Examining inflammation in the CNS: from inflammatory molecules to *in vitro* models

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Abstract

Studies of CNS inflammation typically focus mostly on microglia cells or co-cultures of CNS cells, and not on how each CNS cell type alone can contribute to an inflamed environment. To address this deficiency, the current study investigated the inflammatory potential and *in-vitro* responses of CNS cells following treatment with pro-inflammatory stimuli (lipopolysaccharide (LPS); Interferon-γ (IFN-γ)). To determine cells' potentials for responding to these stimuli, expression of their cognate receptors was determined using qPCR, while inflammatory responses were determined using immunoassays to measure the secretion of inflammatory and regulatory cytokines. For non-microglial CNS cells, despite expression of TLR-4 and IFNγR1 being detectable, limited responsiveness to LPS or IFNγ was observed.

In order to study microglia, microglia(-like) cell models are often used; these are generally produced through complicated methodologies involving multiple steps. The current study aimed to develop a novel (and simpler) procedure for generating an *in-vitro* model for microglia-like cells. Specifically, a one-step process was developed and optimised, by which monocytic THP-1 cells were differentiated into microglia-like 'mgTHP-1' cells. The microglia-like nature of mgTHP-1 cells was confirmed using a panel of microglial markers, while their molecular and functional properties, and aspects of their metabolism and epigenetics, were also investigated. mgTHP-1 cells were shown to be capable of pro-inflammatory M1-like responses, as indicated by significant secretion of pro-inflammatory cytokines when challenged with LPS or IFN-γ. Conversely, pre-treatment with the anti-inflammatory flavonoid Vicenin-2 significantly reduced IFNγ- or LPS-triggered secretion of pro-inflammatory cytokines, and increased secretion of anti-inflammatory cytokines, indicating that mgTHP1 cells can also shift towards an M2-like anti-inflammatory phenotype.

In conclusion, this study reports the establishment of a novel protocol for generating microglia-like (mgTHP-1) cells, and demonstrates that CNS cells have significantly varying inflammatory response profiles *in-vitro*, which may be associated with their physiological roles *in-vivo*.

"The woods are lovely, dark and deep,

But I have promises to keep,

And miles to go before I sleep,

And miles to go before I sleep."

Robert Frost *Stopping by Woods on a Snowy Evening*

This PhD is dedicated to the PhD candidates whose PhD stole their mental health and lives, and never got to write their own dedication page. You were not weak, too sensitive, nor unworthy and I am sorry you were failed.

This is your dedications page.

This PhD is dedicated to my family, my Liz, and my friends, who are the reason I have my own dedications page.

To my dad who went away too soon.

You inspire me every day.

I hope this makes you proud.

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(c)DNA	**(complementary) Deoxyribonucleic acid**
5-mC	**5' methyl-Cytosine**
ACM	**Astrocyte conditioned media**
AD	**Alzheimer's disease**
Aldh1L1	**Aldehyde dehydrogenase 1 member L1**
ALS	**Amyotrophic lateral sclerosis**
ANOVA	**Analysis of variance**
AQP4	**Aquaporin 4**
BBB	**Blood brain barrier**
BCA	**Bicinchoninic acid**
BMP	**Bone morphogenic proteins**
BSA	**Bovine serum albumin**
C1q	**Component subunit 1q**
CDx	**Cluster of differentiation x**
CNS	**Central Nervous System**
COX	**Cyclooxigenase**
CSF	**Colony simulating factors OR cerebrospinal fluid**
Ct	**Cycle threshold**
DMEM	**Dublecco's modified eagle medium**
DMSO	**Dimethyl sulfoxide**
EAE	**Experimental autoimmune encephalomyelitis**
EB	**Embryoid body**

EGF/FGF	Epidermal/Fibroblast growth factor
ELISA	Enzyme-linked immunosorbent assay
ESC	Embryonic stem cell
EVs	Extracellular vesicles
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate transporter 1
HD	Huntington's disease
HRP	Horseradish peroxidase
HSC(s)	Hematopoietic stem cells
Iba1	Ionized calcium binding adaptor molecule 1
ICC	Immunocytochemistry
IFNγ	Interferon gamma
IL-x	Interleukin x (where x=1, 6, 10 etc)
iPSC	Induced pluripotent stem cells
IκB	Inhibitor of (NF) kappa beta
LDS	Lithium dodecyl sulfate
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharides
MCP-1	Monocyte chemotactic protein 1
MDM	Monocyte derived macrophages
mg-	Microglia-
miRNA	Micro Ribonucleic acid
MMPs	Metalloproteinases

MP	Mononuclear phagocytes
mRNA	Messenger Ribonucleic acid
MS	Multiple Sclerosis
NEAA	Non-essential amino acids
NF-κB	Nuclear Factor kappa beta
NGF	Nerve growing factor
NIS	Neural induction supplement
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NPC	Neuronal precursor cells
NSC(s)	Neural stem cells
O(2)AP	Oligodendrocytes/type 2 astrocyte progenitor cell
OD	Optical density
OPC	Oligodendrocyte precursor cells
PAMPs/DAMPs	Pathogen/Damage associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PMA	Phorbol myristate acetate
PPARα	peroxisome proliferator-activated receptor α
qPCR	Quantitative Polymerase chain reaction
RAGE (s)	Receptor for advanced glycation end products (soluble)
RCF	Relative Centrifugal Force
RGP	Radial glia progenitor cells
RPMI	Rosewell park memorial institute

RT	**Room temperature**
SD	**Standard deviation**
SDS	**Sodium dodecyl sulfate**
Shh	**Sonic Hedgehog (signalling pathway)**
SVZ	**Subventricular zone**
TBP	**Tata(-box) binding protein**
TBST	**Tris-buffered saline-Tween**
TGFβ	**Transforming growth factor beta**
TLR (2, 3, or 4)	**Toll-like receptor (2, 3, or 4)**
TMEM119	**Transmembrane protein 119**
TNFα	**Tumour necrosis factor alpha**
TREM2	**Triggering receptor expressed on myeloid cells 2**
v/v	**Volume/volume**
V-2	**Vicenin-2**
WB	**Western Blot**
XR	**Receptor for X (e.g. IL-6R)**
Δ	**Delta (change/difference)**

Chapter 1

General introduction
Inflammation and the CNS: from molecules to cells, and from
development to pathology

1. General Introduction

1.1 Neural immune system: Origins, development, and neuroinflammation

Neuroinflammation is a term used to describe the inflammatory responses of the central nervous system (CNS) (O'Callaghan et al., 2008). Other researchers, however, depending on the angle they look at it from, define neuroinflammation as *"CNS-specific, inflammation-like glial responses that do not reproduce the classic characteristics of inflammation in the periphery but may engender neurodegenerative events"* (Streit et al., (2004)), thus including a cellular element (glial) and a resulting element (neurodegenerative events). All studies, however, agree on the fact that neuroinflammation is initiated and mediated by the presence of a variety of stimuli, including pathogens, tissue-damage associated mediators, as well as cytokines, and chemokines. Neuroinflammation has positive and negative aspects, depending on its duration (transient vs chronic), and intensity (high vs low), as well as the developmental stage in which it presents, all of which are reviewed by DiSabato et al., (2016). At any point, during the development of the brain, as well as in the fully developed, mature brain, a not well understood (especially in humans) highly organized choreography takes place between astrocytes, microglia, oligodendrocytes, and neurons, as well as other cells (such as infiltrating peripheral cells, and neural stem cells), whose aim is to keep the CNS environment stable (Marc, 2013). This stability is especially crucial during inflammatory imbalances, either internal (e.g. resulting from injury), or external (caused by infection of the brain tissue from pathogens). The main mediators of the immune response are the microglial cells, with the astrocytes having a more regulatory role, and the neurons and oligodendrocytes being the cells most affected by the response.

Epigrammatically, and as shown in figure 1.1, embryonic stem cells follow either the ectodermal or the mesodermal/myeloid lineage to give rise to the cells of the CNS. In vertebrates, the neurons and macroglial cells (i.e. astrocytes and oligodendrocytes) of the

central nervous system (including the retina of the eye) derive from the part of the ectoderm which forms the neural tube (Greene &Copp, 2009), while those of the peripheral nervous system derive mainly from the neural crest which is part of the ectoderm as well (Prendergast & Raible, 2014).
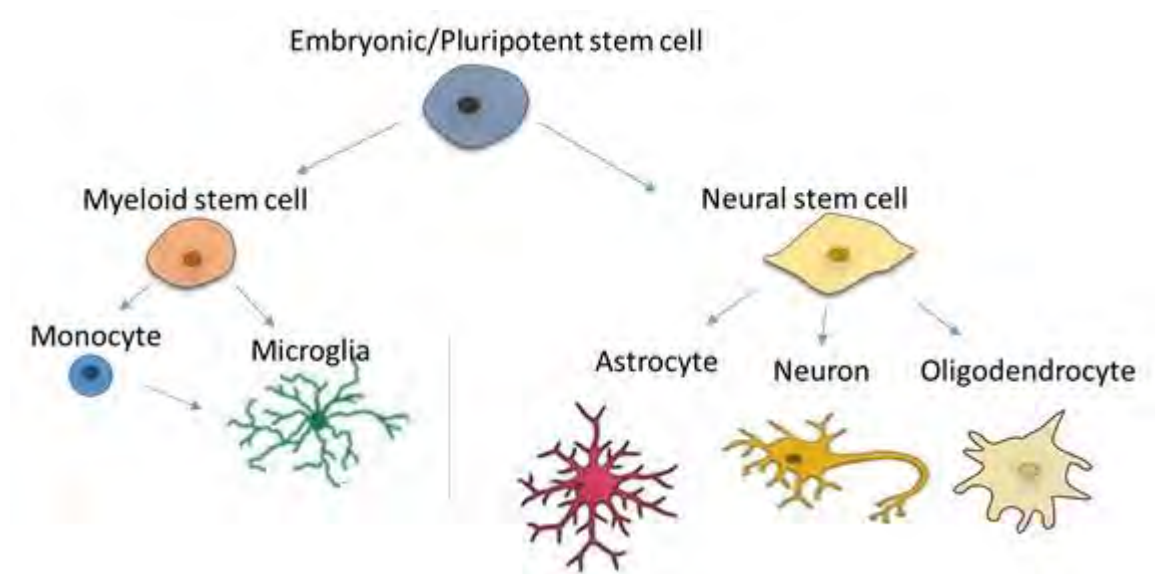
Figure 1.1 ***A simplified outline of the development of the cells of the central nervous system***. Embryonic (or pluripotent) stem cells differentiate into myeloid or neural stem cells. Two lineages are thus created: the ectodermal/neural, which includes cells derived from the neural stem cells, and the myeloid, which includes cells derived from the myeloid stem cells. CNS cells included in the neural lineage include neurons, astrocytes, and oligodendrocytes, while CNS cells included in the myeloid lineage include microlgial cells, as well as infiltrating monocytes that have been shown to differentiate into microglia-like cells.

With the focus on the origins and development of the CNS (as this thesis does not focus on the peripheral nervous system) most of the brain's resident cells develop from the ectoderm, including astrocytes, neurons, and oligodendrocytes. On the other hand the microglial cells are formed from the yolk sac, at almost the same time as the hematopoietic stem cells (HSC- which originate from the mesoderm) and then settle in the CNS; infiltrating monocytes and macrophages (which can subsequently differentiate into microglia-like cells) arise from the mesodermal/myeloid lineage as well (Weiskopf et al., 2016). Such cells of the myeloid lineage are the primary cells that form the brain's immune system, however the cells of the ectodermal lineage are also known to play an important role, not only in defending the CNS from pathogenic invasion and tissue damage, but also orchestrating this defense and the subsequent healing phases, as described in later sections of this chapter.

Neurons are in continuous communication with macroglial cells (astrocytes and oligodendrocytes), which provide a supporting framework and create an enclosed, protected environment in which the neurons can perform their functions. Therefore, it is no surprise that during their development, maturation, and even after these events, these different types of cells always communicate, either via cytoplasmic "bridges" between membranes, or through soluble factors secreted and received through appropriate receptors. As an example of a cytoplasmic bridge, are the gap junctions. These formations consist of proteins named connexins, which form a pore between the membrane of two cells (a semi-pore in each cell, which then connect). In the brain, such connections have been found between the same or different cell types (e.g. oligodendrocytes and either oligodendrocytes (o-o) or astrocytes(o-a) or neurons (o-n), or astrocytes and astrocytes(a-a)) which can be either beneficial or detrimental depending on the health and function of the cells connected (Vejar et al., 2019; Papaneophytou et al., 2019).

The role of DNA methylation and epigenetic changes in cell differentiation

One of the ways in which cells have the necessary plasticity which allows them to differentiate into other cell types, without changes in their DNA sequence, is via epigenetic changes. As such, in response to differentiation signals, chromatin (essentially DNA-protein complexes formed in the nucleus) changes. Epigenetic changes include but are not limited to structural/chemical modifications of chromatin, which in turn allows or prevents transcription factors from accessing specific areas. Chemical modifications include methylation of the DNA, as well as histone modifications. In general, DNA hypomethylation of a region is correlated to euchromatin (active genes), whereas heterochromatin regions are methylated; nevertheless, in some cases global DNA hypomethylation is correlated with gene silencing (Hon et al., 2012). This could be due to the different roles of the DNA sequences that are methylated and the

regulatory elements that exist in those regions. Another reason could be issues in methylation related genes such as TET-2 (López-Moyado et al., 2019), the protein of which is involved in demethylation.

Epigenetic changes can be observed not only in the differentiation of, for example stem cells when they commit to a specific fate (e.g. neural line, myeloid line etc) (Falrik et al., 2016; Sawai & Dasen, 2018; Ziller et al., 2018), but even within those lines (e.g. when monocytes differentiate into macrophages) (Dekkers et al., 2019) in addition to the fact that epigenetics control the plasticity observed in some cell types (e.g. microglia (Cheray & Joseph, 2018)). Arney & Fisher (2004) suggest two models in which these changes could affect the differentiation of cells, focusing on where usually DNA is methylated. Jackson et al., (2004) found that lack of the ability to induce DNA methylation (via lack of methyltransferases) leading to an expected global hypomethylation, also blocks the ability of stem cells to differentiate. Overall, as discussed by Suelves et al., (2016), an increase of global DNA methylation is found and expected in differentiating cells which is reduced terminally differentiated cells. This could be explained by reduction in the expression of the "old" lineage specific cell type-specific markers via hypermethylation to avoid the expression of non-appropriate lineages, and subsequent loss of methylation occurs the "new" lineage-specific genes to define cellular identity. Nevertheless, epigenetics are still not fully studied when it comes to the differentiation of all cell types, and their maintenance, but from the above it is to be expected that DNA methylation, as all epigenetic processes are dynamic, rather than static.

1.1.1 Neural and myeloid stem cells: Origins and inflammatory responses

An organism starts as a totipotent "stem" cell, that divides, and during development and organogenesis gives raise to different groups of multipotent stem cells, which are lineage committed (Mikkelsen et al., 2007). Of the cells found in the adult CNS, neural stem cells

(NSCs) are a progenitor cell type that can self-renew, and differentiate into both neurons and glia (astrocytes and oligodendrocytes). In the periphery, myeloid hematopoietic stem cells (HSCs) produce macrophages and monocytes, which can potentially infiltrate the CNS. As for microglial cells, although they cannot be derived from either adult hematopoietic or neural stem cells, other cells have been discovered in the CNS that have been dubbed as microglia progenitor cells; these are activated usually in cases such as microglia depletion, and do not usually contribute in the renewal of resident microglia (Elmore et al., 2014). A more recent study by Bruttger et al., (2015) showed that genetic ablation of microglia revealed clusters of these progenitor cells. As it will be discussed in 1.1.5, and as also recently described by Masuda et al., (2020), and Sankowski et al., (2019), and as discussed by Kierdoff & Prinz (2019), microglia has not only distinct temporal and spatial subtypes and states, but also disease specific subtypes in the CNS; these subtypes proliferation is favoured under certain conditions (e.g. demyelination/neurodegeneration). However whether different progenitors give rise to different subpopulations, or the environment subtype is yet to be determined. These findings show that microglia is a largely heterogenous group of cells, and that focusing on the heterogeneity could help us understand better not only how these cells develop, but also how they change in health and disease.

Macrophages were thought to originate from myeloid progenitors (HSCs) through the intermediate of monocytes. These progenitors could be found in the bone marrow, develop transiently into circulating monocytes, and then after colonizing different tissues, they would develop into macrophages. However, this view on macrophage development, has been recently challenged with findings that show that resident brain macrophages (i.e. microglia) (Ginhoux et al., 2010), as well as macrophages found in other tissues, such as the liver, epidermis and lung in mice (Perdiguero et al., 2015) were found to originate directly from the yolk sac during embryonic development, and thus have a different origin from macrophages found in other

tissues. These findings could be explained by the fact that some tissue macrophages can renew independently of HSC depending on myeloid progenitors that originate from the yolk sac, and therefore have the same origin as the tissue macrophages they produce; i.e. they are tissue specific. Moreover, a relative order of development of tissue macrophages/progenitors/HSCs has been suggested, with differentiated macrophages (i.e. microglia) originating earlier in development than HSC, or HSC derived cells (Elmore et al., 2014; Perdiguero et al., 2015).
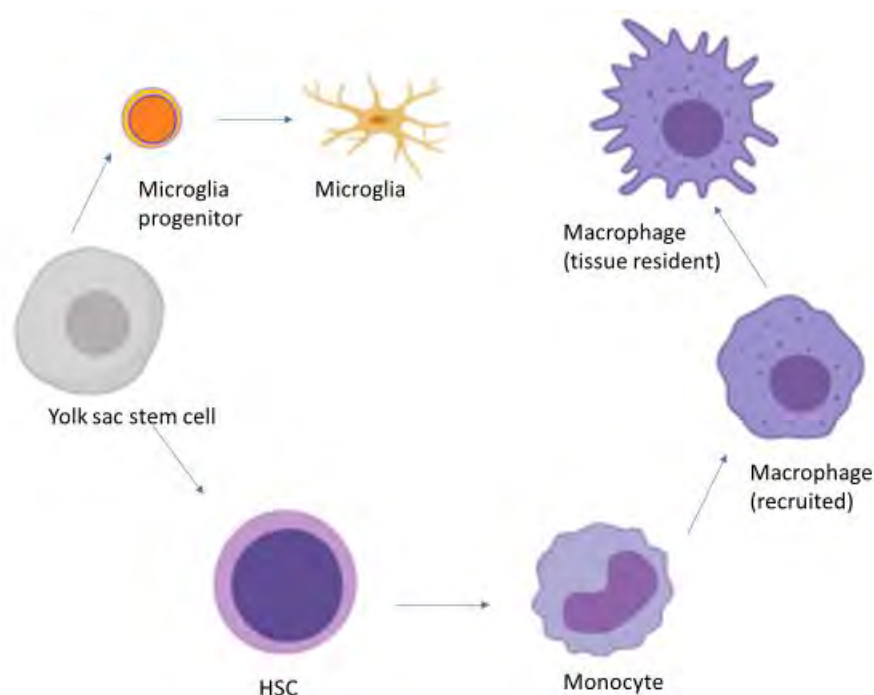
Figure 1.2*: **A synopsis of the development of the myeloid lineages that can be found in the brain***. Yolk sac stem cells give rise to microglia progenitors, and microglia cells directly, as well as other tissue macrophages. They also give rise to hematopoietic stem cells, which in turn can differentiate into circulating monocytes, which are recruited in different tissues where they can in theory act as tissue resident macrophages, or help the resident macrophages (more in the microglia and infiltrating monocytes sections below).

Nevertheless, most cell origin studies have been undertaken in mice, and there have been differences in results. These differences include those reported by Ginhoux et al., (2010) who found yolk sac microglia progenitors at embryonic day 7 in mice versus Kierdorf et al., (2013) and Perdiguero et al., (2015) who observed microglia at day 8-8.5 as erythromyeloid

progenitors that invade the neural folds that are essentially the developing brain. These results, should however, be interpreted cautiously, particularly with regards to their relevance to human biology, as the majority of the studies investigating microglia development *in vivo*, are animal studies. The development of microglia, and myeloid stem cells are described in more detail in figure 1.2.

NSCs have long been identified both in the adult and developing brain: at least two groups of pluripotent neural stem cells have been identified, namely neuroepithelial, and neurosphere stem cells, that give rise to at least five types of restricted precursors of the neural system (Rao, 1999). Although typical and atypical NSC niches have been found throughout the adult brain (Bernstock et al., 2014), neurogenesis, and thus neurogenic neural stem cells persist only in certain areas of the brain, such as the dentate gyrus. There, NSCs proliferate, and give rise to young neurons that migrate short distances and differentiate into hippocampal neurons (Gage et al., 1998). In the other NSC area of the brain, the subventricular zone (SVZ), neural stem cells have been shown to migrate, and shift between progenitor forms, ending up to finally differentiate into neurons (Lois & Alvarez-Buylla,1994; Doetsch et al., 1997). It is of interest, that one of the progenitor forms observed in the SVZ is essentially identical to mature astrocytes, which under certain conditions can act as NSCs, express nestin (usually found in neuroepithelial stem cells, but not in mature astrocytes in other brain regions), and differentiate into neurons and glia, as has been shown in vitro and in vivo (Doetsch et al., 1999). As will be discussed below, astrocytes do not have a developmental end point per se, however the fact that they possess neural stem cell properties is still surprising. Nevertheless, it is to be noted that radial glia, one of the primary neural stem cell types in the developing brain differentiate into astrocytes after neurogenesis ceases, and exhibit glial characteristics; transition between the two types has also been shown (Pixley & de Vellis, 1984). Radial glia are either generated by neuroepithelial cells (so the two cell types coexist), or neuroepithelial cells can completely

transform into radial glia in the beginning of neurogenesis (where upon radial glia is essentially acting as the only neural stem cell type). Thus, radial glia have been shown to be a rather heterogenous population, containing pluripotent and lineage-restricted progenitors that all express, however, astroglial markers (Malatesta et al., 2008). Therefore a more complicated (although by no means complete) diagram of the neural stem cell development, and origins of neurons, and macroglia is presented in the figure 1.3. Although the full details of it are not included in figure 1.3, it is worth to mention a recent review by Obernier & Alvarez-Buylla (2019) which focuses on the development and persistence of NSC and subsequent adult neurogenesis in the mouse SVZ. In this review it is discussed how the NSCs of the adult mouse brain do indeed constitute a heterogenous population, which contains type B1 and B2 astrocytes, radial glia, and NSC with astroglial properties. It is further discussed how these cells can be divided in additional subgroups, some of which are only transient (cells type C) which then differentiate into cells type A, which then migrate and end up differentiating into neurons. In summary, the regulation of the function of the SVZ NSCs is done by both neighbouring cells, as well as by cells found in more distant areas via neurotransmitters. This becomes more obvious when discussing the development of the CNS cells from NSC, which will be discussed in the following sections of this chapter.

Last, while it has been shown that Hematopoietic Stem Cells can differentiate into microglia and astrocytes *in vivo* (Eglitis & Mezey, 1997) it is also the case that microglia share many of the same markers with macrophages (which have been shown to differentiate into microglia-like cells in the brain), and peripheral hematopoietic progenitors have also been shown to differentiate into NSCs (Wang et al., 2015), which can further differentiate into astrocytes under microenvironmental cues. Alternatively, as discussed above, and shown by Ahmed et al., (2012), NSCs can express GFAP (Glial fibrillary acidic protein) and resemble glial cells. From the above it can be concluded that the nervous system and its development is complex;

also it should be reinstated that most studies are done in mice, and that therefore, the human nervous system remains largely unexplored (Zhao, & Bhattacharyya, 2018). Notably, some progress is being made in translating animal studies findings to human-related results, via bioinformatics (Clancy et al., 2008), but again, *in silico* analyses (or even *in vitro)*, do not always translate to *in vivo* as expected.
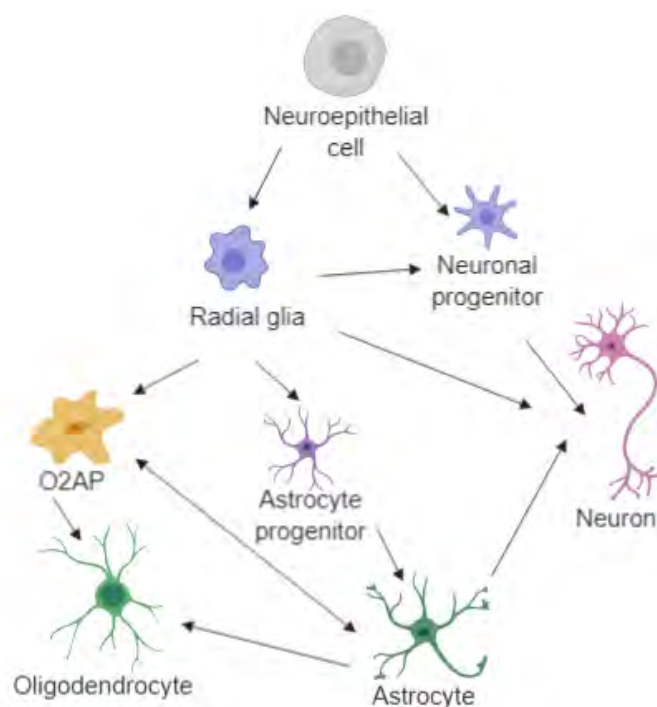
Figure 1.3*. **Diagrammatic representation of the development of neurons, and macroglia (astrocytes, oligodendrocytes) from neural stem cells (Neuroepithelial cells, Radial glia).*** A summary of the text found on this chapter which focuses on the development of the CNS cells. O2AP: oligodendrocyte/type 2 astrocyte progenitor cell.

Inflammation and stem cells

It has been shown that inflammatory events *in utero*, and in early human (as well as rodent) foetal development, can lead to CNS disorders, such as schizophrenia (Allswede & Cannon, 2018), hippocampal dysfunction (Giovanoli et al., 2015), autism (Madore et al., 2016), and has even been correlated with Alzheimer's disease (AD)-like neuropathology (Krstic et al., 2012)

and brain lesions (Yoon et al., 2000) *in vivo*. These events can be correlated with the dysfunctional reactions to inflammatory mediators of developed cells (as will be discussed in the following chapters), as well as the response of neural and myeloid stem cells to inflammatory cues, via expression of CNS-specific receptors on their cell surfaces.

NSCs have been shown to express immune system related receptors, such as Toll-like receptor 4 (TLR4 a member of the class of proteins that play a key role in the innate immune system (Takeda & Akira, 2005)), and depending on the brain area TLR2 at varying levels with TLR4 being the dominant receptor affecting NSC proliferation and differentiation (Grasselli et al., 2018). TLR4, as will be discussed below, is a pattern recognition receptor, that when activated usually leads to inflammation-related responses. Interestingly these receptors are also involved in neurogenesis. For example, Rolls et al., 2007 showed that TLR4 activation with lipopolysaccharides (LPS which is one of the main ligands of TLR4, and is used widely in inflammation related studies (Lu et al., 2008)), inhibits the differentiation of NSCs into neurons in mice. Another study found that NSCs found in neurospheres (a NSC culture system) express the TLR3 receptor, and that its activation via viral insult negatively impacts the proliferation of these cells by inhibition of the Sonic Hedgehog signalling pathway (Shh) (Yaddanapudi et al., 2011) an important signalling cascade involved in a plethora of developmental processes, as well as regeneration, repair, neuroplasticity and neurogenesis.

Moreover, it has been shown that TLR3 is involved with the numbers of neurospheres produced, and thus with the proliferation of NSCs, supporting these findings (Lathia et al., 2008). Therefore, as TLRs are present in the brain in early developmental stages, where the NSCs need to proliferate, migrate, and differentiate in different neural populations, disturbance of the TLR signalling could have negative effects on the development of the CNS.

Interferon gamma (IFNγ), a cytokine secreted predominately after viral insults, but also in other inflammatory conditions, acts primarily via the JAK/STAT pathway (Gil et al., 2001) and the

IFNγ receptor heterotetramer (2xIFNγR1 and 2xIFNγR2) (Ramana et al., 2002) (although other pathways have been recognized). IFNγ has inflammation independent roles in the developing brain; for example, in a study focusing on non-inflamed animal models ,it was demonstrated that IFNγ regulates neurogenesis and proliferation in a negative way via the Shh pathway (Li et al., 2010). However, Sun et al., (2010) showed that IFNγ/Shh activation leads to proliferation of neuronal precursor cells, while Walter et al., (2012) showed disturbance in cell fate regulation via the same pathway in mice. IFNγ has also been shown to induce apoptosis *in vitro* (Ben-Hur et al., 2003), however findings by Wong et al., (2004) disagree with that effect, and find IFNγ non-toxic under proliferative conditions, and they also found that it induced neurogenesis, a finding supported by Song et al., (2005). The reasons for the differences could be the disease models used, with the former study focusing on an inflammatory disorder that mimics human Multiple Sclerosis (MS), while the latter did not. While studies in adult NSCs have shown mixed results in response to IFNγ, most agree on the fact that it promotes neuronal differentiation/neurogenesis. In contrast, in embryonic NSCs it has been found that exposure to IFNγ leads to suppression of neuronal differentiation (Ahn et al., 2015). As such, IFNγ can be considered as a pleiotropic cytokine, where its effects depend on a variety of factors, such as NSC age, location, and whether inflammatory conditions are present or not.

Age-dependent differences in impact on NSCs have also been found for other cytokines, such as IL-6 (Interleukin 6), but for cytokines belonging to the IL-1 family, receptors are found in all NSCs, independent of the age of the organism they were derived from. For example, it has been shown that IL-6R is more expressed in adult derived cells, thus making them more responsive to that cytokine, while IL-1R was present independent of age (McPherson et al., 2011). Moreover, the effect of the cytokines showed differences in function depending on age: IL-1 stimulated the proliferation of young NSCs, while IL-6 inhibited the proliferation independent of age. IL-6 has been shown to be produced from both microglia/macrophages, as

well as astrocytes, and activation of these cells with LPS (which leads to release of IL-6 amongst other cytokines) has also been shown to inhibit the proliferation of NSCs (Monje et al., 2003). Interestingly, Leukaemia inhibitory factor (LIF), a cytokine known for its ability to preserve a stem cell like phenotype, and for its ability to promote regeneration via promoting the proliferation of NSCs, belongs in the IL-6 family of cytokines (Bauer et al., 2003). However, administering IL-6 *in utero*, resulted in an increased NSC pool, but also disturbed neurogenesis *in vivo* (Gallagher et al., 2013) possibly via an autocrine/self-renewal pathway. Confounding findings regarding the response of NSCs to different cytokines have been reported for other cytokines as well. For example, Tumour Necrosis Factor alpha (TNFα) has been shown to induce apoptosis (Chen et al., 2016) as well as proliferation (Widera et al., 2006) in NSCs. These effects could be attributed to the differential expression of TNFα's receptors, with TNFR1 predominant expression leading to suppression of neurogenesis via suppression of NSC proliferation, while TNFR2 expression and signalling having the opposite effect (Chen & Palmer, 2013).

Thus, human NSCs have been found to release multiple cytokines under normal conditions, belonging in both pro and anti-inflammatory categories, such as IL-6, IL-1, TGFβ, TNFα, and IL10 with some differences in mice, where most *in vivo* studies focus on (Klassen et al., 2003; Yang et al., 2009). How these change under inflammatory conditions, and the meaning of the changes is still unclear.

Another inflammation-related receptor which will be discussed further in the following sections, namely the receptor for advanced glycation end products (RAGE), has been shown to be involved in the migration of NSCs (Xue et al., 2018) as well as proliferation and differentiation into neurons (Meneghini et al., 2010; Zhang et al., 2017). While a soluble form of that receptor has been shown to be secreted by mesenchymal stem cells, and lead to protection of neural cells against RAGE-mediated cell death (Son et al., 2017), NSCs have not

been investigated for a similar function.

It may be concluded that, overall, NSCs have a neuroprotective role (Rafuse et al., 2005; Carletti et al., 2011), and therefore their secretions and thus ways of interactions with other cells are worthy of notice and investigation. This was recently demonstrated by Peruzzotti-Jametti et al., (2018), where they found that the effects of NSCs directly on macrophages is not only anti-inflammatory, but also that a specific metabolite secreted by pro-inflammatory macrophages, namely succinate, resulted in change of gene expression in NSCs in order to promote an anti-inflammatory effect and cessation of secretion of that metabolite from the macrophages.

Finally, in summary, HSCs have been found to express various immune system related receptors (such as TLRs), and to respond differentially to cytokines, by either inhibition of proliferation, and the responses are often age-related (Pietras, 2017; Mann et al., 2018). As for microglia, and microglia progenitor cells, it has been shown that early developmental inflammatory events as well as aging and neurodegeneration can potentially "prime" cells, and make them more reactive to future insults (O'Loughlin et al., 2017), and that cytokines such as IL-1β, might be partly responsible for that effect (Bittle & Stevens, 2018). Priming has also been observed in monocytes (Kim et al., 2011) which could potentially emphasize the interrelated roles of peripheral inflammation and neuroinflammation, via the infiltration of monocytes in the CNS. Primed microglia is often described as dystrophic (which includes changes in morphology such as in general shorter processes), and pro-inflammatory (Niraula et al., 2017). In addition, a study focused on the molecular signature of priming in microglia cells (as that was induced by either aging or neurodegenerative disorders), and although similar features were found between the different conditions (e.g. changes in the phagosomal, lysosomal, and antigen presentation aspects of these cells), each condition had its own molecular features (Holtman et al., 2015). In the case of in utero microglia priming, molecules

such as TLR4, and CD36 have been found to be altered, along with transient changes in anti-inflammatory molecules, such as IL-10 (O'Loughlin et al., 2017). Finally, Norden et al., (2014) find that overall primed microglia has three elements that separate it from the non-primed ones: 1) a higher baseline expression of inflammation-related genes and mediators, 2)a lower threshold to which a switch to a pro-inflammatory phenotype is activated, and 3)an exaggerated and unregulated response following activation.

