocytes derived from iPSC cultures (Juopperi et al., 2012) and lymphoblasts from HD patients (Nagata et al., 2004) in which they occur in a CAG length dependent manner (Nagata et al., 2004). The appearance of vacuoles may be indicative of an accumulation of autophagic structures in these cultures (Juopperi et al., 2012).

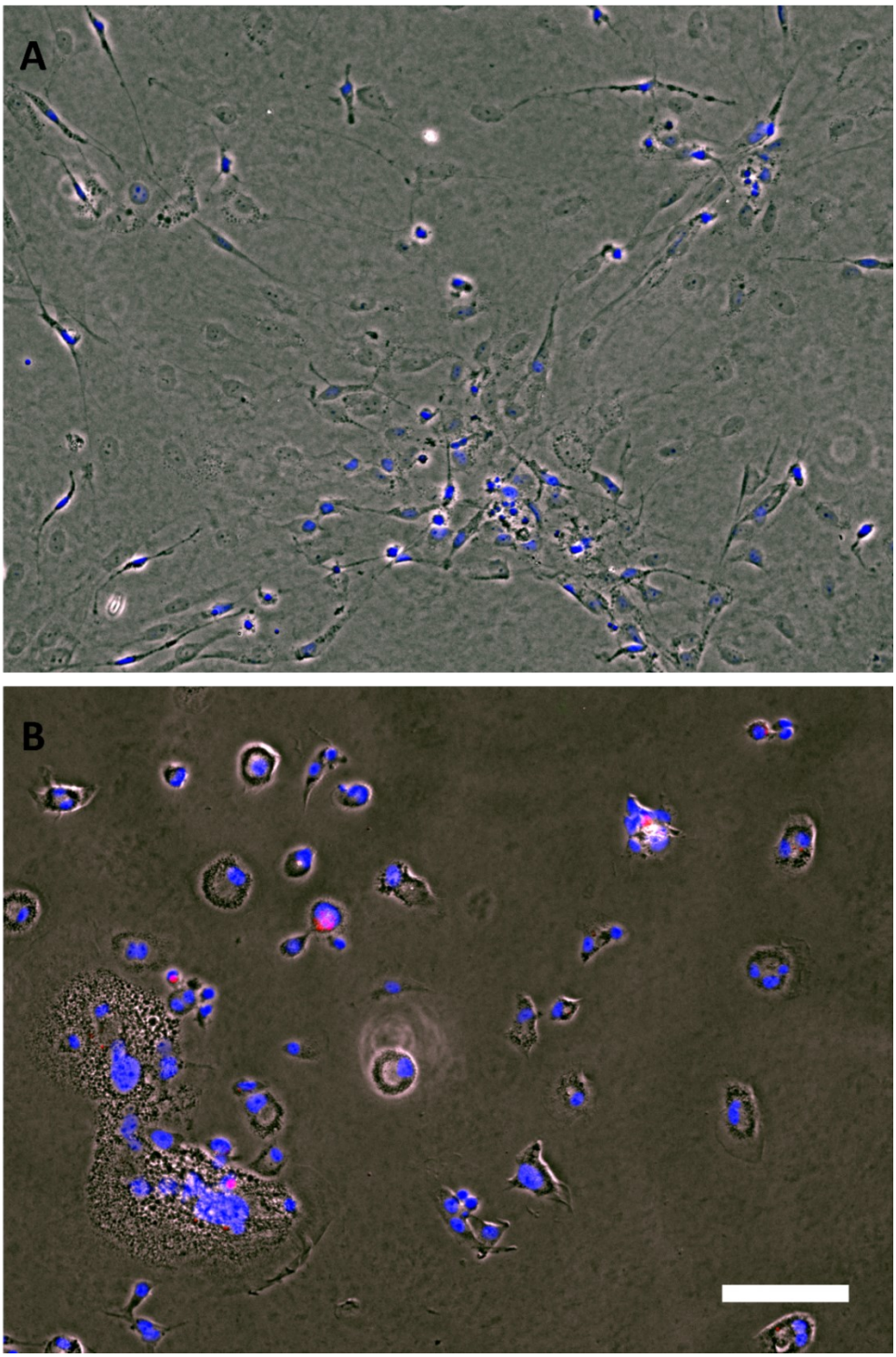

Figure 4. HipSci and HD 109 microglia stained with a HTT-targeting antibody*. On day 15 of culture microglia were fixed and stained with DAPI (blue) and HTT (red). (A) HipSci, (B) HD 109. Images were taken at X20 magnification and are representative. Scale bar 50 µm. (n=2).*

Both cytoplasmic and nuclear inclusions are observed in post-mortem HD patient brains (DiFiglia et al., 1997; Sieradzan et al., 1999). Immune cells express high levels of mHTT (Moscovitch-Lopatin et al., 2010) and mHTT inclusions have been detected in microglia (Jansen et al., 2017). Here, potential cytoplasmic and nuclear aggregation of mHTT is observed in HD 109 microglia with immunoreactivity of a huntingtin targeted antibody (Figure 4). Although precise location within the cytoplasm is unable to be determined due to the lack of co-labelling. Aggregation of HTT appears to be absent in HipSci microglia.

3.2.3. Functionality

3.2.3.1. Control and patient microglia demonstrate phagocytic competency

Phagocytic activity is an important function of microglia in the healthy CNS and during challenge (Janda et al., 2018). In this project, the ability of microglia to engulf and phagocytose material was assessed by treatment with *E.coli* particles conjugated to a pH sensitive fluorescent dye. Due to the pH sensitive dye conjugated to the *E.coli* particles, upon engulfment by cells and entry into the low pH environment of the phagolysosome, the bioparticle conjugate fluoresces indicating the process of phagocytosis. HipSci and HD 109 cells both demonstrate this phagocytic function as indicated by the red fluorescence observed within the cells upon addition of pHrodo labelled *E.coli* bioparticles (Figure 5).

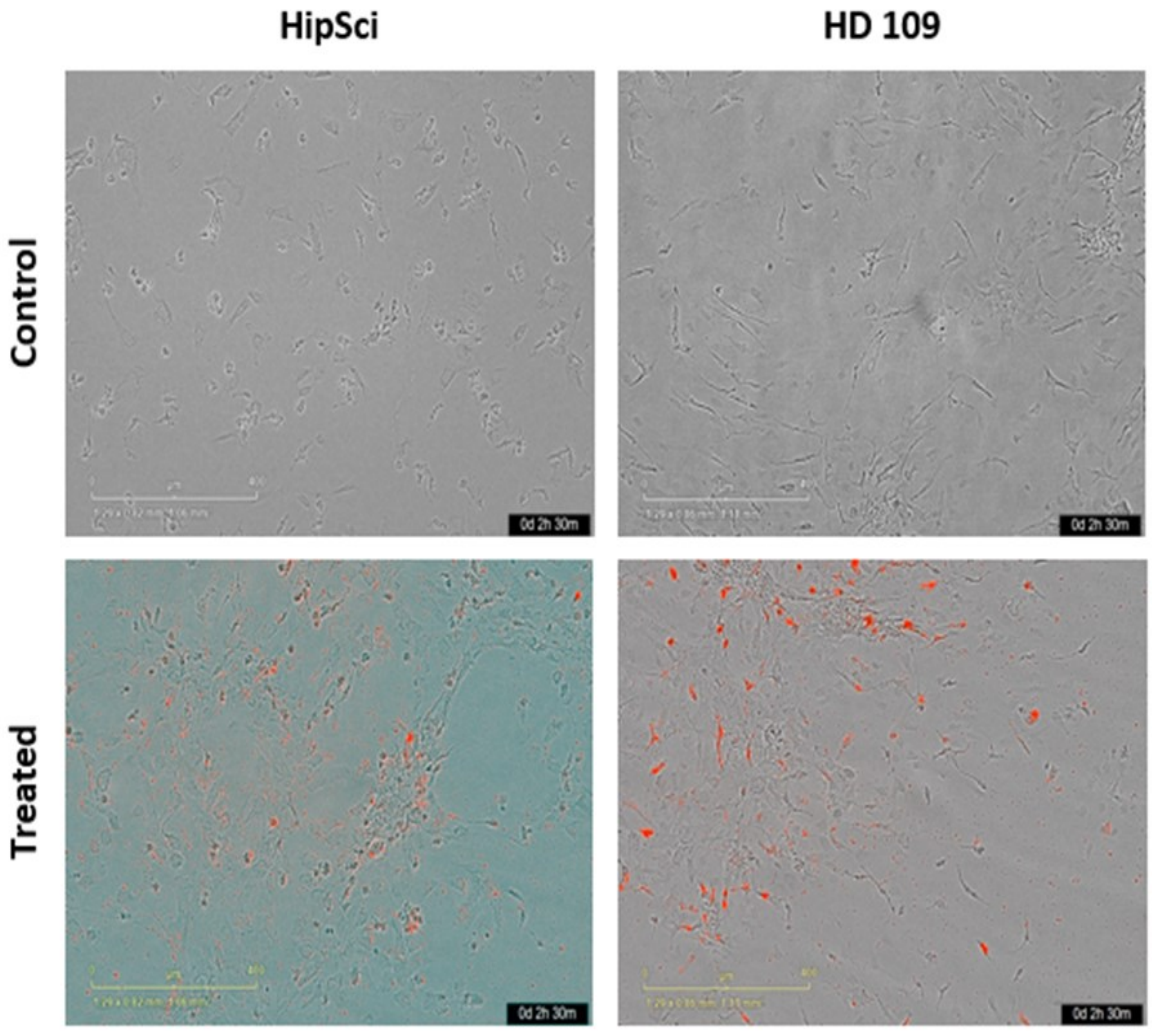

***Figure 5. Phagocytic activity of iPSC-derived microglia**. Macrophage precursor cells were plated onto PDL (50 µg/mL) coated glass coverslips at 3.5 10^4 cells/cm^2 and terminally differentiated to microglia. At day 15, microglial cells were treated with 10 µg/mL pHrodo™ E.Coli Bioparticles and imaged using an Incucyte Live Cell Imaging system. Two images per well were taken every 15 minutes for a total of 10 hours at X10 magnification. Images displayed are representative of cellular uptake at 2 hours and 30 minutes.*

3.2.3.2. Microglial responses to inflammatory stimuli

Microglia were treated with LPS, a known activator of immune receptors expressed in microglia including TLR4, resulting in pro-inflammatory cytokine release (Ciesielska et al., 2021). LPS was used at 70 ng/mL due to observations across the literature that concentrations below 100 ng/mL reliably produce a response without inducing toxicity (Lively and Schlichter,

2018). The interleukin, IL-4, was used to promote polarisation of microglia towards an anti-inflammatory profile following treatment with LPS due to its potent anti-inflammatory properties (Hart et al., 1989), and concentration of 20 ng/mL chosen based on the literature (Hu et al., 2012; Lively and Schlichter, 2018). Across cytokine release experiments, conditioned media collection was carried out at time points commonly indicated in the literature. This includes an earlier time point at 4 hours which is based around the observations of a sufficient detectable response in microglial cultures (Lively and Schlichter, 2018; Lund et al., 2006).

Treatment with 70 ng/mL LPS for 24 hours promotes reduced process extension and increased clustering of both HipSci and HD 109 cells indicative of immune activation (Lively and Schlichter et al., 2018). Addition of IL-4 (20 ng/mL) appears to trigger process extension which is more noticeable in HD 109 cells that typically have a larger proportion of cells exhibiting few or no processes under ‘’resting’’ state conditions. Removal of LPS (70 ng/mL) after 4 hours and addition of IL-4 (20 ng/mL) appears to promote process extension of cells that were previously clustered with shorter processes in response to LPS (Figure 6).

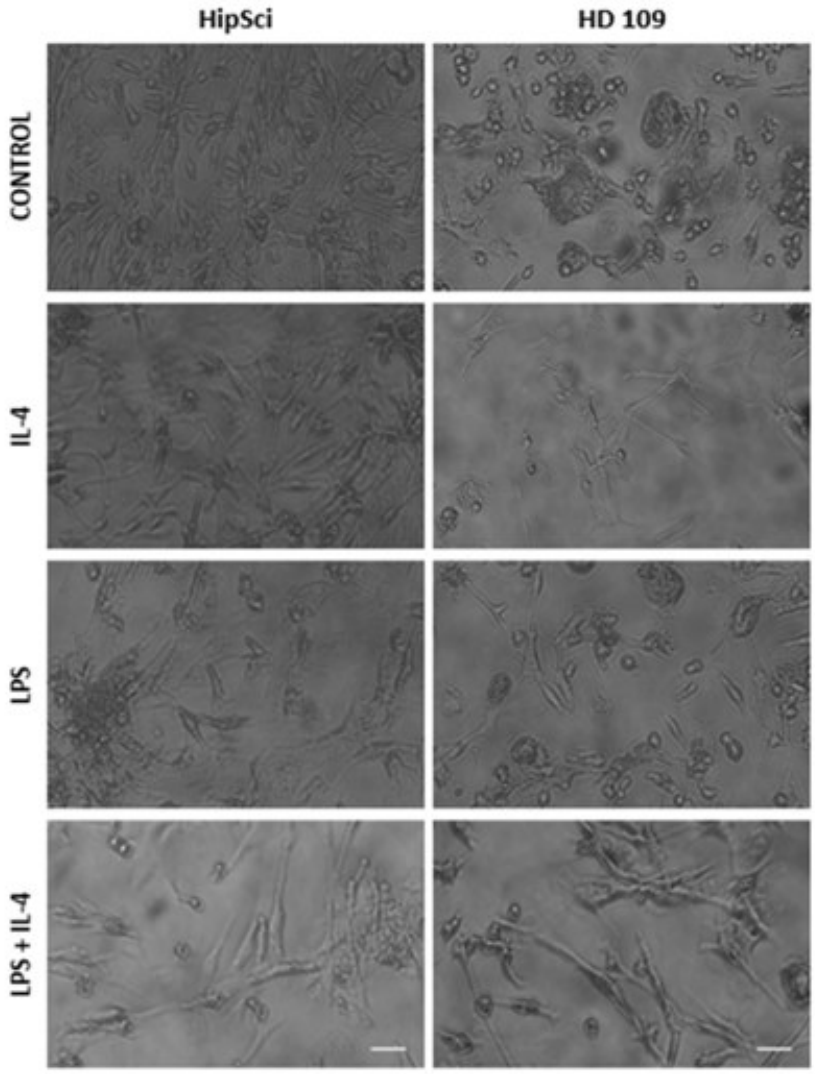

Figure 6. Representative images of microglial morphology in response to LPS and IL-4*. HipSci and HD 109 microglia were treated with 70 ng/mL LPS, 20 ng/mL IL-4, or treated with 70 ng/mL LPS for 4 hours followed by removal and addition of 20 ng/mL IL-4 until the end of the experiment (24 hours). Images were taken at 24 hours following addition of treatments at X20 magnification. Scale bar 50 µm.*