1.1.2 Neurons: the influence of inflammation on their development and functions

Neurons are one of the most specialised cell types found in the CNS. Neuron specialization comes from their site of development and birth, rather than their origin (i.e. the location/source of the NSC they come from). Neurons invariably develop to fully functioning neurons and neuronal systems due to their interactions with glial cells (e.g. oligodendrocytes for myelination, microglial cells for pruning etc.), although in general neurogenesis precedes gliogenesis in the developing brain (Qian et al., 2000). As discussed above, neurons originate from NSCs, the same cells from which most glial cells (with the exception of microglia) originate. In the human cortex, the "cognitive" part of the human brain, the progenitor cells are called radial glia progenitor cells (RGPs), which originate from neuroepithelial cells (which are the primary NSCs in the CNS and are the major progenitor cells for neurons and most glial cells (Gotz & Huttner, 2005)). Cortical neuron development relies on signals that include physical (e.g. mechanical stress) as well as chemical (molecules secreted from cells, but also communicated between cells via gap junctions) (Elias & Kriegstein, 2008).

Studies investigating these signals often focus on specific gene switches, and what signals can lead to these changes. For example, changes in genes such as Wnt (Aubert et al., 2002), Pax6 (Schuurmans & Guillemot, 2002), as well as BMPs (Bone morphogenetic proteins) (Kasai et al., 2005) have been shown to promote a neuronal path of differentiation from NSCs, rather

than an astrocytic one. Mimicking these signals *in vitro* has allowed the study of neurons, particularly the aspects of neurogenesis and neurodegeneration/neuroregeneration, outside the brain, and outside the context of their interactions with glia. Indeed different types of neurons have been generated from stem cells, and studied for their properties. These include serotonergic (Alenina et al., 2006), and dopaminergic (Woodard et al., 2014) neurons depending on the neurotransmitter they secrete, hippocampal (Yu et al., 2014), hypothalamic (Yao et al., 2017), striatal GABAergic (Lin et al., 2015), and striatal dopaminergic (Peng et al., 2016) neurons depending on their location as well as their function. As these neurons develop in different stages during the development of the CNS, (with some being correlated to specific disorders), studying how these cells respond to inflammation during development, and whether this leads to dysfunction is a research area of great significance. Neurons can also be studied as non-specialised types, which can give greater insights regarding their responses, as the findings can apply to multiple subtypes; conversely findings on certain subtypes often don't apply to others.

Several conditions that are accompanied by inflammation, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), as well as traumatic brain injury, stroke, as well as physiological conditions including aging have been shown to affect neurogenesis and induce neuronal cell death both during development, and in adult brains (Fan &Pang, 2017). While a great amount of these actions are due to glial cells, which will be discussed later on in this chapter, there are a few actions that are neuron-specific, and can be attributed to the reaction of the neurons to a pro-inflammatory environment. Such actions can often be attributed to receptors that neurons express, and their upregulation or downregulation during inflammatory events.

RAGE isoforms and their functions in the CNS

One such receptor is RAGE which can be expressed in neural cells under different conditions, with its expression increasing during neuroinflammation, and being relatively low in physiological conditions (Brett et al., 1993). RAGE consists of an extracellular domain (V-type followed by two C-type regions), a single transmembrane domain and a short cytosolic tail, which is responsible for the signal transduction. RAGE was first discovered as a membrane bound protein, which was eventually identified to have 19 isoforms, including soluble isoforms (sRAGE1, 2, and 3) (Sterenczak et al., 2013; Schlueter et al., 2003), membrane-bound full length isoforms, as well as the dominant negative isoforms, which are membrane-bound, but lack the intracellular part of the receptor, and with a variety of splice variants that are tissue and/or disease specific. These sRAGE variants have been shown to act as anti-inflammatory mediators, as they bind the RAGE ligands in plasma or tissues that would otherwise activate the RAGE inflammatory pathway resulting in most cases in a pro-inflammatory cascade (Hudson et al., 2003). Ligands of interest for neuroinflammatory conditions include the Aβ peptide, advanced glycation end-products (AGEs), as well as the S100 proteins, and HMGB1. Depending on the ligand and the condition, cell type or location, RAGE can be either beneficial or detrimental for neurons, as discussed by Sorci et al., (2013): RAGE activation can be involved both in neurodegeneration, and also in repair of neural tissue, and could also prove to be protective in some aspects. For example, RAGE has been shown to be upregulated in neuronal cells in later stages of AD, and while the exact role of RAGE in the pathogenesis of AD is complicated, the receptor has been linked to induction of neuronal loss; several of the findings linking AD and RAGE and AGEs are discussed by Srikanth et al (2011). In general, activation of RAGE in cells of the immune system, leads to downstream activation of the Nuclear Factor κB (NF-κB) signalling pathway leading to the expression and release of a range of pro-inflammatory and immunoregulatory mediators including cytokines IL-6, TNFα, as well

as cytokines of the IL-1 family. However, depending on the isoform of the RAGE that is predominately expressed, its harmful effects can be attenuated. Arancio et al., (2004) showed that overexpression of both RAGE and APP (the protein which after cleaving produces Aβ) in mouse neurons produced cognitive issues and induced neuropathologic changes in mice; both effects were attenuated if the overexpression was that of the dnRAGE isoform (which lacks the cytosolic tail). Similarly, Fang et al., (2010) showed that overexpression of the full form of the membrane-bound RAGE in microglia with additional overexpression of the mutant pre-Aβ protein in neurons in mice lead to harmful effects in the animals, including accumulation of Aβ, and neuroinflammation; if the overexpression was that of dnRAGE, the effects of Aβ accumulation were not as harmful. The roles of RAGE and its isoforms in the brain is discussed fully in a review by Ding & Keller (2005), where among others different splice forms are discussed per CNS cell type (with a mention in alternative RAGE splicing in astrocytes and neural cells), and mentions how not only different splice forms, but also their polymorphisms may play a role in CNS pathologies. The same authors in another paper (Ding & Keller, 2004) focus on the splice variants of the 6 more prominent RAGE isoforms found in the human brain. The 6 isoforms they focus on are the membrane bound RAGE, the membrane bound RAGEΔ (with a deletion within the exons 10 and 11), the soluble sRAGE, and sRAGEΔ (with a deletion within the exons 5 and 6), as well as the N-turnicated (Nt)RAGE (contains intron 1) and NtRAGEΔ, which additionally contained intron 6. It is also worthy of mention that unlike in humans, where sRAGE is a product of alternative splicing of the RAGE gene, in mice sRAGE is likely additionally produced by carboxyl-terminal truncation (Handford et al., 2004), in addition to the tissue and disease specific splicing that the RAGE gene undergoes (Kalea et al., 2009). In the next sections more information on RAGE will be included, depending on the brain cell type and condition.

NFκB and the brain

NF-κB is a family of dimeric transcription factors and its composition and main functions have been previously described (Baeuerie & Baltimore, 1996). The most common subunits of the NF-κB dimers are p50, p65, and IκBα; in the CNS, neuronal-specific NF-κB subunits have also been described (Moerman et al., 1999). The activation of the pathway depends on the molecular composition and localisation of the NF-κB dimers, with usually the inactive forms staying in the cytoplasm and interacting with inhibitory proteins, namely IκB family proteins. The active forms after stimulation of the cell e.g. by inflammatory stimuli – and after phosphorylation and degradation of IκB- move in the nucleus where they bind on the DNA and activate the transcription of their target genes (Baeuerie & Baltimore, 1996). However, the same pathway is involved in different functions in non-immune system cells, such as neurons (Bierhaus et al., 2005) and can be activated with CNS specific stimuli, such as nerve growing factor (NGF) (Carter et al., 1996).

In addition, Meneghini et al., (2013) showed that the NF-κB pathway which has been shown to contribute to neurotoxicity via activation of glial cells, also promotes neuronal differentiation, and thus an increased turnover of the neurons lost in AD. RAGE, which has been shown to be activating the NF-κB pathway, is also implicated in Aβ peptides (primarily involved in AD) mediated neurotoxicity, as it is involved with the Aβ peptides translocation and subsequent Aβ-related mitochondrial dysfunction (Takuma et al., 2009; Tóbon-Velasco et al., 2014). Another pathway that shows how differential activation can lead to different results, is that of ERK1/2: Huttunen et al., (2000) showed that low level activation of the RAGE/ERK pathway leads to trophic events, while high level leads to toxicity. Further discussion on NFκB can be found in the next sections, in the appropriate context per cell type/CNS condition.

TLR receptors

Another group of inflammation related receptors whose activation in neurons might lead to different results, is the toll-like receptor (TLR) family. Studies about the TLR-2, TLR-3 and TLR-4 members of that family will be discussed as examples. Although in leukocytes these receptors have been long associated with inflammatory activation (Muzio et al., 1998), and they have been found in glial cells (Jack et al., 2005) their role in neurons has only relatively recently started to emerge. For example, after viral insult, TLR-3, which is related to virus-related immune response is upregulated (Lafon et al., 2006), which can lead to neurons adapting a more immune system cell role, as they were shown to secrete proinflammatory cytokines and mediators (TNFα, IL-6, as well as interferons). This establishes that neurons have the potential to contribute to a pro-inflammatory environment, when triggered with viruses. It has also been shown that in response to IFNγ, neurons upregulate the expression of TLR3, as well as TLR2, and TLR4 (Tang et al., 2007); upregulation of the specific TLR members by IFNγ has also been demonstrated for other cell types (Yu et al., 2016).

TLR2 and TLR4 are highly expressed in leukocytes, and their ligands include several viral proteins, lipids, lipopeptides. TLR2 usually acts synergistically with TLR4, or other members of the TLR family, such as TLR1, something which results in the binding of different ligands (Yu et al., 2010). For example, when acting with TLR4, TLR2 has been shown to bind LPS, a potent pro-inflammatory stimulus, which is widely used in inflammation-related studies. However, while activation of TLR2/4 usually leads to pro-inflammatory cytokine production, in the study by Tang it led to neuronal death, independent of microglia activation (Tang et al., 2007). Another study showed LPS induced pain-related activation of trigeminal neurons, however this study did not focus on cell death, or neuronal cytokine secretion (Diogenes et al., 2011). Interestingly neurons have been shown to modulate the glial LPS response in vitro, by

reducing the pro-inflammatory response, however the mechanism underpinning this effect was not investigated (Chang et al., 2001).

When focusing on the role of TLRs on neurogenesis, there have been various studies showing the effect of TLR deficiency in adult and developing brain: TLR2 has been shown to be essential for neurogenesis, as TLR2 deficient mice exhibit reduced neurogenesis in the hippocampus (Rolls et al., 2007); also, TLR3 deficient mice seem to have a larger number of neurons that express mature neuronal markers (Okun et al., 2010) indicating that TLR3 may be involved in neural progenitor cell proliferation (Lathia et al., 2008). Interestingly, immune activation of TLR3 alone *in utero* has been shown to lead to reduced neurogenesis, which in turn can lead to subsequent behavioural issues in mice (de Miranda et al., 2010). How this correlates with the fact that neurons assume a more immune-cell-like role, as well as the fact that they use their energy to produce cytokines (Lafon et al., 2006), has not been investigated. TLR4 has been shown to have more complex results, depending on whether neurogenesis takes part in the adult or the developing brain. Rolls et al., (2007) found that TLR4 has a negative effect on proliferation and neuronal differentiation, an effect which is reduced by TLR2 activation. Focusing on immunity rather than neurogenesis, Leow-Dyke et al., (2012), showed that TLR4 activation with LPS can lead to inflammatory cytokine production and release from primary neuronal cultures (including TNFα, and IL-6).

To complicate things further, pro-inflammatory mediators have been shown to have rather paradoxical effects in neurons. For example TNFα and IL-1β, two cytokines that are primarily pro-inflammatory, are trophic for certain types of neurons as shown by neurite number, as well as GDNF production (Gougeon et al., 2013). During development and prenatally, however, TNFα alone and in synergy with IL-1β show a negative effect not on viability per se, but in nodes and neuronal dendrite length (which are important for neuronal function and connectivity) up to a certain concentration, however in higher concentration neurotoxicity and

reduced neuronal survival were observed (Gilmore et al., 2004). Notably, TNFα has two different receptors, whose correlation with its different activation pathways has been shown by Iosif et al., (2006): TNFR1 deficient mice had more new mature neurons compared to TNFR2 deficient mice, which showed reduced neurogenesis. TNFR1 has been shown to have a so called "death domain" (Gaeta et al., 2000), and its effects are considered to be detrimental for the cells. As already mentioned above, neurons do secrete TNFα under inflammatory conditions where neurons are intact, however TNFα secretion has been shown from injured/ischemic neurons as well (Liu et al., 1994). In the ischemic brain, however, it has been shown that TNFα signalling can have a neuroprotective effect (Bruce et al., 1996). As neurons secrete both the ligand and the receptors, there could be autocrine feedback mechanisms in place, in order to produce these different actions.

Similar findings have been demonstrated for other cytokines such as IFNγ (Barish et al., (1991) which showed a positive effect on neurogenesis and neuronal survival. However in another study IFNγ induced neuronal damage (Suzumura et al., 2006) when used with IL-1β; in the latter study, single cytokines on their own did not affect neuronal viability, but did so when used synergistically. Lastly, IL-1β, has been shown to be correlated directly with neuronal function rather than viability (e.g. Kelly et al., 2001), although it has been shown to have indirect effects on viability, via enhancement of β-amyloid cytotoxicity (Li et al., 2003); IL-1β induced NF-κB activation and subsequent cytokine release from neurons has not been demonstrated (Srinivasan et al., 2004).

From the above it can be concluded that neurons can, under certain conditions, contribute to a neuroinflammatory environment by secreting pro-inflammatory cytokines, as well as expressing their receptors. However, variations do exist depending on the type of neuron, or organism, or conditions under investigation. Whether the cytokines secreted have a positive or negative effect on the cells that secrete them, and on the cells expressing their receptors, is a

fascinating -albeit as yet incomplete- area of research where the focus of many different studies lies and will continue to require further investigation in the future.

1.1.3 Astrocytes: inflammatory regulators in development and disease

Along with microglia, astrocytes are thought to be the other main cell type that are considered key players in neuroinflammation; they comprise up to 50% of the CNS cell population and they have a variety of morphologies and functions (Herculano-Houzel, 2009). Astrocytes are not a homogenous type of cells, and initially Miller and Raff (1984) described them as being found in two different forms in the brain, fibrous in the white matter, and protoplasmic in the grey matter, with differences in morphology and function; others have reported that there are up to nine different forms in a brain region at any given time (Mattyash & Kettleman, 2010). More recent findings indicate an even greater diversity among the cell type, due to differences in morphology, function, and physiology. Miller, (2018) in a review on the heterogeneity of astrocytes discusses how these differences are essential for the maintenance of a microenvironment prone to changes. Karpuk et al., (2011) show that neuroinflammation related changes in astrocytic function, morphology, as well as communication, are region dependent. Whether these changes can be attributed to source or environment is still under debate, however there have been various sources of mature cortical astrocytes identified, including Radial glia cells, glial progenitors, as well as the oligodendrocyte/type-II astrocytes progenitor cells (Howard et al., 2008). From their diversity of origins, as well as subtypes, it can be assumed that their roles are diverse; indeed, astrocytes contribute to vital CNS functions, such as neurotransmission, Blood brain barrier (BBB) formation, as well as essential roles in the CNS immune responses (Mattyash & Kettleman, 2010).

Although progress has been made in pinpointing astrocyte functions, and developmental origins such progress often reveals challenges. These challenges include differences in

astrocyte function and biology between species (although it is to be noted that certain functions are preserved across species (Oberheim et al., 2012)), as well as the fact that astrocytes do not seem to have a specific developmental maturity stage. For the former, differences have been found for example in polarisation/localisation of proteins, such as Aquaporin-4 between humans and mice (Eidsvaag et al., 2017), or the accumulation of copper between more "related" species as mice and rats (Sullivan et al., 2016); copper accumulation is implicated in neurodegeneration. Thus, the fact that subsets of astrocytes in rats brains stain positive for copper, and in mice brains the same cells do not, may have implications on neurodegeneration studies, as in humans accumulation of copper in astrocytes has been repeatedly shown as discussed by Dringen et al., (2013), however it is not known whether that is in the same clusters. For the latter it is argued that unlike mature neurons and oligodendrocytes that are essentially post-mitotic, mature and fully differentiated astrocytes have mitotic potential (Ge et al., 2012) with functional integration of the new cells in the existing glial network. Mature/reactive astrocytes are widely identified by the expression of the glial fibrillary acidic protein (GFAP), however, Walz and Lang (1998) showed that the protein might be undetectable in certain populations in some cases, while Trias et al., (2013) showed that under specific conditions, microglia cells might assume astrocytic characteristics and functions, and start expressing GFAP as well as microglial markers. Additional markers such as Aquaporin-4 (AQP4), Glutamate transporter (GLT-1), as well as aldehyde dehydrogenase 1 family member (Aldh1L1) have been successfully used to identify astrocytes (Cahoy et al., 2008). Additionally, due to their functional and morphological diversity, it is quite difficult to attribute certain functions to certain populations, as depending on the CNS area the cells originate from, their metabolic and genetic profile, and thus their potential for certain actions could be limited. Subsequently, the diversity and importance of astrocytes can be better observed if one focuses primarily on one of their functions. As this thesis focuses on neuroinflammation, the astrocytic

contribution to neuroinflammation will be discussed below.

One of the primary roles of astrocytes, is the establishment and maintenance of the BBB; during inflammatory events as well as under normal conditions, astrocytes have been shown to modulate the function of the BBB endothelium. Further, astrocytes have been found to create scar-like formations using BBB elements, in order to prevent entry of leukocytes after acute injury (Voshkuhl et al., 2009), with the formation of the scar being beneficial to neuronal survival and axonal regeneration (Anderson et al., 2016). However the BBB/astrocyte relationship and interactions can end up having negative effects, especially in pathological conditions, as discussed in a review by Abbot et al., 2002. This is a result of changes in the transcriptome, and thus function of astrocytes, induced by acute or chronic inflammation; the cells in these cases are dubbed as reactive astrocytes. Zamanian et al. (2012) investigated these changes in different astrocytic populations, using models of acute and chronic inflammation, and although two different states of activation were observed (namely A1 and A2, in accordance to the microglial/macrophage M1 (proinflammatory) and M2 (anti-inflammatory) which will be discussed in 1.1.5 below), the authors raise questions about the potential multiple subtypes of the activation states observed. In the A1 state, sets of genes linked to harmful functions (e.g. synapse destruction) were found to be overexpressed, while in the A2 state, genes that included trophic factors as well as genes beneficial to synapse formation and repair were induced. Interestingly, a correlation between A1 with chronic inflammation (e.g. via systemic LPS injections in animals) and NF-κB activation, and A2 with acute inflammation (e.g. trauma or ischemic conditions) can be claimed (Clarke et al., 2018; Liddelow & Barres, 2017), although it is not known whether this is always the case, especially in vivo.

Interestingly, Liddelow et al., (2017) succeeded in creating an A1 state model, by using inflamed microglia medium (cell culture medium collected from microglia cells that had been triggered with a pro-inflammatory stimulus). By analysing its contents, they found that three

specific microglia-derived cytokines (namely IL-1β, TNFα, and Component subunit 1q (C1q)) were sufficient in eliciting responses mimicking in vitro the gene expression that is observed in A1 astrocytes in vivo. Studying those cells, Liddelow et al. (2017) uncovered formerly unknown functions of the A1 astrocytes, such as their ability to induce apoptosis of neurons, as well as oligodendrocytes, however not microglia. Moreover, in the same study it was shown that the activated microglial cells used to activate the astrocytes, were unable to induce cell death to neurons or oligodendrocytes.

As will be analysed in the microglia section in further detail, microglia are generally able to kill neurons, however this could be dependent on certain conditions which were not present in the Liddelow et al., 2017 study and in absence of this microglial role, it appears that A1 astrocytes can make up this deficit in cell-killing. An explanation on how astrocytes can kill neurons is through phagocytosis: although microglia are the main phagocytic cells of the CNS, it has been shown by different studies (e.g. Morizawa et al., 2017; Wakida et al., 2018) that astrocytes can have phagocytic roles in certain conditions. In what seems to be in contrast to the A1/A2 paradigm mentioned above, astrocytes can respond to acute inflammation (i.e. injury) essentially by eating up injured but not dead cells, especially neurons and other astrocytes. Although this does not seem in accordance with the A2 role of astrocytes, which is to aid in the repair of the tissue, it could be argued that the phagocytic response observed could be a clean-up of cells that could potentially increase local neuroinflammation, and possibly lead to an increased microglial response. Wakida et al., (2018) showed that astrocytes selectively had a phagocytic response to cells they had membrane connections with, and hence the astrocytes were able to respond to single cell damage.

With a focus on the A1 phenotype, which is associated with the NF-κB pathway activation, it would be useful to focus on the function of that pathway in astrocytes (Liddelow et al., 2017). In contrast to neuronal NF-κB activation, which was discussed above, and has been shown to

have potentially beneficial effects for the neurons, in astrocytes things are more complicated. A study by Lian et al., (2015) showed that Aβ peptide induced activation of astrocytes, triggered the NF-κB pathway, and resulted in activation of the complement pathway with the release of the C3 protein, which acted on the C3aR neuronal receptor and caused neuronal dysfunction. Aβ has also been shown to lead to release of other directly or indirectly proinflammatory mediators. As such, Johnstone et al., (1999) showed that astrocytes treated with Aβ promoted microglia/macrophage migration; this was attributed to the secretion of molecules such as RANTES and MCP1/CCL2, as well as MIP family chemokines which are known to act as chemotaxins for these cells. Aβ stimulation of astrocytes also resulted in the production of several pro-inflammatory cytokines, including IL-1β, as well as TNF-α and nitrite. IL-1β, as well as IL-18 have also been shown to be produced by astrocytes, using both a model system of Japanese encephalitis as well as in vitro (Das et al., 2008). The expression, processing, and eventual release of IL-18 and IL-1β are known to be regulated by the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome. Inflammasomes are multi-protein complexes, which trigger the activation of inflammatory mediators, such as Caspase-1 and the aforementioned cytokines. Activation of the inflammasome is regulated in a multi-signal cascade that involves among others the TLR/NF-κB pathway, as well as pathogen and damage/danger associated molecular patterns (PAMPs such as LPS, and DAMPs, such as the RAGE-ligand HMGB1, ATP, and extracellular DNA) (Jo et al., 2015). Johann et al., (2015) showed that astrocytes have the potential to regulate the expression of IL-18 and IL-1β via the inflammasome in an in vivo model of an inflammatory/neurodegenerative disorder, namely Amyotrophic lateral sclerosis (ALS). Using a mouse model of HD, which is known to be a neurodegenerative disease with a major neuroinflammatory role, Hsiao et al., (2013) showed that LPS activation of mice brains lead to an increase of pro-inflammatory cytokines, such as TNFα and IL-1β in both wild type and HD animals (with an augmented

increase in HD animals). By investigating the origin of the cytokines, they found that activation of the NF-κB was co-localised with GFAP+ cells, giving evidence that the cytokines were released from astrocytes, rather than microglia or neurons. In turn, it has been shown that in astrocytes exposed to TNFα and IL-1β, not only is there a positive feedback loop created (as these cytokines can autoregulate their own release), but also other inflammatory and immunoregulatory cytokines are released. These cytokines include IL-6, IL-8, and various colony stimulating factors (CSFs) (Aloisi et al., 1992; Sawada et al., 1992); however that loop has been shown to be antagonised by negative feedback mechanisms, as shown by Krasowska-Zoladek et al., (2006). Such positive feedback could be attributed to the fact that TNFα and NF-κB activation leads to increased expression of TLR2 in astrocytes, as demonstrated by Phulwani et al., (2008) and Esen et al., (2004), which in turn could result to additional release of TNFα should a ligand (including DAMP/PAMPs such as LPS, HMGB1, or S100) be available.

Interestingly, a member of the S100 protein family, namely S100B, is expressed and released by astrocytes constitutively, as it is implicated in physiological functions of these cells, such as shape, and migration (Brozzi et al., 2009). S100B then affects in a paracrine way neurons and microglia, and can be either toxic or trophic (Zimmer et al., 1995). Its roles on neurons, microglia, as well as astrocytes have been shown to be mediated by RAGE (Adami et al., 2004; Ponath et al., 2007), where especially for astrocytes, S100B induced the release of IL-6 and TNFα. RAGE and its ligands have been shown to be expressed in a disease stage dependent manner in astrocytes from HD patients brains (Kim et al., 2015), where RAGE's downstream activation leads to an increase of the HD pathology and progression (the study focused on protein-interaction based assays, therefore the different isoforms of RAGE were not distinguishable-as a further comment, RAGE was shown to exist in the extracellular area using immunofluorescence). However, it has also been shown that a secreted splice variant of the

receptor (sRAGE) is produced and released by astrocytes (Park et al., 2004). sRAGE has been shown to act as a decoy, as ligands that could trigger the membrane bound RAGE and thus lead to enhanced inflammation, bind to the secreted form, something which has been shown *in vivo* (Juranek et al., 2016) to reduce inflammation and disease progression. In another study, however, sRAGE appeared to have pro-inflammatory effects (Pullerits et al., 2006). The differences could be attributed to the different sRAGE, and overall RAGE isoforms that have been recognised (Miranda et al., 2017; Moriya et al., 2014), as previously discussed in section 1.1.1.

As baseline expression of TLR2 as well as TLR3 and TLR4 has been found in astrocytes almost throughout the CNS (Park et al., 2006; Bowman et al., 2003), and that expression has been shown to change under chronic and acute inflammatory conditions, also due to interactions between the receptors (Farina et al., 2005; Jack et al., 2005; Bsibsi et al., 2006), targeting these receptors, as well as their interactions holds much promise. As an example, it was shown that a flavonoid (curcumin) interfered with the TLR4 pathway, resulting in anti-inflammatory results, such as a decrease of secretion of the pro-inflammatory cytokines TNFα and IL-6, and an increase of the anti-inflammatory cytokine IL-10 (Yu et al., 2016). Nevertheless, it should be kept in mind that, due to the pleiotropic effects of cytokines, modulating the release and expression of pro-inflammatory cytokines can be beneficial, but it can also be harmful. One example can be that of TNFα: while in specific cases TNFα signalling has shown to have negative effects on astrocyte/neuron signalling, which in turn can lead to cognitive effects such as memory impairment (Habbas et al., 2015), in other cases it has been shown to have a neurotrophic effect. In an NF-κB dependent way, TNFα has been shown to induce the secretion of the neurotrophic factor BDNF (Saha et al., 2006) which is essential for neuronal growth and has even been shown to promote neurogenesis (Quesseveur et al., 2013).

With everything mentioned taken into account, more studies focusing on the secretome of the

cells under inflammatory conditions would be useful, as well as the possible pathways activated, and the meaning of the activation, not only for astrocytes, but for the cells they interact with. As astrocytes can be found abundantly in the brain, and interact with all other cell types, investigating the characteristics of these cells, from receptors, to responses, and focusing on how these responses can affect adjacent cells (from phagocytosis, to cytokine-mediated communication) is extremely interesting, as these cells can be considered the most versatile in function within the CNS.

1.1.4 The effect of inflammation on oligodendrocytes

Although oligodendrocytes are not directly under investigation within this PhD, an overview of their involvement in neuroinflammation, as well as development of the CNS is discussed in Appendix 3. While these cells are important for the CNS, their importance can be shown only when other CNS cells are present (i.e. neurons), oligodendrocytes do not secrete pro-inflammatory cytokines including IL-18 (Cannella, & Raine, 2003), however they have the receptors for the messages sent by other cells during neuroinflammation.

1.1.5 Microglia: The brain's Trojan Horse?

As discussed in section 1.1.1, microglia develop from myeloid progenitors that migrate via a complex regulatory mechanism to the CNS from the yolk sac during embryonic development as already discussed (Ginhoux et al., 2010). They account for up to 16.6% of the total cells of the brain, depending on which area is studied (Mittelbronn et al., 2001), and have been shown to be involved in the development, activity, surveillance, and homeostasis of their occupied areas as discussed below.

Many theories exist on how the adult microglial population establishes and maintains itself. In

a recent study using lineage labeling/tracking, it was shown that, although there might be CNS infiltration of monocytes during various developmental stages, the microglial population doesn't include any of these cells; rather, self-renewal is maintained by selective proliferation and apoptosis (with the two events being temporally and spatially coupled), and the overall microglial population remains stable over the lifetime, although the microglial sub-types per area might change (Askew et al., 2017).

The principal functions of the microglial cells, both during development and in the adult brain are immune-system related, although non-immune system related roles have also been reported. For example, investigations have suggested that microglia have a function in synaptic pruning, which is the sculpting of neuronal circuits through the elimination of excess neuronal synapses throughout the brain's development, carried out in a predetermined and highly regulated manner (Paolicelli et al., 2011; Ekdahl 2012; Hong, et al., 2016). Thus, it would suggest that not only does microglial function aid in development, but also that their dysfunction could lead to neurodevelopmental disorders.

Microglia can be considered as the main cell type of the brain's immune system; with the help of astroglia they regulate changes in the neural environment, and maintain the brain's homeostasis. In the early years of brain development a significant amount of cell death occurs as the brain affirms its complex network of synaptic connectivity (Kuan et al., 2000). Cell death also occurs in the developed brain, usually in a strictly programmed and controlled procedure; however pathological conditions (e.g. extensive neuroinflammation) change this dramatically to a greatly dysregulated process (Cecconi et al., 1998; Martin, 2001; McTigue & Tripathi, 2008;). Microglia's role in both regulated and pathological cases is to clear primarily dead neurons, but also glial and myeloid cells from the milieu (Janda et al., 2018).

Neurons interact with the cells that surround them mainly by expressing and either presenting or secreting specific molecules that are used as signals of communication. Two interesting

groups of the molecules that neurons present, are the 'eat me' and the 'don't eat me' signals that essentially determined if the cell will be phagocytosed or not. These signals include the phospholipid phosphatidylserine, which is an 'eat me' signal of lipoic nature found as part of the cell membrane, and CD47, a transmembrane protein which acts as the 'don't eat me' signal (Li, 2017). As phosphatidylserine is found on the inner part of the plasma membrane, in order for it to be presented and therefore flag the cell, it needs to move to the outer part of the membrane –as occurs for example during apoptosis-, and then be detected by surrounding cells. Conversely, as CD47 is a transmembrane protein, it is constantly presented in healthy neurons, essentially telling that the cell is not to be eaten; interestingly this protein is involved in pruning as well, where it is thought to protect neurons from unnecessary pruning (Lerhman et al., 2018). Thus, phagocytes will select and remove the cells that expose the 'eat me' signals from the tissue unless the balance between these signals and the 'don't eat me' signals leans towards the latter. Microglia constantly inspect the surface of neurons for specific 'eat me' signals (such as phosphatidylserine) that when detected, flag the cell that presents them for microglial phagocytosis. Whilst the 'eat me' signals generally appear due to permanent cellular changes such as neuronal damage, microglia have also been shown to actively phagocytize dying, injured, or even stressed but still viable neurons that temporarily expose eat me signals (Brown, et al., 2015), in a process termed phagoptosis. During this process, specific soluble proteins bind the target cell to the phagocyte, which engulfs and phagocytizes in a lysosomal-dependent manner (Hochreiter-Hufford et al., 2013).

Further to the pruning function described above in the developing brain, microglia have also been shown to actively eliminate neuronal precursor cells in a variety of organisms. Specifically, Cunningham et al. (2013) demonstrated that the number of neuronal precursor cells (NPC) correlates with the quantity of activated microglia present in prenatal and postnatal developing macaque brains. With regard to the NPCs, microglia have been shown not only to

regulate their number through cell death, but also can influence their proliferation (Noctor et al., 2019), differentiation, and migration (Aarum et al., 2003).

Despite the plethora of studies indicating that microglia belong in the macrophage lineage, the cells have many distinct morphological, molecular and developmental features that set them apart from other -non CNS- tissue macrophages (Ginhoux & Prinz, 2015). As these cells are established early in the brain's developmental process (as discussed in section 1.1.1), and given that the brain is isolated from the rest of the body with the help of the blood brain barrier (BBB), an interest has been shown in how microglial precursor cells are guided to the brain, specified, and how their number and function is maintained thereafter. Although several regulatory factors have been identified, these are not specific to microglia alone. As an example, Xpr1b, a zebrafish phosphate balance related transmembrane protein, also characterized as an atypical G-protein coupled receptor, and a human XPR1 homologue, is essential for the development of microglia and other types of macrophages (Meireles et al., 2014). Although this gene's precise *in vivo* function remains unknown, its lack of function leads to problems with microglial development and colonization, problematic osteoclastic function and reduced Langerhans cells number. Unravelling more information about microglial origins, a more recent study (Xu et al., 2015) shows that there is not one but multiple sources from where microglia arises, that the sources are development dependent, and that more than one microglial population exists (at least in zebrafish). The study indicated that embryonic microglia originate from a different region than the adult microglia in the zebrafish (rostral blood island vs ventral wall of dorsal aorta region in the adult), with the two populations being regulated differently (Xu et al., 2015). However, as mentioned above, differences between species development should prompt caution when it comes to generalizing results regarding gene function.

Microglial maintenance

When it comes to their maintenance, it has been shown that in the healthy brain, microglial cells are being continuously renewed, and that the contribution of monocyte infiltration to the microglial population happens –usually- only in pathological conditions (Hashimoto et al., 2013). In two separate studies, manipulation of experimental conditions demonstrated that some bone marrow derived microglia were observed in the brain (Mildner et al., 2007; Lund et al., 2018), and that in extreme circumstances such as an empty microglial niche, bone marrow cells entered the CNS and developed into microglia (Beers et al., 2006). In both studies transplant models were used (i.e. in order to follow the bone marrow cells' fate, bone marrow and microglia were of different genetic origin, with bone marrow being transplanted from a donor), in conditions not reflective of normal physiology. More studies have shown that self-renewal can be achieved from either the resident microglial population (Bruttger et al., 2015), or infiltrating monocytes (Varvel et al., 2012). In a relatively recent study, Yao et al., (2016) showed that acute partial depletion is followed by infiltration and establishment of microglia-like cells, which are subsequently replaced by resident microglia. However, as shown in the lineage study by Askew et al., (2017) as well as in a review by Stratoulias et al., (2019), microglia is rather a heterogeneous population of cells, and to our knowledge, the renewal potential of each subpopulation under normal conditions has not yet been fully investigated.

Microglial function

Microglia are usually in a resting state, called M0, whereby they inspect their environment for changes (Davalos et al., 2005; Nimmerjahn et al., 2005); surveillance is an active process, which can lead to rapid changes of the microglial state. If change does occur, microglia then can assume a diversity of phenotypes (i.e. are polarized), in order to maintain the homeostasis of their environment. Interestingly, RAGE and its ligand S100B are involved in the migration

of microglia, as well as its polarization and activation (Bianchi et al., 2011; Zhang et al., 2010). The two most prominent states they assume are the M1 and M2 phenotypes (see Orihuela et al., 2016 for a review of the states from a polarization and metabolic point of view). Another aspect of the microglial actions of the neuro-immune system are the inflammatory processes which are triggered by infections, diseases, and injuries both in the brain, and as a response to peripheral immune system activation (Biesmans et al., 2013). Inflammation's fundamental role is to protect tissues from harm, by attracting the appropriate cells (in the case of the CNS microglia and astrocytes) through molecule secretion from the injured neurons on the site of inflammation. These cells subsequently respond by producing molecules such as pro-inflammatory cytokines (e.g. TNFα, IFNγ, and pro-inflammatory interleukins such as IL-6) (Boche et al., 2013; Suzuki et al., 2005).

Cytokines activate immune system cells, and lead to a classically activated M1 polarized microglial phenotype that has essentially a proinflammatory role (but one which is tightly controlled and is auto-regulated). M1 cells have the role of killing pathogens through a range of mediators such as nitric oxide production, additionally to their phagocytic role (Boje & Arora 1992; Nau et al., 2014). As an example of regulation, it has been shown that one of the processes that regulate this phenotype, is apoptotic cell death (Cheon et al., 2017). Thus, regulation is implemented by sets of intrinsic and extrinsic factors, which change gene expression, and can lead to different phenotypes, functions, and metabolism (Kierdorf & Prinz, 2013; Ghosh et al., 2018).

In contrast to the M1 cells, the alternatively activated M2 cells have an anti-inflammatory role, as well as a neuroprotective one. Classification of the two types is in theory based a) on the cytokines that activate the maturation of macrophages to the M1 or M2 phenotype, b) membrane markers, and c) what the respective secretory products are (Varnum & Ikezu, 2012), especially *in vitro*. It is to be stressed however, that *in vitro* does not necessarily equal *in vivo*.
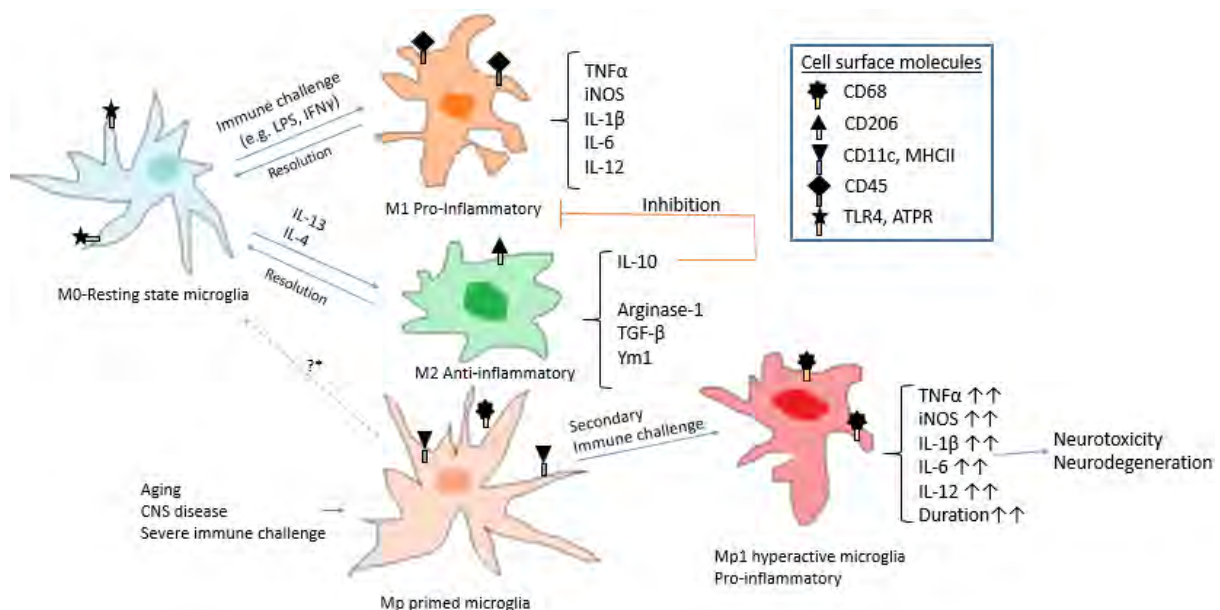
Figure 1.4 . _A brief overview of the various microglial states_ _(see text for further detail and references)._ These states can be distinguished by using either their morphology, membrane molecules, or secretion products. The best way to distinguish between them is using all three factors: morphology, secretion/behaviour, and membrane molecules. Resting state microglia are branched in appearance, and are the predominant type of microglia in healthy adult brains. This type inspects its environment for changes. When change is found the microglia polarizes into other states. The M1 state can be activated after an immune challenge, and is pro-inflammatory in nature, as it secretes pro-inflammatory cytokines amongst others. Its function is to fight pathogens, and to clear up dying or dead neurons. The M2 on the other hand, is anti-inflammatory in nature, and it generally has a repair role. Both the M1 and the M2 phenotypes are amoeboid in appearance. After resolution of the inflammatory process, the main type of microglia is again the M0. Due to aging, CNS disease, or severe immune challenge, another type of microglia appears: the primed microglia, which is more sensitive to immune challenges, and whose active state is more reactive to changes, in both amount of secretion products, and in duration of reaction. This hyperactive type can lead to extensive neurodegeneration, and neurotoxicity. *Signifies reversal of priming, which has been shown experimentally by Ramaglia et al., (2012).

In the case of the microglial states *in vivo* classification is more difficult, as the states are not 'locked', and microglia as a cell type is both plastic in nature, and diverse in phenotypes, and sub-phenotypes have also been observed (Cherry et al., 2014). M1 and M2 cells co-exist during inflammation, and are up or downregulated accordingly: M1 cells are usually in the most abundance during the initiation of the inflammatory process, and are succeeded by enhanced levels of the M2 phenotype during the resolving of the response and the tissue-repair phase (Thawer et al., 2013). This process does not always develop in a favourable way, as certain conditions and situations may favour one cell type over the other. When the M1 phenotype is more dominant, such as in acute inflammatory conditions that result from tissue injury or

ischemia, the tissue damage may not result in repair but neurodegeneration which could be extended beyond the injury (Taylor et al., 2013).

Extension of brain damage beyond the initial injury point has been shown in imaging studies where patients with traumatic brain injury were examined. It was demonstrated that the white matter damage was progressing over time rather than it being a static event (Greenberg et al., 2008; Farbota et al., 2012). On the other hand it has been shown experimentally, when the conditions are manipulated so that the M2 phenotype expression is induced, anti-inflammatory M2 action that leads to tissue repair and even recovery is observed (Shechter et al., 2013). An overview of the microglia polarization states, (as well as the relevant markers) are presented in figure 1.4. Primed microglia, as described by Perry & Holmes, (2014) are also included in the figure. Essentially, priming occurs after repeated exposure to inflammatory insults, or chronic inflammation, and can lead to an exaggerated pro-inflammatory response. Although priming is not fully molecularly characterized, one of the proteins implicated in priming in some cases is RAGE (Franklin et al., 2018).

Although generally the M2 phenotype is deemed to be the desired phenotype in neurodegenerative disorders, this is not always the case. For example, studies exist that indicate that in brain tumours, especially gliomas, the M2 phenotype supports tumour growth and progression for example via secretion of growth factors (Wu & Watabe, 2017; Kennedy et al., 2013; Chen et al., 2017); it is however to be noted that in gliomas this is more true for tumour associated macrophages, a heterogeneous population of cells that derives from both infiltrating monocytes, and microglial cells in varying amounts, depending on the tumour (Müller et al., 2017; Chen et al., 2018).

The many faces of microglia

From the above, one could conclude that microglia is a cell type as versatile as its functions.

Indeed, as a cell type, microglia is extremely plastic, changing its morphology rapidly depending on activity, environment, and even pathology. Microglia were thought to have three primary distinct morphologies: compact, longitudinal, and ramified, as identified by Lawson et al., (1990); although this is the general paradigm when it comes to microglia morphological phenotypes nowadays, studies have shown that microglia have more than these morphologies. For example, Fernández-Arjona et al., (2017) showed more than 10 morphotypes of rat microglia, depending on brain region, inflammatory state, and other factors as shown after analysis with mathematical modeling. Another study which focused on microglial remodeling, discussed four different models of morphological change, which included over ten different observed post-injury microglial morphologies, and proposed that each microglia remodels itself depending on its microenvironment in a rather complex and multifactorial fashion (Walker et al., 2014). However, this has been shown to be true in a different way as well, as not just morphological diversity exists, but also functional: different types of microglia exist in the brain at the same time, in any given moment (Hanisch & Gertig, 2014). The microglia population should be viewed as a network of cells, with specific characteristics depending on their spatial distribution, rather than a single cell population all over the brain. Recent studies (Askew et al., 2017) have shown that apoptosis and proliferation maintain a stable microglial population, but that there is more to be told in this story. Indeed, Bilbo (2018) showed that microglial cells, depending on the region they are in, have different functions, even with regard to phagocytosis. Tay et al., (2018) showed that not only there are distinct microglial populations, but also that they behave differently in health and pathological conditions. Using a novel mapping method, as well as mathematical modeling, they were able to follow microglial cells under normal circumstances, and they found out that even in the same region there are distinct cell groups which seem proliferate randomly and not in a specific manner per group. Under pathological conditions, only certain clones/sub-populations are expanded, in

order to overcome what causes the disturbance in the microenvironment. After the balance has been recovered, the excess microglial cells die in a specific way, and apoptosis is regulated and focused on certain clones, rather than random (Tay et al., 2018). This not only makes sure that an unwanted and potentially harmful microgliosis is quickly eliminated, but also that the microglial sub-population diversity which translates to functional diversity, is maintained. Clearly, given the complexity and unresolved nature of the above, further work is required to elucidate the full nature of this process.

1.1.6 CNS (Infiltrating) Monocytes and monocyte derived macrophages

The notion that the brain is an immune-privileged organ was long standing, due to the fact that the BBB separates the brain from the periphery (Pachter et al., 2005). However, under certain conditions (e.g. pathological/inflammatory conditions) the BBB loosens its structure, and allows the entrance of blood leukocytes, in a highly organized way (Paul et al., 2016). Therefore, a) in an inflamed brain, it is established that microglia are not the only type of myeloid cell, and b) in order for infiltration to happen there has to be inflammatory disruption of the BBB.

During the breakdown of the BBB, monocytes are actively recruited from the periphery and migrate into the CNS; this involves the CCL2(MCP1)/CCR2 axis (Chu et al., 2014). CCL2, the ligand of the CCR2 receptor which is responsible for the adhesion and migration of monocytes, has been shown to be increased in a variety of neuroinflammatory conditions, for example in Alzheimer's disease it is correlated with the amount of amyloid present, and by promoting endothelial dysfunction leads to the loosening of the BBB (Roberts et al., 2012). The cells that secrete CCL2, and thus recruit peripheral monocytes, have been shown to be microglial cells, as well as astrocytes. In a study by Persidsky et al (1999) it was shown that after HIV infection, microglia were activated and secreted CCL2, which attracted monocytes

through an experimental BBB model; in the same study, media from infected microglia, induced the secretion of chemokines from astrocytes. Interestingly even neurons have been shown to secrete CCL2/MCP1 in inflammatory conditions, such as AIDS-related dementia (Conant et al., 1998), thus correlating the development of dementia with both neuroinflammation, and monocyte infiltration. Weiss et al (1998) showed that CCL2 alone is enough to promote migration of monocytes through the BBB in a dose dependent manner, using a co-culture of endothelial cells and astrocytes as a model for the BBB. In the same study they showed that exposing the cells of the BBB to inflammatory conditions also leads to monocytic transmigration, and that the main source of CCL2 were the astrocytes. This hints to the BBB having an active, rather than passive immunoregulatory function, as its components actively recruit leukocytes, but also shows the importance of the crosstalk between the glial cells of the CNS. Liu, H., et al. (2009) further emphasized that monocytes need to be recruited, and do not passively migrate in the brain, even if the BBB is disrupted: focused ultrasound, and superparamagnetic iron oxide MRI (Magnetic resonance imaging) were used in mice to a) disrupt the BBB, and b) monitor the infiltration of leukocytes. It was shown that under conditions that disrupted the BBB but did not cause inflammatory events, monocytes did not enter the brain *in vivo*.

Finally, the fact that other molecules seem to be needed for the migration of the peripheral monocytes into the CNS, was revealed by a pharmacological study: Lovastatin was shown to decrease or even eliminate the inflammatory response in experimental autoimmune encephalitis (EAE, a multiple sclerosis model in rodents (Constantinescu et al., 2011)), hallmarks of which are mononuclear cell CNS infiltration, and subsequent induction/release of inflammatory cytokines and iNOS. Stanislaus et al. (2001) used Lovastatin treatment to downregulate the expression of LFA-1, which is needed for leukocyte-endothelial interaction; this indicates that not only chemotaxis, but also interaction between cells, are needed for the

infiltration.

After monocytes enter the CNS they differentiate into microglia-like cells, but whether these cells contribute to the microglia pool, or even replace microglia is under debate. In an interesting study on EAE it was shown that infiltrating monocytes are a transient cell type found in the brain during inflammatory events, and although morphologically they are similar to microglia, they have a more aggressive role than microglia in the progression and pathogenesis of the disease (Ajami et al., 2011). In this study, it was also found that infiltrating monocytic cells are no longer detectable after the inflammatory event is under control, something which is also supported by a study by van Ham et al (2014). They showed that in zebrafish following injury, a mixed population of macrophages (resident and infiltrating) clear dead cells, after adapting an M1-like phenotype which is accompanied by an increase in microglia-like phenotype. However, during the resolution of the neuroinflammatory event, the numbers of overall myeloid cells decreases, infiltrating monocytes seem to die, and resident microglia appear to engulf their remains (van Ham et al., 2014).

Using another useful methodology, that of microglia depletion, similar results were exhibited by Yao et al., (2016): the resident microglia population left over after depletion was responsible for the re-population of the CNS (in this case the spinal cord). Using Mac-1-saporin (a toxin that specifically targets microglia), 50% of the spinal microglial population in mice were depleted, and infiltration of monocytes was observed; the depletion consequently causes neuroinflammation, as well as a relatively rapid re-establishment of the microglial population within 14 days. The cells exhibited a hypertrophic morphology (larger cell bodies, and thickened/shortened processes), clusters of Iba1+ cells were presenting (Iba1 is a marker of both microglia as well as monocytes and macrophages (Nakamura et al., 2013)) after 3 days, which were almost non-existent in day 14. In day 14, the cells exhibited ramified morphology, typical for microglia, however not fully identical to the pre-depletion state. Using GFP tagging

for the peripheral cells, Yao et al. also showed that infiltrating monocytes were almost non-existent in day 14, while they peaked in day 5. These cells differentiated into microglia-like cells in days 5-14 of the experiment, but eventually disappear and do not contribute to the resident microglia pool, probably due to competition between cells, or due to the hypothesis that the CNS environment does not favour the survival of circulating/infiltrating monocytes (Yao et al., 2016). Taking into account a study by Elmore et al., (2014) which identified a microglial progenitor population in the brain (nestin-positive cells which indicates some level of stem cell-like properties), which gets activated and gives rise to microglia cells when needed, this hypothesis seems plausible, and is now widely accepted.

Conversely however, other studies have shown that the circulating monocytes, do end up contributing to the microglial pool post inflammation/depletion. Varvel et al. (2012) showed that after depletion of microglia cells, the brain regions that were affected were repopulated within 2 weeks, however the source of repopulation was the infiltrating cells rather than the resident microglia. Although this study has been criticised for creating conditions favourable for the circulating monocytes to occupy the microglial niche, it is nevertheless a strong indicator that infiltrating cells have the potential to take up the roles of microglia in the brain. Although microglia and infiltrating monocytes have overlapping actions, several studies have focused on their differences. As discussed below (and as mentioned in section 1.1.5), infiltrating monocytes can have either a positive or negative role with regard to the outcome of a disease. For example, as discussed above, infiltrating monocytes negatively affect the outcome of EAE/MS (e.g. Stanislaus et al., 2001). In other cases, such as acute injury it is not as clear whether their actions are beneficial or detrimental. For example, macrophages play several extremely important beneficial roles, such as that of myelin clearing as well as promoting neural regeneration after injury (Keilhoff et al., 2007). In theory, this could be attributed to the M1 and M2 states these cells can be activated into; generally they follow the

same M1/M2 rules microglial cells follow, as discussed above (Sun & Nan, 2016). Indeed, transplantation of M2 cells improved clinical features in EAE in the aspect of immunomodulation and suppression of ongoing severe EAE episode, as well as aided in the recovery (Mikita at al., 2011). Miron et al., (2013) showed that CNS macrophages switch spontaneously from an M1 dominant phenotype to an M2 around 10 days after an inflammatory insult, which results in oligodendrocyte differentiation and the instigation of remyelination. It was also shown that M2 related molecules protected the oligodendrocytes from apoptosis, while the M1 did not have such an effect, and that depletion of M2 polarised cells alone inhibited the differentiation of OPC to oligodendrocytes (Miron et al., 2013). In contrast, however, the M2 phenotype is disastrous in cases such as brain tumours, and more specifically gliomas. In brain tumours, a mixed group of macrophages consisting of both microglia as well as infiltrating monocytes make up more than 40% of the mass of the tumour in case of gliomas and are referred to as tumour-associated monocytes (TAMs) (Kennedy et al., 2009). These cells are predominately in a M2 state as they promote an immunosuppressive and tumour-friendly environment, while the M1 state is considered tumoricidal (Li & Graeber, 2012).

However, things are not as simple as this dichotomy suggests: Miron et al. (2013) demonstrated that it is a specifically timed switch between states that promoted remyelination, rather than a specific state; interestingly, this mechanism (and study) didn't appear to distinguish between infiltrating and resident macrophage populations. Focusing on separate populations, there are papers that indicate that infiltrating and resident cell types have different functions, where for example infiltrating cells perform actions microglia can't. An important example of this is the role of microglia in Alzheimer's disease: studies on mice showed that microglia not only not clear the amyloid deposits, but also promote fibrillary plaque formation and development (Wegiel et al., 2001). In contrast, macrophages originating from infiltrating monocytes are suggested to restrict plaque formation, at least in mouse models (Butovsky et al., 2007).

Studies that focus on the depletion of bone marrow derived cells, rather than microglia cell, show the importance of the function of infiltrating cells, and their possible neuroprotective roles. This is exemplified by studies by London et al., (2011) (who focused on the retinal neural tissue), and Greenhalgh et al (2018) (who focused on spinal cord injury) showed not only that microglia and infiltrating monocyte derived monocytes have different roles, but also that they communicate and regulate each other. London et al.'s study, which focused on the retina and its regenerative/self-healing properties due to the presence of normally dormant progenitor cells, showed that infiltrating monocytes that are recruited after an inflammatory event differentiate into macrophages in the neural tissue, and promote the activation and survival of the progenitor cells, as well as the establishment and maintenance of an anti-inflammatory milieu at a specific subsequent time point (London et al., 2011). This is essential for the healing of the area, something which is dependent on the amount of infiltrating cells, and not observed in the absence of the infiltrating cells. In the above study, the mechanism of how these cells have an anti-inflammatory/neuroprotective effect was also investigated, and findings suggested an immunoregulatory role for these cells, as they don't allow pro-inflammatory immune cells to accumulate in the regenerating tissue (London et al., 2011). The role of infiltrating monocyte derived macrophages (MDM) in the retina, therefore, seems to be in contrast with the neurodegenerative role of the retinal microglia, as reviewed by Silverman & Wong (2018). Greenhalgh et al. (2018) showed in a spinal cord injury study that the time point of MDM CNS infiltration and activation coincided with that of microglia down-regulation. MDM and microglia follow similar distributions after an inflammatory event, such as injury, and are expected to communicate as they are in close proximity. To investigate possible communication, and role of that communication, mixed populations of microglia and MDM were introduced into a de-myelination assay. In these assays, the amount of neurotoxicity and neurodegeneration depends on the activity of the microglia. It was shown that demyelination

decreased with the increase of the MDM present in the assay, and identified day 3 after injury as a key day in the amount of MDM present (Greenhalagh et al., 2018). In order to assess the MDM-microglia communication further, an in vitro system was developed where microglia and MDM were cultured in separate compartments which allowed controlled communication. By essentially co-culturing the cells under different conditions it was shown that MDMs suppress microglial phagocytic activity by suppressing pro-inflammatory gene expression (as well as the expression of IL-10 which is predominately an anti-inflammatory cytokine) via prostaglandin E2 signalling, and surprisingly that microglia upregulate the phagocytic activity of macrophages (Greenhalgh et al. (2018)). It was further shown that MDMs promote microglial cell death under inflammatory conditions, but do not promote the proliferation of the cells.

Importantly, it is obvious from the above that the role of infiltrating MDM is an important and complex one, therefore investigating these cells would not only increase our knowledge about neuroinflammation, regulation of CNS healing, and cell development and communication, but could also lead to treatments that are based on cells that are more easily accessible, such as the circulating monocytes. However, isolating and studying these cells is difficult after they are in the neural tissue, as they tend to look like microglia, express similar markers to microglia, and their presence there seems to be in most cases transient, and limited to about 2 weeks. Gene expression studies have focused on finding markers that are specific to the MDM or microglia populations. Although specific genes or sets of genes have been identified for each category, these markers can still be difficult to use, as some exhibit developmental stage specificity (i.e. they appear in mature microglial cells but not developing cells), or exhibit conditions-specific differences (e.g. they respond differently to specific stimuli or respond differently, and show different characteristics depending on whether they are investigated *in vitro* or *in vivo*). For example TMEM119 (Transmembrane protein 119) (a transmembrane protein with currently

unknown function) has been indicated as one of the most accurate microglia markers in the CNS, and is extremely useful in immunohistochemistry and tissue related work (Bennett et al., 2016). However, Satoh et al., (2016) showed that that expression was limited to a subset of human microglia, and was expressed in over 50% of the Iba1+ (Ionized calcium binding adaptor molecule 1) CNS microglia. Finally, although TMEM119 is useful in studies involving tissue, it has been shown that cultured microglial cells seem to lose their "mature" marker expression profile, and assume a gene expression profile similar to that of M1 cells, or cells derived from patients with neurodegenerative disorders, such as AD (Bohlen et al., 2017). Expression of TMEM119, albeit in low levels has also been shown in macrophages in a more recent study by Qian et al., (2018); therefore contamination of MDM cultures with microglia cells and vice versa is possible, and hence examining the exact function and potential of the MDM may be problematic. Nevertheless, protocols for studies using cells of different origin (e.g. different organism for periphery and CNS macrophages), and therefore different markers do exist, and can help in the discrimination of the cells, therefore it is not impossible (Bennett et al., 2016; Fyfe, 2019).

As a final remark, it should be noted that discrimination between microglia and MDM is often difficult within the literature, due to the markers issues mentioned above and due to the fact that brain macrophages are heterogenous in nature, but also exhibit enormous plasticity. So although *in vitro* models do exist for microglia-like cells (see table 4.1 in chapter 4 for some examples), it is not known whether these cells represent a specific brain macrophage subtype, microglia subtype, or can have infiltrating MDM characteristics. Nevertheless, the environment (including inflammatory-related mediators and signals, and cells that the monocytes interact with when they enter the brain) plays a major role in the differentiation of monocytes into morphologically microglia-like cells; therefore establishing a similar environment (by employing a BBB model and inflammation-related mediators) in future *in*

vitro models, might produce cells that are more relevant to infiltrating MDMs.

1.2. Inflammation in the CNS and the periphery: A crosstalk

After focusing on the different cell types that can be found in the CNS, we will now turn our attention to the interactions (or cross-talk) between cells, and also between the CNS and the periphery. The central nervous system is relatively isolated from the periphery due to the existence of the BBB. Because of this, one would assume that it is immunologically isolated as well, and that inflammation that begins in the periphery does not affect the CNS, and vice versa. However, this has been proven wrong in peripheral inflammatory disorders such as diabetes and cancer, and even peripheral infection where not only peripheral organs are being affected, but the CNS as well (Dantzer et al., 2008).

Interestingly, inflammation that begins in the CNS (neuroinflammation) has been shown to affect the periphery as well: several inflammatory cytokines have been found to increase in patients' plasma during neuroinflammatory episodes or conditions, and the increase has been shown to correlate with the progression of the disorder. As an example, in a study by Motta et al., (2007), the blood of AD patients was tested in order to measure pro-inflammatory cytokines and correlate the findings with disease progression. The highest amount was found in the mild-AD group, whereas there was no difference observed in severe-AD patients. It was concluded that this was due to a decline in immune responsiveness in AD, but, it could also be correlated with the progression of a neurodegenerative disorder as follows. In mild pathogenesis, the neuro-immune system is overactive, something which leads to neurodegeneration, but as the degeneration progresses, so does the decline of immune responsiveness. Additionally it was recently shown that mouse astrocytes are involved in regulating the peripheral inflammatory response (Dickens et al., 2017). Specifically, astrocytes were found to communicate with the

periphery using extracellular vesicles (EVs) in mice. These EVs were detected after using a proinflammatory cytokine as a trigger, and were targeting a receptor belonging in a family of receptors involved in a plethora of immune responses (namely, peroxisome proliferator-activated receptor α (PPARα)). PPARα activation triggered the liver to produce a variety of proinflammatory cytokines, and eventually assisted in the migration of leukocytes from the periphery to the CNS. Another study by Schmitt et al., (2012) also showed leukocyte infiltration in neuroinflammatory conditions, and further showed that there are specific brain areas in the forebrain, and midbrain that are preferred when that specific process takes place. Thus, it is well established that cross-talk between the CNS and the periphery takes place; therefore, the following section will explore the precise mechanisms and consequences of such cross-talk.

1.2.1 Neuroinflammation and neurodegeneration

Neurodegenerative events are accompanied by neuroinflammation, and in a relatively recent review by Kempuraj et al., (2016) it was concluded that neuroinflammation is a crucial factor in how neurodegenerative disorders begin, and proceed –but importantly, not the only factor. This is not a surprising finding, as brain cell functions depend on a well-balanced and optimal milieu. The neuronal microenvironment relies for this balance on the BBB, and the glial cells, and both elements are affected severely in neuroinflammatory conditions (Erickson et al., 2012; Takeuchi, 2013). More specifically, the BBB has been shown to be dysfunctional in neuroinflammatory and neurodegenerative disorders such as Alzheimer's disease, stroke and multiple sclerosis (de Vries et al., 2012). The BBB's 'structural' element is endothelial cells whose function is regulated mainly by astrocytes. In neuroinflammatory conditions such as the ones mentioned above, the permeability of the BBB is disrupted, allowing leukocytes

(especially monocytes, and monocyte derived macrophages) to enter the CNS. This often leads to uncontrollable neuronal cell death and enhancement of the neuroinflammatory activity of the glial cells (Varvel et al., 2016). The infiltrating cells have been shown to be involved in the clearance of dying cells, as their main role is to assist the CNS resident macrophage, the microglia. However as the resident microglial cells are themselves vulnerable to neuroinflammation (Ji et al., 2007), and their population decreases, the infiltrating cells have been shown not only to assist, but even to transform into microglia-like cells, although with some characteristics somewhat different to the resident cells (Varvel et al, 2016). Indeed, Yao et al., (2016) demonstrated the dynamics of such an event, by depleting a high percentage of the resident microglia; as the resident microglial cells have a relatively low proliferation rate, circulating monocytes infiltrated the area, and assisted with the microglial functions until the homeostasis was re-established.

Not surprisingly, cytokines secreted during neuroinflammation have shown potential pro- or anti-neurogenic effect which contribute in a positive or negative way to the different steps of neurogenesis, and some of these have been discussed in the previous sections of this chapter. Many of these effects are correlated with differential microglial activation: for example, low levels of neuroinflammation, and effects such as microglia activation by low level of IFNγ, have been associated with neuroprotection. Shaked et al., (2005) showed that microglial cells, after IFNγ induction, are able to take on a new ability, and clear glutamate, and thus inhibit glutamate neurotoxicity. Wong et al. (2004) demonstrated that low levels of IFNγ lead to neural stem cell activation, as well as survival of new-born neurons. However, Vass et al., (1992) showed the other face of the IFNγ, which is essentially destructive for the CNS on an *in vivo* study which focused on demyelination induced by the cytokine. Overall, IFNγ is considered proinflammatory, with some anti-inflammatory effects that depend on cell type, dose, and even disease stage, as indicated by Arellano et al., (2015).

Another example of a molecule of great interest in the brain, is IL-6. Summarizing and adding to what was discussed above, IL-6 in the CNS has roles in neurogenesis, and the physiological function of neurons and glial cells under both normal and inflammatory conditions. Its expression and secretion is affected in many CNS disorders, making it both a potential marker and therapeutic target for these disorders (Erta et al., 2012). As in the periphery, it has both pro- and anti-inflammatory properties, depending on the downstream activation of either the classical (anti-inflammatory) or trans-signalling (pro-inflammatory) pathways. These pathways are activated depending on the form of the IL-6 receptor: a membrane bound receptor activates the classical pathway, whereas a soluble receptor activates the trans-signaling pathway (Rothaug et al., 2016).

From the above it can be concluded that neuroinflammation has the potential to trigger neurodegeneration; however, it also triggers repair mechanisms, and the same signaling molecules can initiate either repair or destruction. To contribute to the pro-neurogenic/reparative effect of neuroinflammation, Nakatomi et al., (2002) showed migration of neural stem cells to inflamed and injured areas of the brain, and also their subsequent activation and neuronal differentiation. Additionally, a recent paper demonstrates how neural stem cell transplantation with the purpose of nervous tissue repair, can suppress neuroinflammation (Peruzzotti-Jametti et al., 2018). Repair of brain tissue and essentially replacement of damaged or degenerated cells has been the focus of a great amount of research, and its potential as a future therapy is becoming more and more obvious (Lindvall, & Björklund, 2004), as transplanting cells with anti-inflammatory properties, such as NSC or progenitors, can have beneficial effects that expand beyond simply replacing cells, as these cells can produce an anti-inflammatory environment.

1.2.2 Neuroinflammation on a molecular level: response and agents

On a molecular level, inflammation is regulated by numerous molecules and factors, such as chemokines, cytokines (e.g. IL-6, TNF-a), proinflammatory enzymes (e.g. cyclooxygenase-2(COX-2), matrix metalloproteinases (MMPs)), and proinflammatory transcription factors such as the NF-κB (Aggarwal, 2004).

NF-κB is the central intracellular regulator of inflammation. *In vitro*, NFκB activation is a downstream consequence of treatment with Lipopolysaccharides (LPS) (Covert et al., 2005), which mimic a bacterial infection initiating an inflammatory response, or with advanced glycation end products (AGEs) (Tobon-Velasco et al., 2014) whose receptor, RAGE has been implicated in the pathogenesis of diabetes, neuroinflammatory conditions, and thus induction of proinflammatory cytokine cascades. All of these target protein responses are mediated through NF-κB-dependent signaling pathways, with more than 500 genes having been implicated in NF-κB related inflammatory responses (Gupta et al., 2010). Normally, NF-κB is active in glutamatergic neurons; NF-κB in glia has a lower basal activity but upon stimulation can be highly activated, which plays a crucial role in brain inflammation (Kaltschmidt, & Kaltschmidt, 2009). Moreover, inhibition of astroglial NF-κB can reduce inflammation as demonstrated by a study that showed functional recovery after spinal cord injury in mice (Brambilla et al., 2005). Thus, as this specific transcription factor has been found in abundance in the brain where it has a plethora of functions, it's not surprising that various CNS disorders are linked to inflammation-activated NF-κB signaling effects.

With regards to extracellular triggers, cytokines are some of the main molecular regulators of inflammation. Indeed, as discussed in the previous section, in the CNS they are involved greatly in the cell-to-cell communication in inflammation, as well as the normal brain function and development (Deverman & Patterson, 2009; Stolp, 2013). Cytokines have been the focus of mostly microglia studies, or microglia/neuron interaction studies (Eyo et al., 2016), as

microglia is the cell type that due to its role produces most cytokines. The secretome of astrocytes has also been studied, and a focus has been given to the secretome during inflammatory conditions *in vitro* (Choi et al., 2014). Summarizing the above studies, a different set of cytokines is secreted under different condition, with the main inflammatory cytokines that the CNS uses to communicate information to and from cells being IL-6, TNF-α, and the IL-1 family (including the inflammasome triggered proinflammatory IL-1β, and IL-18 which has been indicated as of great importance in neuroinflammation and neurodegeneration (Felderhoff-Mueser et al., 2005)).