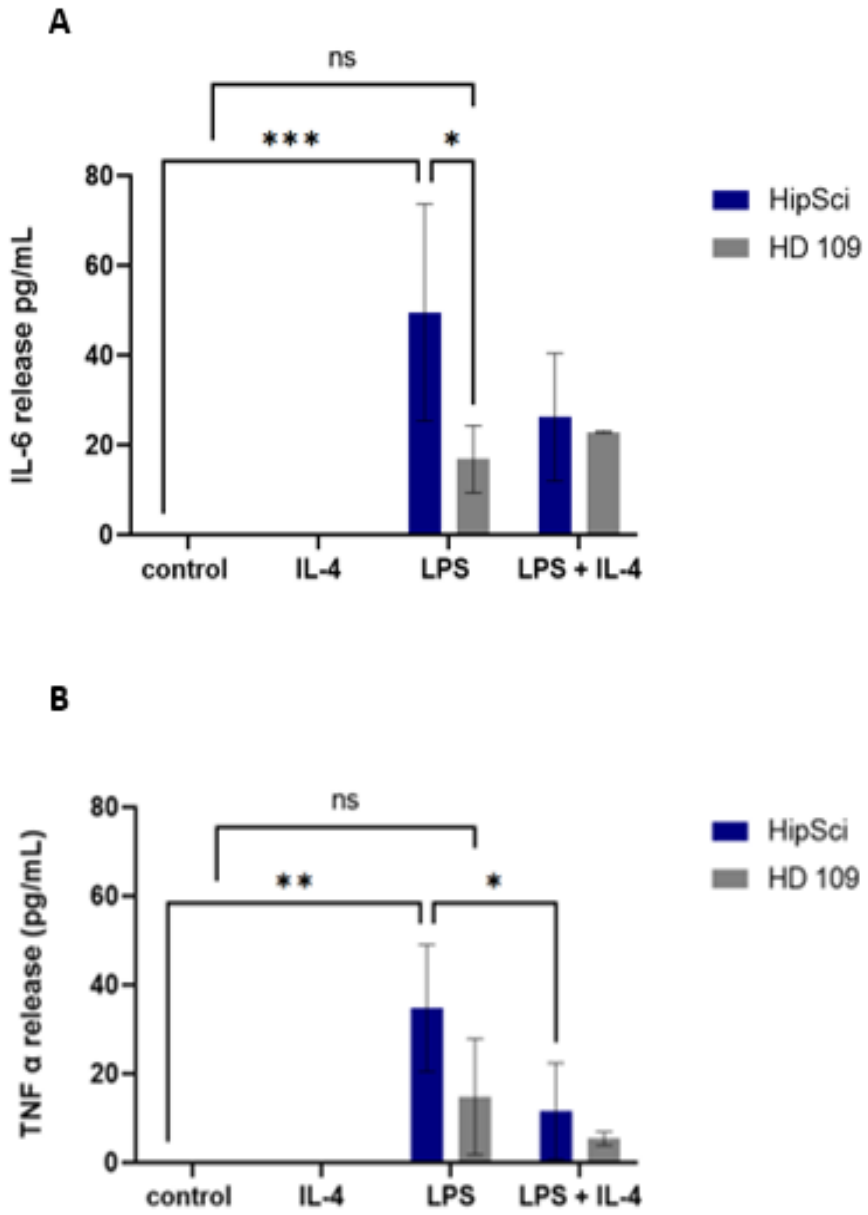

Figure 7. Release of proinflammatory cytokines following LPS stimulation*. HipSci and HD 109 microglia were treated for 24 hours with 70 ng/mL LPS, 20 ng/mL IL-4 or treated with 70 ng/mL LPS for 4 hours followed by removal and addition of 20 ng/mL IL-4 for the remainder of the experiment. Conditioned medium samples were collected for analysis by ELISA and cells lysed for total protein quantification. Cytokine release from HipSci and HD 109 microglia is represented as amount (pg/mL per µg of total protein) in graph (A) IL-6, (B) TNF α. Statistical analysis was carried out where appropriate using 2-way ANOVA with Tukey's multiple comparisons test.*

Graph A (Figure 7) displays the provoked IL-6 response when cells are treated with LPS (70 ng/mL), IL-4 (20 ng/mL) for 24 hours or treated with LPS for 4 hours followed by removal and addition of IL-4 for the remainder of the experiment. IL-6 was not detected in the conditioned medium from both HipSci and HD 109 cells under control or IL-4 alone treatment conditions. In response to 24-hour LPS treatment HipSci and HD 109 cell lines secrete 49.6 (±24.17) and 16.9 (±7.47) IL-6 (pg/mL), respectively. Although the secretion of IL-6 by HipSci cells is significant compared to the control condition ($p=0.008$), the change in IL-6 release from HD 109 cells is not significant ($p=0.5387$). However, the difference in IL-6 between LPS treated HipSci and HD 109 cells is significant at 24 hours ($p=0.0296$). Secretion of IL-6 is lower in LPS + IL-4 treated HipSci cells compared to LPS treated cells at 26.3 pg/mL (±14.23). There is, however, a slight increase in IL-6 in HD cells when treated with LPS + IL-4 at 22.9 pg/mL (±0.189) compared to LPS treatment alone.

Graph B (Figure 7) shows the TNF α response to treatments. TNF α secretion was not detected in either cell line in control or IL-4 alone treatment conditions. In response to LPS, HipSci cells release a higher amount of TNF α at 34.76 pg/mL (±14.29) compared to 14.82 pg/mL (±13.08) by HD cells. Both HipSci and HD 109 show reduced secretion of TNF α when treated with LPS + IL-4 compared to LPS alone at 11.6 pg/mL (±10.85) and 5.4 pg/mL (±1.49) respectively. The difference between these conditions for HipSci cells was significant ($p=0.05$).

As HipSci cells show a significant reduction in TNF α release when treated with IL-4 following LPS treatment compared to HD 109 microglia, this may be suggestive of an impaired anti-inflammatory network and return to normal resting conditions, and initiation of anti-inflammatory networks for microglia expressing mHTT following activation. However, the level of significance in HipSci cells is only marginally detectable. Overall, the observation of reduced pro-inflammatory cytokine release upon stimulation by HD 109 microglia compared to control microglia is in contrast to much of the literature which largely demonstrates an upregulation in pro-inflammatory signalling in HD microglia with elevated levels of cytokines including IL-6 and TNFα observed (Björkqvist et al., 2008; Chang et al., 2015; Podlacha et al., 2022; Sillvestroni). Perhaps most importantly, a comparable model in terms of the iPSC-derived nature of the cells, corroborates with the pro-inflammatory consensus providing evidence of an exaggerated inflammatory response in iPSC-derived HD microglia in

comparison to controls (O'Regan et al., 2021). Although differences in treatment parameters must be considered in drawing conclusions from these findings.

3.2.2.3. Release of IL-6 and TNF α over time in response to LPS

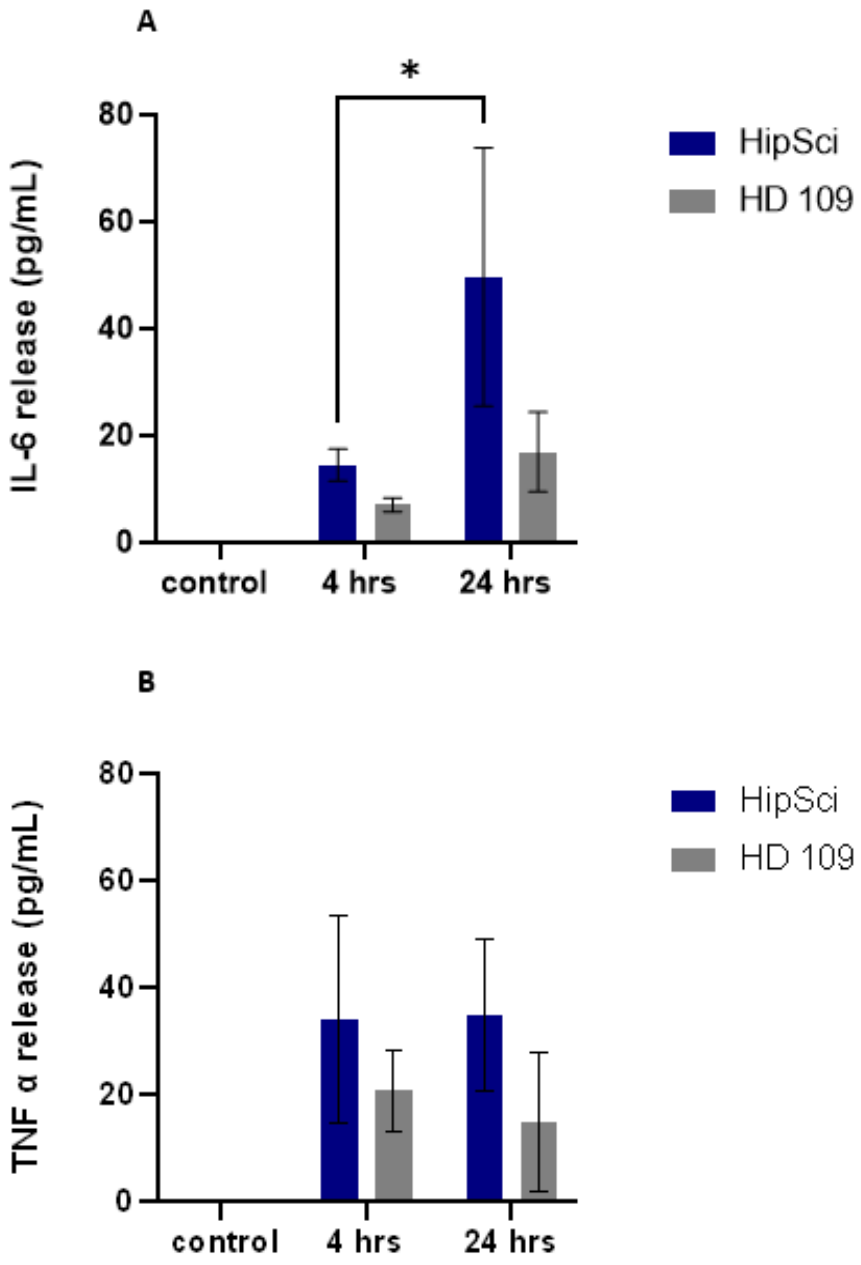

Figure 8. Microglial IL-6 and TNF α response to LPS over time*. Comparison of IL-6 and TNF α secretion at 4- and 24- hour treatment with 70 ng/mL LPS. Where appropriate (n=3) 2-way ANOVA with Tukey's multiple comparisons test was carried out.*

IL-6 release increases between 4 and 24 hours for both HipSci and HD 109 cells with HipSci cells secreting higher amounts compared to HD cells (Figure 8A). HipSci cells release 14.44

pg/mL (±3.02) at 4 hrs, increasing to 49.60 pg/mL (±24.17) at 24 hours. This increase is significant (p=0.0206). HD cells demonstrate a smaller increase in IL-6 between 4 and 24 hours, secreting 7.02 pg/mL (±1.30) and 16.91244805 pg/mL (±7.47) respectively.

The change in release of TNF α is smaller in HipSci cells compared to the change in IL-6 overtime. At 4 hours HipSci cells release 34.13 pg/mL (±19.39) and 34.76 pg/mL (±14.29) TNF α at 24 hours. HD 109 cells show a decrease in TNF α release with 20.70 pg/mL (±7.55) at 4 hours compared to 14.82 (±13.08) at 24 hours (Figure 8B).

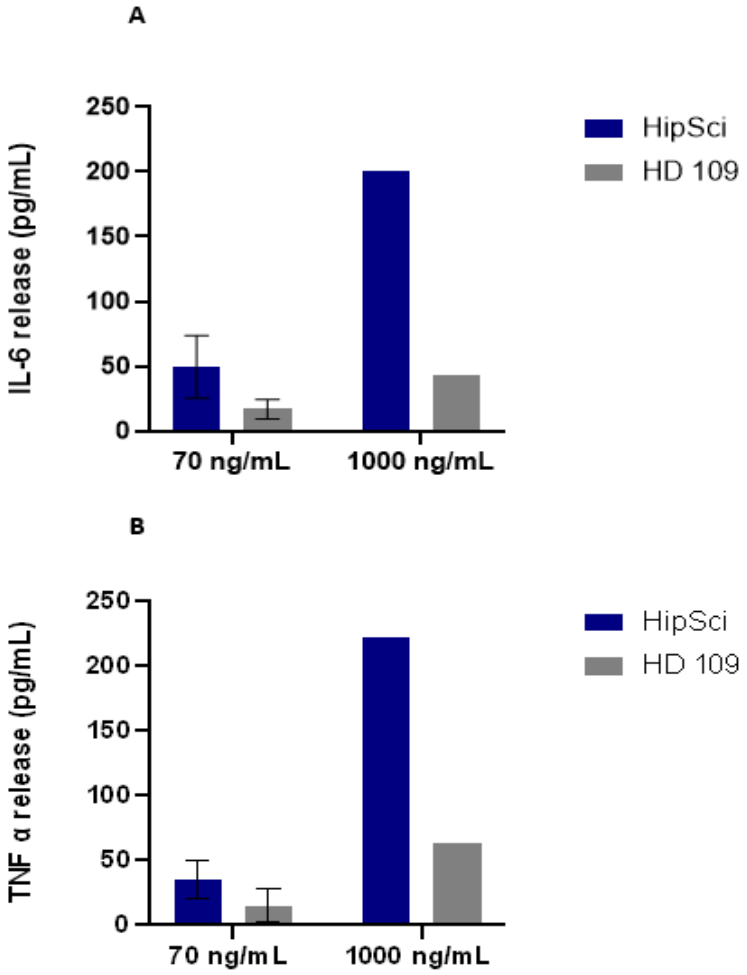

Figure 9. IL-6 and TNF α release in response to low dose and high dose LPS*. Comparison between 24-hour treatments with either 70 ng/mL (low dose) and 1000 ng/mL (high dose) LPS. Cytokine release is represented in pg/mL per µg of total protein. Cytokine release following 70 ng/mL LPS treatment (n=3), release following 1000 ng/mL (n=1).*

Control and patient cells demonstrate an increase in secretion of both IL-6 and TNF α when treated with high dose (1000 ng/mL) LPS compared to low dose (70 ng/mL) (Figure 9). HipSci cells show a larger increase in IL-6 secretion from 49.6 pg/mL (±24.17) to 200.9 pg/mL when treated with the higher dose (Figure 9A). The high dose LPS condition was only carried out once for each cell line due to time constraints and so standard deviation is not indicated. IL-6 secretion from HD 109 cells increases to 43.7 pg/mL from only 16.91 pg/mL (±7.47) (Figure 9A). Similarly, a larger increase in TNF α secretion is observed in HipSci cultures from 34.76 pg/mL (±14.29) to 221.51 pg/mL, compared to an increase from 14.82 pg/mL (±13.08) to 62.68 pg/mL for HD cells (Figure 9B).

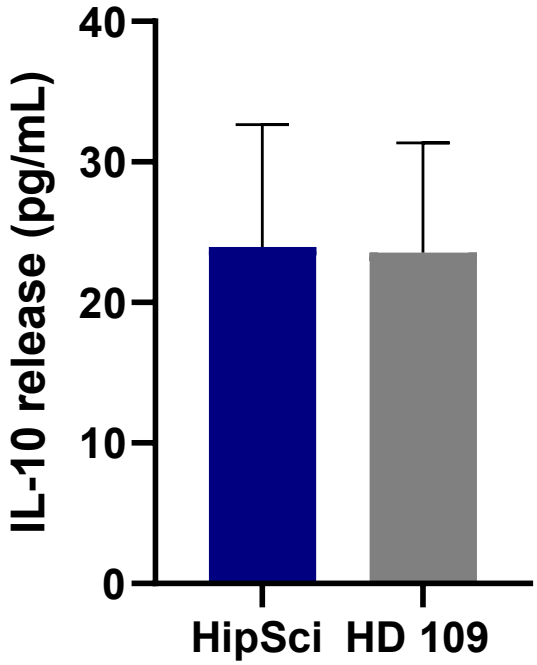

Figure 10. IL-10 response to LPS stimulation*. Comparison of IL-10 release from HipSci and HD 109 microglia when treated for 24 hours with 70 ng/mL LPS. Cytokine release is represented in pg/mL per µg of protein. Statistical analysis was carried out using paired t-test. (n=3).*

IL-10 is a key anti-inflammatory cytokine that is upregulated early in an immune response (Couper et al., 2008). Both HipSci and HD 109 microglia demonstrate similar IL-10 responses to LPS stimulation (Figure 10). HipSci microglia secrete 23.96 pg/mL IL-10 (±8.70) with HD 109 microglia secreting 23.54 pg/mL (±7.82). Statistical analysis using paired t-test indicated no

significant difference between the different cell lines (p=0.9644) suggesting anti-inflammatory networks with regards to IL-10 release are functioning similarly between control and patient cells.

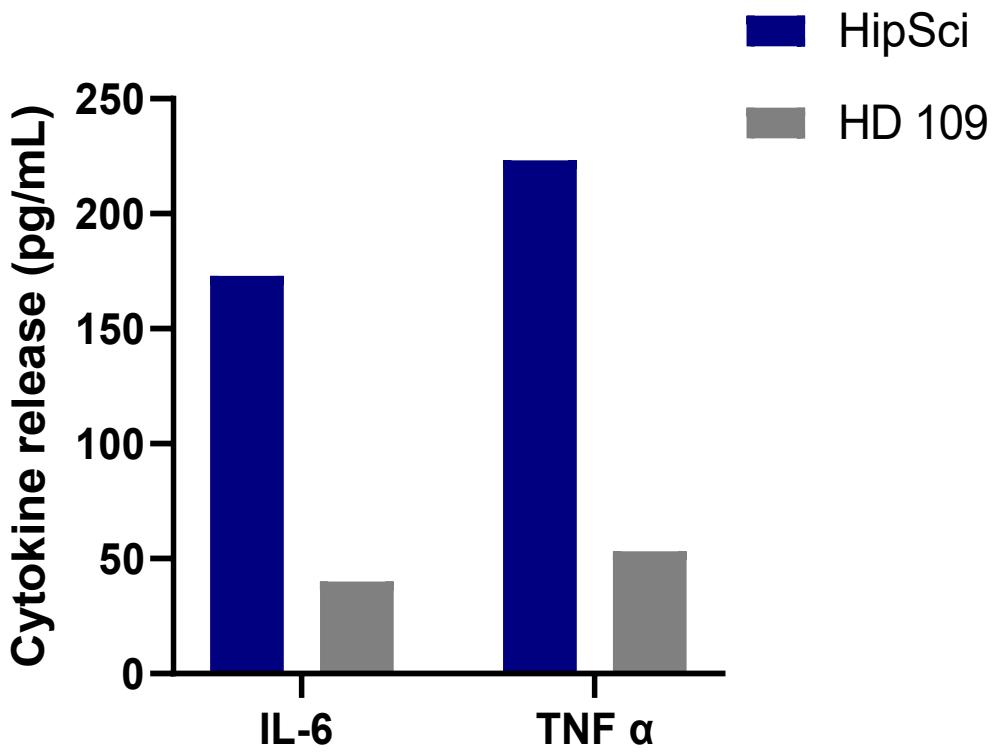

Figure 11. Cytokine release in response to amyloid*. HipSci and HD 109 microglia were treated with 1 µM amyloid (Aß 1-42) for 24 hours before collecting conditioned medium samples for analysis by ELISA and lysing the cells for total protein quantification. Cytokine release is represented as pg/mL per µg of total protein. (n=1).*

To enhance the neurodegenerative relevance of this microglia model it was decided to treat microglia with a neurodegeneration relevant protein which in this case is amyloid (Wolfe and Cyr, 2011). The concentration of Aβ 1-42 (1 µM) and time point of media collection was selected based on previous literature (Caldeira et al., 2017; Quiroga et al., 2022).

Microglia were treated with 1 µM amyloid (Aβ 1-42) for 24 hours (Figure 11). Controls are omitted from the graph as IL-6 and TNF α were undetectable, falling below the threshold for the ELISA kits used. HipSci microglia show a more pronounced response to amyloid compared to their HD counterparts secreting 173 pg/mL IL-6 and 223.2 pg/mL TNF α, compared to 40.1 pg/mL IL-6 and 53.3 pg/mL TNF α by HD cells. Although this was only carried out once due to

the failure of some cultures prior to treatment and subsequent time constraints. The experiment, therefore, requires repetition to draw any conclusions.

For all inflammatory stimuli conditions mentioned, levels of IL-1β were analysed, however, all treatment conditions failed to produce an IL-1β response that was detectable by the ELISA kit used (working standard range: 3.91 – 250 pg/mL). Therefore, IL-1β has been omitted from individual graphs.

3.2.3.3. Response to ATP

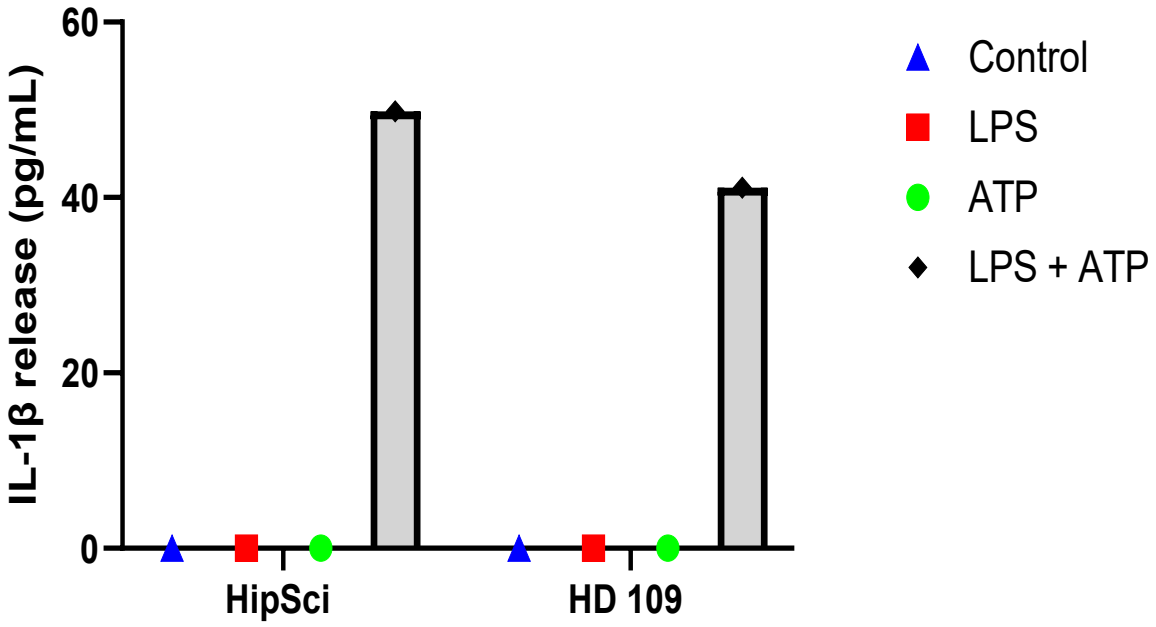

Figure 12. Microglial IL-1β release in response to ATP*. Microglia were either treated for 24 hours with 70 ng/mL LPS alone or with the addition of 2 mM ATP in the final 20 minutes of the 24-hour experiment time course and compared to controls and cells treated with 2 mM ATP only for 20 minutes. Following treatment, conditioned medium was collected, and cells lysed for total protein quantification. IL-1β release was determined by ELISA and is represented as pg/mL per µg of total protein. (n=1).*

Both HipSci and HD 109 microglia produce no IL-1β in response to 24-hour LPS (70 ng/mL) stimulation. Similarly, no response is observed when the cells are treated with 2 mM ATP for 20 minutes (Figure 12). ATP is shown to not evoke an IL-1β response unless a priming LPS

stimulus is also provided in microglial cells (Clark et al., 2010) and as such was applied following LPS priming. Addition of 2 mM ATP for 20 minutes following LPS (70 ng/mL) treatment for 24 hours evokes IL-1β release from both HipSci and HD 109 microglia with HD cells secreting slightly lower levels. This was only carried out once and must, therefore, be repeated to assess the role of ATP in activating microglia.

3.3. Discussion

Generally, the literature indicates prominent pro-inflammatory mechanisms in Huntington's disease across animal models, patients, and cell lines derived from patients including iPSC (Bjorkqvist et al., 2008; Chang et al., 2015; O'Regan et al., 2021; Rodrigues et al., 2016; Silverstroni et al., 2009). Findings in this chapter conflict with this consensus. Although some experiments were only conducted once and conclusions cannot be extrapolated, the microglial response to 24-hour LPS treatment (n=3) supports a stunted immune response in HD patient cells with decreased release of IL-6 and TNF α compared to controls.

Data derived from patients including brain tissue, blood, and CSF, along with animal models all represent sources where immune cells are essentially primed by the biologically rich environment, with cells receiving a multitude of signals from other cells and signalling mediators that are challenging to replicate *in vitro* (Crehan et al., 2012). The role of immune reactivity in the periphery in shaping immune responses and microglial function is becoming increasingly recognised (Greenhalgh et al., 2020; Zhang et al., 2022) with evidence of peripheral immune activation occurring prior to clinical manifestation of HD (Bjorkqvist et al., 2008).

Microglia express a multitude of receptors allowing them to react to a broad range of signals and are now considered to exist on a much more nuanced spectrum of activation that is dependent upon cellular context (Ransohoff, 2016). Thus, experimental factors including the applied stimulus may explain discrepancies in observed microglial responses in culture. In this chapter, a reduced immune response in terms of IL-6 and TNF α release is observed in response to LPS with no IL-1β detected at either 70 ng/mL or 1000 ng/mL. Contrasting literature that also presents an iPSC-derived model of microglial function in HD shows elevations in pro-inflammatory cytokine (IL-6, TNFα, IL-1β) release from HD cells in response to LPS (O'regan et al., 2021). This difference may be due to the dose of LPS in experiments presented here (70 ng/mL) compared to the study which applied 1 µg/mL LPS for 24 hours (O'Regan et al., 2021). Although this high dose of LPS was carried out in this thesis only once, a reduced pro-inflammatory cytokine response was observed but requires repetition to determine if the trend in response remains the same at varied dosages. Another factor that could explain the different findings is the application of IFNγ in conjunction with LPS (O'Regan et al., 2021). IFNγ enhances macrophage activation and is, therefore, commonly used to prime

macrophages, to amplify pro-inflammatory cytokine production in response to an activating stimulus such as LPS (Hu and Ivashkiv, 2009) and is shown to potentiate the release of IL-1β (Masters et al., 2010). This highlights the need for consistency in the applied activating stimulus to allow rigorous testing across the literature to determine the role of mHTT in shaping microglial responses that propagate disease.

Impaired anti-inflammatory signalling including reduced levels of IL-10 has been described in the literature, although pro-inflammatory cytokines including IL-6, TNF α are increased (Podlacha et al., 2022). This is observed in the periphery of R6/1 HD mice, however, myeloid cells in the periphery are ontogenically distinct from microglia (Ginhoux et al., 2010) and thus responses in the periphery cannot necessarily be extrapolated to the CNS. Differences in responses to activating stimuli between humans and mice are also demonstrated highlighting the need for consideration when extrapolating data from mice to humans (Mestas and Hughes, 2004).

Other morphological observations in HD microglia include the appearance of vacuole like structures that have been noted in other models of HD with other cell types including astrocytes and lymphoblasts (Juopperi et al., 2012; Nagata et al., 2004). Autophagy is a cellular mechanism by which large proteins and damaged organelles are degraded by lysosomal mechanisms (Todde et al., 2009) and alterations to this process affects the clearance of aggregate-prone proteins in neurodegenerative disorders including HD (Plaza-Zabala et al., 2017). Induced neurons (iN) derived from HD patient fibroblasts show differences in protein expression related to autophagic mechanisms (Pircs et al., 2022). In these iN cells increased accumulation of autophagolysomal structures in neurites occurs but autophagic activity is reduced, suggestive of a failed autophagosome transport mechanism (Pircs et al., 2022). Expanded polyQ regions in HTT modulate autophagic activity by altering the interactions between the deubiquitinating enzyme, ataxin-3 interactions and BECN1, an essential autophagy initiator, ultimately leading to reduced autophagy (Ashkenazi et al., 2017). The vacuole structures described here in HD 109 microglia are striking and appear to be widespread. Further investigation is, however, required to determine their identity and role in HD pathology such as labelling with LC3, a protein essential in the formation of autophagosomes (kabeya et al., 2004). Importantly, aberrant autophagic function in microglia may have consequences for inflammatory function (Plaza-Zabala et al., 2017).

The stunted inflammatory response observed here may also be indicative of receptor dysfunction. HTT interacts with a large number of proteins with the presence of an expanded CAG sequence altering such interactions (Saudou and Humbert, 2016). TLR4 mediated responses have been shown to be dysfunctional in mast cells expressing mHTT from R6/1 mice leading to the attenuated release of both pro- and anti-inflammatory cytokines (Pérez-Rodríguez et al., 2020). This response was shown to be related in part to the lack of TLR-4 receptor trafficking via dynamin-dependent interactions (Pérez-Rodríguez et al., 2020).

Chapter 4

4: Progress towards a model of neuroinflammation in HD incorporating additional CNS cell types

4.1. Introduction

As demonstrated in Chapter 3, differentiation of HipSci and HD 109 iPSC using defined factors generates microglia in a monoculture setting that display typical microglial protein expression and functional properties. Microglia, however, receive a number of signals from the CNS microenvironment including those produced by neurons and astrocytes such as TGFβ and CX3CL1 (Butovsky et al., 2014; Cardona et al., 2006). Some of these signals attenuate microglial activity (Cardona et al., 2006; Liang et al., 2009) and so monocultures may express heightened inflammatory responses *in vitro* that are not typical of the *in vivo* situation. Indeed, protocols to generate microglia from iPSC have also been further improved to include secreted neural factors (Banerjee et al., 2020; McQuade et al., 2018). Microglia are also shown to display a morphology more reminiscent of mature microglia with an increased number of processes and secondary branching patterns when transplanted into mouse brains, demonstrating the importance of CNS derived signals and the physical presence of other cell types in the proper functioning of microglial cells (Bennett et al., 2016; McQuade et al., 2018).

Unlike microglia which arise from mesoderm, neurons, and astrocytes develop from a neuroectodermal origin, with neurons appearing before astrocytes via a common neural precursor cell (NPC) that lines the neural tube during embryonic CNS development (Bayer and Altman, 1991). Neurogenesis and the production of different subpopulations of neurons is determined by the expression of proneural genes such as Ngn 1/2 (Parideaen and Huttner, 2014). Ngn1 is in fact essential for neuronal development and prevents astrogenesis by blocking the transcription of astrocytic genes (Sun et al., 2001). The timely generation of neurons is under the control of the Notch-Delta signalling pathway with a reduction in notch signalling promoting neurogenesis (Gaiano et al., 2000). Activation of the ciliary neurotrophic

factor (CNTF) receptor initiates JAK-STAT signalling, promoting astrogenesis and transcription of astrocytic genes (Bonni et al., 1997). The *in vivo* ligand of CNTF was later determined to be a factor secreted by neurons, cardiotrophin 1 (CT-1), and is required for the neuron-glia switch (Barnabe-Heider et al., 2005). BMP signalling and notch activation also act in concert to promote astrogenesis (Chambers et al., 2001; Gross et al., 1996).

Protocols to generate NPC and neurons from iPSC have been described in the literature (Chambers et al., 2009; Shi et al., 2012) and mimic *in vivo* development by the specification of neuroectoderm, and prevention of mesoderm and endoderm by antagonism of BMP and TGFβ signalling networks to generate NPC (Chambers et al., 2009). Neuronal subtype specification can be achieved using additional factors. Addition of the wingless-related integrated site (WNT) inhibitor XAV939 promotes specification towards a cortical neuronal fate (Qi et al., 2017). Appropriately timed exposure to sonic hedgehog (SHH) directs cells towards striatal lineages (Amimotto et al., 2013; Delli Carri et al., 2013). Subsequent addition of the gamma-secretase inhibitor, compound E, blocks Notch-Delta signalling and accelerates differentiation to post-mitotic neurons (Li et al., 2011). HD iPSCs have been successfully differentiated into neural precursors followed by striatal lineage determination using SHH, DKK1 (dickkopf-1), and BDNF, producing DARPP32 positive neurons carrying 72 CAG repeat that demonstrate increased caspase activity (Zhang et al., 2010). Both striatal and cortical neurons derived from HD patient iPSC have been shown to display abnormalities during development including delays in both neuronal specification and appearance of mature electrophysiological properties (Mathkar et al., 2019; Mehta et al., 2018).