Further to what was discussed in the cell-specific sections above, RAGEs are involved in a variety of normal functions throughout the body, and in the brain it is involved in neuronal development and repair (Alexiou et al., 2010). Furthermore, it is involved in functions such as neuroinflammation; one good example of how RAGE is involved in neuroinflammation, comes from a neuroinflammatory disease, Huntington's disease (HD). HD is an autosomal dominant disease, caused by a CAG repeat expansion over a specific limit in the Huntingtin gene (HTT). It is characterised by neurodegeneration accompanied by neuroinflammation in the striatum. RAGE has been shown to be expressed in both neurons and astrocytes in HD brains and there is a suggestion that the pattern of expression of RAGE correlates with that of cell loss (Kim et al., 2015; Ma et al., 2004). RAGE binds multiple types of ligand to produce either neurotrophic or neurotoxic effects. In human HD brains a high degree of co-localization of RAGE with its ligands S100B and N-carboxymethyllysine (CML) has been reported, especially in astrocytes and medium spiny neurons –the type of neurons primarily affected in HD-, but not so much in microglia or other types of neurons (Shi et al., 2018).

1.2.3 (Neuro)inflammatory conditions of the brain

As mentioned before, neuroinflammation and neurodegeneration go hand in hand most of the

time (Ransohoff et al., 2016). Focusing on the inflammatory conditions that start in the brain, neuroinflammatory processes have been extensively linked to the progression and possible initiation of a plethora of neurodegenerative diseases, even though inflammation on its own, as for example caused by LPS, has not been shown to induce acute neurodegeneration (Morimoto et al., 2002). Additionally, Calabrese et al. (2015) have hinted the possibility of degeneration progressing without the need for inflammation, in a disorder that has been long established to have an inflammatory element, multiple sclerosis (as also discussed by Losy, 2013); however this still needs to be further investigated in other disorders to establish whether it is a valid widespread phenomenon. If it is valid in the majority of cases, then a major revision of the relationship between neuroinflammation and neurodegeneration will need to take place. Nevertheless, the prevailing view is that neuroinflammation and neurodegeneration are invariably linked.

In chronic inflammatory conditions or ageing (as opposed to for the neurons that die due to the neurotoxicity of either their internal or external environment), some cells may temporarily present distress signals for various reasons, such as injury or stress. As mentioned in previous sections, these may include 'find-me' and 'eat-me' signals: molecules such as nucleotides released by dying cells (Chekeni et al., 2011), or phosphatidylserine (Li W., 2012) appearing in the outer layer of the cell membrane. Macrophages, including microglia, detect these signals, and essentially kill the cells that produce them and engulf their remains. Brown et al., (2015) suggested that in the CNS when the microglial cells are involved, in a type of cell death termed phagoptosis, microglial cells attack neurons that show the 'eat me'/distress signals but are otherwise viable. Neuronal death in phagoptosis, therefore, is caused by inflammation-related stress of neurons, and probably primed/over-reactive microglia, and does not precede microglia clearance, but it happens because of it.

Finally, genetic and epigenetic factors are regarded increasingly as having a role in

neurodegeneration/neuroinflammation (Garden, 2013; Hwang et al., 2017). Although neurodegenerative diseases have distinct patterns of gene expression and feature different inflammatory responses, there are elements that are similar between them (Ugolini et al., 2018; Richards et al., 2018). When focused on genetics, a plethora of studies revealed that mutations in phagocytosis related genes were risk factors for neurodegenerative disorders. One of the most studied genes is the microglial surface receptor TREM2 (Triggering receptor expressed on myeloid cells 2). Genetic studies demonstrate that specific mutations of the gene are correlated with increased risk of developing Alzheimer's disease. As an example, Wang et al., 2015 demonstrated the role of TREM2 as a lipid pattern damage detector through the TREM2 R47H variant, and its contribution to the microglial response to Aβ accumulation (a hallmark of AD) was revealed; the same variant has been linked to amyotrophic lateral sclerosis (ALS) (Cady et al., 2014). Other TREM2 variants have been linked to Parkinson's disease, as well as fronto-temporal dementia (Rayaprolu et al., 2013); for more regarding the genetics and epigenetics of neuroinflammation, please see Yeh et al., (2017)'s analysis in their review of substantial bodies of data and evidence regarding how variants of one gene can lead to a plethora of neurodegenerative diseases, and how microglia is essential for maintaining a healthy brain, as well as Garden's (2013) review on microglia, neuroinflammation, and epigenetic mechanisms.

1.2.4 Targeting neuroinflammation: from genes to molecules and cells

An increasing understanding of the major role that the immune system plays in neurodegenerative disorders has occurred over recent decades (Dantzer, & Wollman, 2003; Labzin et al., 2018; Stephenson et al., 2018). Thus, targeting components of the immune system that are key players of the inflammatory processes of the brain could aid in treating these disorders, or their symptoms. Indeed, the use of anti-inflammatory factors and drugs, whether

that is their primary function or a secondary function, has been proposed to aid in the prevention or even treatment of neuropsychiatric neurodegenerative disorders *in vivo* (as will be discussed further below). It is worth mentioning that most neurodegenerative disorders do have neuropsychiatric symptoms, either after the diagnosis or preceding it: Murray et al., 2014 discuss psychosis as a symptom Alzheimer's disease, while Halperin & Korczyn (2008) show that depression appears before the diagnosis of dementia, and discuss their overlapping biological causes. Last but not least, neuroinflammation and neurodegeneration are a common finding in both depression (Hurley et al., 2013), schizophrenia (Archer, 2010), and bipolar disorder (Shioya et al., 2015).

Many treatments used for neurological or psychiatric disorders have been shown to possess anti-inflammatory properties (for relevant review see the anti-inflammatory properties of anti-depressants by Galecki et al., 2018). It is worth mentioning that those actions take place through microglia signaling involving molecules and pathways, such as IFNγ and its receptors (Kubera,et al., 2001), and the Toll-like receptors 4 and 2, whose primary ligand is LPS (Tynan et al., 2012). The use of anti-inflammatory factors and drugs, whether as their primary function or a secondary function, has been proposed as aids in the prevention or even treatment of neuropsychiatric neurodegenerative disorders *in vivo*, but also in *in vitro* as described below.

The example we will focus on is aspirin, as, Laan et al., (2010), Nitta et al., (2013), and Müller (2019) show that aspirin and other anti-inflammatory drugs can aid in the treatment of neurodegenerative as well as neuropsychiatric diseases with an inflammatory element such as schizophrenia and depression. Although the exact mechanisms have not been elucidated when it comes to the treatment of such disorders, aspirin's actions have been shown to have a synergistic effect with other anti-inflammatory compounds, such as omega-3 fatty acids (Emsley et al, 2003), that demonstrate positive effects as an adjunct treatment in schizophrenia and other psychiatric disorders (Bozzatello et al., 2016). As a drug aspirin has been found to

have a positive effect on the pathology of a variety of neurodegenerative disorders (both neurological and psychiatric in nature), but also a direct effect on microglial cells and their inflammation related processes (Berk et al., 2013). Moreover, aspirin has been shown *in vitro* to stimulate the M2 alternatively activated microglia by activating anti-inflammatory signalling pathways (Laan et al., 2010). In this study an AD animal model was used, and in addition to reduced pro-inflammatory and increased anti-inflammatory cytokine production, the Aβ deposits were reduced, due to the fact that phagocytic function of the M2 phenotype was improved, as was cognitive function. The mechanism by which this occurred was through Lipoxin A_4 (LXA_4) being produced, after cyclooxygenase-2 was inhibited through acetylation after aspirin administration (Laan et al., 2010). LXA_4 is normally produced via cell-cell interactions in inflammatory conditions and promotes an M1 to M2 switch, something which is indicated by the upregulation of anti-inflammatory cytokines such as IL-10, and Transforming growth factor beta (TGFβ) and the downregulation of pro-inflammatory cytokines including IL-6, TNF-α, and IL-1β (Laan et al., 2010).

An additional example of a treatment used for neurological or psychiatric disorders (as reviewed by Wada et al., (2005)) is lithium, which is a mood stabilizer, and is used to treat the manic phase of bipolar disorder. It has not only anti-psychotic properties, but also anti-neurodegenerative and anti-inflammatory properties. Furthermore, and possibly hinting a mechanism of how it achieves the aforementioned effects, lithium, when used *in vitro*, has been shown to have the ability to inhibit microglial activation, when this is caused by LPS (Dong et al., 2014). The mechanism behind that inhibition is by suppressing the pro-inflammatory TLR-4 expression which was induced by LPS. Studies on the neuroprotective effect of lithium *in vivo* show that when used in micro doses and as an adjunct treatment it successfully improved the symptoms of neurodegenerative disorders such as AD, PD, and HD, and showed its neuroprotective effect even in acute brain injury (Sarkar, et al., 2008; Lazzara et al., 2015;

Forlenza et al., 2014; Leeds et al., 2014). However, the studies that make the connection between antipsychotics and microglia modulation are relatively few and *in vitro*, so the results should be interpreted with caution, and the negative side-effects of these drugs should be taken into account as well (additionally to the fact that not all medication is well tolerated by all patients). Thus, while caution is suggested as results seen in *in vitro* studies, and even animal studies don't always translate to disorder symptoms improvement, and the possible negative symptoms of the treatment should always be taken into account, effective use of anti-inflammatories in the treatment of neurodegenerative and/or psychiatric diseases emphasizes the important role of inflammation in determining the overall health of the CNS.

In addition to the above, studies that use modulation of gene expression, blocking of specific cellular processes involved in neuroinflammatory processes are useful, as they provide more targets for treating neuroinflammatory-related neurodegeneration. For example, polyglutamine expansion (expansion of the CAG repeat that encodes glutamine) is the primary cause behind nine human neurodegenerative disorders, including HD, and the ataxias (Fan et al., 2014). The expression of certain microRNAs (miRNAs) in a polyglutamine-induced toxicity model of neurodegeneration (Bilen et al., 2006) were studied, and by modulating either miRNA processing as a whole, or focusing on the upregulation of a specific miRNA (bantam) they were able to enhance neurodegeneration in the first experiment, or prevent degeneration in the case of the bantam miRNA experiment. In another study, by differentially expressing a miRNA (miRNA-146a) that has been found to be overexpressed in AD, and whose targets include genes that are involved in inflammatory processes, it was reported that a different response to the miRNA was seen in the different cell types that were used, (i.e. neuronal, microglial, and astroglial cells) something which indicates the pleiotropic effect of the specific regulator (Li et al., 2011). Similarly, as more and more miRNAs have been shown to be involved in inflammatory and microglial processes (e.g. M1 and M2 phenotype regulatory miRNAs have

been found by Orihuela et al., 2016), studies focusing on either enhancing or downregulating the expression of these miRNAs could be beneficial for controlling or even treating certain aspects of neurodegenerative disorders; this could be achieved for example by using either gene editing technologies (e.g. CRISPR) or antisense nucleotides for the miRNAs of interest (however results should be interpreted with caution as miRNAs are often involved in more than one processes and regulate more than one gene).

Moving on from genes and molecules to cells, a ground-breaking study led by Luca Peruzzotti-Jametti (2018), it was shown that primary NSCs, and induced neural stem cells (converted in vitro into NSCs from other cell types, such as fibroblasts, somatic cells, or embryonic stem cells) have the potential to modulate the inflammatory phenotype and actions of mononuclear phagocytes (MP) (i.e. microglia and infiltrating monocytes and macrophages) in an anti-inflammatory way. As mentioned in sections 1.1.5 and 1.1.6, MPs depending on their environment can polarize into different phenotypes with different functions (the main of which are M1 and M2, although evidence as reviewed by Martinez and Gordon (2014) suggest that the M1/M2 paradigm is oversimplified and might need review), and NSCs' ability to influence such polarization could be of great potential in the future treatment of neuroinflammatory conditions.

Succinate has been shown to be one of the metabolites that positively regulates the M1 phenotype in both autocrine (internally regulating a pro-inflammatory phenotype), and paracrine (by secreting succinate the cells activate the receptors of neighbouring cells, turning them into a M1 phenotype as well) ways (Tannahill et al., 2013). Accordingly, in the Peruzzotti-Jametti (2018) NSC study it was shown that NSCs not only reduce the succinate produced by the MPs through active uptake, thus making it less available for the MPs, and reducing the inflammatory milieu, but also that they suppress production of pro-inflammatory cytokines, produce anti-inflammatory molecules, such as prostaglandin 2 as a response to

succinate.

In summary, an increasing understanding of the major role that the immune system plays in neurodegenerative disorders has occurred over recent years. Thus, targeting components of the immune system that are key players of the inflammatory processes of the brain could aid in treating these disorders, or their symptoms. For example, Neniskyte et al., (2011) showed that inhibiting the phagocytic action of microglia in a cell-based model of inflammatory neurodegeneration was sufficient to prevent neuronal death and loss of neurons during inflammation. This was achieved without actively interfering with the inflammatory process per se, but by stopping the phagocytic process of viable neurons that were presenting 'eat me' signals (see phagoptosis above).

Clearly, in all the targeting solutions proposed here, it is always necessary for the molecules (or cells) to reach the affected area. Although the BBB in most pathological conditions is more permeable than under normal conditions, some molecules (especially larger ones) cannot traverse it, and so can't reach their targets (Pandey et al., 2016). Focus must be, therefore, given to molecules that have been known to do so. All the pharmacological treatments mentioned above have been shown to enter the brain, however they might have more than one target, and their anti-inflammatory action might be a side-effect rather than their primary effect; or in many cases, they might induce even more side-effects, which may cause more harm than the benefits of their anti-inflammatory actions.

Flavonoids

Recently, a focus has been given to naturally derived molecules, with a variety of beneficial effects, and –unlike their pharmaceutical concentrated counterparts (e.g. aspirin) - few side effects in physiological doses. For example, flavonoids have been shown to not only get through the BBB, but also be beneficial for not only treating, but also even preventing inflammation (Pan et al., 2010; Faria et al., 2014; Ferri et al., 2015). Flavonoids are plant

derived natural products that chemically are characterized as benzo-γ-pyrone derivatives; they have relatively simple chemical structures, but over 4000 derivatives have been reported, each chemically diverse (Hollman & Katan, 1997). Their size and chemical structures allow them to pass through the blood brain barrier, making them great candidates for targeting not only peripheral inflammation, but neuroinflammation (Youdim et al., 2003). They possess various biopharmacological activities, of which the anti-inflammatory activity is the one we will focus on in the current thesis. Several animal models have proven that flavonoids aid in acute and chronic inflammation and a variety of mechanisms of actions have been suggested for their *in vivo* anti-inflammatory activity, including that of downregulating the NF-κB pathway, as well as the secretion of pro-inflammatory cytokines (Vafeiadou et al., 2007). Middleton et al., (2000) summarized the possible cellular mechanisms which may be involved in regulating the activity of inflammation related cells such as macrophages which have been found to inhibit the expression of pro-inflammatory genes, and attenuate the inflammatory response that was triggered by LPS. This fact is of interest, as microglia, as well as infiltrating monocytes, could be affected by flavonoids, as could in theory the other glial cells that are actively participating in neuroinflammation, such as the astrocytes. Indeed, evidence for modulation of proinflammatory cytokines produced by astrocytes has been recently shown (Sharma et al., 2007), as well as of microglia (Zheng et al., 2008; Lee et al., 2003). Flavonoids have also been shown to have neuroprotective properties by acting directly on neurons, as shown by Schroeter et al., 2001, and a beneficial effect in patients with neuropsychiatric disorders, as summarized by Lopresti (2017). Recently, Hassan et al., (2018) showed that the flavonoid Vicenin-2 (V-2) has the potential to modulate the polarisation of macrophages towards an M2/anti-inflammatory phenotype. Vicenin-2 (see Figure1.5 for structure) is a flavonoid 8-C-glycoside, which are compounds containing a carbohydrate part linked to the 8-position with a 2-phenylchromen-4-one flavonoid backbone. V-2 is a solid, highly soluble in water and can be

found in a number of foods, such as fenugreek, sweet basil, oat, and wheat.V-2 has been shown to have anti-inflammatory effects *in vitro* and *in vivo*. Kang et al., (2015) showed that post-treatment of cells with the compound after treatment with an inflammatory compound (either LPS or PMA) reduced the effects of the inflammatory compound. Although the effects of V-2 in the CNS have not been elucidated, the flavonoid can travel through the BBB and potentially act on the various CNS cells (Figueira et al., 2017; Faria et al., 2014; Yang et al., 2014), both intact, but also as its metabolites (Gasperotti et al., 2015; Youdim et al., 2003). In addition to the fact that V-2 has been patented as having a "beneficial effect on neurological and/or cognitive function" (Buchwald-Werner & Fujii, 2014), Sampath et al., (2014) found that sweet basil extract, which contains V-2 among other flavonoids had a beneficial effect on cognition. A 2020 study found that extract from *Saccharum officinarum* L. also containing V-2, induced anti-nociceptive effects *in vivo* (Gomes et al., 2020), while V-2 has also been detected in a dubbed "traditional treatment for AD" (Wang et al., 2016). More about V-2 will be discussed in chapter 5 and 6, as part of the discussion of the results of chapter 5.
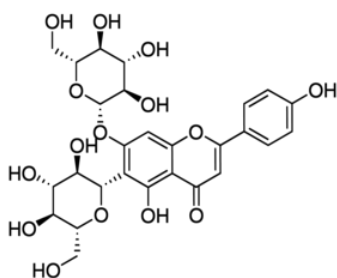
Figure 1.5 *The molecular structure of V-2*

1.2.5 Studying neuroinflammation *in vitro*

A great diversity of experimental conditions have been used in the literature in order to assess the neuroinflammatory response of cells *in vivo* and *in vitro*. Although both have merits, both also have disadvantages. Depending on the organism the cells come from, and depending on

whether they are a primary culture, a mixed/co-culture, or cells that have been differentiated from progenitor or stem cells, the results, as well as the optimal experimental conditions are expected to be different. However, an in vitro approach gives us the advantage of focusing on a specific aspect of the brain, be it a developmental stage, a cell type, or a specific disorder. For example, a plethora of inflammatory conditions can be mimicked using different inflammatory stimuli: if LPS is used, the focus can be on bacterial infection, but also on fever, as a low dose of LPS produces fever *in vivo* (Klir et al., 1994) whereas if IFNγ is used, this mimics viral infection (Chesler et al., 2002) or even a tumour related environment (Castro et al., 2018). Moreover, LPS interacts with receptors that are highly involved in neuroinflammatory studies, namely, the Toll-like receptors. For example, both TLR-2 and TLR-4 (which both bind to LPS) are suggested to be involved in amyloid clearance (Richard et al., 2008); similar involvement has been shown for the IFNγR1 receptor of IFNγ (Yamamoto et al., 2007). Conversely it has also been shown that lack of the aforementioned TLR receptors, might extend the life expectancy in a Huntington's disease mouse model (Griffioen et al., 2018). Consequently, studying the receptors involved in neuroinflammatory conditions can be achieved through the use of molecules not necessarily involved in the conditions *in vivo* (such as LPS); in other words, targeting the receptors in an *in vitro* setting is able to produce results relevant to the conditions in question.

Moving from conditions to the cells themselves, the fact that different cell types can be removed from their native environment holds both advantages and disadvantages. For example using primary lines (e.g. neuronal, microglial) instead of induced cells/cell lines, gives the advantage of a more accurate idea on how the cells can react in vivo, as they maintain markers of interest and their morphology (for the most part) that is observed *in vivo* (Lorsch et al., 2014). Cell interactions are also more "realistic" when studied in primary cells, as the connections have already been established, as well as the microenvironment via cell/cell

interaction and coupling for example. Nevertheless, as the cells are not in their natural environment any more, they might act differently, and a lot of adjustments are needed in order for them to react as they do in vivo (special substrates, media, etc.). Moreover, primary cell lines have a limited lifespan, and expansion potential, and their response is more sensitive to manipulations. The latter can be negative or positive; as an example of the former even different brands of the same serum/medium, might give different results. Additionally, primary cell lines have limited availability, and this may impact on the replicability of results: not only does there have to be a donor of tissue (in our case, brain tissue), that donor has to have specific characteristics in order to "fit" the profile needed. For example, in order to study neurodegenerative disorders, the donor has to have not only that disorder, but lack co-morbid conditions (as they might influence the neuroimmunological and neurodegenerative processes), as well as fall in specific categories for other characteristics (e.g. age, sex, and race) which might otherwise make interpreting the results more challenging. Furthermore, many primary cells are not well characterised, they proliferate slowly, and can be quite heterogeneous in nature.

Some of these issues can be addressed using immortalized cell lines, and their derivatives. Although recently there has been some negative interest in such cell lines, due to queries as to how fully they replicate the characteristics of the primary cells in question, or to contamination with cells from other cell lines, or to misidentification, they remain an effective way to study a great range of conditions (Kniss et al., 2014; Lin et al., 2018). Moreover, by employing gene editing technologies, and using immortalized cell lines, we can see the effects of certain genes on certain cell types with otherwise stable characteristics. Especially using diseases that are caused by certain genes (e.g. HD), we can investigate the effects of the disease on how cells function and even interact with other cells, by knocking in the defective gene, or knocking out its wild-type/normal form. Moreover, often there are multiple cell lines for the same cell type

(e.g. THP-1 and U937 are both monocytic cell lines; H9 and H1 are both human embryonic stem cells), allowing us to get more information by comparing results between lines, or using certain lines depending on the focus of the study and the characteristics under investigation. Cell lines are well characterised, allow for a better manipulation, and control of the conditions, easier to culture, and have "adapted" to laboratory conditions, giving scientists a bigger time frame for experiments, and larger number of manipulations (Kaur et al., 2012; Maqsood et al., 2013). Nevertheless, in vitro studies on cell lines alone cannot give us all the information needed. In vitro models of diseases can be made using cell lines, and they are valuable for research. But, only when used in conjunction with in vivo results, or results from primary cells will the information that experiments and studies give us will be more accurate and relevant to the in vivo scenario relating to the disease/process in question.

Usually, in neuroinflammation studies, the focus is on co-cultures of different types of cells (one of which is usually microglia) and their interactions or synergistic effect (Gresa-Arribas et al., 2012). Therefore, questions such as the contribution of a single cell type and the synergistic contribution of different cell type combinations on a variety of conditions, such as neuroinflammation, need to be addressed.

Importantly, in the context of the current thesis, primary human microglial and neural cells are difficult to obtain in large enough numbers needed for studies, and there are senescence and proliferation issues after a relatively low number of passages (Guo et al., 2016). Although some cell lines have been produced (such as the HMO6 microglia cells (Nagai et al., 2001)), there are complications in using them, such as instability of their genome due to the inclusion of viruses in their genome, as well as cost of obtaining and maintaining them. Breaking it down further, focusing on subpopulations of brain macrophages, which include resident and infiltrating cells, it is obvious that the infiltrating macrophages play just as an important role as the resident cells. Moreover, as monocytes can be isolated from the body, they can be used

for example as mediators of immunomodulation, as well as pharmacological targets, or even as vehicles of drug/gene-therapy delivery. Knowing the properties of these cells, and of their differentiation process after they enter the CNS, would be useful in understanding better the neuroinflammatory processes in the CNS, as well as help us with focusing on and interfering with specific elements of the processes. Therefore, an important starting point for the current thesis is that it would be extremely useful to have *in vitro* models of microglia-like cells (see sections 1.1.5 and 1.1.6). Monocyte derived microglia-like cells have been generated in the past (e.g. Leone et al., 2006 and table 4.1) in a multi-step method using blood derived monocytes but the authors of the study admit issues with cell survival and division (which is expected as the cells reach a terminal differentiation point, in which proliferation ceases).

Thus, as neuroinflammatory processes depend majorly on the heterogeneous nature of the brain macrophages, it is clear that there is an urgent need to decipher how this is happening. It is crucial to be able to easily generate such *in vitro* models of microglia (or microglia-like) cells and to be able to study and research these cells and their neuroinflammatory responses and mechanisms, while keeping in mind that microglial cells (as well as all brain macrophages) exhibit incredible plasticity, the proof of which is their heterogeneous morphology and functions (Lawson et al., 1990; Suzumura et al., 1991; Glenn et al., 1992; Hailer et al., 1996).

1.3 Aims and objectives

The overall focus of the present study will essentially be to investigate the contribution of different brain cell types to neuroinflammation *in vitro*. As discussed above, previous studies that focus on neuroinflammation have shown how cells respond to a variety of inflammatory stimuli in different ways, with changes in inflammation-related molecule secretion being one of them. However, there are no studies investigating the respective roles of different cells originating from the same genetic source. Moreover, most of the studies which focus on

developing microglia(-like) cell models are often complicated, with regard to either the cell source, or the process; thus one of the aims of this study is to develop such a model using both a relatively simple and easy to find cell source, as well as an efficient but not overly complex method of differentiation.

a) First, NSCs, and neurons and astrocytes derived from them, will be used to explore how cells respond to the same inflammatory stimuli. The stimuli selected were LPS and IFNγ as they are established pro-inflammatory agents for in vitro microglia studies, and their receptors have been indicated as involved in neuroinflammation and potentially neurodegeneration (Lively et al., 2018; Papageorgiou et al., 2016; Pintado et al., 2012). As cells use cytokines to communicate with their environment and with each other, it will be investigated how different types of brain cells communicate with their neighbours as well as themselves (autoregulation). The cytokines in focus are IL-6, IL-1β, TNFα, as they have already been the focus of various studies as mentioned above. Moreover, IL-18 will be used (alongside IL-1β), as an indicator of inflammasome activation, as will RAGE which has been shown to be expressed in most cells of the CNS as discussed above (Derk et al., 2018; Ding & Keller, 2005). The interest lies in its soluble form (RAGEs), which has been shown to be secreted in some cases from CNS cells and has been shown to have potentially anti-inflammatory properties. However, as microglia are the main immune cell type of the brain, and thus secretes the majority of the volume of the pro-inflammatory cytokines it is expected to see relatively low secretion levels from the non-microglial cells which are the focus of the first chapter of this PhD.

b) Secondly, an additional aim is to produce a microglia-like model, by exposing an established monocytic cell line to a CNS-like environment. It has been shown, as

already discussed that cells of myeloid origin, can differentiate into microglia-like cells upon exposure to a neural environment, both *in vivo* (e.g. infiltrating monocytes, and yolk sac progenitors) and *in vitro* (see table 4.1 for examples). The THP-1 cell line will be used (Tsuchiya et al., 1980). The rationale behind using these cells is that this specific cell line is commercially available, well characterized, relatively easy to maintain, and is extremely useful with regards to its characteristics. For example, it has been shown that under specific conditions THP-1 monocytic cells can be differentiated into macrophage-like "dTHP-1" cells (Auwerx, J., 1991), and it is a cell line often used in studies for modeling inflammation and immune modulation. The resulting dTHP-1 cells are often used as macrophage-like cells, and have been found to have such characteristics as they can assume both an M1-like and M2-like phenotype (Genin et al., 2015; McFarland et al., 2017). In addition THP-1 and dTHP-1 cells have been used to model microglia-like responses (e.g. Giri et al., 2003), and studies using both microglia and THP-1 cells have found similarities between the two cell types in their responses (Klegeris & McGeer, 2003). Other studies have used monocytic cells in a neural environment to generate microglia-like cells (e.g. Ohgidani et al., 2014), therefore, THP-1 cells, despite their limitations, are a good cell line to be used in this instance. The methodology for this differentiation will be optimized, and the cells produced will be characterized by investigation of their gene expression, as well as their morphology under different conditions.

c) Third, the cells produced in (b) will be exposed to conditions similar to the conditions to which the non-microglial neural cells were exposed in (a), and their immune response will be investigated. The secretome of the cells will also be investigated under non-inflammatory conditions. By exposing the cells to Vicenin-2 (V2), a flavonoid with potential anti-inflammatory properties that has been shown to produce M2-like

characteristics in macrophage-like THP1 cells in the past (Hassan et al., 2018), the potential anti-inflammatory effect that agent has on these microglia-like cells will be investigated. V-2 will be used alone, and in combination with the same pro-inflammatory factors used in (a). Therefore, it will be investigated whether these cells can shift towards both an M1 and an M2 phenotype, and thus are CNS-macrophage-like.

In conclusion, this thesis aims to set a base for future studies investigating the effects of anti-inflammatory agents on the CNS cells by focusing on establishing their pro-inflammatory potential, and possible response, as essentially it will establish a baseline for cellular response to inflammatory stimuli in a CNS setting. Moreover, in the second part of the thesis, the differentiation of a well-established monocytic line into cells that are microglia-like will be attempted; these cells will be characterized and compared to the literature's "image" of microglia. A further aim is to then investigate the pro-inflammatory potential and response of this novel cellular model and establish that it is indeed microglia-like, as well as to elucidate its responses to anti-inflammatory modulators. Essentially therefore, this study will provide the scientific community with beneficial insights regarding the contribution of different CNS cells to neuroinflammation.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 General chemicals and reagents

In Tables 2.1 and 2.2 the materials used for the majority of the experimental work are listed. More details, as well as additional information can be found in the materials and methods section of each experimental chapter.

Chemical	**Supplier**
Methanol (13218003) Isopropanol (11308471) Tween20 (BP337-100) Ethanol (BP2818-4) TrizolReagent (12044977) SuperSignal West Pico PLUS Chemiluminescent Substrate (15669364)	Fisher Scientific, UK
Chloroform (C2432-2.5L) Western Blotting Developer and Fixer (Z354147) Phorbol myristate acetate (PMA) (P8139-1MG) Dimethyl sulfoxide (DMSO) (D8418-100ML)	Sigma Aldrich, UK

Table 2.1 *List of chemicals used*

2.1.2. Tissue culture reagents

Reagent	**Supplier**
10x Phosphate buffered saline (PBS) (12579099)	Fisher Scientific
Foetal Bovine Serum (FBS) (15595309)	
Dulbecco's Phosphate-Buffered Saline (DPBS)- with and without Calcium and Magnesium (11590476 and 11580456)	
Dublecco's Modified Eagle Medium (DMEM)- with and without F12, and Knockout F12 included (12023479 and 11995-040)	Gibco
Roswell Park Memorial Institute (RPMI) 1640 Medium (11530586)	
Neurobasal™ Medium (21103-049)	
GlutaMAX™ Supplement (35050-061)	
Non-essential Amino acids (NEAA) (11350912)	
Sodium Pyruvate (12539059)	
Lipopolysaccharides (LPS) (L7770-1MG)	Sigma Aldrich
Interferon gamma (IFN-γ) Recombinant Human Protein (10474733)	Gibco
Vicenin-2 (V2) (03980585-10MG)	Sigma Aldrich
Epidermal Growth Factor (EGF (Human Recombinant)) and basic Fibroblast Growth Factor bFGF (Human Recombinant) (PHG0024 and PHG0314)	Gibco
StemPro® Neural Supplement (A10508-01)	
B-27™ Supplement (17504-044)	
N-2 Supplement (17502-048)	
PSC Neural Induction Supplement (NIS) (Part of the PSC Neural induction Medium pack) (A1647801)	
Antibiotic-Antimycotic (Pen/Strep) (100X) (15240-062)	
TrypLE™ Express Enzyme (1X) (12604-013)	
Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (12760-021)	
CTS™ CELLstart™ Substrate (A1014201)	
Poly-L-Lysine (PLL) (A3890401)	
Trypan blue stain (15250-061)	

Table 2.2 *List of reagents used for tissue culture*

2.2 Methods

2.2.1 Neural stem cells culture and THP-1 cells culture

Ethical approval was obtained from the Cardiff School of Health Sciences Research Ethics Committee (project ref number 7181approval in appendix). H9 derived human neural stem cells (NSCs) were obtained from Invitrogen (GIBCO® Human Neural Stem Cells (H9 hESC-Derived, Cat no N7800-100). Cells were thawed and established according to the provider's instructions. After thawing, cells were plated in a 6 or 12 well plate, coated with cellstart (Gibco). Cells' medium was changed every second day with the appropriate media and supplements (Knockout DMEM/F12 1x, FGF 20ng/ml, EGF 20ng/ml, Stempro Supplement 2%, Glutamax Supplement 2mM, Pen/Strep 1%). After reaching ~90% confluence (9-12 days post seeding), cells were treated with TrypLE, lifted from their substrate, and sub-cultured in a density of 5×10^4 cells per cm^2 for up to 3 passages before differentiation.

THP-1 cells (Tsuchiya et al., 1980) are a well-established human cell line, that has monocytic characteristics and is often used as a model for monocytes (Bosshart, & Heinzelmann, 2016), were obtained from the American Type Culture Cultures (ATCC). The cells were maintained in 6 well plates or flasks, uncoated. As cells were non-adherent, they were sub-cultured when reaching ~80% confluence ($1x10^6$ cells/ml), seeding approximately $3x10^5$ cells/ml, for up to 30 passages; their supplemented medium (Supplemented RPMI/THP-1 medium) consisted of RPMI 1640, FBS 10%, 1% sodium pyruvate, 1% NEAA, and 1% Pen/strep. Cells were cultured under standard culture conditions (37^oC and 5% CO2) throughout the experiments.