HD astrocytes have been generated from iPSC using various methods (Emdad et al., 2010; Krencik and Zhang, 2010; Shaltouki et al., 2013) with the inclusion of developmentally relevant factors such as CNTF and BMP2 (Shaltouki et al., 2013). Additional factors have also been employed to regionally specify astrocytes (Holmqvist et al., 2015; Roybon et al., 2013). HD iPSC-derived astrocytes demonstrate a reduced ability to provide support to neurons including protection against glutamate-mediated excitotoxicity when co-cultured with both control and HD neurons (Garcia et al., 2019). HD astrocytes also display altered metabolism and dysregulated transcriptional networks including those associated with inflammation (Lange et al., 2023).

Research into the crosstalk between brain cells has mostly focussed on communication between neurons and glia with signalling between non-neuronal cells less defined. From an inflammatory perspective, astrocytes as well as microglia are capable of eliciting immune responses and secreting common immune signalling molecules (Colombo and Farina, 2016). Signalling and communication between astrocytes and microglia is not fully defined, however, appropriate signalling between microglia and astrocytes is essential for the health and proper functioning of neural networks**.** Activated microglia are shown to be capable of polarising astrocytes to a reactive phenotype resulting in an expression profile of pro-inflammatory cytokines similar to microglia, and consequently results in accelerated neuronal and oligodendrocyte death (Liddelow et al., 2017). Astrocytes and microglia along with glutamatergic neurons form the quad-partite synapse, a unit which is responsible for normal circuit operation with communication heavily related to neuro-immune signalling (Schafer et al., 2012). Thus, platforms that incorporate multiple CNS cell types are better able to recapitulate the responses of microglia *in vivo*. Combining multiple cell types, however, poses challenges as the most suitable culture conditions for optimal functionality of all cell types is necessary.

The aims of this chapter are:

1) To differentiate iPSC into neurons and astrocytes for subsequent co-culture with microglia.
2) To assess the compatibility of co-culture BrainPhys (coBP) medium with microglial differentiation and functional responses to inflammatory stimuli.
3) To determine if HD 109 co-cultures of microglia and astrocytes display a synergistic cytokine response due to the presence of both microglia and astrocytes in comparison to monocultured microglia when treated with pro-inflammatory stimuli.

4.2. Results

4.2.1. Differentiation of iPSC to generate neural precursor cells and neurons

4.2.1.1. NPC

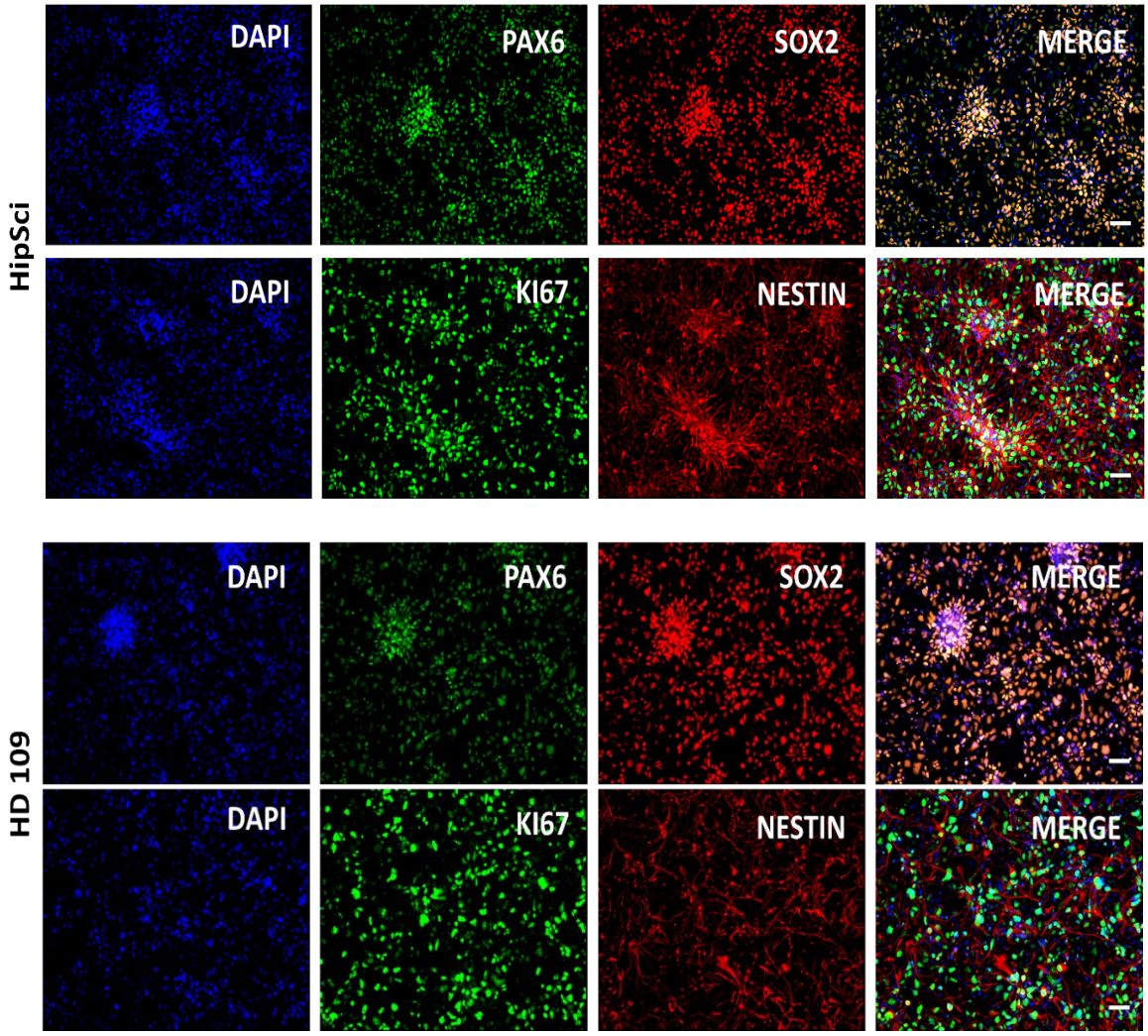

Figure 13. Differentiation of iPSC to NPC*. iPSC were differentiated in Neural Induction Medium (NIM). At day 14 cells were transferred to Neural Maintenance Medium (NMM) and expanded for up to a week before plating onto glass coverslips at 1.0 x 10^4 cells/ cm^2. After 48 hours cells were fixed and co-stained with Nestin (red) and Ki67 (green) with DAPI (blue) or, Sox2 (red) and Pax6 (green) with DAPI. Images were taken at X20 magnification and are representative. HipSci (n=2) and HD 109 (n=1). Scale bar 50 µm.*

Each line differentiated into NPC with typical neural rosette morphology reminiscent of *in vivo* neural tube formation (Dhara and Stice, 2008) and expressed key markers including *Nestin*,

Pax6, and *Sox2* (Osumi et al., 2008; Vinci et al., 2016). The cells also express high levels of *Ki67* (Gerdes et al., 1984) indicative of a proliferative population of cells. The NPCs also showed minimal staining for the neuronal marker, *Map2*, and the commonly used astrocytic marker, *Gfap* (Supplementary figures S1, S2). *Oct4* staining was absent (Figure S1). Taken together this indicates a relatively pure population of proliferative neural progenitor cells.

4.2.1.2. Neurons

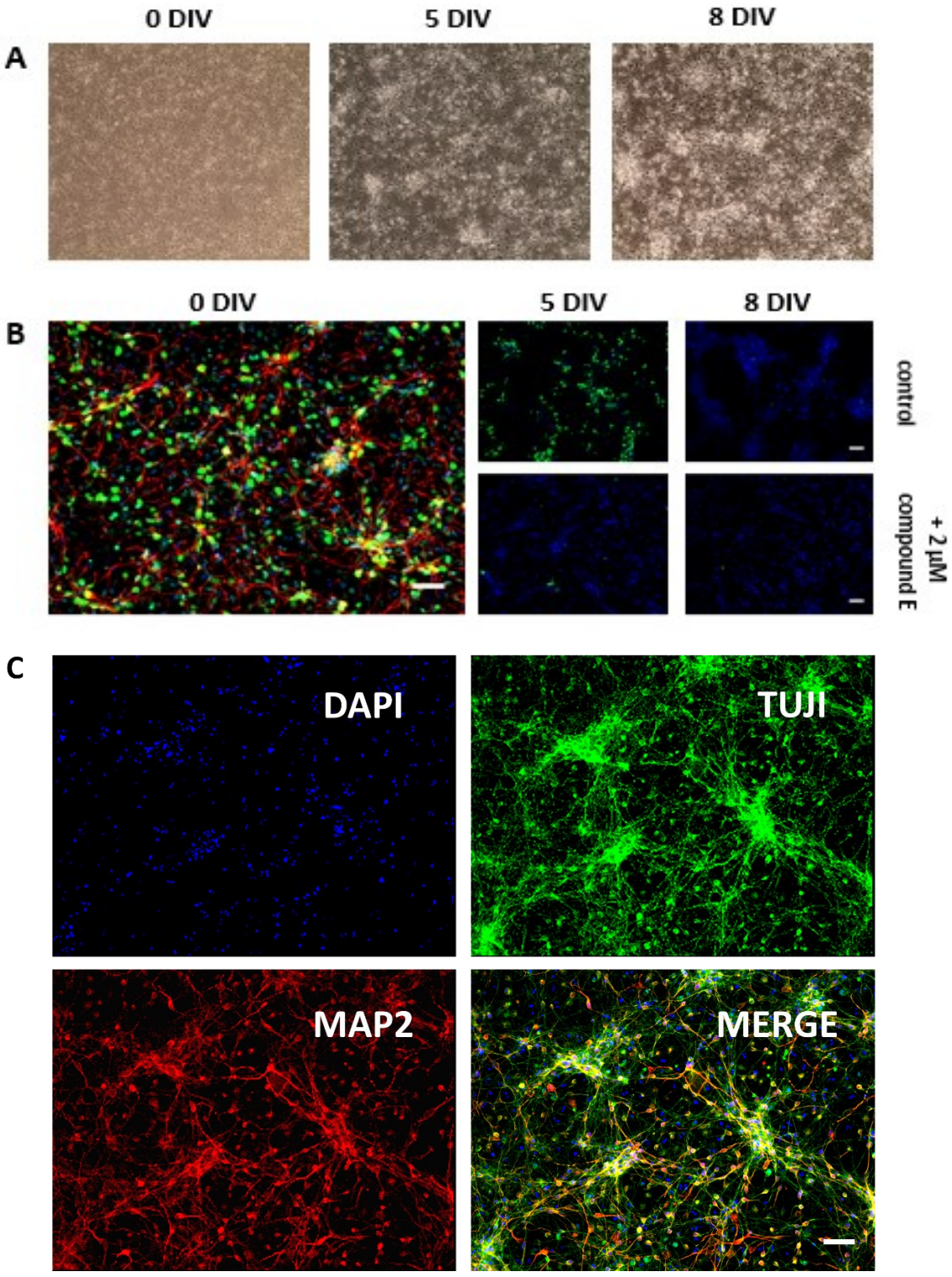

Figure 14. Neuronal differentiation of HipSci NPC*. To induce neuronal differentiation, NPC were treated with the gamma secretase inhibitor, compound E. (A) displays representative phase images of the differentiation process at D0, 5, and 8. (B) displays the expression of Ki67 throughout differentiation with comparison to untreated cells. (C) shows the expression of the neuronal markers, Tuj and Map2. Scale bar 50 µm.*

Only HipSci neurons (Figure 14) were generated due to rapid differentiation of HD 109 NPC cultures. Microtubule-associated protein (*Map2*) labels microtubules and is used as a neuronal marker (Dehmelt and Halpain, 2004). It demonstrates very weak expression in neural precursors but becomes strongly expressed soon after the expression of another neuronal marker, beta III tubulin (*Tuj*) (Menezes and Luskin, 1994). Visually, the lower expression of *Map2* relative to *Tuj* follows this pattern of appearance of each marker with *Tuj* preceding *Map2* (Menezes and Luskin, 1994). Immunoreactivity of *Map2* has, however, been observed in astrocytes (Papasozomenos and Binder, 1986) and oligodendrocytes (Vouyiouklis and Brophy, 1995). The intermediate filament protein, *Nestin,* is highly expressed in neuroepithelial precursor cells and is therefore, considered an essential marker of neural stem cells (Hockfield and McKay, 1985; Lendahl et al., 1990). A marked reduction in *Nestin* immunoreactivity is indicative of differentiation towards a neuronal or glial state (Frederikson and McKay, 1988). *Ki67* staining is also absent with no colocalization with the nucleus observed (Figure 14B) by 8 days *in vitro* (DIV) following compound E treatment.

However, neurons generated from HipSci cells also posed challenges and will not be considered further. Off-target differentiation within cultures prior to the addition of astrocytes and/or microglia (Supplementary figure S3) and time limitations meant it was not possible to set up subsequent replacement cultures of neurons. It has been demonstrated that iPSC retain a degree of DNA methylation from the parental cell type from which they were derived, meaning they have an increased propensity to differentiate into the original cell type prior to reprogramming (Kim et al., 2010).

4.2.2. Astrocytes

4.2.2.1. Astrocyte differentiation

HipSci and HD 109 iPSC NPC both differentiate into astrocytes expressing *S100β* and *Gfap* which are commonly used as astrocytic markers in the literature (Raponi et al., 2007). Interestingly, HD 109 microglia display an altered morphology with a smaller soma in comparison to control astrocytes (Figure 15). Astrocytes from HD patient brains immunolabelled with *Gfap*, however, display larger soma and thicker processes in comparison to control striatal astrocytes (Faideau et al., 2010). This difference between iPSC-derived

astrocytes and primary patient astrocytes may be due to age differences and the microenvironment of the cells. The astrocytes produced in this thesis using a commercially available kit are also more cortical-like and this regional difference may impact morphology as astrocytes demonstrate broad regional heterogeneity in morphological properties and phenotypes (Matias et al., 2019). Other literature demonstrates a reduced surface area of mouse and human astrocytes that express mHTT (Octeau et al., 2018; Osipovitch et al., 2019).

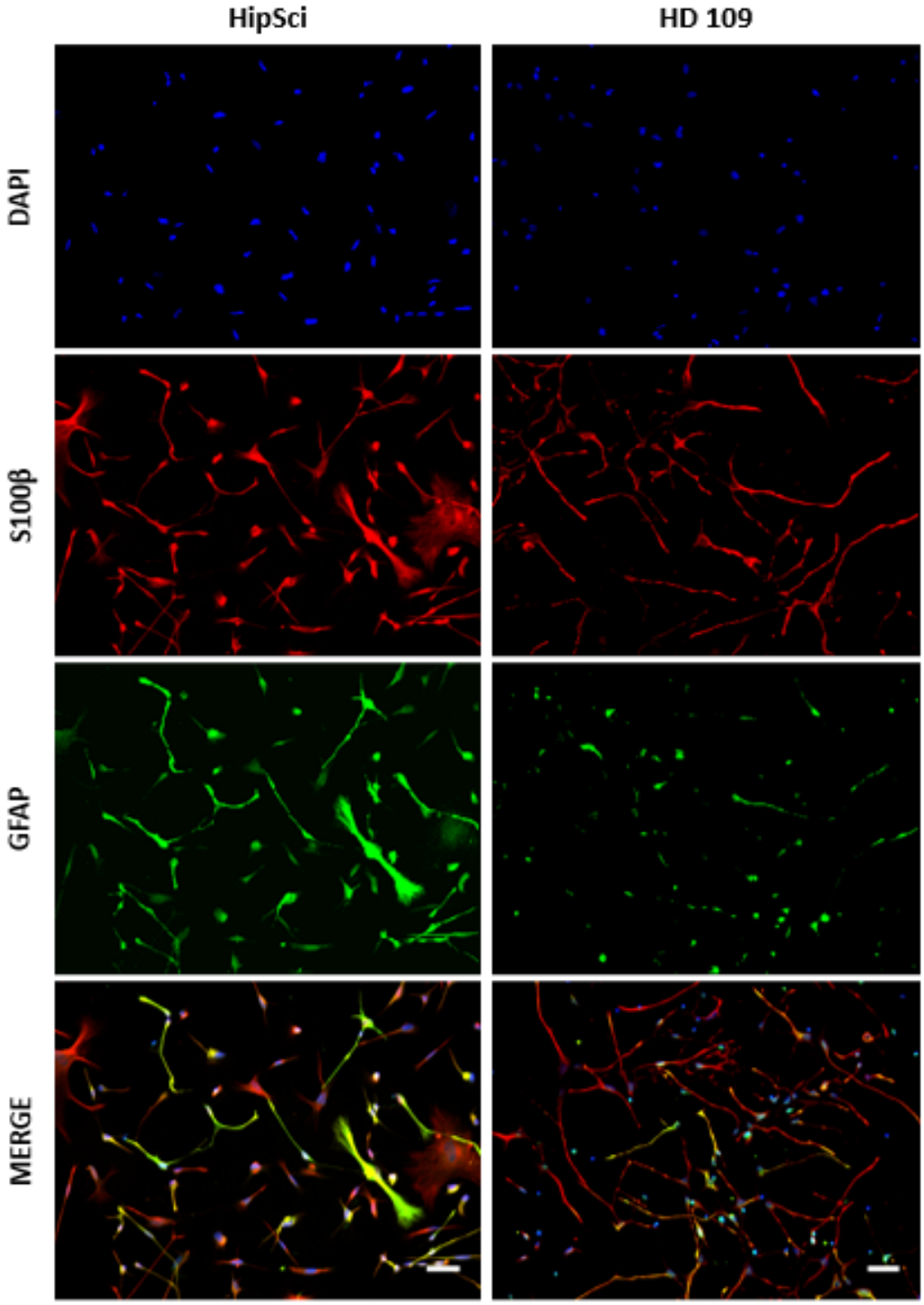

Figure 15. Generation of astrocytes from control and patient iPSC lines via an NPC intermediate*. HipSci and HD 109 NPC were further differentiated to astrocytes using a commercially available kit from STEMCELL Technologies for 45 days and then maintained in serum-free Astrocyte Medium (AM) thereafter. D65 astrocytes were stained for S100β (green), Gfap (red), and DAPI (blue). Images were taken at X20 magnification and are representative. Scale bar 50 µm. (n=1).*

4.2.2.2. HipSci and HD 109 astrocytes do not release cytokines in response to inflammatory stimuli, including the neurodegenerative relevant protein, amyloid

Both HipSci and HD 109 astrocytes display changes in morphology including slight clustering when treated with either LPS or Aβ for 24 hours (Figure 16 A). No obvious change in morphology of astrocytes treated with LPS is documented in the literature (Sheng et al., 2011), however, these were primary and immortalised astrocytes treated with a lower dose of LPS (100 ng/mL) than is used here (1 µg/mL). Observations in rat astrocytes may also not extrapolate to those of human astrocytes (Zhang et al., 2016). Changes in astrocyte morphology are observed in the presence of amyloid (Garwood et al., 2011).

Neither HipSci nor HD 109 astrocytes release the pro-inflammatory cytokines IL-6, TNF α, or IL-1β in response to LPS or Aβ 1-42 (Figure 16 B). There is variation in the literature regarding inflammatory cytokine release from astrocytes following LPS stimulation with some observing no cytokine response (Tarassashin et al., 2014; Zhang et al., 2017) while others record modest cytokine release (Dozio and Sanchez, 2018; Rutkowska et al., 2018). Differences across the literature may be due to dosage, exposure time, and the limits of detection for individual assays. It may also be due to age of the astrocytes with adult and fetal astrocytes demonstrating different responses to stimuli (Zhang et al., 2016) (Figure 16 B).

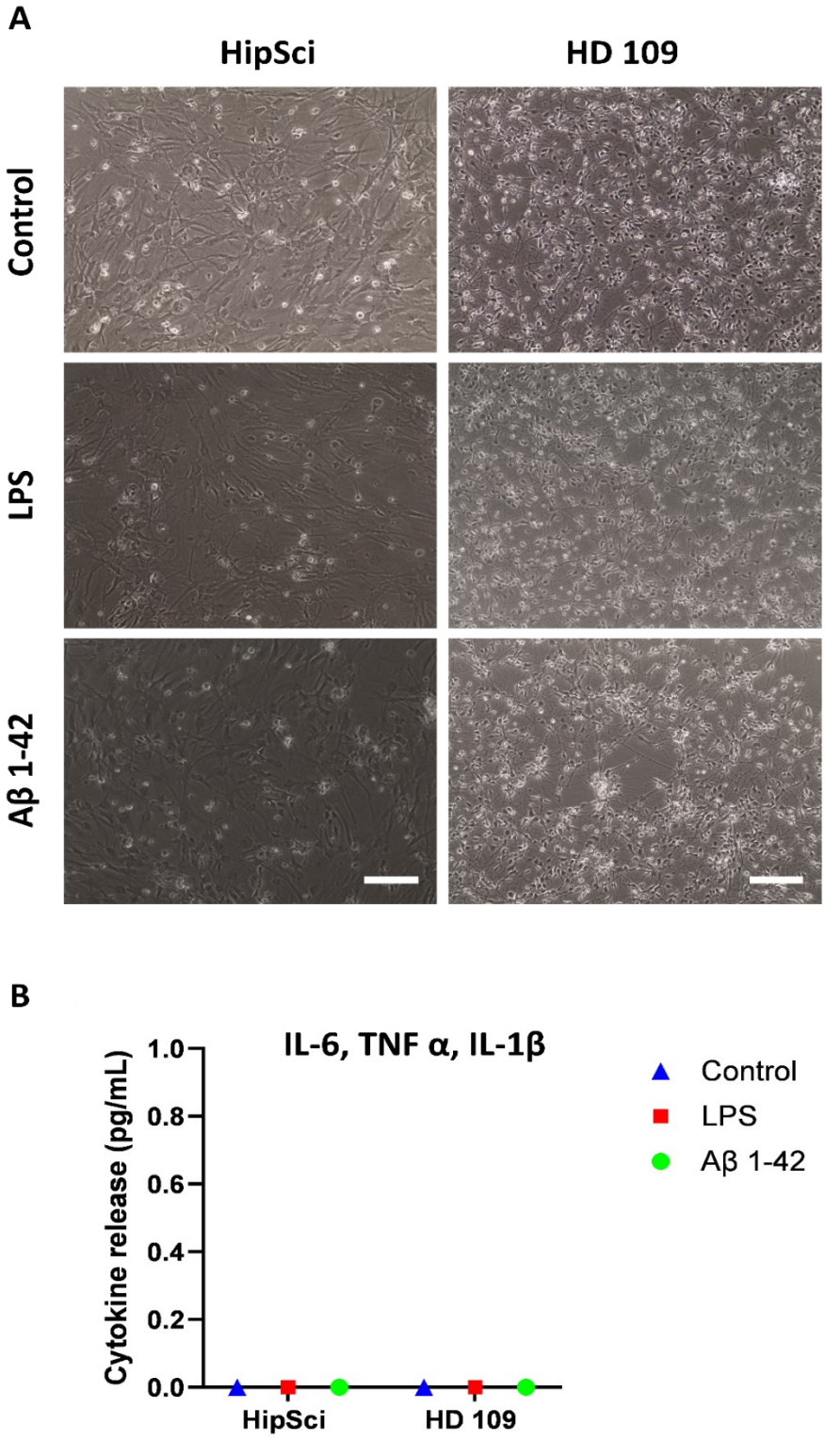

Figure 16. iPSC-derived astrocyte response to inflammatory stimuli*. HipSci and HD 109 astrocytes (D65) were treated with either LPS (1 µg/mL) or amyloid (Aϐ 1-42; 1 µM) for 24 hours. Conditioned medium was collected, and the cells lysed for total protein quantification. (A) phase images of HipSci and HD 109 astrocytes in response to LPS and amyloid in comparison to controls (B) IL-6, TNF α, and IL-1ϐ release determined by ELISA. Scale bar 50 µm. (n=1).*

4.2.3. Co-culture of microglia and astrocytes

4.2.3.1. Co-culture of microglia and astrocytes in microglial medium (MM)

Co-cultures of astrocytes and microglia were generated by first plating astrocytes at a density of 1.5 x 10^4 cells/ cm^2 onto pORN-LAM 511 coated plates with Astrocyte Medium (AM). The AM used throughout this thesis is composed of the same base media and supplement as Microglial Medium (MM) with Advanced DMEM/F12 and 1X N-2. The only additional factor is IL-34. As such, MM was selected as the co-culture medium to support both cell types. 24 hours after initial plating of astrocytes, the medium was removed and replaced with AM containing 2 µM cytosine arabinoside (Ara-C). Following 24-hour treatment with Ara-C, the medium was replaced with AM only for a further 24 hours before addition of microglial progenitors at a concentration of 3.0 x 10^4 cells/cm^2. The glial cells were then co-cultured for a total of 14 days before characterisation by fluorescent microscopy and assessment of cytokine release in response to inflammatory stimuli including LPS and amyloid (Aβ 1-42) using enzyme-linked immunosorbent assay (ELISA).

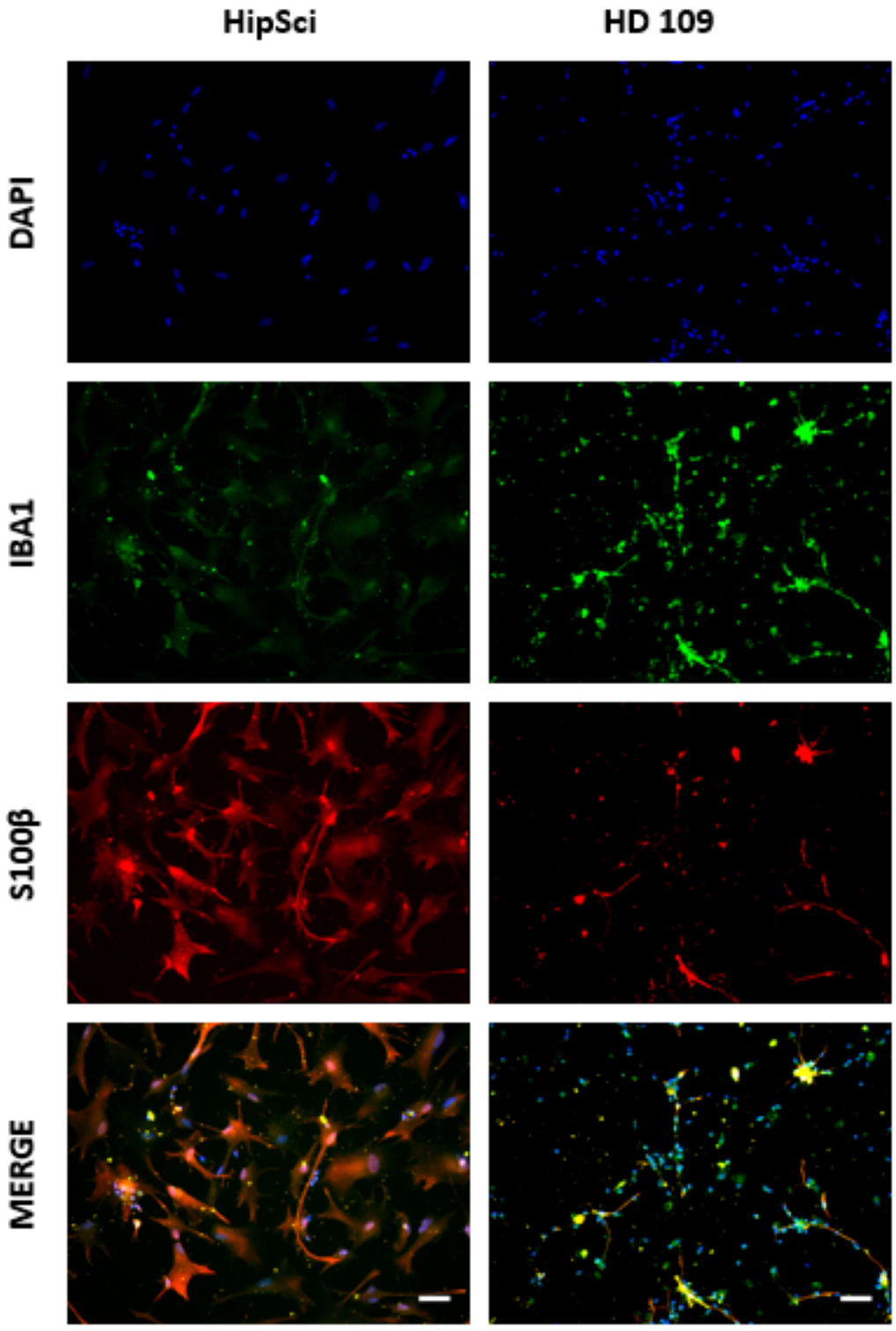

***Figure 17. Co-culture of microglia and astrocytes in MM**. Astrocytes and microglia were co-cultured for 14 days. Cells were plated at a density of 1.5 x 10^4 cells/cm^2 and 3.0 x 10^4 cells/ cm^2 for astrocytes and microglia respectively (ratio 1:2). At day 14 cells were fixed and stained for the microglial marker Iba1 (green) and astrocytic marker, S100β (red) along with DAPI (blue). Scale bar 50 µm. (n=1).*

HipSci and HD 109 co-cultures of astrocytes and microglia stain positively for the microglial marker, *Iba1* and the astrocytic marker, *S100β (Figure 17).* HipSci co-cultures showed an even distribution of cells as opposed to HD 109 astrocyte and microglia co-cultures where cells appear to cluster together. Clustering of microglial cells is documented as a feature of their reactivity (Paasila et al., 2020), thus clustering may indicate glial activation in the HD 109 co-cultures. Reactive gliosis is observed in HD patient striatal tissue where both astrocytosis and microglial reactivity is detected (Vonsattel et al., 2008). Although co-cultures stain positively for *Iba-1* and *S100β*, the two markers seem to overlap and so one or both cell types may to some degree be expressing both markers. Indeed, *S100β* expression has been noted in microglia (Adami et al., 2001) and may explain a degree of overlap in protein expression.