2.2.1.1 Differentiation of NSCs to neurons or astrocytes

Cells' medium was changed to differentiation medium 2 days post splitting, up to passage 7. Cells were seeded at approximately $2.5 - 5 \times 10^4$ cells/cm^2 in the appropriate matrix as indicated in the Gibco differentiation protocol (appendix 1); Geltrex (neurons) or Cellstart (astrocytes) were preferred, as more stable and consistent results were observed. After cells were washed once with DPBS, they were placed in the appropriate medium, which was changed every 4 days. After approximately 2 weeks differentiation was observed in the majority of cells, and the neural stem cell phenotype/morphology (morphology of the undifferentiated neural stem cells: not many characteristic extensions, cells tend to cluster together -as seen in the light microscopy image of NSC in fig.3.1-) changed towards the characteristic neuronal (small soma, few large (usually 1 large one-axon and a few smaller ones) thin processes) or astrocytic phenotype (larger soma, many processes -see fig. 3.1). Gage (2000) shows such morphologies (Figure 1 of his paper for a sketch of representative morphologies) and discusses further phenotypic changes observed in the NSC differentiation towards other cell types, one of which the majority of the cells expressing the characteristic phenotypic markers per cell type (i.e. Tuj-1 in neurons and GFAP for astrocytes) which was also shown here. The two weeks' timeframe was included in the differentiation guidelines of the protocol, and confirmed via personal communication with the provider's representatives. The neuronal differentiation/maintenance medium consisted of Neurobasal 1x, B27 Supplement 2%, Glutamax Supplement 2mM, Pen/Strep 1%; the astrocyte differentiation/maintenance medium consisted of DMEM 1x, N2 supplement 1%, Glutamax Supplement 2mM, FBS 1%, Pen/Strep 1%. More details on the formulations of differentiation/expansion media can be found in appendix 1.

2.2.1.2 Differentiation of THP-1 cells to dTHP-1 macrophage-like cells, or to microglia-like cells

For differentiation towards microglia-like cells, THP-1 cells were incubated with supplemented RPMI, enriched with 50x Neural induction supplement (NIS) (Gibco) (to final dilution of 1x, so used at a final concentration of 2%), and seeded at $3x10^5$ cells/ml. Medium was changed every 3-4 days. More conditions and different media were tested during optimization experiments which will be explained in the appropriate chapter (chapter 4), however the aforementioned conditions produced the most stable and repeatable results. The different conditions included using Astrocyte conditioned medium instead of RPMI as the basal medium, as well as using THP-1 cells that were differentiated into dTHP-1 cells via exposure to PMA (25ng/ml). PMA has been shown to differentiate monocytes, and especially THP-1 cells into M0 macrophage-like cells (dTHP-1 cells). To differentiate towards dTHP-1 cells, after an extensive literature search (e.g. Park et al., 2007; Daigneault et al., 2010; Spano et al., 2013), as well as optimisation by previous members of the lab (including Isa et al., 2011; Ruffino et al., 2016), THP-1 cells were exposed to 25ng/ml of PMA for 24 hours, and then the medium was changed to supplemented RPMI/THP-1 medium; this made the cells adherent. After the 24 hours in THP-1 medium, cells were incubated in either NIS/RPMI or NIS/ACM (so the overall protocol can be summarised as $(PMA)_{24} \rightarrow (THP-1\ medium)_{24} \rightarrow (NIS\ treatment)$). Astrocyte conditioned medium was collected from astrocytes grown in our lab, every 2 days and frozen until use.

2.2.2 Cell viability

Cell viability was measured pre and post treatment with trypan blue (exclusion dye) and a phase contrast microscope. Cells were counted using a haemocytometer or an automatic cell-counter.

For adherent cells, they were lifted using TrypLE, then the reaction was stopped with the addition of medium and the cells were placed in a 15 ml tube; for non-adherent cells, they were placed in a 15 ml tube in their medium. Then in both cases, the cells were centrifuged for 4 min at 200RCF, resuspended in 1ml of the appropriate medium, and after a series of dilutions, mixed with trypan blue, and placed in a haemocytometer. Dead cells appeared blue, due to the dye entering through their membrane, which had started to break down, whereas alive cells did not allow the dye to enter, as their membrane was intact (Strober, 2001). Viability was expressed as % alive cells at different time points. The formula used was alive cells counted/overall cells counted.

2.2.3 Treatment of cells with LPS and IFNγ

In order to investigate how cells respond to inflammatory stimuli, and thus how they contribute to neuroinflammation on their own, the cells were exposed to two potent inflammatory mediators: IFNγ and LPS. After the cells were differentiated, and maintained their phenotype for over a week (for neurons and astrocytes), or in the appropriate day after the addition of NIS to the THP-1 medium (i.e. days 3, 7, and 14), standard media were replaced with the appropriate medium (i.e. the medium the cells were growing in) containing either LPS or IFNγ in different concentrations, and for different incubation times (more details in the appropriate chapters). After each time point, cell supernatants as well as cells were collected for further analyses (ELISA/cytokine arrays, viability, RNA extraction).

2.2.3.1 (Pre)-Treatment of cells with Vicenin-2

For the experiments that included Vicenin-2 (V2) (50 ng/ml, reconstituted in DMSO), the differentiated microglia-like THP-1 cells (mgTHP-1) were incubated in V2 for an hour prior

to either performance of the assay in question, or their treatment with a pro-inflammatory stimulus. V2 remained in the media of the cells during their treatment with the pro-inflammatory stimulus (INFγ, or LPS).

2.2.4 Cytokine and protein analysis

Measurement of cytokines is of great interest in the study of cell to cell communication/interaction, as cell-to-cell interaction can be investigated by focusing on proteins/protein isoforms the cells secrete under certain conditions. Cytokines and proteins are secreted from cells under different conditions, and their receptors can either be found on the surface of cells or be soluble. Quantification of proteins and cytokines can be quantitative (ELISA), or semi-quantitative (Western blotting, cytokine arrays). Here for cytokine analysis we used both a quantitative and a semi-quantitative method (therefore using the quantitative as a control for the semi-quantitative), and for protein analysis we used ELISA for one of the proteins of interest (soluble form of RAGE), while Western blotting was employed for the rest. Details for the products used, can be found in the appropriate chapters.

2.2.4.1. Enzyme-Linked Immunosorbent Assays (ELISAs)

Cytokine analysis was done with ELISA. Specifically, supernatant from untreated and treated cells were collected, and placed in individual 1.5ml Eppendorf tubes. The tubes were then centrifuged at 10000g for 15 min at 4°C to remove debris prior to analysis or storage. Supernatants were then transferred to a new Eppendorf tube. Supernatants were stored at -20 until use. For the ELISAs kits for the cytokines TNFα, IL-6, IL-1β, IL-18, and sRAGE were obtained from R & D systems (full protocols can be found in appendix 1). As per manufacturer protocol, a 96-well plate was coated with the capture antibody specific to the respective

cytokine overnight, and then blocked using 1% BSA in PBS 1X for 1 hour. Standards and supernatants were added for 2 hours. Wells were then incubated with the detection antibody for 2 hours, after which Streptavidin conjugated-horseradish peroxidase (HRP) and TMB Substrate Solution were added to wells for 20minutes before the addition of Stop Solution. The optical density was read at wavelength specified by manufacturer and the concentration of cytokines in supernatants determined using calibration values depending on the cytokine examined. *N.B. for chapter 5 a different type and manufacturer of ELISA is used as well as this type described here. This will be described in the appropriate chapter.*

2.2.4.2 Cytokine arrays

Cytokine arrays for 42 targets were obtained from Abcam (ab133997). This specific array was chosen as it included 3 of the targets that were examined with ELISA (IL-6, Il-1beta, and TNF-α), meaning that comparison and corroboration between the different experimental procedures (i.e. the results from the ELISAs, and the changes between conditions can be used as confirmation for the changes observed in the membranes) would be possible. The other targets detected were additional anti- and pro-inflammatory targets (see chapter 5 for full details). Each membrane consisted of antibody array chips pre-arranged on a membrane, and in duplicate. The method consisted of blocking the membranes with the blocking buffer provided by the kit for 30 minutes, then adding the cell supernatants overnight, and then paired biotinylated detector antibodies (overnight incubation) and streptavidin HRP (with appropriate washes between steps); volumes that were suggested by manufacturer were used-no changes were made in the protocol. Detection was done using HRP chemiluminescent substrate and membranes were exposed to Hyperfilm ECL film (Sigma Aldrich). The film was developed using 1x developer and fixer solutions (Sigma Aldrich). Different exposure times were used in

order to get the clearest images in each case (see chapter 5 for full details). The same methodology was used briefly in chapter 4, in order to potentially detect the ingredients of the differentiation medium used.

2.2.4.3 Western blotting

Western blotting is one of the most widely used protein identification and quantification methods. It relies on epitope-antibody interactions, and therefore more than one antibodies can be used for one protein, or more than one form of protein (e.g. phosphorylated/unphosphorylated or isoforms).

2.2.4.3.1 Protein extraction and quantification

In order to extract total proteins from cells in different differentiation stages, the cell culture medium was removed, and Radio-Immunoprecipitation Assay Buffer (RIPA) was added to the cells on ice. RIPA buffer is a lysis buffer containing sodium dodecyl sulfate (SDS, and essentially disrupts cell membranes as well as nuclear and organelle membranes. After the addition of RIPA, the cells were scraped and homogenised with a pipette (P1000) tip, and then the homogenised samples were sonicated (VCX500 Ultrasonic processor (Sonics & Materials Ltd) for a better protein extraction. The samples were then centrifuged ($14000RCF/15min/4^{o}C$) in order to remove debris, and stored at $-20^{o}C$.

To quantify proteins, and ensure equal loading of wells in terms of total protein, we used the BCA Protein Assay Kit (Cell Signaling technology, cat no 7780S), which relies on the ability of the proteins to reduce copper (Cu) in alkaline solution, which then reacts with bicinchoninic acid (BCA), forming an intense purple colour. This can be quantified in a colorimetric way,

using the absorbance in 562 nm, and a standard curve using BSA as standard, and serial dilutions.

2.2.4.3.2 Protein Electrophoresis and transfer

Proteins in the samples were separated using polyacrylamide gel electrophoresis. NuPage Bis-Tris 10% or 15% gels were used (precast, Thermofisher UK, cat no NP0321BOX), and the same amount of protein was loaded per well (20μg in all cases), after mixing with NuPage 10x reducing agent, and the 4x NuPage LDS (Lithium dodecyl sulfate) Loading buffer (Thermofisher UK, cat no NP0007). Samples were then heated for 5min at 90°C in order for the proteins to be denatured, and centrifuged for 5min at 13000RCF and 4°C. The gels were then placed in an XCell-SureLock Mini Cell tank, filled with MOPS (3- (N-morpholino) propane sulfonic acid) running buffer (Thermofisher UK, cat no NP0001). Samples were subjected to electrophoresis for 50 min, at 180 V. Proteins were then transferred from the gel onto a nitrocellulose membrane using wet transfer (i.e. the gel/membrane sandwich is placed between filter papers and sponges, and submerged in transfer buffer, where an electrical field was then applied), for 2 hours at 40V. This was done so that proteins of interest could be detected on a solid matrix (in this case nitrocellulose).

2.2.4.3.3 Protein detection

The primary antibodies used were: beta-actin (Cell Signalling Technology, β-Actin (13E5) Rabbit mAb #4970), GAPDH (Cell Signalling Technology, GAPDH (14C10) Rabbit mAb #2118, Iba1 (via alphalabs, UK, prod.no 019-19741), or TMEM119 (Biolegend, A16075D). Secondary antibodies were obtained from Biolegend (anti-mouse HRP), or CST (anti-Rabbit HRP). The dilutions were optimised for each antibody, according to the literature,

manufacturer's suggestions, as well as different trials for different concentrations per antibody. With the exception of TMEM119 detection which was used in a 1:500 dilution for the primary antibody, all other proteins of interest were detected using a 1:1000 dilution.

The steps followed for immunoblotting were: after the transfer the membrane was washed with Tris-Buffered Saline/0.1% v/v Tween (TBS-T) for 5 minutes, and then blocked for 1 hour with blocking buffer (BSA (3%)/TBS-T) in room temperature. The membrane was then washed three times for 5 minutes each in TBS-T, and then incubated in the appropriate dilution (see above) of primary antibody in blocking buffer overnight at 4°C. After washing three times for 5 min each, the membrane was incubated in the appropriate dilution of HRP conjugated secondary antibody (1:2000 worked well in all conditions) in blocking buffer, for 1 hour at RT. All steps were under gentle agitation. Visualisation was achieved by incubating the membrane with SuperSignal West Pico PLUS Chemiluminescent Substrate according to manufacturer's instructions, and exposed to Hyperfilm ECL film (Sigma Aldrich). The film was developed using 1x developer and fixer solutions.

To allow re-probing, and detection of further proteins, after exposure, the membrane was washed with TBS-T, then stripped of the initial antibody set using the Restore™ Plus Stripping Buffer (Thermofisher) for 10-15 min. Then, after an additional wash step in TBS-T, the membrane was placed in TBS, and at 4°C until further use (i.e. re-probing with a different primary antibody). In some uses, stripping efficiency was confirmed by re-exposing the membrane, and/or re-incubating with the appropriate secondary antibody, and exposing the membrane.

2.2.4.4 Image analysis and quantification

ImageJ (Rashband, 1997-2019/NIH; Schneider et al., 2012) was used in order to quantify the results of cytokine arrays, where the Protein Array analyser extension (Gilles Carpentier, 2010-2018 v.1.1.c) was used; for western blots the gel analysis function was used.

2.2.5 Immunocytochemistry

Immunocytochemistry (ICC) is widely used to examine morphological cell characteristics, including shape, and size of the cell, shape and size of the nuclei, as well as expression and localisation of certain proteins, as well as for quantification of the number of specific cell types within a population. To do so, cells of interest are grown on coated (coating not needed for microglia-like cells) coverslips, and then fixed, permeabilized (step not necessary for cell surface targets), and then using protein/antibody interactions tagged with fluorescence. Here we used ICC to confirm the identity of neural stem cells, to monitor their differentiation to neurons and astrocytes, and to examine the characteristics of the mgTHP-1 cells during their development. Coverslips were coated with 50 µg/mL Poly-L-Lysine at room temperature for an hour (for mgTHP-11 cells this step was omitted). After washing with sterile water and drying, cells were placed on the coverslips, and were allowed to grow to desirable density. The cells were either differentiated on the coverslips, or were seeded already differentiated. After density was reached, cells were briefly rinsed in PBS, and fixed using 4% paraformaldehyde. Cells were then washed in PBS, and maintained in it, in 4°C until staining.

After permeabilization with EtOH 100% for 5 minutes, cells were washed with PBS (3x5min). Then samples were then blocked in 3% BSA (Bovine Serum Albumin, Sigma Aldrich, cat no A9418-500G)/3% normal serum for the appropriate secondary antibody in PBS for an hour, to block unspecific antibody interactions, and were then washed with PBS three times, for 5 mins

per wash. Cells were then incubated overnight with the appropriate antibody in 3%BSA/PBS

with 3% appropriate serum at 4°C; concentrations and further details are provided in the table

below (table 2.3).

Primary Antibody	Info	Secondary antibody	Labels
Tuj-1 mouse anti-human	Neuromics, Dilution 1:500	Life technologies Alexa Fluor® 488 or 594 Anti-Mouse (250X)	Neurons
GFAP rabbit anti-human	Abcam, Dilution 1:200	Alexa Fluor® 488 or 594 Anti-Rabbit (250X)	Astrocytes
Nestin mouse anti-human	Santa Cruz, Dilution 1:500	Life technologies Alexa Fluor® 488 Anti-Mouse (250X)	Neural stem cells
Cd11b rabbit anti-human	Abcam, Dilution 1:250	Life technologies Alexa Fluor® 488 Anti-Rabbit (250X)	Myeloid cells
Iba-1 rabbit anti-human	Wako, Dilution 1:1000	Life technologies Alexa Fluor® 488 Anti-Rabbit (250X)	Myeloid cells
TMEM119 mouse anti-human	Biolegend, Dilution 1:1000	Life technologies Alexa Fluor® 488 Anti-Mouse (250X)	Microglia subsets
Hoechst 33342	Invitrogen, 1:2000	n/a	Nuclei

Table 2.3: *ICC antibody details*

Cells were subsequently washed in PBS three times for 5 mins each. Cells were then incubated

with the appropriate secondary fluorophore-conjugated antibody in blocking buffer for 2 h at

room temperature in the dark (see Table 2.3 below for concentrations and further details), and

then washed 3 x 5 mins with PBS in the dark. Nuclei of the cells were then counterstained with

Hoechst 33342 (used at 1μg/ml, Invitrogen, cat no H3570), and washed with PBS once more.

Coverslips were mounted on microscope slides with a drop of glycerol:PBS 1:1, sealed with

nail polish to prevent drying and movement under microscope, and stored in the dark at +4

until observation. Observation was performed using a Nikon Eclipse 80i at 40x magnification

and images were captured using Volocity 5.5.using appropriate filters for each secondary

antibody.

2.2.6 qPCR

In order to examine the changes in gene expression under different conditions, Real Time/quantitative reverse transcriptase Polymerase Chain reaction (qPCR) was used. This method is the primary method used in gene expression analysis, as it is highly sensitive, accurate, and with high replication rates between experiments.

2.2.6.1 RNA collection and cDNA conversion

In order to lyse cells, 0.4 ml of TrizolReagent per 10^5 cells was added to cell samples after the various treatments/timepoints (treated and control wells) and after aspirating the supernatant; different cell densities were used in different cell types/treatments (e.g. for mgTHP-1 cells the cell density differed per day, as explained in chapters 4 and 5, but differences were found between experiments (range of densities for mgTHP-1 cells day 14 varied from 60000-150000/ml; all cells per well were collected for RNA extraction in each case). Lysed cells were scraped and mixed with the reagent, according to manufacturer's guidelines. Afterwards, as Trizol's main ingredient is phenol, the phenol/chloroform protocol was followed (full protocol is included in appendix 1). Accordingly, chloroform (0.2ml per 1ml of trizol) was added to the cells/reagent homogenate, and after centrifuging, two phases were produced: an organic, and an aqueous which contains the RNA. After collecting the aqueous phase, isopropanol (0.5ml per 1ml of Trizol) was used in order to precipitate the RNA which forms a pellet. This was then washed in ethanol as described in the protocol in appendix 1, dried, and resuspended in 50µl of nuclease free distilled water. The concentration and purity (A_{260}/A_{280} and A_{260}/A_{230} measurements-with acceptable values ~2; if the values were lower a second round of EtOH washes was done to eliminate phenol residues) of each sample was measured using nanodrop (Nanodrop ND-1000 spectrophotometer, ThermoFisher). Yield differed per tube, but in general

for mgTHP-1 cells it was over 1000ng/μl, and lower for neural cells. The RNA samples were stored at -80°C.

RNA was converted to cDNA (complementary DNA) using the High Capacity cDNA Reverse Transcription kit (ThermoFisher, UK), with the RNA concentration adjusted to 1μg/reaction for each sample, using the Nanodrop measurements, in order to produce 1μg of cDNA. According to the protocol (appendix 1), Reverse transcriptase, random primers, dNTP mix ad buffer were added to the samples, and were incubated in a thermal cycler in the following conditions: 25°C/10 min, 37°C/120 min, 85°C/5 min, and then 4°C until collection. The resulting cDNA samples were stored at -20°C.

2.2.6.2 Taqman qPCR
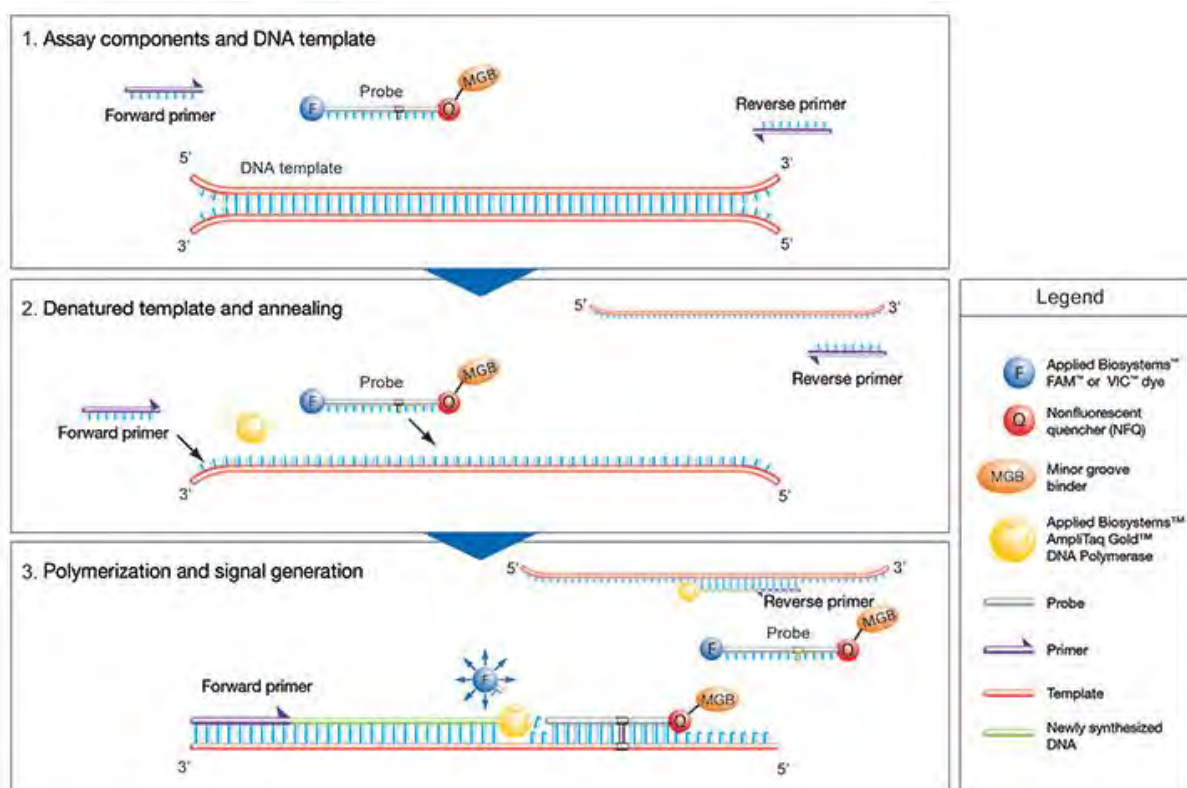
Figure 2.1: *The principle behind the Taqman methodology* (adapted from https://www.thermofisher.com/uk/en/ home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html)

The Taqman methodology is based on the use of fluorescence-tagged oligonucleotides (see van der Veldden et al., 2001 for an overview and figure 2.1 adapted from Thermofisher.com). The probes consist of a reporter and quencher fluorophores, which when they are close to each other, the reporter is quenched. During the extension phase of the PCR, the reporter is released, thus generating fluorescence. Therefore the higher the fluorescence measured, the more abundantly a gene is expressed.

The Taqman probes employed are pre-designed, already optimised, and used successfully in studies, therefore optimization, and efficiency determination were not required, according to provider's instructions. More specifically, the document on the guarantee of the products used (https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1500-PJ9167-CO017361-TaqMan-Guarantee-WhitePaper-Global-FHR.pdf) states that: "Efficiency: Every assay will exhibit 100% ±10% amplification efficiency when tested in reactions over five orders of magnitude of input template" and " Reproducibility through manufacturing quality: TaqMan Gene Expression Assays are made using validated manufacturing processes that include stringent manufacturing QC criteria: assay identity is confirmed by mass spectrometry and assay concentration is determined using quantitative spectrophotometry. TaqMan Assays are formulated ready-to-use right out of the tube, eliminating the need to optimize primer and probe concentrations". Similar instructions of no need for optimisation or efficiency determination were given during calls to the provider to confirm the above. Due to that, optimisation and efficiency determination were mistakenly not carried out, as such validation would confirm that the targets and housekeepers amplify at similar efficiencies; therefore, not running the validations is a deficiency of the thesis, and should be taken into account when interpreting the qPCR results. However, due to the fact that a variety of cell types, as well as genes were used, different housekeeper genes were evaluated for suitability for use. GAPDH and actin were found to be suitable housekeeping genes for already differentiated/mature cells (e.g. neurons

and astrocytes (differentiated from NSCs and not changing phenotypes, as investigated with ICC), neural stem cells (differentiated from H9 cells)), but for differentiating cells they were not as suitable, as during differentiation, the metabolism and shape of the cells changes (see chapter 4 for more details). Therefore, the TBP (tata-binding protein) gene was selected as a housekeeper in the mgTHP-1 cells, as its expression didn't change between differentiation conditions.

The expression of the genes was determined using the comparative Ct method (Livak, K.J, & Schmittgen, T.D., 2001), where the fold change was calculated comparing the gene examined versus the housekeeping gene:

- ΔCt = Ct (gene of interest) – Ct (housekeeping gene), where Ct=cycle threshold, where the fluorescence generated by the PCR reaction is higher than the background noise.

- $\Delta\Delta$Ct = ΔCt (treated sample) – ΔCt (untreated sample)

- Fold change= $2^{-\Delta\Delta Ct}$

All PCRs were performed using the Applied Biosystems Fast 7500 Real-Time PCR system, using the TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (Thermofisher, cat no 4352042).

2.2.7 DNA collection and methylation analysis

In order to investigate epigenetic DNA methylation as a potential mechanism behind the changes in gene expression that we observed during the differentiation of the mgTHP-1 cells, we employed methylation analysis on the DNA of cells at different time points (days 0, 3, 7, and 14 of differentiation). To do so, DNA from the cells was extracted using the Genomic DNA Extraction Kit (ab156900, Abcam) according to manufacturer's instructions (appendix 1). The

DNA was then quantified using nanodrop spectophotometry, and in order to measure overall methylation, the MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (colorimetric) (P1030-96, epigentek), was used. As changes in gene expression can be sometimes attributed to epigenetic changes, this method was employed to show us if there are overall methylation changes in differentiating cells. For example, NIS, which was used to differentiate the THP-1 cells, is used to differentiate Pluripotent stem cells into neural stem cells, a change which has been shown to be accompanied by epigenetic changes (Meshorer & Misteli, 2006; Williams et al., 2006; Bártová et al., 2008). The assay used is a modified ELISA assay, using DNA instead of proteins/cytokines, and capture and detection antibodies directed against methylated nucleotides being quantified colorimetrically. The percentage of DNA methylation (5mC) is proportional to the optical density (OD) intensity measured.

2.2.8 ATP/metabolic measurements

Intracellular ATP content was measured in order to determine the metabolic activity of cells during the different stages of the differentiation process via the Cell Titer-Glo assay which is used generally as a luminometric viability assay, based on the differences in the cellular ATP concentrations ([ATP]cell) between live and dead cells. This assay, employs a reagent that consists of a luciferase/luciferin system and has cell lytic properties (the reagent is produced after mixing the substrate with the buffer provided), which in the presence of ATP produces luminescence. The schematics of the reaction are presented in figure 2.2.
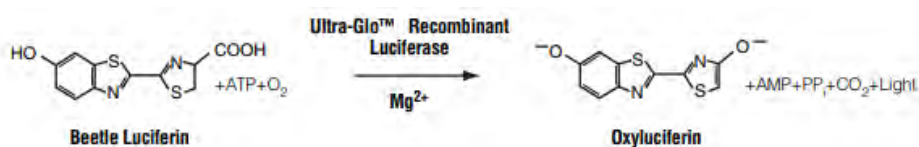
*Figure 2.2.: **The reaction of luciferase/luciferin that makes measuring ATP possible** (as the ATP used is proportionate to the light produced with the reaction. Adapted from the CellTiter-Glo® Luminescent Cell*

Viability Assay Technical Bulletin (https://www.promega.co.uk/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-luminescent-cell-viability-assay/?catNum=G7570)

In general, taking into account the number of cells (as measured with using a haemocytometer-see section 2.2.2), this method can be used to measure the cellular ATP concentrations in different samples or condition, thus providing information about the metabolic activity of the cells. Cells were grown in different conditions in 12 well plates. The cells were then harvested and counted, and then as per manufacturer instructions, added to an opaque white-walled 96 well plate, with the appropriate controls (medium with no cells control, untreated cells control). The plate was then equilibrated at room temperature, and the CellTiter-Glo Reagent was added. After mixing the plate, so that cell lysis was achieved, luminescence was measured, and [ATP]cell results were calculated using a standard curve.

Note: Prior to the ATP measurements, the cells number was determined using a viability assay (Chapter 2.2.2). The amount of ATP was then normalized against the cell number per well, and the results were presented as [ATP]cell for each condition.

2.3 Statistical analysis

In order to determine differences between control and treated groups, ANOVAs with Bonferroni correction or t-tests were conducted. Statistical significance was set at $p < 0.05$, with values < 0.05 set as statistically significant. Data is expressed as mean (M) $\pm$ standard deviation (SD). Error bars represent SD of at least 3 replicates.

Chapter 3

Investigating the pro-inflammatory potential and response of CNS cells, part 1: Neural stem cells, neurons, and astrocytes

3.1 Introduction and aims

In recent times, the focus of the majority of studies about the neuroinflammatory profiling of CNS cells, has been on the microglia (Grabaer et al., 2011). It is also known that in an inflamed brain there are a plethora of different pro-inflammatory cytokines secreted by mostly brain macrophages (including microglia), but as already mentioned in Chapter 1, other neural cells may also secrete cytokines, therefore contributing to neuroinflammation (Turner et al., 2014). As it is not known how or to what extent the non-microglial cells contribute, we wished to investigate whether and how other CNS cells respond to an inflamed environment.

We focused on responses targeting the secretion of IL-6, IL-1β, and TNF-α, as they have been shown to be secreted by macrophages (including microglia) upon inflammatory challenge (Smith et al., 2011), IL-18, as it is involved with the NLRP3 inflammasome (Alboni et al., 2010), and the soluble form of RAGE (RAGEs), as it has been recently proposed to play an important role in CNS disorders (Ding & Keller, 2005).

The inflammatory stimuli selected to mimic the milieu of the inflamed CNS in this instance were IFNγ, and LPS, as they are both potent activators of microglia, and have been shown to have effects on the CNS similar to pathological conditions. For instance, LPS produces fever-like effects, and is used in fever studies (Roth & Blatteis, 2014), as well as studies of bacterial infections; IFNγ is secreted from cells during viral infections, and is used to study these and other CNS-related conditions where it activates secretion of inflammatory molecules from its targets (Lee et al., 2012).

Of the CNS non-microglial cell-types described in chapter 1 which have been found to be able to secrete proinflammatory cytokines, and thus are included in this chapter (i.e. excluding oligodendrocytes), interestingly, it has been shown that neural stem cells possess anti-inflammatory properties (Lee et al., 2008), as well as low immunogenicity

(Hori et al., 2003). Therefore they are not expected to contribute in a pro-inflammatory way. However, some of the cytokines that are investigated in this chapter have been known to have both pro- and anti-inflammatory roles, (e.g. IL-6 (Luo & Zheng, 2016)), something which has also been shown for IFNγ (Mühl & Pfeilschifter, 2003). Mouse neurons have also been known to respond to inflammatory stimuli by secreting inflammatory cytokines (Leow-Dyke et al., 2012), however similar studies in human cells have not been undertaken and hence is important to gain an understanding of how human cells would respond in such conditions. Finally, human astrocytes have been shown to have the potential to secrete pro-inflammatory cytokines *in vitro* (Choi et al., 2014).

More broadly, there is a lack of studies have been conducted where cells from the same genetic origin are used, and which focus on the distinct response of the cells to inflammation, and how this changes upon differentiation of the cells, e.g. from NSC to neurons. Having cells from one origin (i.e. NSC) and comparing the responses of differentiation products of NSCs (i.e. neurons and astrocytes) would be advantageous as it would eliminate confounding factors that can be attributed to different characteristics of the different origins of different cells (e.g. immune responses have been known to have differences due to sex (Klein & Flanagan, 2016; Hanamsagar & Bilbo, 2016), race (Haralambieva et al., 2013), age (Giefing-Kröll et al., 2015), and -as many studies are conducted in rodents- species (Mestas & Hughes, 2004)). Moreover, while the ability to produce homogenous cell populations does remove the effect of interactions between different types of cells, it potentially reveals elements about these cells as well (i.e. their potential activities in the absence of influences from other cells).

From the above, one can conclude that there is a need to investigate how non microglial cells respond to inflammatory conditions, and also to remove as many potential confounding factors as possible by using the same genetic source of cells (i.e. by using cell types which have been differentiated from a common source, i.e. NSCs).

Aims

Therefore, the aims for this chapter are:

a) To differentiate NSCs into neurons and astrocytes

b) To confirm the identity of all cell types using ICC for appropriate markers

c) To investigate the expression of the receptors in NSCs, neurons, and astrocytes for the inflammatory stimuli : for LPS (TLR4, TLR2), and for IFNγ (IfngR1) using qPCR.

d) To treat the aforementioned types of cells with different concentrations of pro-inflammatory stimuli for times that have been previously shown in microglia cells to produce an inflammatory effect (as we want to see if and how non microglial cells contribute to neuroinflammation in the same way (secretion of pro-inflammatory cytokines) and in same timeframe (as determined by the literature)), and to measure the cytokine secretion of NSCs, neurons, and astrocytes for neuroinflammation-related markers, such as TNFα, IL-6, IL-1β, IL-18, and RAGEs using ELISA.

By taking the aforementioned steps, the current chapter aims to answer the questions: Do non-microglial CNS cells respond in the same way, and same timeframe as the microglial cells do as these are indicated in the literature? And do they contribute to the cytokine heavy environment of an inflamed CNS?