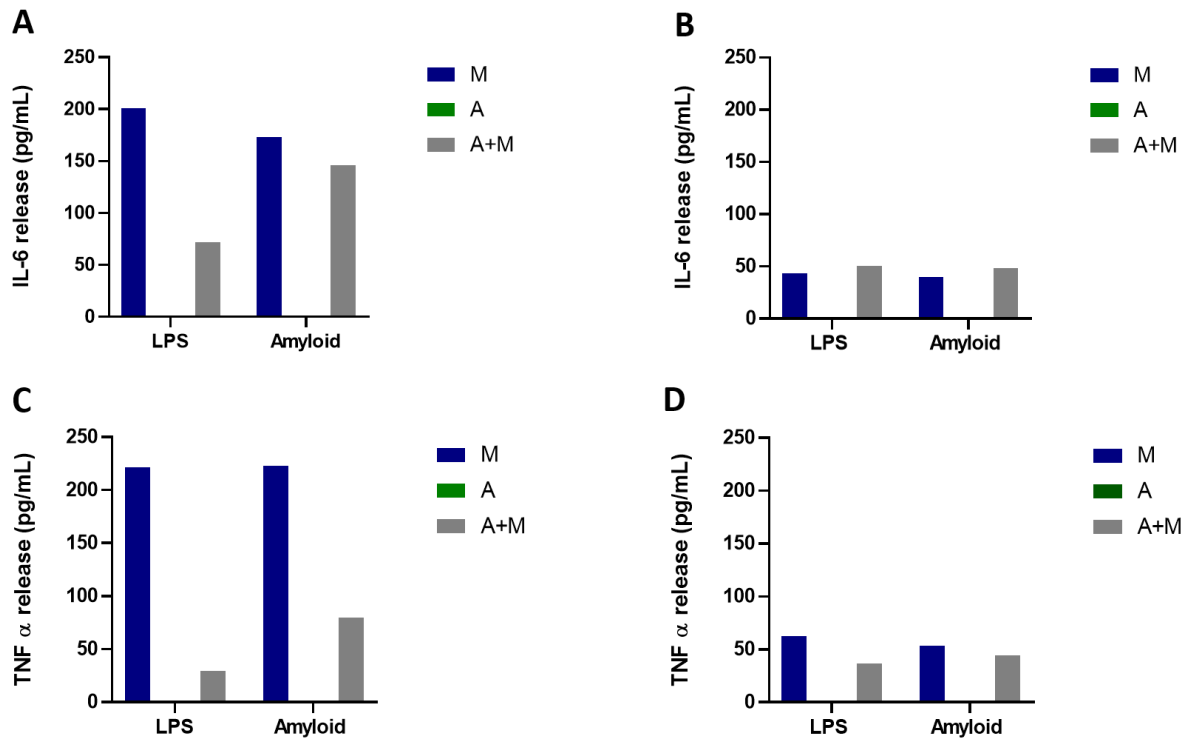

Figure 18. Microglia and astrocyte co-culture responses to LPS and Amyloid in MM*. Comparison of microglia and astrocyte monoculture responses to respective co-culture responses. M – microglia, A – astrocyte and A+M – astrocyte and microglia co-culture. Cultures were treated with either 1 µg/mL LPS or 1 µM amyloid for 24 hours. IL-6 and TNF α responses for HipSci (A, C) and HD 109 (B,D) are displayed. Cytokine release is displayed as pg/mL per µg of total protein. (n=1).*

Both HipSci and HD 109 astrocytes fail to produce a cytokine (IL-6 and TNFα) response to LPS (1 µg/mL) and amyloid β 1-42 (1 µM). Microglia respond to LPS and amyloid with HipSci cells secreting 200.9 pg/mL and 173.03 pg/mL IL-6 for LPS and amyloid respectively, compared to 221.51 pg/mL and 223.18 pg/mL TNF α under the same conditions (Figure 18 A, C). HD 109 microglia released 43.71 pg/mL and 40.06 pg/mL IL-6 in response to LPS and amyloid, and 62.69 and 53.25 pg/mL TNF α respectively (Figure 18 B, D).

Interestingly, the response to inflammatory stimuli is reduced in co-cultures of HipSci microglia and astrocytes for both IL-6 (LPS: 71.9, amyloid: 146.4 pg/mL) and TNF α (LPS: 29.34, amyloid: 79.46 pg/mL) (Figure 18 A, C). HD 109 cocultures showed an increase in IL-6 secretion in response to LPS (50.68 pg/mL) and amyloid (48.62 pg/mL) under co-culture conditions, although the change is small. There was, however, a reduction in the release of TNF α in response to LPS (37.03 pg/mL) and amyloid (44.69 pg/mL) in co-culture conditions compared to monoculture (Figure 18 B, D).

4.2.3.2. Co-culture of microglia and astrocytes in BrainPhys Medium (coBP)

Microglia responses to inflammatory stimuli were compared to an alternative medium: co-culture BrainPhys (co-BP) followed by comparisons to co-culture conditions. BrainPhys Neuronal medium was selected for this based on the previous in-house development of a co-culture system involving neurons and astrocytes. As such, assessing the compatibility of this media with microglial function seemed a logical step in the development of a tri-culture system involving all three cell types.

Firstly, it was important to assess whether macrophage precursors plated into coBP would differentiate successfully into microglia and express key microglial markers. IL-34 is essential for microglial proliferation (Elmore et al., 2014; Wang et al., 2012) and was, therefore, included in both co-culture media compositions. Figure 19 demonstrates that both HipSci and HD 109 macrophage precursor cells differentiate into microglia expressing both *Iba1* and *Tmem119* in this alternative media.

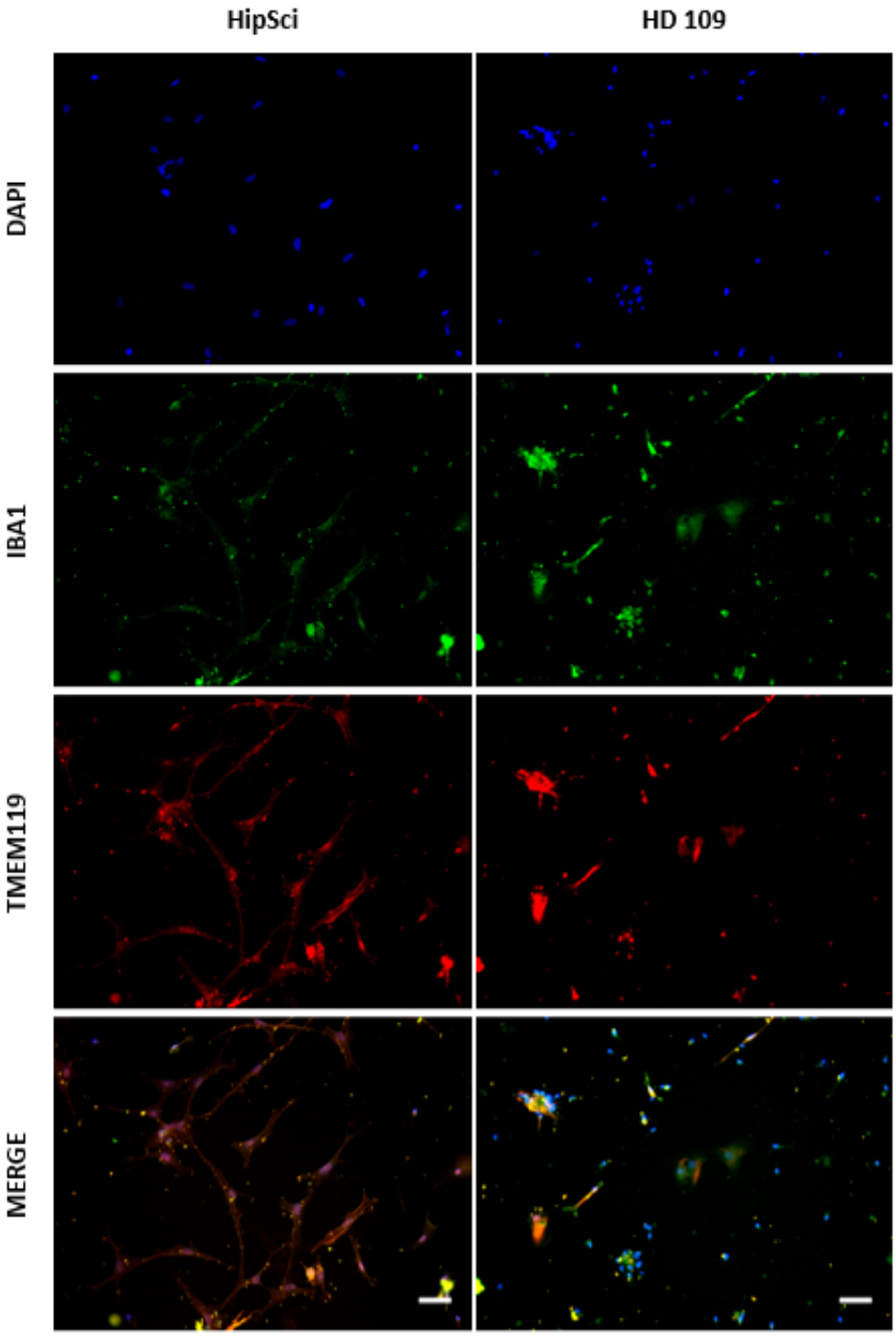

Figure 19. Differentiation of HipSci and HD 109 microglia in an alternative co-culture medium (coBP). *Macrophage precursors were plated onto pORN-LAM 511 coated glass coverslips and differentiated in BrainPhys Neuronal SM1 Medium with BDNF (20ng/mL), GDNF (20 ng/mL), 1X N2 and 100 ng/mL IL-34. At day 15 following initial plating cells were fixed and stained for DAPI (blue), Tmem119 (red) and Iba1 (green). Images were taken at X20 magnification. (n=1).*

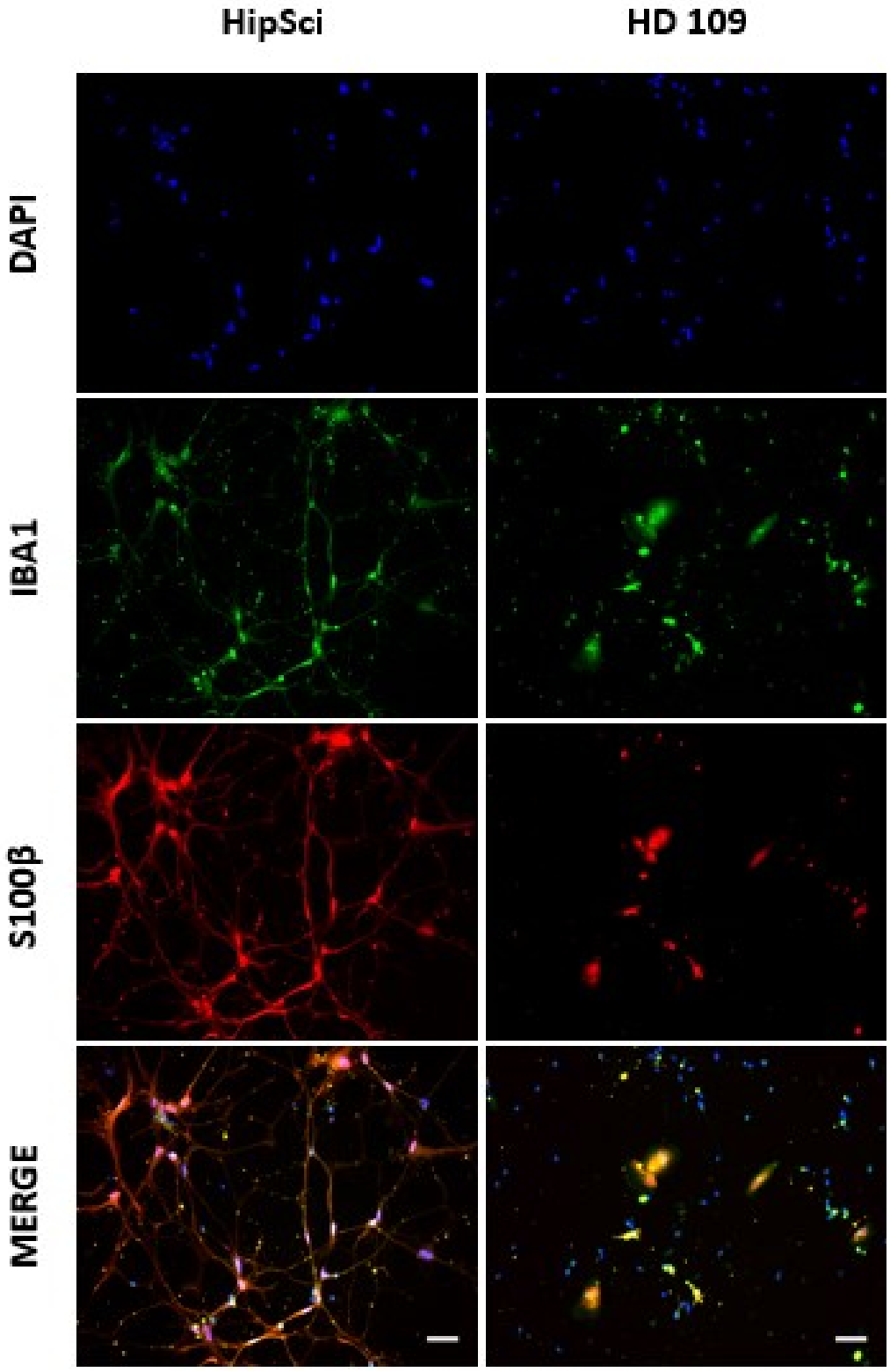

Figure 20. Co-culture of microglia and astrocytes in the alternative co-culture medium, coBP*. Astrocytes were plated at a density of 1.5 x 10^4 cells/ cm^2 followed by addition of microglia after 48 hours at 3 x10^4 cells/ cm^2. At day 14 cells were fixed and stained for Iba1 (green), S100β (red) and DAPI. Scale bar 50 µm. (n=1).*

Both HipSci and HD 109 co-cultures maintained in coBP express the markers *Iba-1* and *S100β* indicative of the presence of both astrocytes and microglia (Figure 20). However, there appears to be overlap of the two stains suggesting one or both cell types may express both of the markers used. Interstingly, the morphology of HipSci co-ultures appears quite different compared to co-cultures in MM, with cells exhibiting longer processes compared to their HD counterparts.

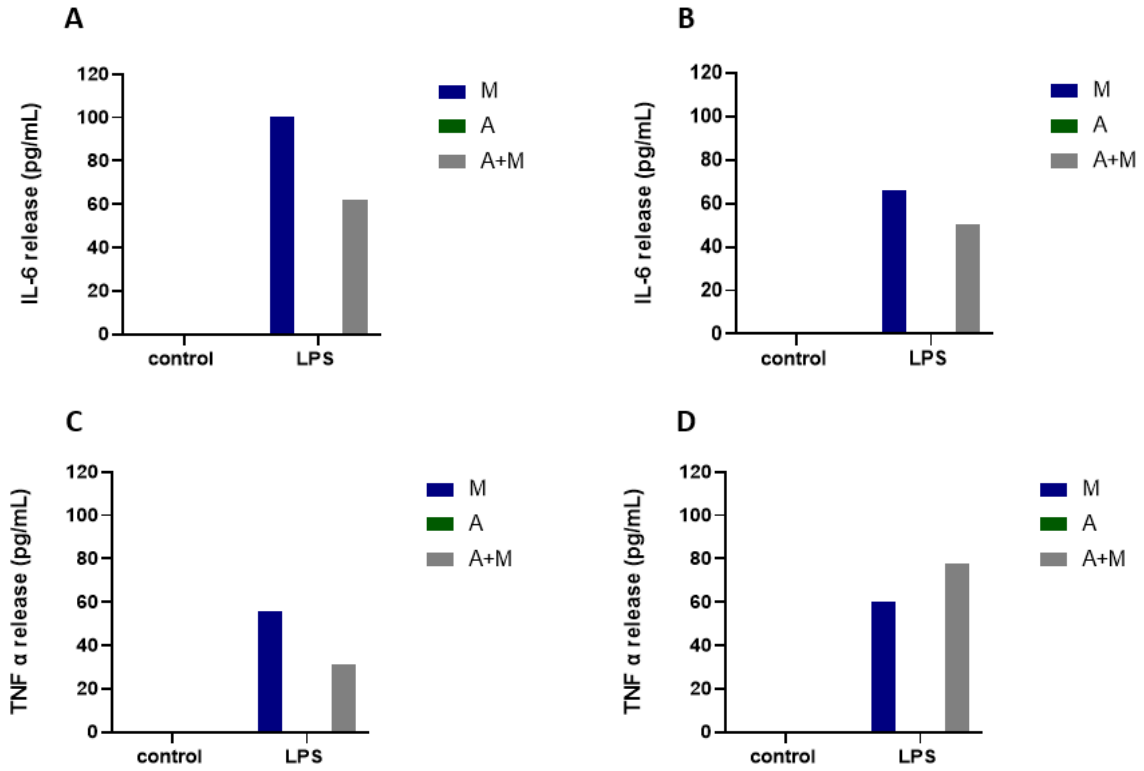

Figure 21. Comparison of glial monoculture and co-culture responses to LPS in an alternative medium composition (coBP)*. Macrophage precursors were differentiated to microglia in BrainPhys Neuronal SM1 Medium supplemented with BDNF (20ng/mL), GDNF (20 ng/mL), and 100 ng/mL IL-34. At day 15, cells were treated with 1 µg/mL LPS for 24 hours before collecting conditioned medium for cytokine analysis and cell lysis for total protein quantification. Cytokine release is presented in pg/mL per µg of total protein. (n=1).*

Similar to results observed in Figure 18, a reduction in cytokine release was found in co-culture conditions compared to monocultured microglia (Figure 21). HipSci co-cultures

produced only 61. 7 and 30.9 pg/mL of IL-6 and TNF α respectively compared to 100.2 and 55.6 pg/mL release in microglial monocultures maintained in coBP (Figure 21 A, C). HD 109 co-cultures demonstrate a similar trend with LPS prompting a reduced IL-6 response in co-culture (compared 50.4 pg/mL) compared to monocultures (66.01 pg/mL) (Figure 21 B). However, there is an increase in the TNF α response in co-cultures (77.46 pg/mL) compared to microglia alone (58.89 pg/mL) (Figure 21 D). Again, astrocytes do not produce a response to stimuli in this alternative medium.

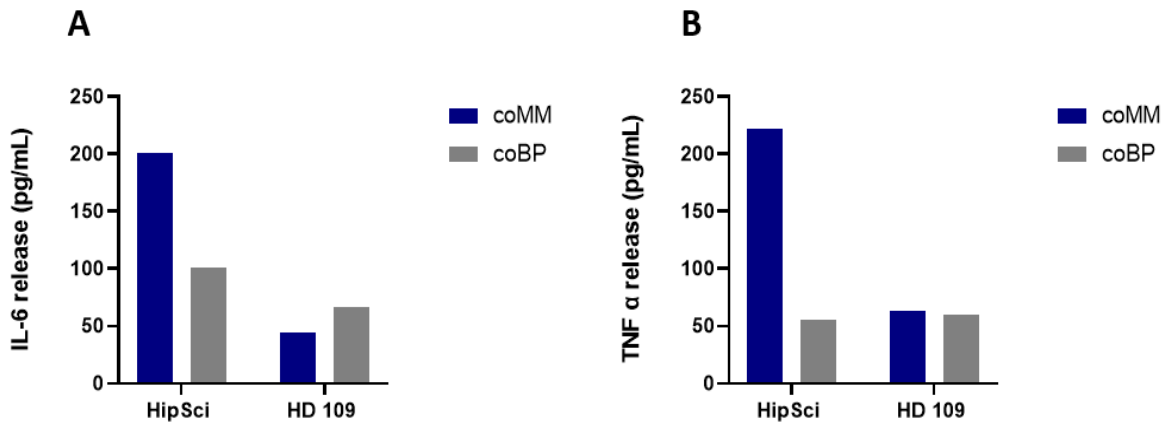

Figure 22. Microglial responses to stimuli in different medium compositions*.. Microglia were treated with 1 µg/mL for 24 hours and conditioned medium samples collected. (A) IL-6, (B) TNF α. Cytokine secretion is represented as pg/mL per µg of total protein. coMM – co-culture microglial medium, coBP – co-culture BrainPhys medium. (n=1).*

Controls are omitted as cytokine release is not detected. HipSci microglia exhibit a reduced IL-6 response when treated with 1 µg/mL LPS in coBP (100.2 pg/mL) medium compared to coMM (200.9 pg/mL) (Figure 22). The same is observed in relation to TNF α, with cells secreting 221.51 pg/mL in coMM compared to only 55.6 pg/mL in coBP. HD 109 microglial responses in the media are more closely related, producing 43.7 and 66.01 pg/mL IL-6 in coMM and coBP respectively. TNF α release by HD microglia is similar in both coMM and coBP, with cells secreting 62.68 and 59.89 pg/mL respectively (Figure 22).

4.3. Discussion

Astrocytes derived from patient iPSC display an altered morphology that is observed in the literature with both R6/2 mouse astrocytes (Octeau et al., 2018) and human astrocytes that express mHTT (Osipovitch et al., 2019), indicating early developmental changes in the HD astrocytes produced in this thesis. The iPSC-derived astrocytes presented here express both *Gfap* and *S100β* which are commonly used in literature, although, debate surrounds their use as specific astrocyte markers (Raponi et al., 2007). S100β expression has been observed in a mature subpopulation of oligodendrocytes (Hachem et al., 2005) and even in neurons (Rickmann et al., 1995). Gfap has been shown to poorly label astrocytes covering roughly 15% of total astrocyte volume despite being the major intermediate filament of the cytoskeleton (Bushong et al., 2002). Depending on astrocyte location, positive *Gfap* staining can also be variable (Walz et al., 1998). Therefore, it is postulated that certain astrocytic markers are better suited depending on the target region (Zhang et al., 2019). An upregulation of *Gfap* staining in disease states has also been observed and may confound results regarding the total number of astrocytes (Middeldorp and Hol, 2011).

Both HipSci and HD 109 astrocytes fail to release cytokines in response to proinflammatory stimuli. The lack of cytokine signalling from astrocyte monocultures may simply be due to the detection limits of the ELISA. From a neuroimmune perspective, responses of astrocytes are under investigated compared to microglia. Some literature using primary human astrocytes does, however, support this lack of a response from an inflammatory stimulus (Tarassishin et al., 2014). Interestingly, there appears to be differences in functional responses of primary mouse and human astrocytes in response to activating stimuli due to differential signalling mechanisms with mouse astrocytes producing a potent response to LPS (100 ng/mL) while human astrocytes are unresponsive (Tarassishin et al., 2014). Further demonstrating the divergent properties of animal and human models. However, some studies suggest that astrocytes require microglial priming for activation (Cunningham et al., 2019) which makes the lack of a synergistic response observed here in co-culture with astrocytes, surprising.

In this chapter, two different medium compositions were compared in the context of microglial inflammatory responses. BrainPhys was chosen as a comparison to see if microglia could be easily incorporated into a co-culture method involving neurons and astrocytes previously developed in house. BrainPhys is a medium optimised for the functionality of

neurons and so to support microglial survival, the medium was simply supplemented with additional factors including the essential microglial cytokine, Il-34 (Elmore et al., 2014; Wang et al., 2012). HipSci and HD 109 cells successfully differentiate into microglia as indicated by expression of *Tmem119* and *Iba1* in this alternative medium. Although the data is yet to be repeated there appears to be differences in responses between the different mediums but determining which one is the most suitable for determining microglial responses requires extensive testing of how medium compositions may impact metabolism, gene, and protein expression, and ultimately how this translates to functional properties.

Immunofluorescence imaging of co-cultures shows overlapping areas of staining, but certain regions do indicate distinctive staining of astrocytes and microglia with their respective markers. Staining cells with specific antibodies is crucial for characterising these cells in co-cultures but some markers are not always particularly selective for a certain cell type and will stain multiple cell types. Microglia are shown to express *S100β* (Adami et al., 2001) and is, therefore, not a specific marker of astrocytes in this context. As microglia and astrocytes are both phagocytosis competent cells (Konishi et al., 2022) it may also be plausible that engulfment of cellular debris by either cell type may lead to overlap in the expression profile.

Lower cytokine release is observed in co-cultures compared to monocultures with the exception of HD 109 where the co-culture condition produced an increased response. This response may not be due to the presence of astrocytes and bidirectional signalling between the two cell types, but instead may arise simply from differences in microglial numbers. Discrepancies in plating and potential removal of microglia during regular maintenance of cultures may have reduced the total number of microglia present at the time of treatment and as a result, a reduced cytokine response when stimulated with amyloid or LPS. Thus, it is difficult to determine the origin of the response in a culture of mixed cell populations. Owing to the fact that monocultured astrocytes appear to not respond to stimuli tested in this thesis, it can be assumed that functionally microglia must be present in all co- cultures discussed in this thesis and are primarily responsible for the observed response. Although it is difficult to determine the impact of astrocytes on these cultures without knowing the end ratio of astrocytes: microglia. Therefore, further interrogation of the system is required.

Chapter 5

5: General discussion and future directions

5.1. Summary of research findings

In this thesis control and HD 109 iPSC are shown to successfully differentiate into microglia expressing typical markers including the microglial specific marker, *Tmem119* (Bennett et al., 2016). Microglia and their role in shaping the neuroinflammatory environment in HD has been the primary focus of this project. Therefore, successful generation of functional microglial cells is critical to developing a model of inflammation in HD. Indeed, the microglial cells generated from patient and HD iPSCs in this thesis demonstrate typical microglial functions including phagocytic activity and production of inflammatory mediators in response to immune challenge. Interestingly, HD 109 microglia appear to demonstrate a stunted pro-inflammatory cytokine response to LPS and amyloid in contrast to HipSci cells. This observation is significant when IL-6 levels are compared in response to 24-hour stimulation with LPS. Neurons and astrocytes are also successfully produced from these cell lines, providing the tools to develop a xenofree platform to model HD.

Although due to challenges including off-target differentiation neurons were excluded from co-culture systems. However, co-cultures of microglia and astrocytes were assessed for inflammatory function and compatibility of culture medium to support microglial function. Co-cultures of astrocytes and microglia demonstrated reduced cytokine release when stimulated with LPS in comparison to monocultured microglia except for HD 109 co-cultures maintained in MM. This was unexpected as typically an amplified response should occur due to secretion of pro-inflammatory factors by microglia and subsequent polarisation of astrocytes to an A1 phenotype (Liddelow et al., 2017). BrainPhys was selected as a comparison co-culture medium to build upon a co-culture method previously developed in house that supports the function of neurons and astrocytes. The medium was simply supplemented with the essential microglial factor, IL-34 (Elmore et al., 2014; Wang et al., 2012). Microglia are generated following differentiation in this

media, expressing *Tmem119* and *Iba-1*, and demonstrating functional responses to immune stimulation. HipSci microglia showed differences in cytokine activity between the two co-culture mediums. Although this data is preliminary it highlights important considerations when combining cell types and optimising culture conditions to support all cell types and determining which media composition more accurately produces human relevant cell responses.

5.2. Inflammatory responses in Huntington's disease (HD): Comparison to the literature

HD 109 microglia were shown to respond differently to pro-inflammatory stimuli including LPS and amyloid. HipSci (control) demonstrate a more pronounced cytokine response to LPS and amyloid compared to HD CAG 109 cells that appear to show a much more muted response. In the case of LPS (70 ng/mL) this difference in response is actually significant at 24-hours for IL-6 release ($p=0.0296$). Between 4 and 24-hour treatment, the HD cells show smaller increases in cytokine production and even a decrease in TNF α response between 4 and 24-hour treatment time points with low dose LPS. Compared to controls the cells show smaller increases in cytokine production for both IL-6 and TNF α when the LPS dose is increased to 1000 ng/mL. Although these higher dose treatments require further investigation.

This observed stunted immune reactivity compared to controls is in contrast to certain literature. Using multiple HD patient iPSC lines to generate microglia, O'Regan et al., 2021 demonstrated a hyper-reactive state of HD microglia compared to controls with cells secreting significantly elevated levels of pro-inflammatory cytokines including TNF α and IL-6 in response to LPS (O'Regan et al., 2021). This conflicting finding may be due to differences in the genetic background of the iPSCs. Despite HD being a monogenic neurodegenerative disorder, it displays variation in the age of onset that cannot simply be attributed to CAG repeat length, with genetic modifiers altering development and progression of the disease (Genetic Modifiers of Huntington's Disease (GeM-HD) Consortium, 2015) and so differences in responses may be attributable to this. In contrast, another research paper suggests a role for TLR4 dysfunction in mast cells expressing mHTT that in turn affects the ability of the cells to produce a normal cytokine

response when stimulated (Pérez-Rodríguez et al., 2020). There is also conflicting evidence surrounding the impact of inflammatory factors in HD mice with the neutralisation of IL-6 improving disease outcomes in one model (Bouchard et al., 2012) and loss of IL-6 exacerbating behavioural phenotypes in another (Wertz et al., 2020).

5.3. Considerations and limitations of this thesis

Despite the well documented phenotypes in iPSC studies using patient cells with larger CAG repeat expansions, larger CAG repeats are typically associated with juvenile onset HD while most cases are adult-onset (Roos, 2010). In this thesis, a patient line with 109 CAG repeats was used with a diagnosis of juvenile onset and so extrapolating findings to adult-onset is difficult. Comparison of only one control with a single patient line also limits the data somewhat. If for example the control line is rather neuroprotective in genotype, the observed significant difference in microglial response may not appear significant when comparisons are made with other controls or an isogenic line. A range of CAG repeat lengths would, therefore, certainly add strength to the study of microglia in the context of HD. Another consideration is the controls upon which comparisons to disease cells are made. Isogenic cell lines whereby mutations are corrected providing a healthy comparison in the same genomic context except a singular causative gene are considered the gold-standard to determine the effects of a particular mutation (Bailus et al., 2017). However, similar to how 'epigenetic memory' of the previous cell state prior to reprogramming is observed with iPSC cultures, much is unknown regarding the correction of expanded CAG repeat sequences in HD iPSC and if pathology is completely reversed and, therefore, corrected and disease cells may not appear to be different under experimental conditions which is further impacted by the wider genetic background of a cell line (Musunuru, 2013; Tousley and Kegel-Gleason, 2016).

The differentiation pathway employed to produce desired cell types via an iPSC-intermediate results in the removal of epigenetic markers during the reprogramming process and thus age-related signatures are lost (Miller et al., 2013) which is an important consideration when modelling diseases in which pathology is so heavily determined by age. As a result of normal aging microglia display a primed phenotype meaning they are prone to an amplified immune response when exposed to a secondary stimulus (Perry and Holmes,

2014). This primed phenotype is observed in neurodegenerative disorders (Cunningham et al., 2005). As such induced models may better model this phenotype in microglia, similar to the expression of HD pathology observed in induced neurons (iN) such as aggregate formation (Victor et al., 2018). Nonetheless, the ability to model diseases at earlier stages of development provides advantages in understanding early dysfunction of which is necessary to prevent or delay the onset of diseases that have such devastating consequences due to profound cell death.

5.4. Future directions

In this thesis, HD microglia were shown to display a lower inflammatory cytokine response compared to controls, with regards to IL-6 and TNF α. Microglia express a wide range of receptors and respond to an array of stimuli (Colonna and Butovsky, 2017). Microglial responses vary across the literature and this may be attributable to species differences and the experimental methods used to activate microglia (Mestas and Hughes, 2004; O'Regan et al., 2021). Responses are also likely to differ dependent on genetic background. More standardised protocols across the literature is, therefore, required to draw conclusions regarding the role of mHTT expressing microglia in HD pathogenesis. As such, testing of various combinations of activating stimuli described in the literature would help determine the extent to which responses differ depending on the activating stimuli rather than a consequence of the presence of mHTT in cells.

mHTT is shown to disrupt many cellular processes ranging from transcription to trafficking of vesicles and receptors (Luthi-Carter et al., 2003; Kang et al., 2019; Perez-Rodriguez et al., 2020). With regards to the findings presented here it would be a logical step to analyse RNA sequencing and proteomic data from controls and patient cells, to determine where potential differences occur that may correlate with these functional observations.

The observations of microglia function in this thesis are predominantly in the context of monocultures. Further development of a model to incorporate microglia along with neurons and astrocytes will better recapitulate microglial function in HD as microglia also respond to mHTT via non-cell autonomous mechanisms (Kraft et al., 2014; Yang et al., 2017). HD astrocyte and neuronal co-cultures exist (Garcia et al., 2019). However, glial interactions in HD are less characterised and determining the role of these interactions

and how they may amplify dysfunction to promote neuronal death will be beneficial. In this thesis, microglia are likely to show differential responses compared to microglia cultured with other CNS cell types, with microglial identity and their subsequent responses shown to be regulated by the synergistic effects of neurons and astrocytes (Baxter et al., 2021). Neurons and astrocytes exert regulatory control over microglial responses to low dose LPS suggesting that monocultures of primary microglia and cell lines are in a primed state, and likely to produce exaggerated responses to inflammatory cues which may not reflect the *in vivo* situation (Baxter et al., 2021). Indeed, determining the regulatory role of neurons and astrocytes in modulating microglial activity is another target for potential intervention of dysregulated microglial responses with reduction in regulatory molecules such as CD200 expressed on neurons and engagement with the microglia expressed CD200R, associated with disease progression in neurodegenerative disorders such as AD (Walker et al., 2009).