3.2 Materials and Methods

3.2.1 Materials

The materials used for this chapter that were <u>not</u> previously mentioned in chapter 2 are the following

Name	Used for	Provider
Human TNF-alpha DuoSet ELISA DY210	ELISA detection of TNFα	R&D Systems/Biotechne Ltd
Human IL-6 DuoSet ELISA DY206	ELISA detection of IL-6	
Human Total IL-18 DuoSet ELISA DY318-05	ELISA detection of IL-18	
Human IL-1 beta/IL-1F2 DuoSet ELISA DY201	ELISA detection of IL-1β	
Human RAGE DuoSet ELISA DY1145	ELISA detection of RAGEs (RAGE isoform sRAGE-delta)	
GAPDH probe (Hs99999905_m1)	qPCR-housekeeping gene	Thermofisher
TLR2 probe (Hs01014511_m1)	qPCR-expression	
TLR4 probe (Hs00152939_m1)	qPCR-expression	
IfngR1 probe (Hs00988304_m1)	qPCR-expression	

Table 3.1 *Materials used for this chapter*

3.2.2. Methods

3.2.2.1 Neural stem cells culture and differentiation

H9-derived NSC (GIBCO® Human Neural Stem Cells (H9 hESC-Derived, Cat no N7800-100) were cultured and maintained as described in section 2.2.1. Cells were kept in liquid nitrogen until use. Cells were thawed by swirling the vial of cells in a 37°C water bath until the ice had melted; cells were then transferred in a 50ml tube, into which pre-warmed complete NSC medium (as described in 2.2.1) was added dropwise. Cells were then centrifuged for 4min at 200RCF; medium (and cryoprotectant) were aspirated and cells were re-suspended in 2ml of complete NSC medium, counted, and seeded at a density of 1×10^5 cells per cm^2. Cells were left to adhere for 24 hrs, after which the medium was changed. 90% confluency was observed after 6-7 days, and after which cells were split, and either remained as NSCs or differentiated, as described in chapter 2.2.1. Differentiation was achieved by changing the medium to neuronal or astrocyte differentiation medium after splitting the cells, and by seeding a different number of cells, depending on the desired cell type. Differentiation was observed visually as change of phenotype to a neuronal or astrocytic phenotype after 10-14 days of incubation in the differentiation medium, and experiments were carried out after an additional 14 days. Only cells which had retained the differentiated phenotype were used in the subsequent experiments.

3.2.2.2 Immunocytochemistry

In order to confirm the identity of neurons and astrocytes post-differentiation, and to ensure that the NSC maintained their characteristics, immunocytochemistry (ICC) was employed. The method is described in detail in section 2.2.5. Briefly, cells were grown in pre-coated round coverslips, in 12-well plates, in the appropriate (differentiation or maintenance) medium. According to the timeframe provided above, cells were fixed, and antibodies for cell

type-specific proteins were used (double staining was used for Tuj-1/GFAP to exclude differentiation to cell types that were unwanted for the experiments); cell nuclei were counterstained with Hoechst 33342. Cells were then viewed using a Nikon Eclipse 80i at 40x magnification and images were captured using Volocity 5.5.using appropriate filters for each fluorophore-conjugated secondary antibody. The antibodies used for this chapter were Tuj-1 mouse anti-human (neurons), GFAP rabbit anti-human (astrocytes), and nestin mouse anti-human (NSCs), with the appropriate secondary antibodies, concentrations and details of which are described in table 2.3.

3.2.2.3 qPCR

As the cells were to be exposed to different stimuli, it was essential to investigate whether the cells that were used were expressing the receptors for these stimuli. To do so, qPCR was performed as described in chapter 2.2.6; details of the probes are described in table 3.1.

3.2.2.4 Treatment of cells with pro-inflammatory stimuli

Cells were treated with IFNγ or LPS as described in section 2.2.3. Based on the literature review, it was decided to treat samples with two IFNγ concentrations (500U/ml and 1000U/ml) for 24 hours, as these conditions are used in a variety of microglia/mixed cell culture studies, and elicited a cytokine secretion-related inflammatory response from the cells investigated in those studies (e.g. Goodwin et al., 1995; Rozenfeld et al., 2003; Mangus et al., 2005) . Similarly, for LPS 3 different concentrations were used (100ng/ml, 1μg/ml, and 10μg/ml) for 4 different time points (1h, 3h, 6h, and 24h), as determined by the literature and additionally in order to cover as many timepoints as possible (e.g. McMillian et al., 1997; Gao et al., 2002; Olajide et al., 2013; Jung et al., 2005). N=3 biological replicates were used per condition (with 2 technical replicates each averaged per condition).

3.2.2.5 ELISA

ELISAs were performed as described in section 2.2.4.1. The kits used are described in table 3.1, and the detailed protocols can be found in the method protocol related appendix (Appendix 1). In summary, supernatants from the treated cell and control samples were collected after the time stated in section 3.2.2.4 and frozen in -20º C until use. After coating the plate with the capture antibodies, and blocking, the supernatants were added. After a wash step, the appropriate detection antibody was used, and then after a subsequent incubation and another wash step, streptavidin-HRP was added. After a further incubation and wash step the substrate to the enzyme (TMB) was added, and after a short incubation the stop solution was added. The concentration of the cytokines in the supernatant was determined by measuring the optical density at a wavelength instructed by the manufacturer, and by using the appropriate standards to produce a standard curve.

3.2.2.6 Cell viability/cell counting

In order to ensure that the results observed were not due to cell death, the viability of the cells was measured under different conditions (control vs 24 h highest concentration for both stimuli) using trypan blue, with both the cell number and the alive/dead numbers taken into account. The methodology is described in 2.2.2.

3.2.2.7 Statistical analysis

Statistical analysis was performed using SPSS, where one-way ANOVAs with Bonferroni post hoc was used in order to determine differences between control conditions and treatments. Significance was set at $p<0.05$, and indicated by asterisks (*) (* = $p<0.05$, ** for

p<0.01, *** for p<0.001, and **** for p<0.0001). Values are expressed as mean ± Standard deviation (SD). Unless otherwise stated, n refers to biological replicates.

3.3. Results

3.3.1 Differentiating NSCs to astrocytes and neurons

3.3.1.1 Astrocytes and neurons-ICC and morphology

Confirmation of differentiation of the cells to desired forms was achieved using morphological observation, as well as Immunocytochemistry for the appropriate markers, namely nestin for neural stem cells, GFAP (Glial fibrillary acidic protein) for astrocytes, and Tuj-1 for neurons. Examples of neural stem cells differentiated into neurons and astrocytes can be found in image 3.1. Tuj-1 is a marker for neurons, and the gene encodes a neuron specific Human β-Tubulin form (Lee et al., 1990; Memberg & Hall, 1995). GFAP is an astrocyte marker, and encodes a cytoskeleton-related protein (Sofroniew & Vinters, 2010). Nestin is an intermediate filament, which is present in neural stem/progenitor cells (it has been found up until the radial glia stage (Moss et al., 2016)), however is not usually present in mature neurons or astrocytes.

The ICC overall confirmed that the cells had the desired identity, as the fluorescence images showed that in the majority of cases they were expressing the protein marker corresponding to their cell type. The % of differentiation or differentiation efficiency is usually calculated by FACS and/or by using multiple markers per cell type (in this case aquaporin-4 could have been used for astrocytes in addition to GFAP, and MAP-2 for neurons in addition to Tuj-1) which was not carried out in this case. However from the ICC experiments, 95% of neurons and 98% of astrocytes were expressing the appropriate markers (i.e. 95 positive for every 100 cells counted), so an estimate of the differentiation is the aforementioned numbers. The cells not showing any fluorescence were few, and that was potentially due to ICC methodology

issues. However, it should be noted that for neurons and astrocytes, double staining was performed to ensure that the cells were differentiating towards one cell type, and there was some GFAP expression detected in a few neurons that were also expressing Tuj-1 (<1%, figure A1 in appendix 2). This could be due to the nature of the cells, as some neurons and neuronal progenitor cells have been shown to express GFAP (Garcia et al., 2004; Bi et al., 2011; Hol et al., 2003), suggesting that the cells do not differentiate entirely in synchrony.
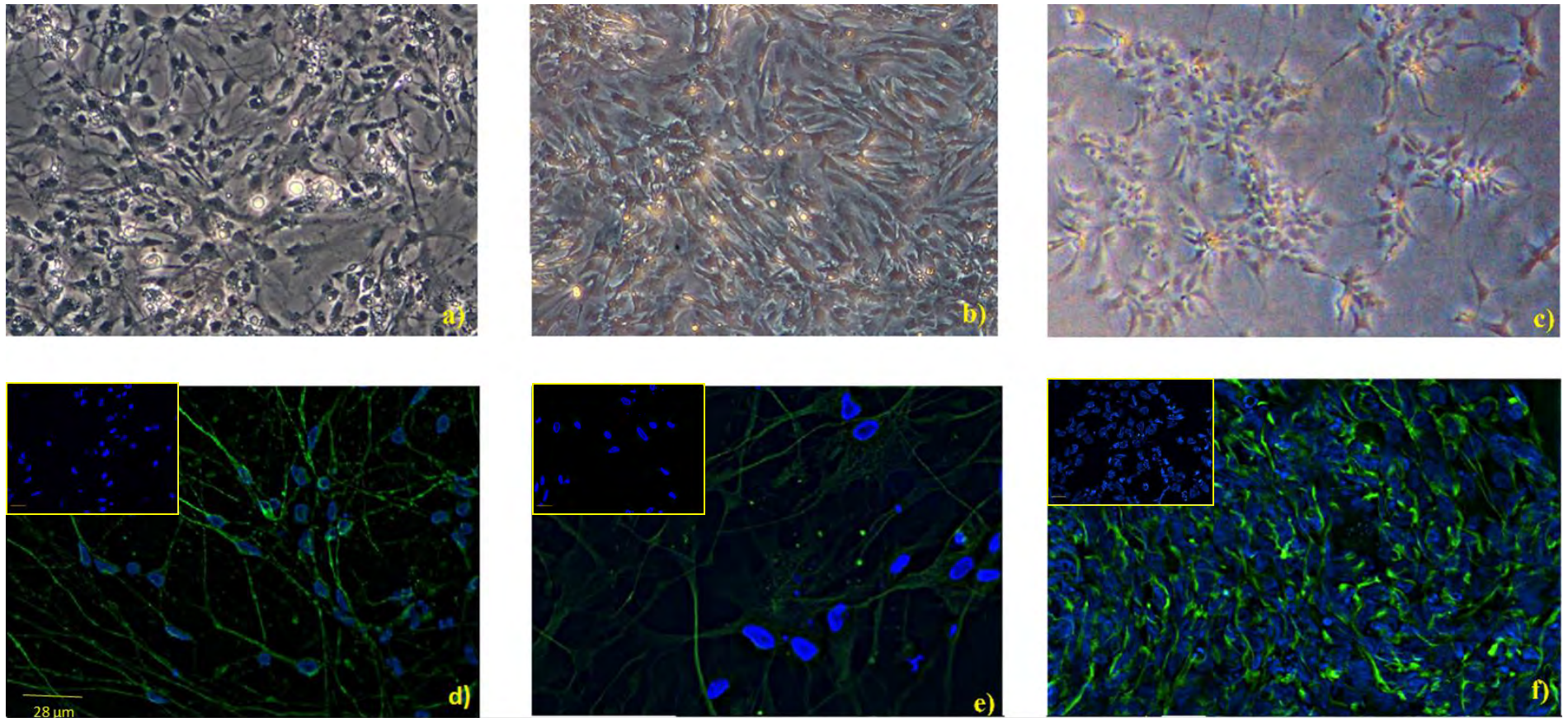
Figure 3.1 Morphological characteristics of Neurons, astrocytes, and NSCs, and ICC results Representative phase contrast (light microscopy) images of neurons (a), astrocytes (b), and neural stem cells(c); d, e, f: representative fluorescent microscopy images of neural stem cells (f) stained with Hoechst (blue), nestin (green), neurons (d) stained with Hoechst (blue) and tuj-1(green), and astrocytes (e) stained with GFAP (green), and Hoechst (blue). Scale bar: 28μm . Negative controls (for primary antibody) are presented at the top left corner of each ICC image (yellow box). Images representative of at least n=3

3.3.2 Effect of LPS and IFN-γ

3.3.2.1 qPCR results

qPCR based gene expression assays for the LPS and IFN-γ receptors TLR4, and IFNγR1 were performed (Figure 3.2). Both receptors were found to be expressed in all of the cells investigated. TLR4 was expressed higher in astrocytes (1) than what it was in NSC (0.31±0.13), while neurons had the highest expression (1.19±0.14). IFNγR1 was expressed the least in neurons (0.35±0.05), and showed similar expression between NSCs (1.04±0.19) and astrocytes (1). TLR2 was also investigated, and while it was expressed in astrocytes (1) and neurons (0.74±0.02), its expression was not detected in neural stem cells. One way ANOVAs were performed for each receptor, and for TLR-4 ($F_{(2,6)}=28.015$, $p<0.001$) a Bonferroni post hoc found that significant differences exist between the expression of NSCs and astrocytes, and NSCs and neurons ($p<0.01$ for both). For IFNγR a one way ANOVA showed a significant difference between cell types in the expression of the gene ($F_{(2,6)}=17.301$, $p<0.01$). A Bonferroni post hoc showed that neurons expressed the gene in lower amounts than both astrocytes and NSCs ($p<0.01$ for both). For TLR-2 a one way ANOVA showed significant differences between different cell types ($F_{(2,6)}=3151.58$, $p<0.0001$). A Bonferroni post hoc found that astrocytes were expressing TLR-2 significantly higher than the other two cell types, while neurons were also expressing TLR-2 higher than NSCs, where it was not detected.

N.B. Astrocytes were used as the reference group (therefore had a value of 1 with no error bars-similar methodology in representation of control is widely used in a variety of fields, e.g. Omran et al., 2013; Withers et al., 2018; Tu et al., 2018), as NSC did not appear to express detectable levels of TLR2 mRNA, and thus calculations using them as reference

would be problematic. GAPDH was used as a housekeeping gene as it showed invariant expression between different cell types.
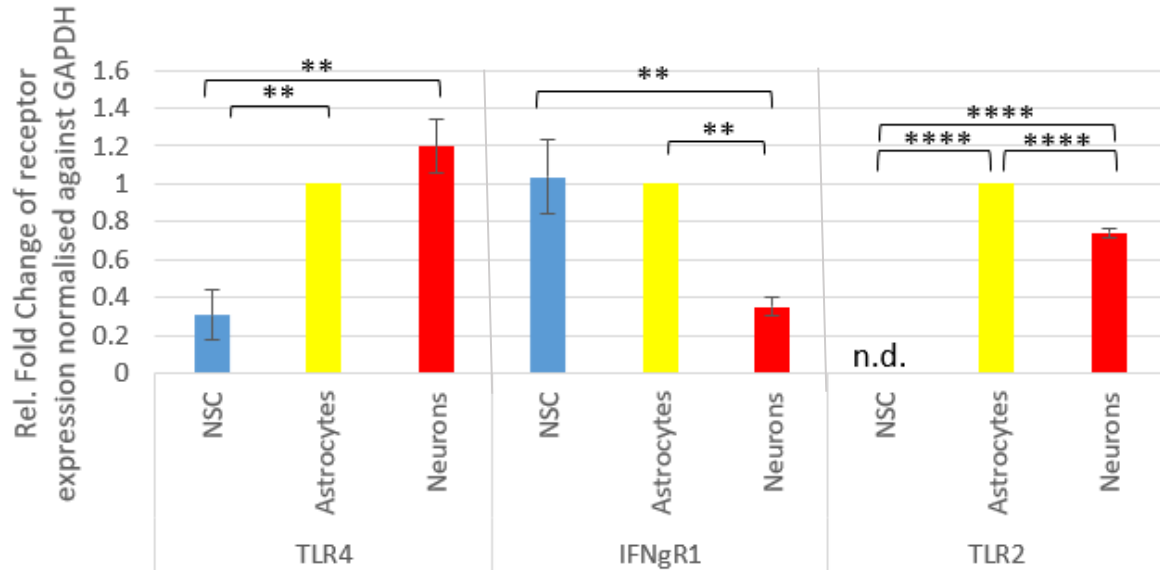
Figure 3.2** Expression levels of the receptors for LPS TLR2 and TLR4, and receptor for IFNγ IFNgR1, as detected using qPCR*. The results are grouped per receptor. Blue coloured bars: NSC, yellow coloured bars: astrocytes, red coloured bars: neurons. Results are expressed as Fold change in expression ± SD when compared the "control group" (i.e. astrocytes), using the housekeeping gene GAPDH. n.d.: Not detected. Significant differences marked with ** (P<0.01), *(P<0.0001), n=3

3.3.2.2. Viability and ELISA results

Viability

No change in viability was observed between pre and post treatment samples when measured with a haemocytometer and trypan blue as an exclusion dye, between all treatment conditions tested. The viability in the control group and after 24h of the highest concentration of treatments (i.e. 10μg/ml LPS and 1000U/ml IFNγ) are represented in figure 3.V. A two way ANOVA showed no effect of treatment or interaction, but there was a main effect of cell type (F(2,18)=75.1378, p<0.001), with a post hoc test revealing that neurons in all cases had a lower viability than the corresponding treatments or controls of the other cell types(Fig 3.V).

However no significant impact of either LPS or IFNγ treatment vs control was observed for the measured samples of either cell type.. This is likely because these cells are more sensitive to any procedure that requires enzymatic detachment from their substrate and/or exposure to the conditions needed for the counting/viability procedure. Cell viability results could have been more accurate should a more sensitive detection method been used. Such methods include methods detecting not only alive/dead cells (the Trypan blue method used here shows essentially whether the cellular membrane is intact or not; cell membrane is not affected in certain forms of cell death, e.g. autophagy), but show whether the cells are proliferative and overall healthy (as for example senescent cells do not proliferate, and react differently to stimuli (van Deursen, 2014)). Examples of methods measuring proliferation include the BrdU/EdU assays which focus on the incorporation of BrdU into newly synthesised DNA and can be measured with ICC/FACS/ELISA. The MTT assay (which measures both proliferation and cytotoxicity) in which alive cells "digest" the yellow MTT and produce a purple product which is then measured in a spectrophotometric way as well as ATP-based assays (such as the celltiter-glo used in chapter 5), live/dead assays (using stains for alive cells (e.g. calcein-AM), dead cells (propidium iodide) and a nucleus stain for the overall cells), antibodies (e.g. Ki67) for cellular proliferation, and other methods such as TUNNEL, which measure whether the cell's DNA is intact are also used.
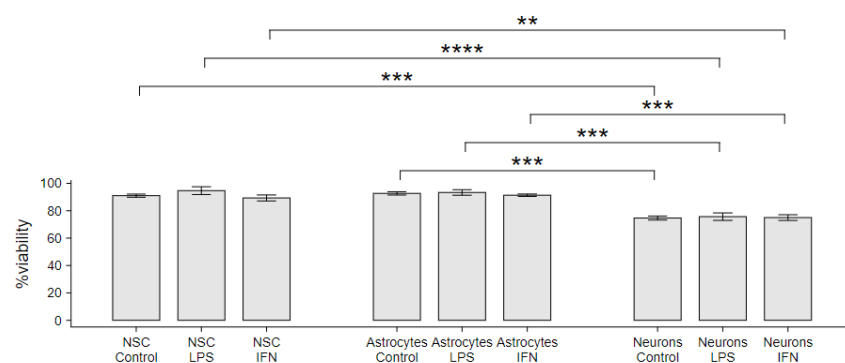

Figure 3.V: Representation of the viability results for the different cell types under different conditions, for 24h. No significant differences were observed between control and LPS or IFNγ treatment groups. N=3

ELISAs

Neural stem cells, astrocytes and neurons were treated with either 100ng/ml, 1µg/ml, or 10µg/ml of LPS for 1, 3, 6, and 24 hours, or treated with 500 or 1000 U/ml IFNγ for 24 hours. All experiments were done in triplicate, and ELISA samples were tested $\geq$ 3 times each. The results are presented (see figure 3.3) for each cell type and cytokine and comparisons within different treatment groups (i.e. 100ng/ml, 1µg/ml, or 10µg/ml of LPS and 24h of IFNγ treatment) are performed using 1-way ANOVAs with Bonferroni corrections when significance is found; no comparison between cell types was undertaken as the aim of the chapter is not to investigate differences between cell types, but to investigate whether each cell type contributes or not) for each cell type/control. Overall the change in cells' cytokine secretion after treatment was limited. For the majority of the cases, secretion was under 20pg/ml (see subsequent pages for more detailed coverage). For all the LPS-treatments, the timepoints are represented from left to right, and colour-wise: light blue: control, orange: 1h incubation, grey: 3h incubation, yellow: 6h incubation, darker blue: 24h incubation. For all IFNγ treatment graphs, and from left to right, treatments are as follow: grey/green: control, light green: 500U/ml IFNγ treatment (24h), dark green: 1000U/ml IFNγ treatment (24h).

__N.B:__ In cytokine amounts as small as the ones in the results below, and differences ~2pg/ml between conditions, statistical significance does not often equate to biological significance. Nevertheless, in highly regulated tissues such as the brain, that might or might not be the case. To our knowledge, such differences and their potential effects have not been investigated.

3.3.2.2a Neural stem cells

TNFα secretion

For TNFα secretion in NSC, for LPS treatments of 100ng/ml and 1μg/ml no significant changes were observed. When NSCs were treated with 10μg/ml LPS, a 1 way ANOVA ($F_{(4,10)}=21.165$, $p=0.0001$) with Bonferroni correction revealed that there was a significant decrease vs the control condition and 1h treatment ($p<0.0001$), between control and 3h ($p<0.01$), and control and 6h ($p<0.05$); significant differences were also found between 1h and 3h treatments ($p<0.05$), 1h and 6h ($p<0.01$), and 1 and 24 h ($p<0.001$). No significant changes were observed upon treatment with IFNγ.

Figure 3.3 (a-d) TNFα secretion from NSCs under different treatments Figures 3.3a and b represent the secretion of TNFα from neural stem cells after 1-24h of treatment with 100ng/ml (a) and 1μg/ml (b) of LPS. Figures 3.3c and d represent the secretion of TNFα from neural stem cells after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Statistically significant differences vs control are indicated by *($p<0.05$), ** ($p<0.01$), ***($p<0.001$) or ****($P<0.0001$). n=3

IL-6 secretion

For IL-6 secretion in NSC, for LPS treatments of 100ng/ml and 1µg/ml no significant changes were observed. A one way ANOVA showed significant differences between conditions for 10µg/ml of LPS treatment, and a Bonferroni post hoc revealed a significant transient decrease ($p < 0.01$) of the secretion of IL-6 when compared to the control (8.18 ± 0.2 pg/ml) was observed after 3 hours of LPS (10µg/ml) treatment (6.7 ± 0.18 pg/ml) and also significant changes between 1h and 3h of treatment ($p < 0.01$), 3h and 6h of treatment ($p < 0.001$), and 3h and 24h of treatment ($p < 0.001$). In contrast, a significant upregulation in NSC-derived IL-6 secretion was observed after 24 h of IFNγ treatment (1000U/ml) (8.02 ± 0.28 pg/ml vs 9.35 ± 0.31 pg/ml; $p < 0.05$) ($F_{(2,6)} = 5.89$, $p < 0.05$, with a Bonferroni post hoc test performed also). However, as the magnitude of both of these effects are of ~1-2pg/ml it's doubtful that they represent any biological significance.
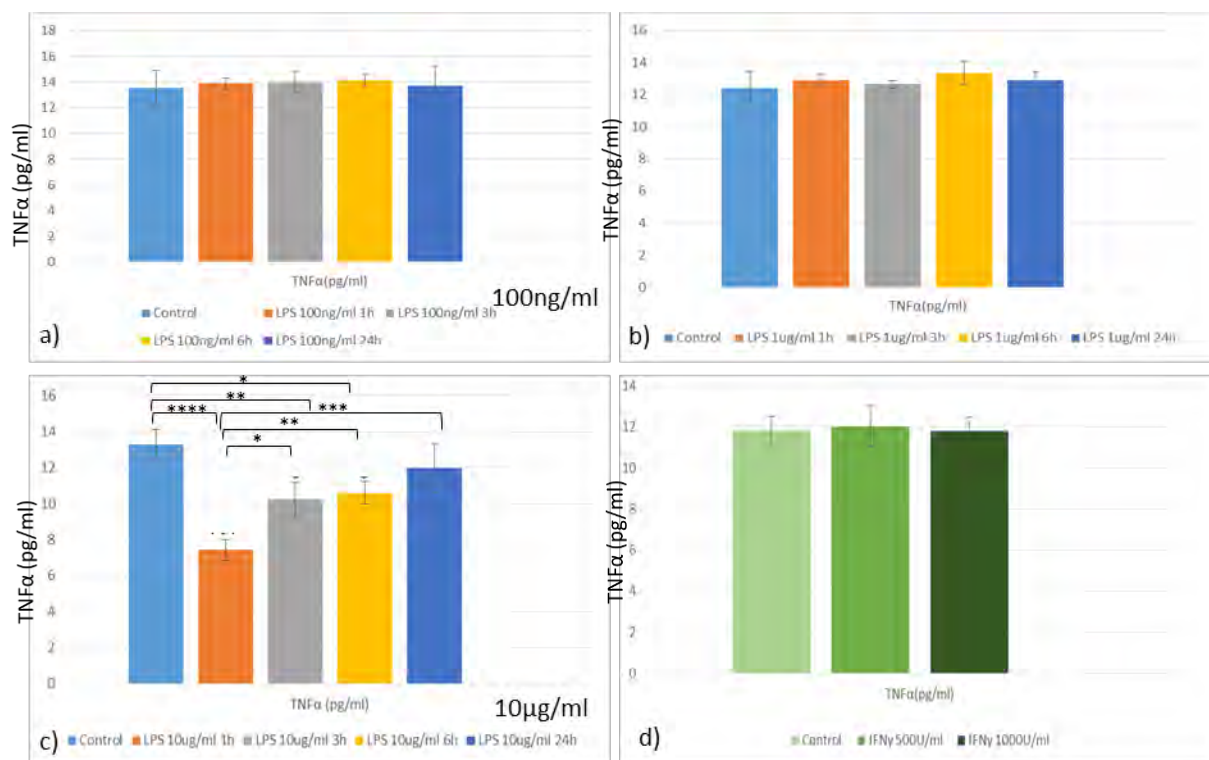

Figure 3.4 (a-d) IL-6 secretion from NSCs under different treatments Figures 3.4a and b represent the secretion of IL-6 from neural stem cells after 1-24h of treatment with 100ng/ml of LPS (a) and 1µg/ml of LPS (b). Figures 3.4c and d represent the secretion of IL-6 from neural stem cells after 1-24h of treatment with 10µg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Statistically significant differences vs control are indicated by *($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). n=3

IL-1β secretion

For IL-1β secretion in NSC, for LPS treatments of 10 μg/ml a one-way ANOVA showed significant differences ($F_{(4,10)}=5.78$, $p<0.05$), which a Bonferroni post hoc test found it to be between control and 3h treatment ($p<0.01$) as well as between 3 and 24h treatments ($p<0.05$). For 1μg/ml LPS treatment, a one way ANOVA showed a significant difference ($F_{(4,10)}=6.4078$, $p=0.008$), and a Bonferroni post hoc showed that there was a significant decrease (marked with an asterisk (*)) ($p<0.05$) observed in the secretion of the cytokine after 3 hours of treatment compared to control, but also 3h compared to 24h treatment ($p<0.05$). No significant changes in IFNγ or 100ng/ml LPS treated cells were observed.

Figure 3.5 (a-d) IL-1β secretion from NSCs under different treatments Figures 3.5a and b represent the secretion of IL-1β from neural stem cells after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.5c and d represent the secretion of IL-1β from neural stem cells after after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results ($p<0.05$) are marked with an asterisk (*), and $p<0.01$ with (**).n=3

IL-18 secretion

For IL-18 secretion in NSC, for LPS treatments of 100ng/ml and 1μg/ml there no significant difference. However, a 1 way ANOVA showed significant changes for LPS (10μg/ml) treatment, with a Bonferroni correction showing significant changes between control (19.8±0.8pg/ml) and 1h (15.6±1.12 pg/ml) but also 3h of treatment (16.04±1.09pg/ml) ($p<0.01$ for both) as well as between control and 6 hours (16.83±0.41pg/ml($P<0.05$). Further differences were observed between 1h of treatment and 24h (19.98±1.23) between 3h and 24h ($p<0.01$ for both). A significant increase between 6h and 24h of treatment was also observed ($p<0.05$). No significant changes in IFNγ-treated cells were observed.
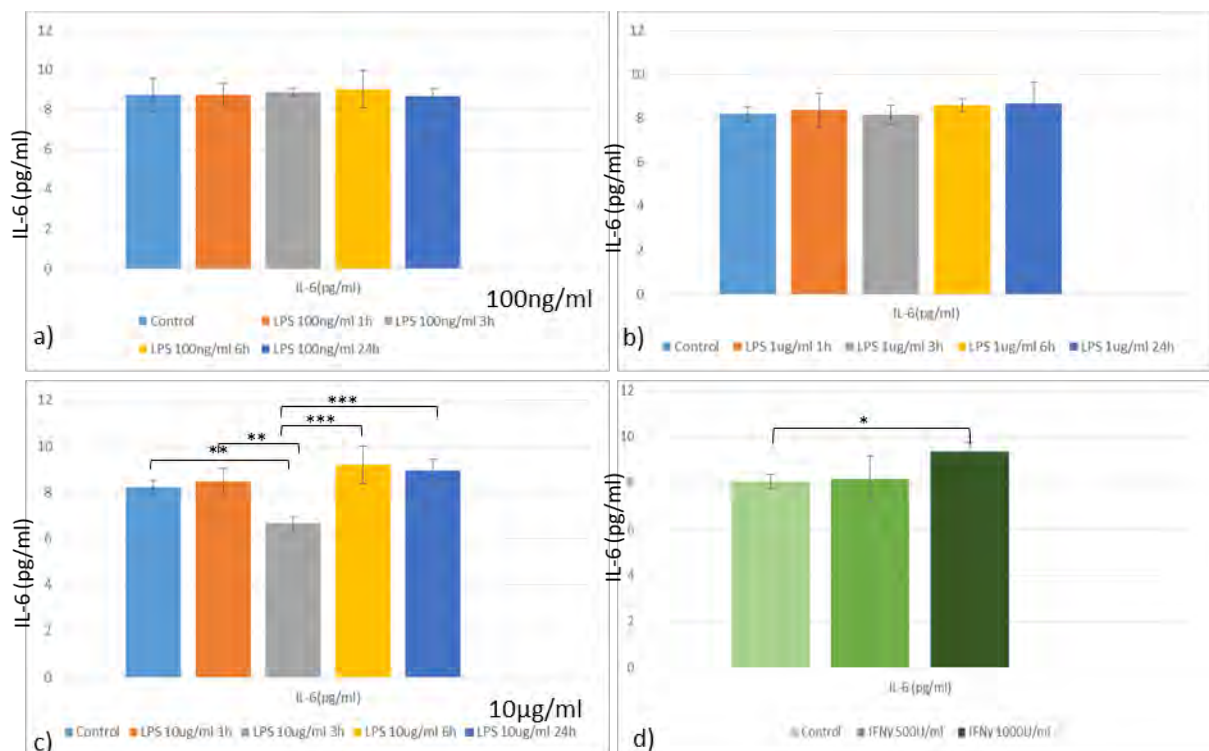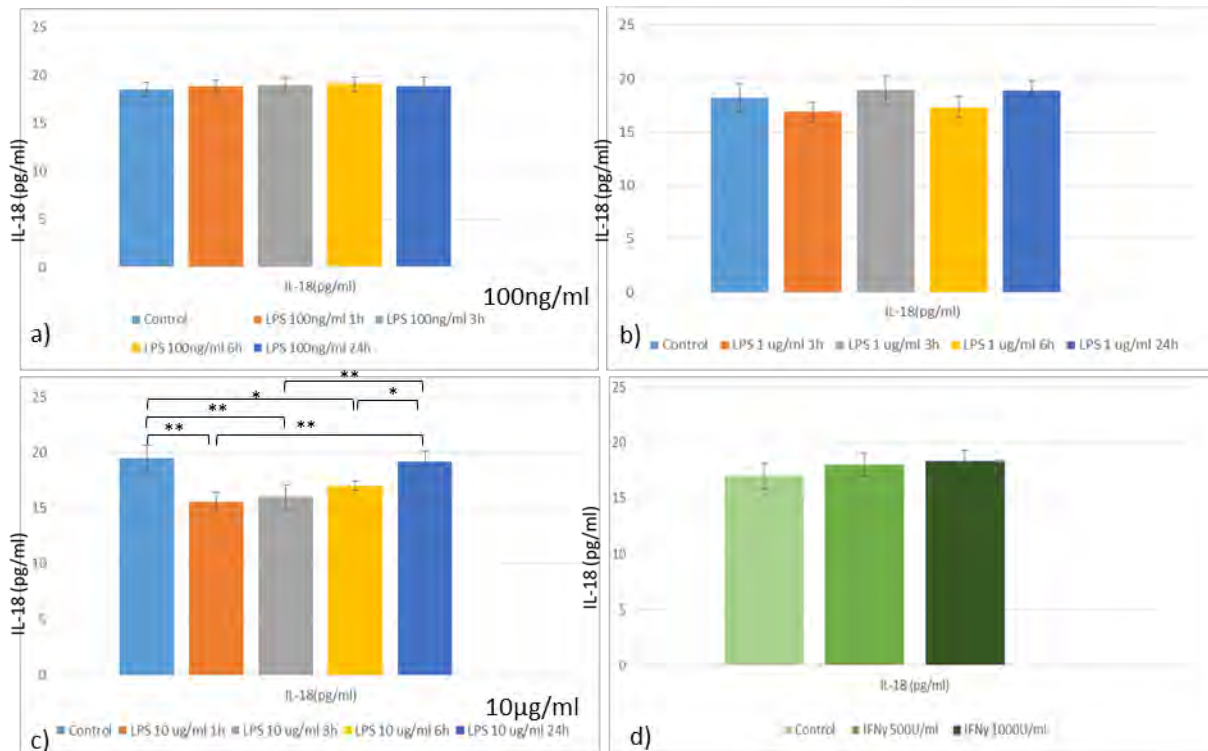

Figure 3.6 (a-d) _IL-18 secretion from NSCs under different treatments_ Figures 3.6a and 3.2.14 represent the secretion of IL-18 from neural stem cells after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). No significant changes were observed. Figures c and d represent the secretion of IL-18 from neural stem cells after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results ($p<0.05$) are marked (*), and $p<0.01$ are marked (**). n=3