5.5. Concluding remarks

Despite being a monogenic neurodegenerative disorder, HD is proving rather complicated to treat which is largely related to the complexity of the protein and its multifunctional and widespread role throughout the body and development (Sadou, 2016). Although a number of animal models exist (Howland et al., 2020; Kaye et al., 2021), the lack of disease-modifying treatments for HD and growing ethical concerns, are raising questions about the translational capacity of animal models in correctly modelling disease phenotypes and predicting clinical outcomes in patients.

A number of compounds and gene targeting technologies that have shown promise in animal models have failed to translate into efficacious outcomes in human clinical trials (Dedeoglu et al., 2003; Ferrante *et al.*, 2000; Ferrante et al., 2002; Hersch et al., 2017; Huntington Study Group, 2001; McGarry *et al.*, 2017). Recent ASO mHTT lowering approaches have failed during clinical trials as the technology has been shown to have no effect or even causes a worsening of clinical outcomes (Rook and Southwell, 2022). As such there is a significant need for the development of alternative models that more accurately recapitulate the biological mechanisms observed in human disease. iPSC technology has provided a platform to model human diseases including HD, with varying

genetic backgrounds and, in different CNS cell types (Garcia et al., 2019; Mathkar et al., 2019; O'Regan et al., 2021; Park et al., 2008).

In this thesis, progress in the development of a xenofree culture system to support microglia, astrocytes and neurons is presented. BrainPhys was selected as a test medium to establish if microglia could be cultured in the same medium that has demonstrated support for the function of neuron and astrocyte co-cultures using a method developed in house. Due to the differences in predictive capabilities of current models of microglia function, more consistent and robust models are required. Obtaining human primary microglia from tissue samples is problematic and are limited in availability. Induced pluripotent stem cell (iPSC) technology offers an opportunity to overcome some of the issues with using primary human tissue and create more relevant human models as animal studies demonstrate clear discrepancies when compared with human microglia. Such a model must encompass a level of complexity that mimics as far as realistically possible the human condition but is also consistently reproducible. To achieve this, cultures involving other CNS cell types alongside microglia are essential.

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7. Appendix

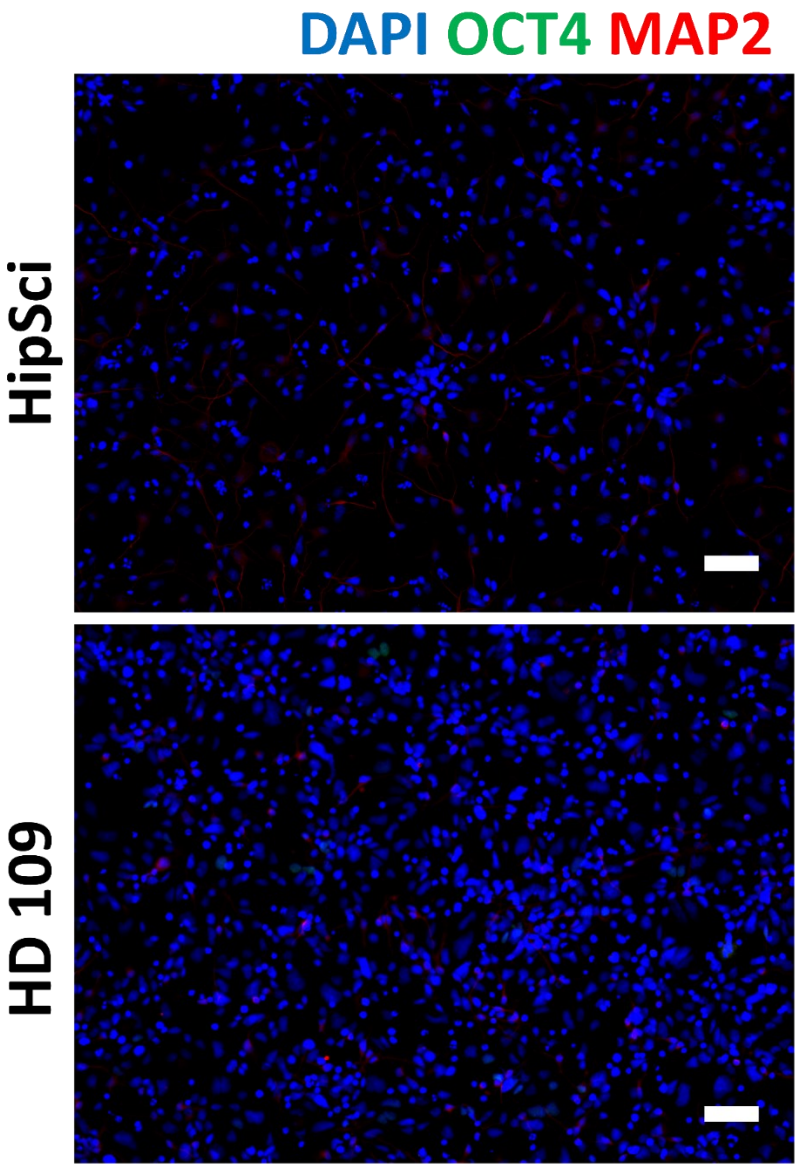

Figure S1. Negative Oct4 and Map2 staining of HipSci and HD 109 neural precursor cells (NPC). *DAPI (blue), Oct4 (green), and Map2 (red).*

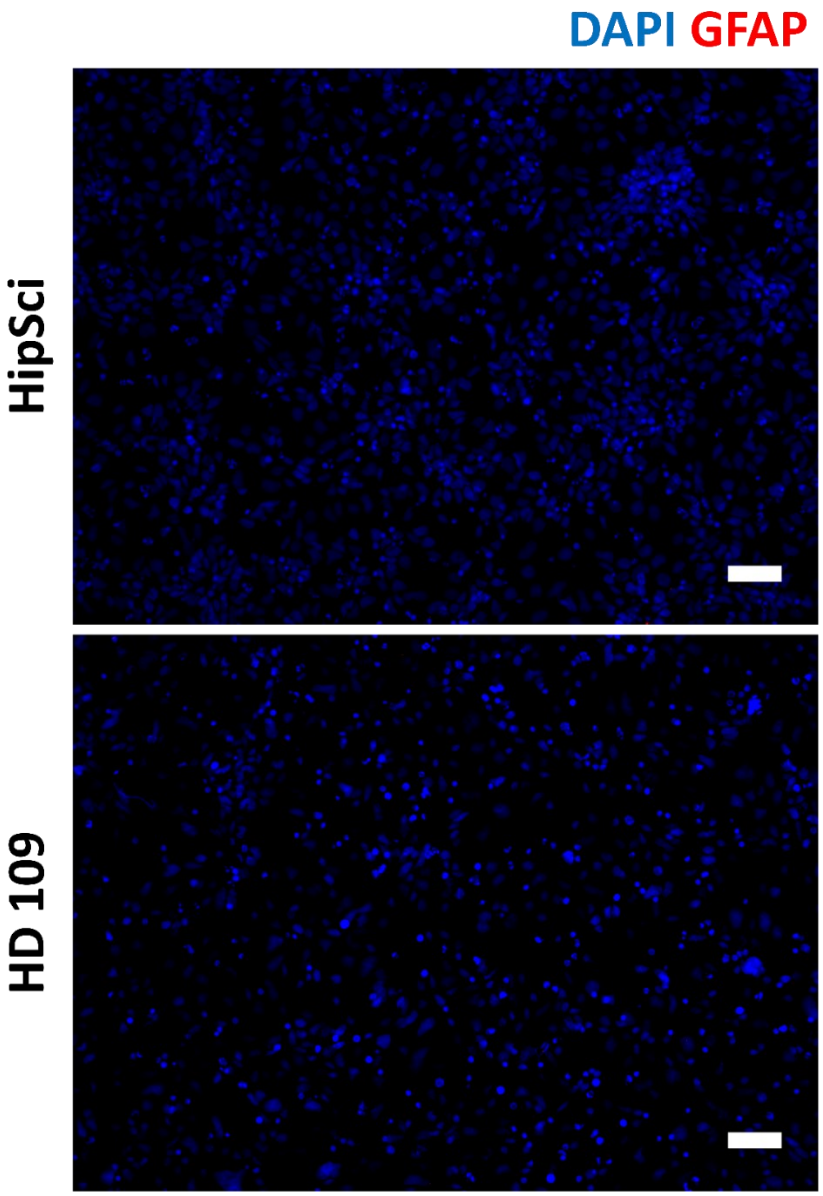

Figure S2. Negative Gfap staining of HipSci and HD 109 neural precursor cells (NPC)*. DAPI (blue), Gfap (red).*

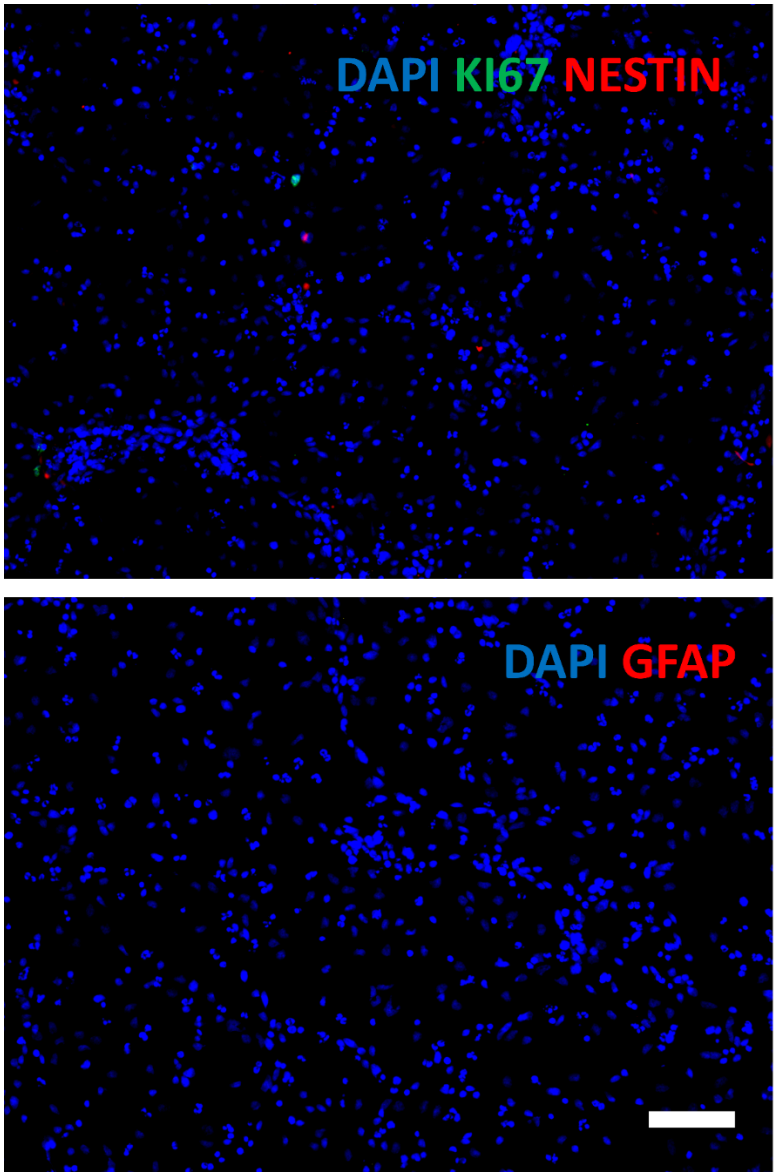

Figure S3. Negative Ki67, Nestin and Gfap staining of HipSci neurons*. (A) DAPI (blue), Ki67 (green), Nestin (red). (B) DAPI (blue), Gfap (red).*

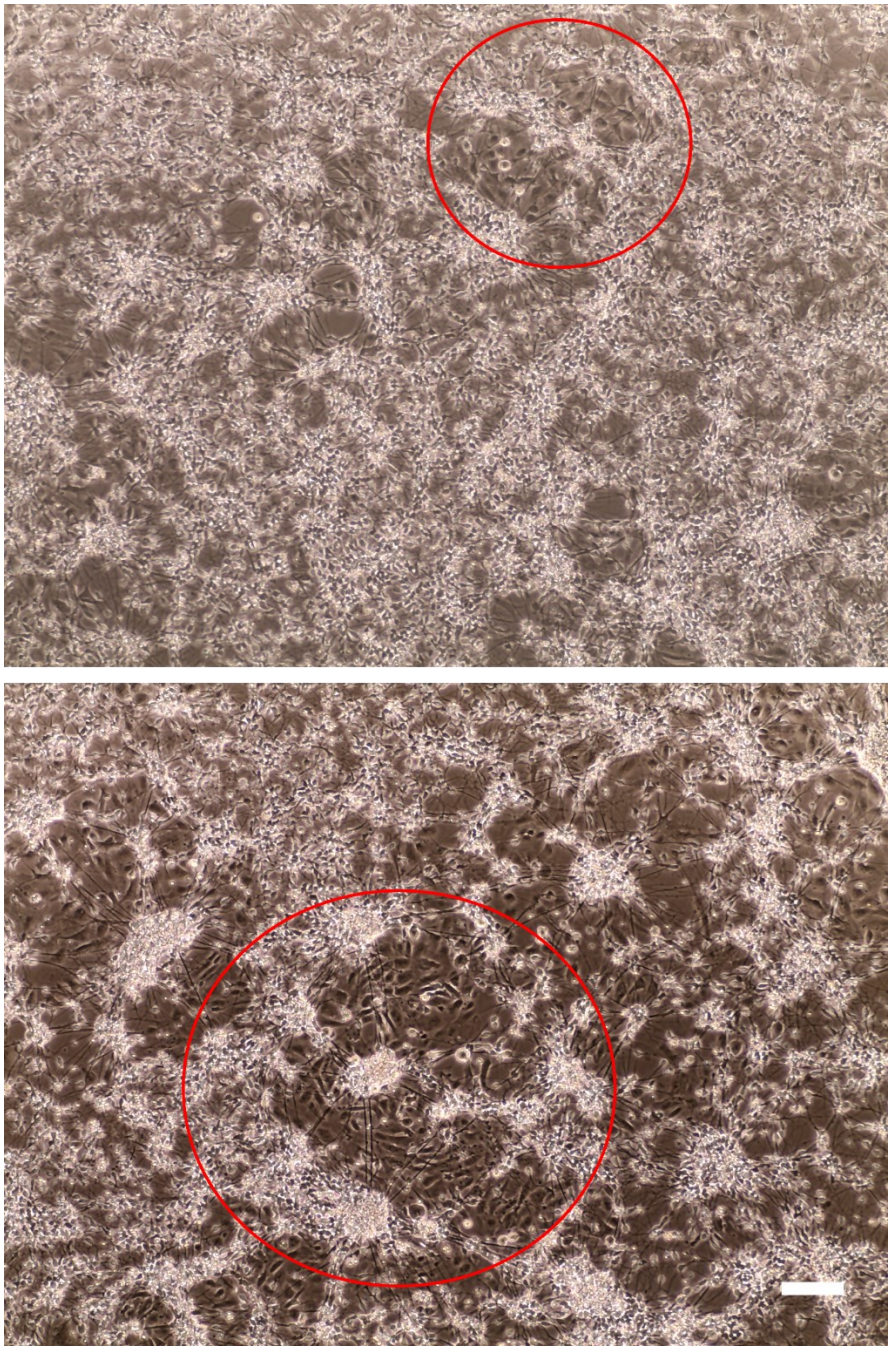

Figure S4. Challenges with off-target differentiation in HipSci neuronal cultures*. Prior to addition of astrocytes and/ or microglia, HipSci neurons displayed off-target differentiation as indicated within the red circle, whereby cells display a 'cobblestone' like morphology.*