RAGEs secretion

For the secretion of RAGEs from NSC, no significant changes were observed with LPS treatments of 100ng/ml or 1µg/ml. For treatment with LPS (10µg/ml) a 1-way ANOVA revealed differences between the conditions (F(4,10)=18.22,p=0.0001). A Bonferroni correction revealed differences between control and 3 and 24 hours of treatment (p<0.001), and further differences between 6h treatment and 24h treatment (p<0.001), as well as differences between 1h treatment and 24h treatment (p<0.01). A one way ANOVA showed a significant change upon treatment with IFNγ (F(2,6)=8.012, p<0.05) with a Bonferroni post hoc test revealing that treatment for 24hrs with 1000U/ml of IFNγ decreases the amount of cytokine secreted vs the 500U/ml condition and the control.
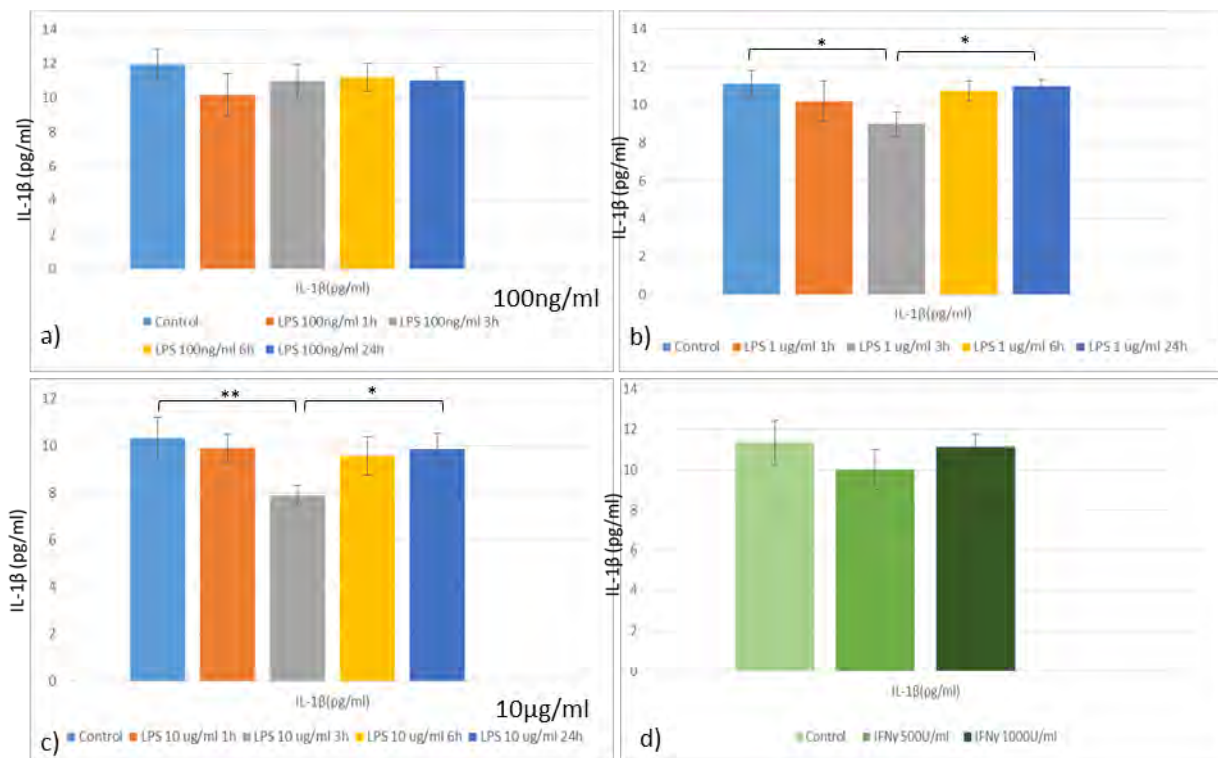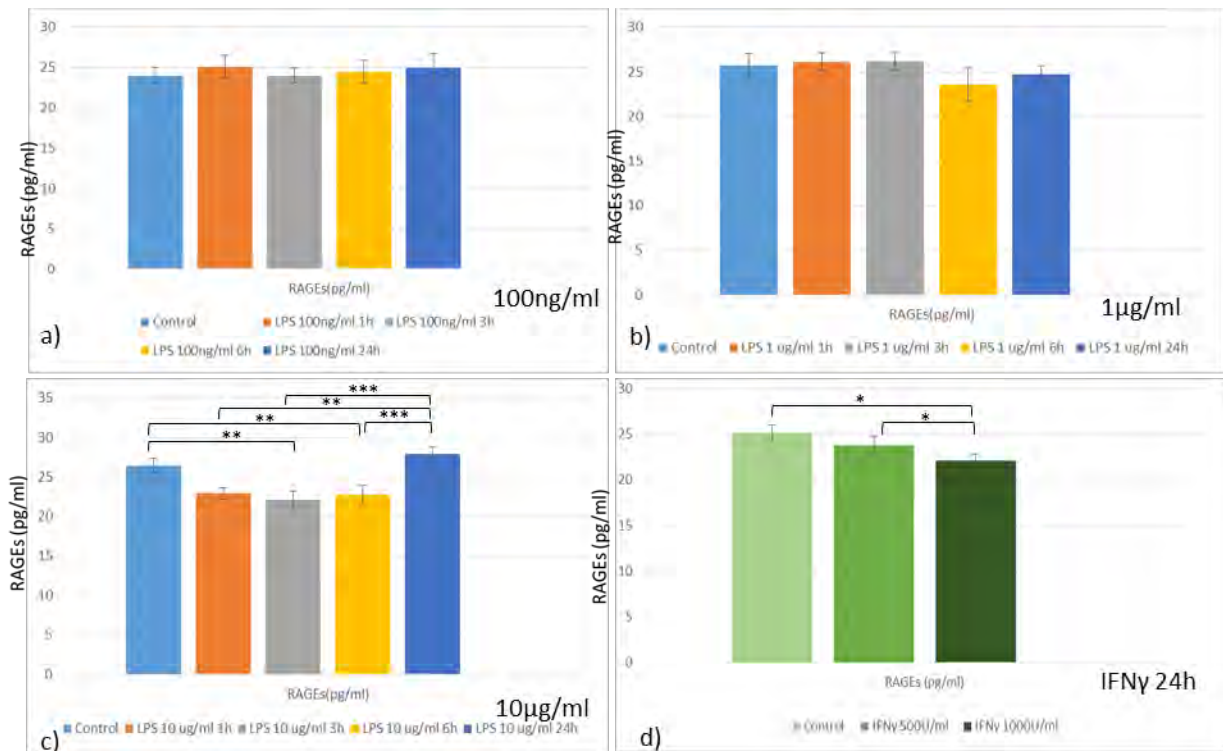

Figure 3.7 (a-d) RAGEs secretion from NSCs under different treatments Figures 3.7a and b represent the secretion of RAGEs from neural stem cells after 1-24h of treatment with 100ng/ml of LPS (a) and 1µg/ml of LPS (b). 3.7c and d represent the secretion of RAGEs from neural stem cells after 1-24h of treatment with 10µg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results (p<0.05) are marked with an asterisk (*), p<0.01 with (**), p<0.001 with (***). n=3

3.3.2.2b Astrocytes

TNFα secretion

For astrocytes, treatment with either proinflammatory stimulus did not affect in a significant

way their secretion of TNFa.

Figure 3.8 (a-d) TNFα secretion from astrocytes under different treatments Figures 3.8a and b represent the secretion of TNFα from astrocytes after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.8c and d represent the secretion of TNFα from astrocytes after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. n=3

IL-6 secretion

IL-6 secretion from astrocytes was not significantly affected by treatment with any of the three LPS doses. There is, however a statistically significant increase of ~2pg/ml after 24hrs of treatment with 500U/ml IFNγ (8.37±0.58 vs 10.55±0.47 pg/ml, and 8.55±1.05 vs 10.55±0.47 pg/ml p<0.05) as determined by a one way ANOVA with Bonferroni correction (F(2,6)= 7.8740, p<0.05).

Figure 3.9(a-d) IL-6 secretion from astrocytes under different treatments Figures 3.9a and b represent the secretion of IL-6 from after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b).Figures 3.9c and d represent the secretion of IL-6 from astrocytes after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results (p<0.05) are marked with an asterisk (*). n=3

IL-1β secretion

Astrocyte secretion of IL-1β was affected by the treatments in different ways. No difference was found when the cells were treated with 100ng/ml or 10μg/ml of LPS. A one way ANOVA revealed significant differences between conditions in the treatment with 1μg/ml LPS ($F_{(4,10)} = 8.36$, $p < 0.01$). A Bonferroni post hoc showed that these differences were between control and 1h conditions ($p < 0.01$), 1h and 3h conditions, ($p < 0.01$), 1h and 6h conditions ($p < 0.01$), and 1h and 24h conditions ($p < 0.05$). There was a decrease of cytokine secretion was observed after 24h of treatment with 1000U/ml of IFNγ, however the small magnitude (≈ 1.5pg/ml) of this effect suggests that although statistically significant ($F_{(2,6)} = 6.928$, $p < 0.05$, and $p < 0.05$ for the differences between the 1000U/ml and both the 500U/ml and the control), it is probably not biologically significant.
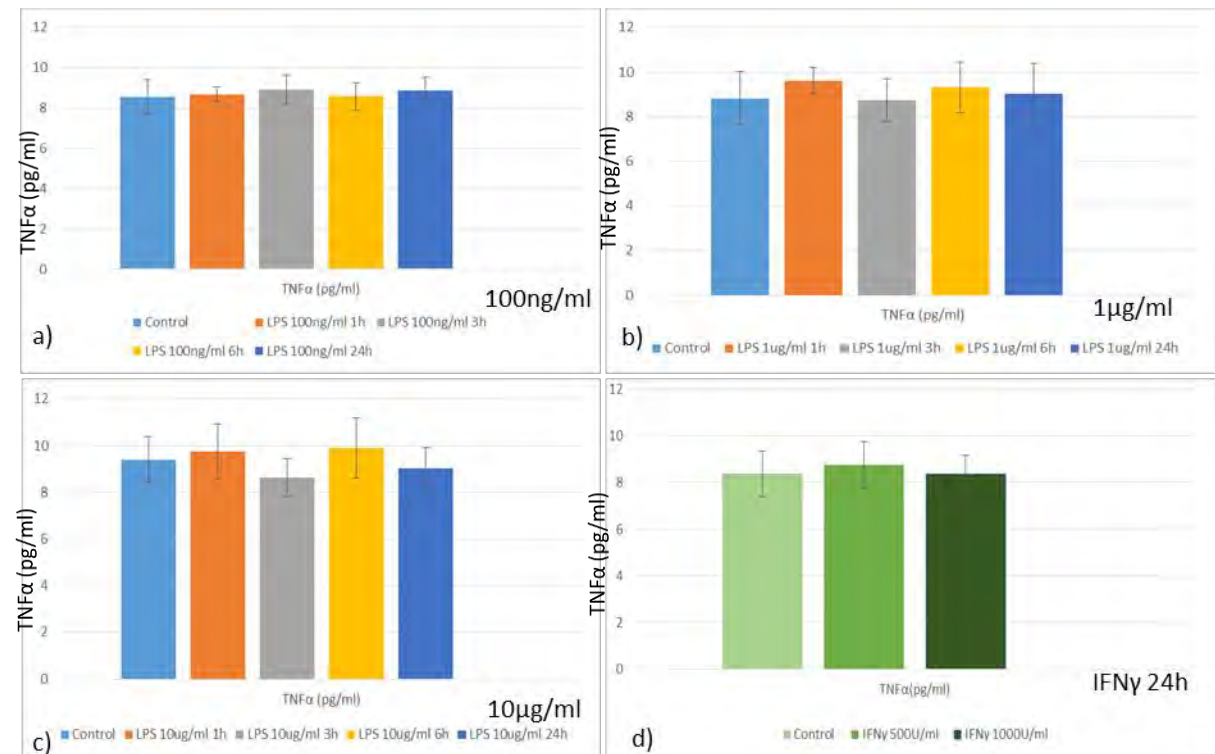

Figure 3.10(a-d) IL-1β secretion from astrocytes under different treatments Figures 3.10a and b represent the secretion of IL-1β from astrocytes after 1-24 of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.10c and d represent the secretion of IL-1β from astrocytes after 1-24 of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant changes marked with an asterisk (*), $p < 0.05$, and (**), $p < 0.01$ n=3

IL-18 secretion

The amount of IL-18 secreted by astrocytes was not affected in a significant manner after treatment with either LPS or IFNγ.
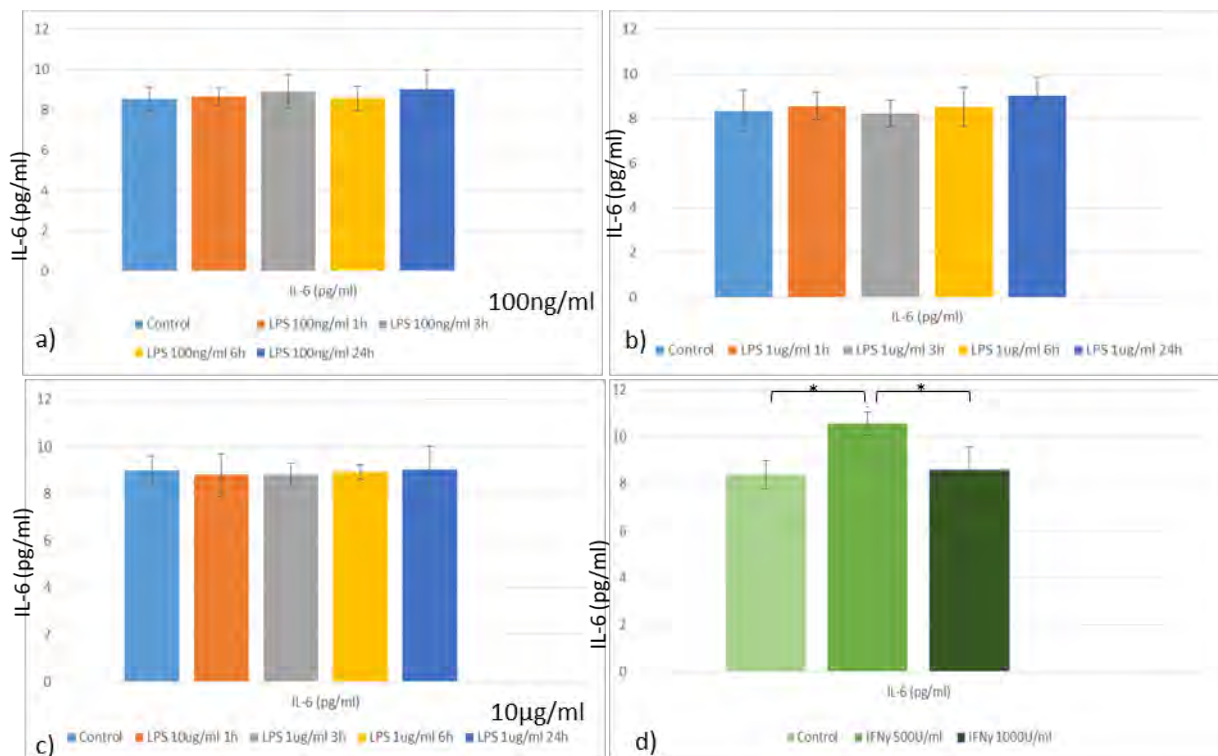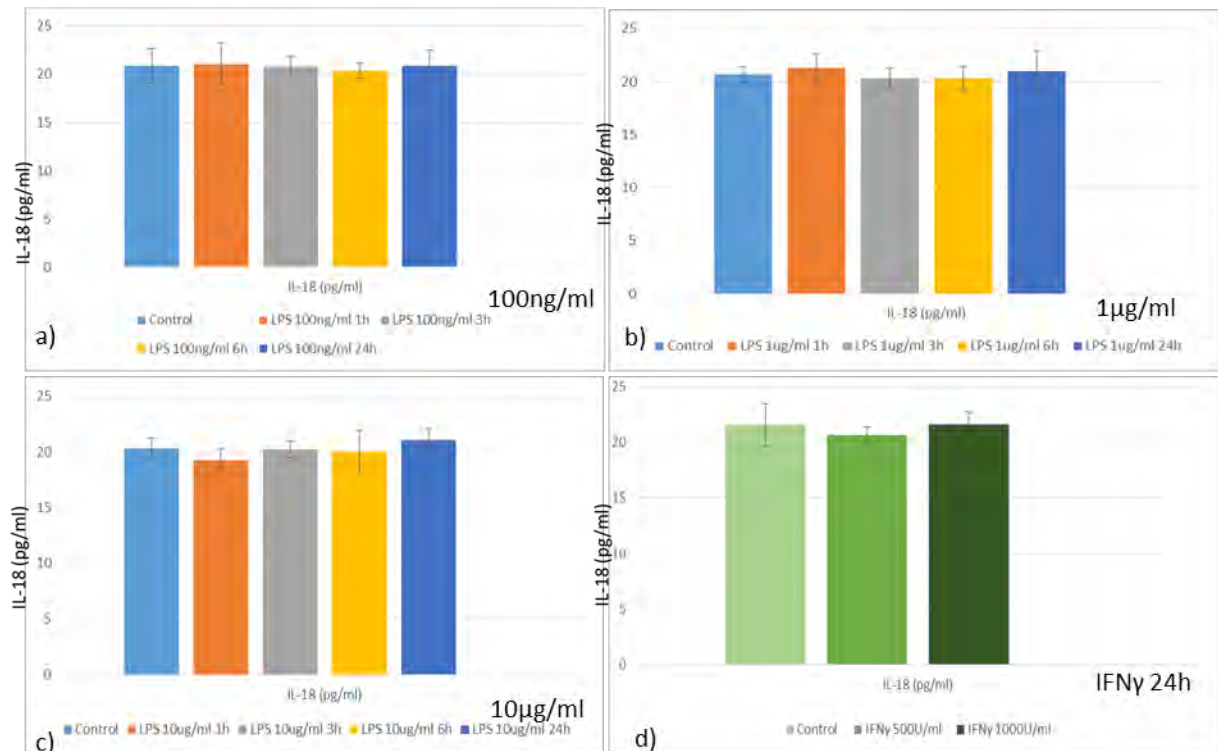

Figure 3.11(a-d) ***IL-18 secretion from astrocytes under different treatments*** Figures 3.11a and b represent the secretion of IL-18 from astrocytes after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.11c and d represent the secretion of IL-18 from astrocytes after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. n=3

RAGEs secretion

The secretion of RAGEs seemed to be unaffected by LPS treatment with no significant effects seen with any duration of dose (with the exception of a significant difference in the 1µg/ml treatment, between 3 and 6h, as shown by a 1 way ANOVA $(F(4,10)=4.96, p<0.05,$ and $p<0.05$ for the difference). A statistically significant increase of RAGEs secretion was observed after 24h of 500U/ml IFNγ treatment (as shown by a 1-way ANOVA with Bonferroni correction $(F(2,6)=10.95, p<0.05,$ and $p<0.05$ significance in difference between 500U/ml and either control or 1000U/ml treatment). Whether that increase is biologically important is debatable, as the value of the cytokine is under 25pg/ml (23.74±0.71pg/ml), and similar to the control condition observed in 3.12b (23.69±1.72 pg/ml). N.B. there are differences of about 3pg/ml between control conditions, which is a miniscule amount and thus the variation is considered negligeble, and could be due to experimental errors.
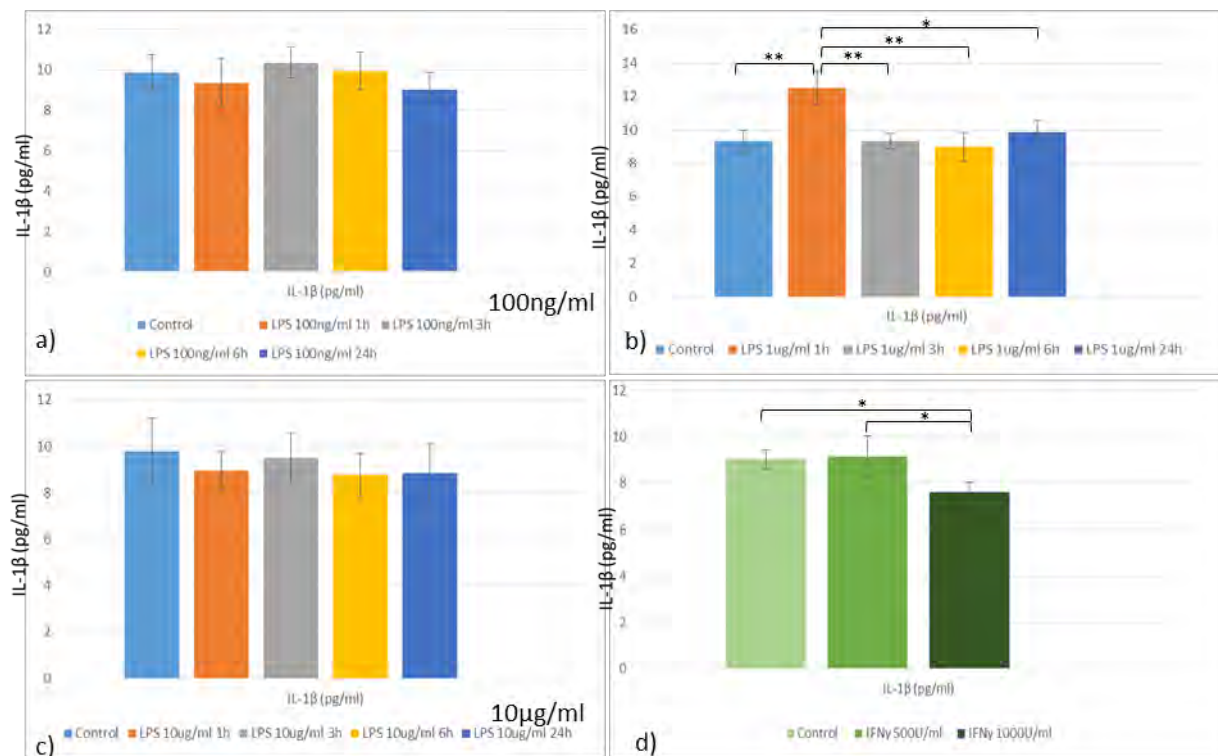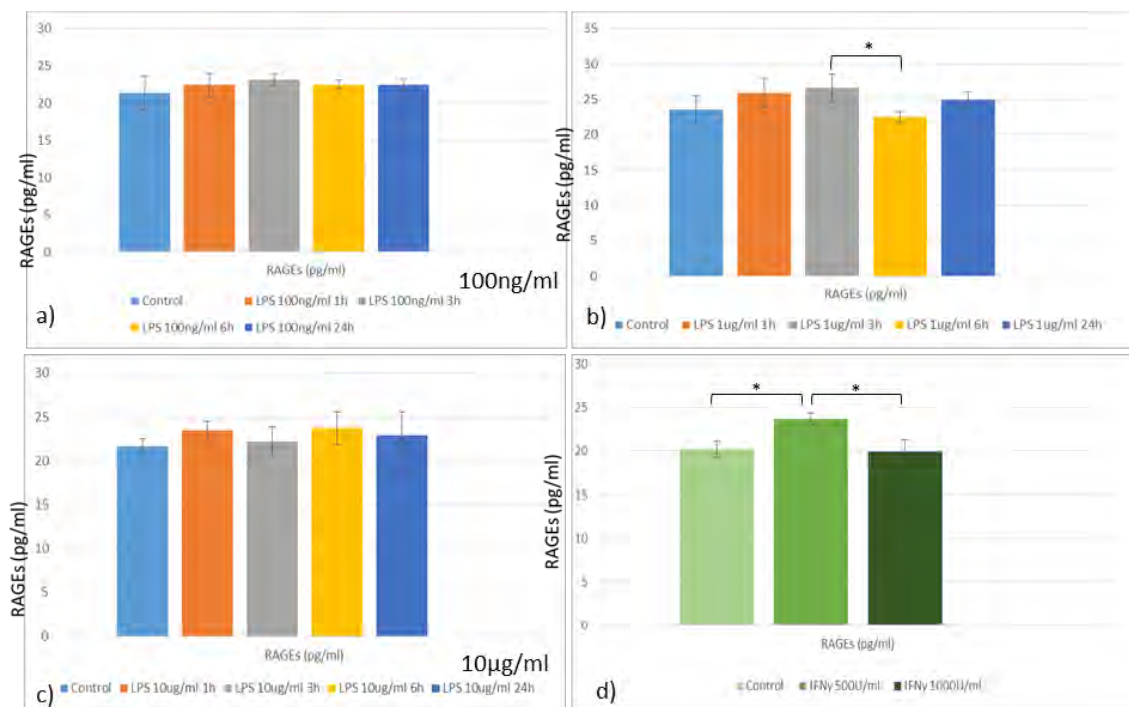

Figure 3.12(a-d) _RAGEs secretion from astrocytes under different treatments_ Figures 3.12a and b represent the secretion of RAGEs from astrocytes after 1-24h of treatment with 100ng/ml of LPS (a) and 1µg/ml of LPS(b). Figures 3.12c and d represent the secretion of RAGEs from astrocytes after 1-24h of treatment with 10µg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant changes (p<0.05) are marked with *. n=3

3.3.2.2c Neurons

TNFα secretion

The secretion of TNFα from neurons was significantly affected after treatment with either LPS or IFNγ: A 1 way ANOVA showed a significant increase ($F_{(4,10)}=5.729$, $p<0.01$) in the observed in the secretion of the cytokine with 1μg/ml LPS treatment, with the 3h timepoint being significantly higher in TNFα secretion than either control, 6h or 24h timepoints ($p<0.05$ for all). A further significant increase of cytokine secretion was observed after 24 hours of treatment with 500U/ml and 1000U/ml of IFNγ as signified by a 1-way ANOVA with Bonferroni post hoc ($F_{(2,6)}=9.8123$, $p<0.05$, with $p<0.05$ between control and both IFNγ concentration treatments). All increases are around 2pg/ml.

Figure 3.13(a-d) TNFα secretion from neurons under different treatments Figures 3.13a and b represent the secretion of TNFα from neurons after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.13c and d represent the secretion of TNFα from neurons after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results ($p<0.05$) are marked with an asterisk (*). n=3

IL-6 secretion

No significant changes in IL-6 secretion were elicited by any of the LPS treatments, but IL-6 secretion from neurons is significantly affected upon treatment with 1000U/ml of IFNγ for 24 hours. A one way ANOVA indicated significant increase of cytokine secretion with IFNγ treatment ($F_{(2,6)}=11.27$, $p<0.01$). A post hoc test revealed that an significant increase was observed after 24 hours of treatment with 1000U/ml of IFNγ, which neared double the control/baseline level approximately 3pg/ml (3.14 ± 1.19 vs 6.09 ± 0.81 pg/ml, $p<0.05$); further significant difference was found between the 500U/ml and 1000U/ml treatments ($p<0.05$).

Figure 3.14(a-d) ***IL-6 secretion from neurons under different treatments*** Figures 3.14a and b represent the secretion of IL-6 from neurons after 1-24h of treatment with 100ng/ml of LPS (fig.3.2.45) and 1µg/ml of LPS (fig.3.2.46). Figures 3.14c and d represent the secretion of IL-6 from after 1-24h of treatment with 10h of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results (p<0.05) are marked with an asterisk (*). n=3

IL-1β secretion

Secretion of IL-1β in neurons was affected in a statistically significant way upon treatment with LPS. A 1way ANOVA with Bonferroni post hoc revealed significant differences between conditions in the 1μg/ml LPS treatment ($F_{(4,10)}=14.429, p<0.001$). A significant increase was observed in the secretion of the cytokine after 6 hours of treatment with 1μg/ml LPS (control 8.14 ± 0.29 vs 10.19 ± 0.53 pg/ml, $p<0.001$). The 6h timepoint elicited a significantly higher response than the 1h, 3h, and 24h timepoints ($p<0.01$). A significant increase of a similar amount (~2.5pg/ml) of cytokine secretion was also observed after 3 hours of treatment with 10μg/ml of LPS as revealed by a 1-way ANOVA with Bonferroni post hoc ($F_{(4,10)}=20.712$, $p=0.0001$, with $p<0.001$ significance in differences between the 3h timepoint and all other conditions tested). IFNγ treatment did not elicit any statistically significant effect on IL-1β secretion.
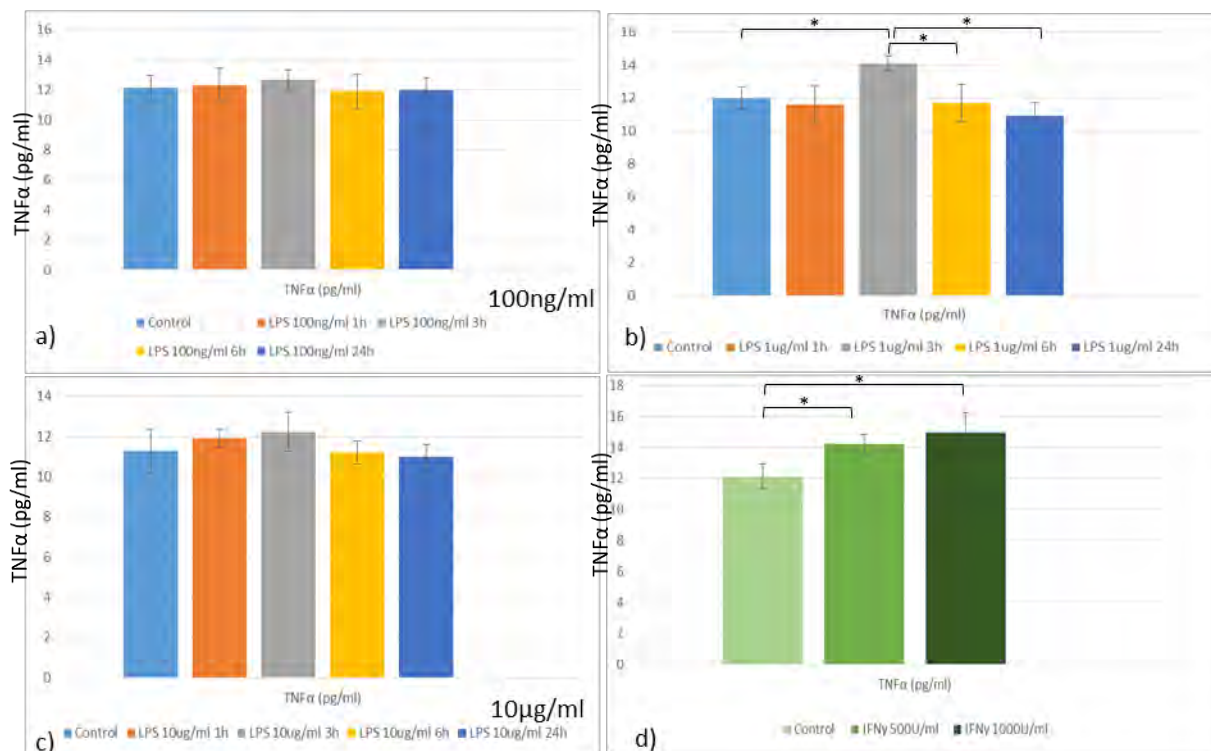

Figure 3.15(a-d) IL-1β secretion from neurons under different treatments Figures 3.15a and b represent the secretion of IL-1β from neurons 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.15c and d represent the secretion of IL-1β from neurons after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results are marked with ($p<0.05$)*, ($p<0.01$) **, ($p<0.001$)*** n=3

IL-18 secretion

While neuronal secretion of IL-18 did not seem to be affected by LPS, it was significantly affected by IFNγ: A one way ANOVA with Bonferroni post hoc showed significant differences ($F_{(2,6)}=6.845$, $p<0.05$), with a significant increase of cytokine secretion observed after 24 hours of treatment with 500U/ml (21.75 ± 0.88 pg/ml) or 1000U/ml (22.81 ± 2.08 pg/ml) of IFNγ between 3-5pg/ml when compared to control (18.53 ± 1.28pg/ml), $p<0.05$ in both cases.
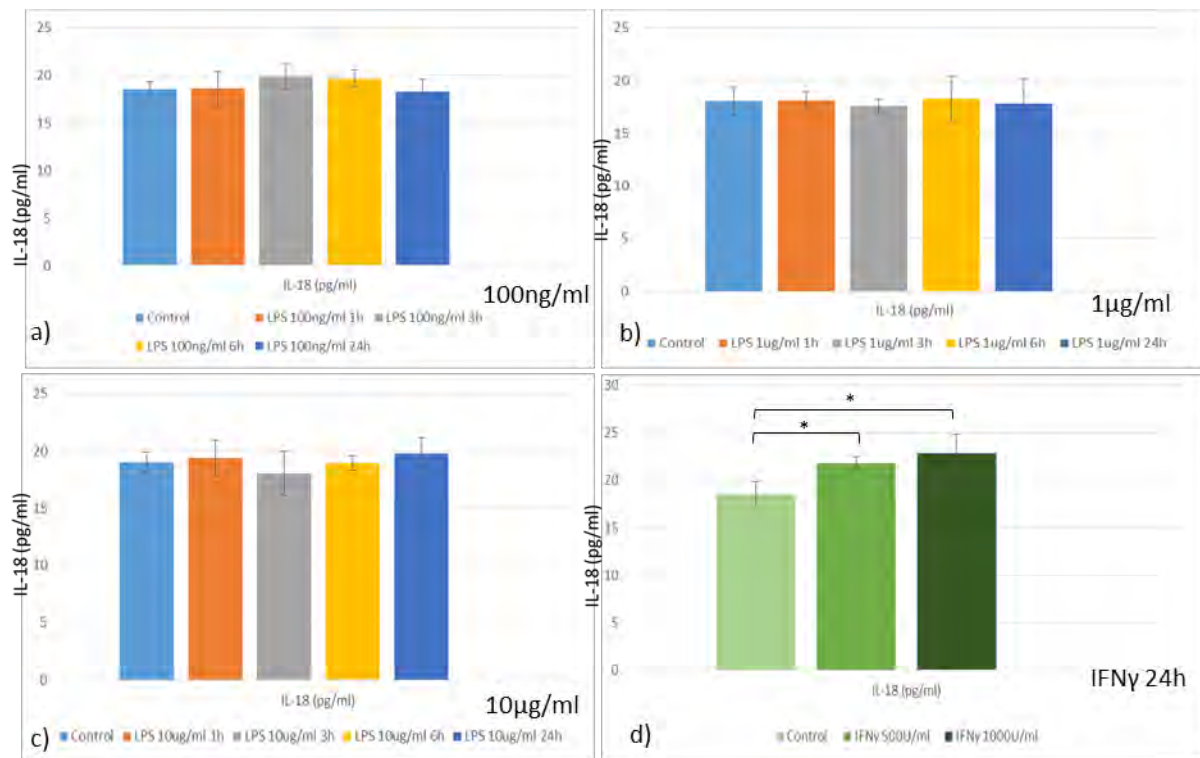
Figure 3.16(a-d) ***IL-18 secretion from neurons under different treatments*** Figures 3.16a and b represent the secretion of IL-18 from neurons after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.16c and d represent the secretion of IL-18 from neurons after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. Significant results ($p<0.05$) are marked with an asterisk (*). n=3

RAGEs secretion

Neuronal secretion of the soluble form of RAGE was not significantly affected in any of the conditions tested.
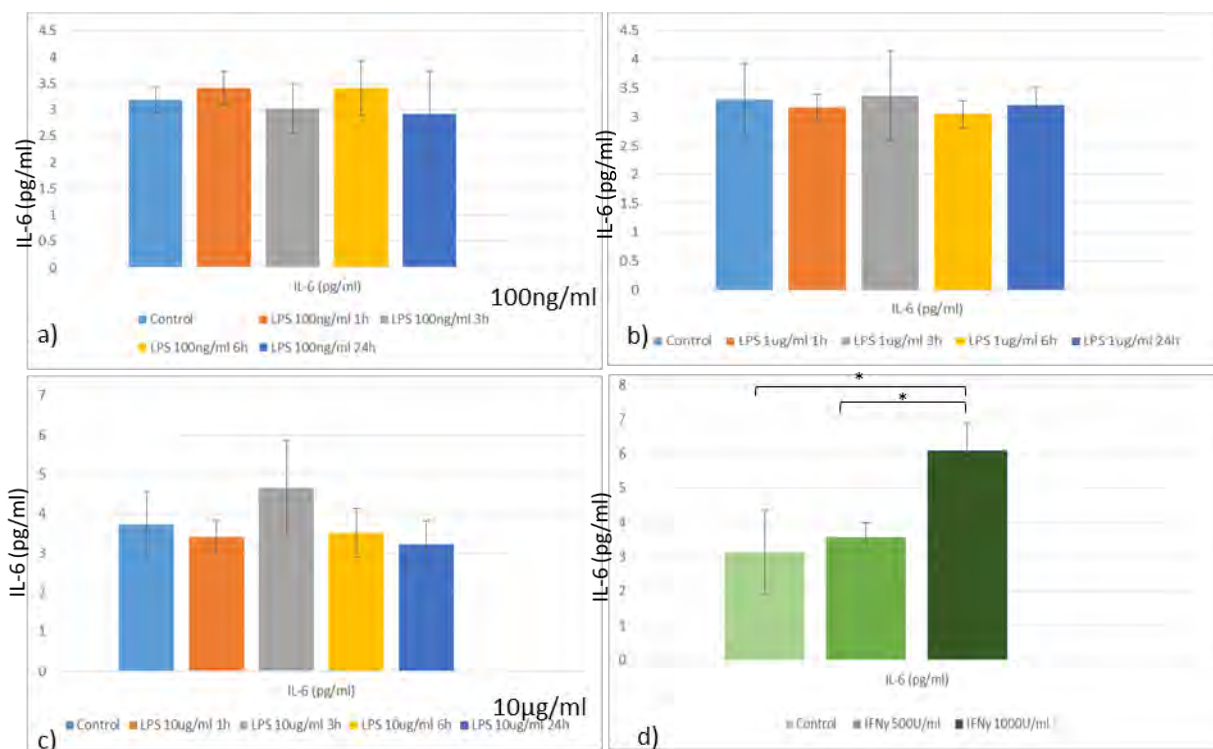

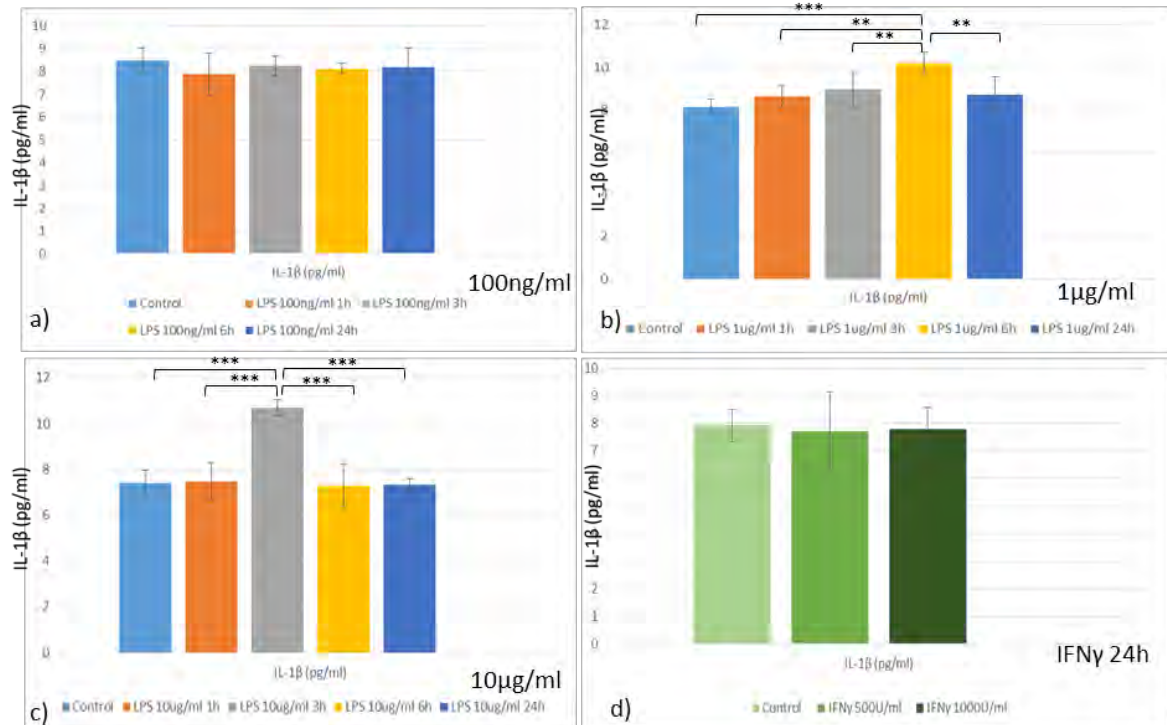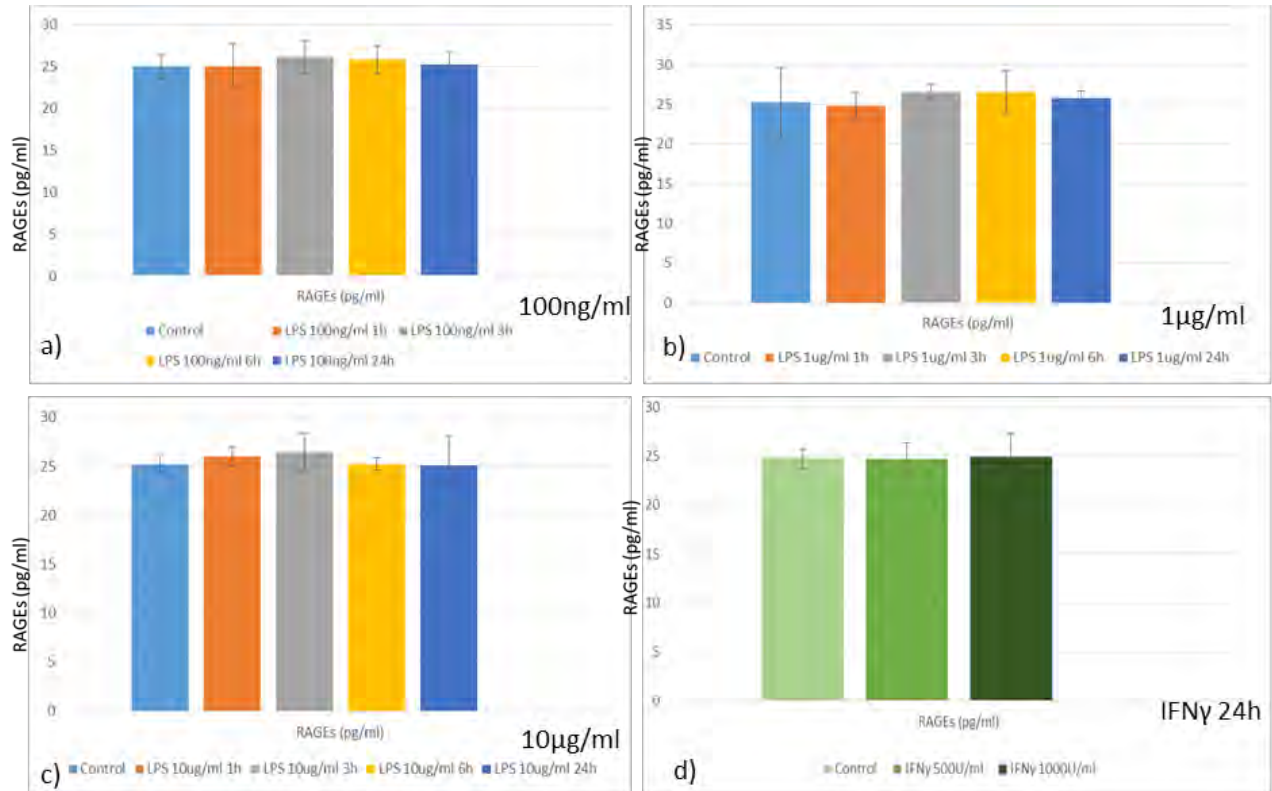***Figure 3.17(a-d) RAGEs secretion from neurons under different treatments*** Figures 3.17a and b represent the secretion of RAGEs from neurons after 1-24h of treatment with 100ng/ml of LPS (a) and 1μg/ml of LPS (b). Figures 3.17c and d represent the secretion of RAGEs from neurons after 1-24h of treatment with 10μg/ml of LPS (c), and 500-1000U/ml of IFNγ (d) for 24h. n=3

The above results based for each individual condition are summarised below in a different format, for an easier way to compare controls and the various treatments/conditions (i.e. increases/decreases are <u>vs control</u> in each case).

IL-1β

IL-6

RAGE-s

IL-18

TNF-α

○ = neural stem cell
△ = neuron
☆ = astrocyte
↑ = upregulation
↓ = downregulation

○ △ ☆ = baseline/control value, no change
● ▲ ★ = significant change from baseline/control value

Table 3.2: _Overall results summary table for LPS treatment:_ Schematic representation of ELISA results, showing cytokine distribution in untreated/control cells, and in cells after treatment for various treatment times and with different LPS concentrations. Neural stem cells are shown in circles, neurons in triangles, and astrocytes in stars. Filled shapes represent significant change from baseline/control samples, and arrows represent the type of change. It is to be noted that the cells, as expected didn't secrete large amounts of cytokines.

Ifn-γ	24 hours			IL-1β
Control	☆	△	○	
500U/ml	☆	△	○	
1000U/ml	★↓	△	○	

Ifn-γ	24 hours			IL-6
Control	☆	△	○	
500U/ml	★↑	△	○	
1000U/ml	☆	▲↑	●↑	

Ifn-γ	24 hours			RAGE-s
Control	☆	△	○	
500U/ml	★↑	△	○	
1000U/ml	☆	△	●↓	

Ifn-γ	24 hours			IL-18
Control	☆	△	○	
500U/ml	☆	▲↑	○	
1000U/ml	☆	▲↑	○	

Ifn-γ	24 hours			TNF-α
Control	☆	△	○	
500U/ml	☆	▲↑	○	
1000U/ml	☆	▲↑	○	

○ = neural stem cell	
△ = neuron	
☆ = astrocyte	
↑ = upregulation	
↓ =downregulation	
○△☆=baseline/control value, no change	
●▲★=significant change from baseline/control value	

Table 3.3. *Overall results summary table for IFNγ treatment:* Schematic representation of ELISA results, showing cytokine distribution in untreated/control cells, and in cells after 24 h treatment with a variety of IFN-γ concentrations. Neural stem cells are shown in circles, neurons in triangles, and astrocytes in stars. Filled shapes represent significant change from baseline/control samples, and arrows represent the type of change. It is to be noted that the cells, as expected didn't secrete large amounts of cytokines (all baselines under 20pg/ml

3.4 Discussion

3.4.1. Main findings

As demonstrated by ICC (fig 3.1), H9 derived NSC can be successfully differentiated into neurons or astrocytes that preserve their morphology and characteristics for at least 14 days post-differentiation. These cells specifically express different characteristic markers according to their cell type, although as discussed briefly in the results section there were some neurons (where neuronal morphology and expression of the neuronal marker Tuj-1 is confirmed with ICC) expressing GFAP (fig. A1, appendix 2). As the cells differentiate from NSC to neurons and astrocytes, changes in the expression of a variety of markers can be observed, including markers characteristic for their cell type (such as Tuj-1, GFAP, and nestin for neurons, astrocytes, and NSCs respectively), as shown by ICC, as well as in markers that relate to their function, such as the receptors TLR2 (not expressed in NSC), TLR4 (expressed in all cell types), and IFNgR1 (expressed in all cell types) as demonstrated by qPCR (see figure 3.2).

Although comparing between cell types is not the focus of this chapter, when exposed to proinflammatory stimuli (namely LPS or IFNγ), these different cell types reacted differently in regards to their inflammatory cytokine (namely TNFα, IL-1β, IL-6, IL-18, RAGEs) secretion-related responses and exhibited different levels of baseline secretion of these cytokines, as demonstrated in figures 3.3-3.17. Overall, decreases in the secretion of IL-1β, RAGEs, IL-18, and TNFα were observed in some conditions and timepoints LPS treated NSCs; in astrocytes there was an increase observed in the secretion of IL-1β in one condition and timepoint, while in neurons there were increases observed in IL-1β in two conditions and timepoints, and TNFα in one condition and timepoint with LPS treatment.

For IFNγ treatment, in NSCs there was an increase of secreted IL-6 observed in one condition, while there was a decrease of RAGEs secretion for the same condition. For astrocytes IL-1β (decrease in 1 condition and timepoint), IL-6 (increase in 1 condition) and RAGEs (increase in one condition) secretion were affected, while for neurons secretions of IL-6 (increase in one condition), IL-18 (increase in two conditions) and TNFα (increase in two conditions) were affected by IFNγ treatment. Moreover, the results were not influenced by cell death, as the cell viability and numbers were similar when comparing the maximum incubation time/treatment concentration and the control condition (fig 3.V). An expected finding was the reduced viability of the neurons when compared to the other two cell types, as neurons are known to be a fragile cell type in general (fig. 3.v)

3.4.2 Discussion on findings

From the current data of treatment of NSCs, as well as NSC derived neurons and astrocytes, cells do not change their secretion of proinflammatory cytokines in a biologically significant amount (as the highest change in secretion vs basal levels observed was ~5pg/ml) in response to either endogenous (IFN-γ) or exogenous (LPS) pro-inflammatory stimuli (tables 3.2, 3.3 for a synopsis of the findings). An important observation overall would be that whether any observed statistically significant differences have a true physiological significance is not known, as the amount of cytokines detected (with the method used, and at the time points used) was minimal, and the changes -where found- were of a magnitude of just a few pg/ml. Nevertheless, as the brain is a tissue where there is a high level of regulation of a variety of functions, even a few pg/ml could be of biological/physiological significance.

With that in mind, it has been shown that non-neuronal cells (i.e. microglia, astrocytes, and oligodendrocyte precursors) are necessary to mediate the effects of inflammatory challenges in neurons, such as exposure to LPS (Hui et al., 2016). On one hand this agrees with our data, as the cultures were homogenous when it comes to cell type (>90% positive for the relevant markers -as shown by co-staining for both neuronal and astrocytic markers and morphological characteristics) and not co-cultures of cells that include both neuronal and non-neuronal cells were included. It should be noted that one of the brain cell types that were not used in this chapter, microglia, has been identified as the cell type that leads other brain cells to their death (Teva et al., 2011), and as the cell that is most responsible for the majority of pro-inflammatory cytokines released in neuroinflammatory and neurodegenerative disorders (Smith et al., 2011). Although not microglia per se, development and characterisation of a novel model of microglia-like cells will be in the focus of chapters 4 and 5, where their response to inflammatory stimuli -among other parameters- will be investigated.

NF-κB activation is related to the majority of the cytokines investigated in this chapter (Baeuerle & Baltimore, 1996). Most neurons are known for their "resistance" to initiating an NF-κB pathway related response in an inflammatory way which could be destructive for the cell. Neuronal NF-κB is not activated by a vast variety of pro-inflammatory compounds, such as LPS or glutamate, however some activity has been shown upon exposure to TNFα (Listwak, et al., 2013). Conversely, a few studies (see review by Meffert & Baltimore, 2005), have shown the opposite, including cases where Ca^{2+} induces the pathway, or having constitutively active NF-κB in specific neuronal types. Interestingly, inhibiting NF-κB activity in neurons has been shown to lead to neuronal cell survival in mice neurons, correlating the NF-κB activity with the damage observed during ischemia (Zhang et al., 2006). Dresselhaus and Meffert in 2019 added to the knowledge

we have about NF-κB activation in neurons; their review focused on the issues that NF-κB studies have when it comes to brain cells: most are not carried out in a cell type specific manner. Indeed, the likelihood of glial contamination in primary neuronal cultures is quite big, something which is also discussed by Massa et al., (2006), so part of the activity found could be attributed to non-neuronal cells. Interestingly neuronal NF-κB activation has been implicated in the survival of motor neurons, by activating the survival of motor neuron (SMN) protein (Arumugam et al., 2017), which indicates some neuroprotective properties of the pathway. From the above, it can be demonstrated that a delicate balance between activation/inhibition of NF-κB activity (which could be due to compartmentalisation and localisation of the subunits (Widera et al., 2016; Dresselhaus et al., 2018)) could be beneficial for the neurons in a variety of ways, including the ones mentioned above (i.e. increased viability).

In the present study, the neuronal differentiation protocol used was not one towards a specific neuronal type, and as spontaneous differentiation is not that common, we cannot be sure how the NF-κB pathway can be activated and what such activation would result in *in vivo*. This could be investigated by a)further characterisation of the neurons, b)directed differentiation of the neurons to specific neuronal types, and c)experiments focusing on the NF-κB subunits and their interactions.

Additionally, it is to be mentioned that in neural cells the relationship between LPS and RAGE (and its downstream NF-κB pathway) activation is quite unclear. Even though some studies have shown direct binding of the former to the latter (Yamamoto et al., 2011), and thus making the release of soluble RAGE that we were detecting (fig. 3.17 a-d) essentially anti-inflammatory (via reduction of available LPS), not many studies agree; as an example, Li et al., 2014 found that LPS increased not only the expression of RAGE

overall, but also the secretion of pro-inflammatory cytokines, making the RAGE/LPS relationship a pro-inflammatory one.

TLR4 is the main LPS receptor in the brain, and it has been found that in primary mouse neuronal cultures IL-6 and TNF-α are induced after treatment with LPS in similar or lower concentrations as those used in the present study (Leow-Dyke et al., 2012). In the same study, they investigated the TLR4 distribution among brain cell types. It was found that interestingly astrocytes have lower levels of TLR4 than other glial cells, but neurons have levels similar to those of microglia. However as the authors note, there was a 1% detected glial contamination in their neuronal cultures, which could have affected the results. Similarly, when comparing the TLR4 expression between astrocytes and neurons as found in this chapter (fig. 3.2), neurons do indeed have a higher expression of TLR4 than astrocytes, however NSC have the lowest among the three.

In neural stem cells, the expression of TLR4 has been shown to be involved in the cells' proliferative ability and differentiation in the hippocampus (Rolls et al., 2007); also, activation of TLR4 reduces both proliferation and differentiation in mouse NSCs. Studies so far have not focused on the contribution of NSCs in the general neuroinflammatory secretome; however some interesting studies demonstrate the role of pro-inflammatory modulators in the fate of NSCs, as reviewed by Breton & Mao-Draayer, (2011). According to Brenton and colleagues, cytokines affect the proliferation, survival, and differentiation of NSCs in a plethora of ways. For example, a variety of cytokines secreted by glial cells, (and which we have investigated in the present study) namely IL-6, TNFα, and IL-1, have been shown to direct the NSCs towards differentiation or cell death. IL-1β has been shown to induce p53 cell cycle arrest and apoptosis in NSCs (Guadagno et al., 2015), promote differentiation of NSC towards a neuronal pathway (Park et al., 2018), while other studies show that exposing foetal NSC to IL-1β interferes

with their proliferation and differentiation (Crampton et al., 2012); IL-1α also interferes with these processes, as one of its functions is to induce proliferation of NSCs *in vitro* (McPherson et al., 2011). TNFα has been shown to stimulate proliferation in adult NSC (via the NF-κB signalling) (Widera et al., 2006), promotes their differentiation towards astrocytes, and inhibits their differentiation towards neurons through the STAT3 pathway (Lan et al., 2012), and it also acts in a protective way in which it induces NSC survival via the activation of the NF-κB pathway (Kim et al., 2018). Last, IL-6 also has pleiotropic effects on NSCs, as it has been shown to inhibit proliferation of NSCs in vitro (McPherson et al., 2011), while Islam et al., (2009) showed that by utilising different receptors, it mediates both neurogenesis and gliogenesis. Moreover, IL-6 has also been shown to decrease survival of the NSCs by Monje et al., 2003, however the last study focused on microglia secreted molecules. Also, many examples of the effects of cytokines on NSC and progenitor cells (as already discussed in chapter 1) show a pleiotropic effect, and are also discussed in reviews by Mousa & Bakhiet (2013), and also by Breton & Mao-Draayer (2011).

Our findings are quite interesting in this regard, as a downregulation of three out of five cytokines was seen in the NSCs, possibly indicating a potential inhibition of the pathways that lead to their secretion, which could cause an overall reduction of the proinflammatory cytokines in the NSC environment. When observing the NSCs pre and post treatment there was no obvious differentiation present, neither was there change in their viability, and therefore the changes observed could not be attributed to those factors. It is possible that what we observe here is another way that NSCs work in an immunomodulatory way, in addition to what has been reported so far (Ben-Hur, 2008) i.e. through their migration and differentiation. However, our results clash with the results of a mouse study by Covacu et al. (2009), which found an increase in TNFα secretion after exposure to similar

concentration of LPS which is in contrast to the findings of the present study. This could be attributed to a variety of factors, including the model organism (human vs mouse), the cell culture (primary vs ESC derived NSCs), as well as differences in growth media, conditions, and homogeneity of cultures. Some microglial cells were found in the cultures of Covacu et al. (2009), and there was measurements of TLR receptors, however they did not investigate a response to stimuli per cell type, which could be a disadvantage when measuring cytokines known to be secreted abundantly from microglial cells. Interestingly, a mouse vs human NSC study by Walter & Dihné (2012) showed differences in response to IFNγ between species, which could extend to other proinflammatory stimuli as well.

Studies focusing on IFNγ have generally shown that it has a neuroprotective role, rather than a neurodegenerative role, as for example was shown in spinal cord neurons in mice (Victório et al.,2010) and hippocampal rat neurons (Lee et al., 2006) as well as in *in vivo* models of neuroinflammation (Sun et al., 2017). However in an Amyotrophic lateral sclerosis mouse model it has been shown that astrocytes can induce neuronal death in some motorneurons through IFNγ, as it is involved in a crosstalk between those cells, via the IFNγR1/2 receptors, and the subsequent activation of the LIGHT protein, and the LIGHT-LT-βR pathway (Aebischer et al., 2011). In another study, IFNγ was correlated to caspase-1 mediated neuronal cell death (Hallam et al., 2000), however again the model used was that of a disorder (trisomy 21), which is correlated with increased IFN sensitivity.

Our findings demonstrate for IFNγ treated neurons that although in some cases there was a small increase in proinflammatory cytokines, namely TNF-α, IL-6, and IL-18 (fig. 3.13d, 3,14d and 3.16d), there was no difference in cell survival (fig. 3.V). No other studies have been done to our knowledge that investigate the influence of IFNγ on the cells and reactions under investigation in the current study. However there are studies that

focus on the effects on TLR4 activation, that show an upregulation of the receptor in rats (Covacu et al., 2009), when using a lower concentration of IFNγ than we used. Another more recent study using 1000U/ml of IFNγ found inhibition of NSC proliferation, however this study did not investigate cytokine secretion (Kulkarni et al., 2016). Although there was a viability assay, and cell counting, this alone would not allow us to gain insights into the proliferative behaviour of the cells we investigated, in addition to the times of incubation being different (24 hours in our study vs 72 in the Kulkarni study), it is not possible to draw any definite conclusions in this instance. Instead a more proliferation-specific method could be used (such as incorporation of BrdU or EdU) in order to draw definite conclusions about the proliferative behaviour of the cells, which was not done in the present study.

Last, it is to be mentioned that the cell type that was expected to react the most, as it is a glial cell (i.e. astrocytes), did not show great reactivity to either stimulus. Human astrocytes have been shown to not express CD14 which is required for optimal response to TLR4 ligands (LPS); in contrast in mice and rodents, where the majority of studies have focused on, CD14 is expressed, facilitating a pro-inflammatory response from these cells (Tarassishin et al., 2014). When it comes to their response to IFNγ, Lee et al., (1993) showed that in rats, the cells respond to the cytokine when this is used as a co-treatment with IL-1β. The response was measured in NO synthetase activity, which is often used as a marker of inflammatory response. Thus repeating the experiments and focusing on factors such as NO, or other enzymatic activity that is related to inflammatory responses could give a better idea on how these cells respond. Interestingly, Lee et al. (1993) showed that LPS had no effect in human astrocytes, even when it comes to NO synthesis, which is in contrast to what is known about rat and mouse astrocytes (where NOS has

been known to be both constitutively active, and further activated by LPS), but in broad agreement with our findings.

To answer the questions set in the beginning of the chapter: the cells examined here do not seem to produce vast amounts of cytokines under the conditions investigated, in order to contribute significantly in an inflamed CNS. Although the cells seem to react under some conditions, these reactions are not constant, they differ for the different cell types, often they are not increases in secretions (thus if anything they contribute less to an already inflamed environment), and they secreted amounts are of a difference of around or under 5pg/ml when compared to the control conditions. Should there have been more profound, significant, or constant differences, more statistical analyses would have been undertaken (e.g. 2-way ANOVAs) comparing the reactivity of different cell types. However, this does not exclude the possibility of a different reaction occurring as part of the response to LPS of IFNγ, which could be investigated using a different methodology (e.g. NOS expression, cytokine gene expression), or of such responses being observable in reaction to different stimuli (e.g. treatment with IL-6 or TNFα) which were not investigated as part of this study.

Overall, the findings of this chapter show that during differentiation, expression of some genes (LPS and IFNγ receptors) involved in the inflammatory response change, which could in turn have implications on the way different cell types respond to their environment, and communicate with each other. It is crucial to mention, however, that the proinflammatory potential of the neural cells examined here did not take into account interactions between cells (as it was a homogenous cell type culture system that was used in each case, which inevitably excludes study of cell types that function via interactions with other cells, for example oligodendrocytes, and CNS macrophages including microglia) that often guide the inflammatory response in the CNS (microglia and CNS

macrophages). Thus it could be argued that the current chapter has focused on the trees rather than the forest. However as the trees are what the forest is made of, the current author would contend that it is useful to investigate the characteristics of separate groups, which are often overlooked in the aspects of their contribution in a pro-inflammatory way to the inflamed brain.

3.4.3 Limitations of chapter

1. The cells used were not primary, so we do not know about the potential of the primary cells to react to pro-inflammatory stimuli. All cells were derived from the H9 embryonic stem cell line (WiCell Research Institute). This gave us the advantage of being able to observe differences during differentiation of the cells to neurons or astrocytes, however the results were limited to the specific cell line, and cannot necessarily be generalised, as factors such as sex, age (the H9 cells come from supplementary embryos from IVF treatment, where the cells are of 46 XX karyotype, with no known abnormalities (hPSCreg description)), and other conditions that although not mentioned in a genetic analysis, have been shown to have significant effects on inflammatory processes. It would be useful to repeat these experiments in different ESC/iPSC derived lines with differences in various factors and primary cells.

2. The time frame and concentrations used were microglia-specific (as decided from the literature and expanded to investigate potential delayed reactions or reactions to higher concentrations as the initial low concentration conditions did not produce any results), and could be inappropriate for the cells used. Although we used different time points in order to avoid "missing" a change in secretion, cytokine half-life is overall short (Aziz et al.,2016) however we used was a broad range of conditions. Nevertheless, focusing on

gene expression rather than secretion of the gene products, and their related pathways (e.g. NF-kB) could give more information on the responses.

3. As already mentioned, the cytokine levels detected were low, as these cells are not immune-systems cells per se (although they might act in an immune way under conditions, as already discussed). Therefore, the results, while statistically significant in some cases might have no biological significance.

Chapter 4

Development and characterisation of a novel model of microglia-like cells from THP-1 cells.

4.1 Introduction and aims

A major source of cytokines in the inflamed brain are the brain macrophages, both resident, and infiltrating. Isolating such cells has proven difficult, and so has been maintaining them, as already discussed in chapter 1. As a solution to that, several *in vitro* models have been developed, dubbed "microglia" or "microglia-like" cells, with different origins. A synopsis of such *in vitro* models can be found in Table 4.1.

In this chapter it was attempted to produce CNS macrophage/microglia-like cells using THP-1 monocytic cells as an origin, and to characterise them. For that we used components of a medium that is used to differentiate iPSC to NSC. The reasoning behind such plan is a)in the developed CNS, monocytes enter the brain and differentiate into microglia-like cells (Simard & Rivest, 2004; Wang et al., 2016), b) monocytic progenitors (Hoeffel & Ginhoux, 2018) enter the CNS early in development and differentiate into microglia cells, and c) that a subpopulation of peripheral blood monocytes has been found to have pluripotent stem cell-like properties (Zhao et al., 2003; Kuwana et al., 2003). These cells, are of hematopoietic nature and origin, and upon entering the brain maintain their hematopoietic gene expression signatures (e.g. expression of the transcription factor PU.1), and do not completely turn into CNS cells (i.e. neurons, astrocytes, oligodendrocytes, or NSC) (Massengale et al., 2005); for instance,they are negative for NSC/neural gene expression, such as Sox-2 (Ungefroren et al., 2015).

Ungefronen et al (2015) further discuss that differentiating the stem cell like cells that are a subpopulation of monocytes into ESC or iPSC-like cells, would require complete reprogramming and silencing of PU.1, and also induced (possibly via vector) expression of Sox-2. To show how much difference the expression of a single transcription factor can make, it is worth to mention that cells that neural stem cells that express the PU.1 transcription factor, lose their neural stem cell phenotype and expression profile, and differentiate into monocytes

(Forsberg et al., 2010). Focusing on the last point, there are models that differentiate monocytes into cells with neuronal -however not neural stem cell- morphological and functional characteristics: for example, (Bellon et al., 2018) used butylated hydroxyanisole (BHA) among other ingredients; BHA has been known to differentiate mesenchymal stem cells into neuron-like cells (Mareschi et al., 2006). The produced cells, are neuron-like in nature, and do allow the study of neuronal characteristics (e.g. electrophysiological properties, ion channel excitability etc) in models that are relatively easy to produce.

For microglia-like cells, the cells produced in this type of *in vitro* model (as shown in the studies in Table 4.1) originate either from iPSC, ESC, or monocytes. The differentiation process is often complex and long, and includes in some cases cell reprogramming using e.g. viral vectors. Characterisation of these cells can be either simple (e.g. functional response to stimuli), or complicated (involving multiple tests that examine the properties of the cells produced). The time of differentiation also varies, however it usually is around 2 weeks.

In our case, the two main components of the differentiation are the cells and the differentiation medium. The medium used will be primarily THP-1 medium (supplemented RPMI as described in Chapter 2.2.1), with the addition of neural induction supplement (Catalog number A1647801 Gibco, UK), thus creating an environment which guides towards neural differentiation

Rationale behind using THP-1 cells

As discussed in Chapter 1 and 2, THP-1 cells have been known to resemble peripheral blood monocytes in a variety of aspects (Bosshart & Heinzelmann, 2016; Rebelo et al., 2018). They can be differentiated into other cell types such as macrophages (Park et al., 2007) and dendritic cells (Berges et al., 2005), and are relatively easy to culture and maintain as they are a well characterised immortalised cell line (Bosshart et al., 2016; Riddy et al., 2018). Moreover, as

they are a cell line they are more homogenous than, for example, PBMC-derived monocytes (which have been differentiated into microglia-like cells (Table 4.1)) there is in theory, a higher chance for more cells to differentiate into the desired cell type. The above in addition to the rationale behind using these cells as mentioned in 1.3 (b) show that THP-1 cells are good candidates for the aim of this chapter.

Source	Methodology of differentiation	Timeframe	Methodology of characterisation	Compared to microglia & similarity	Reference
THP-1 cells	PMA	5-8 days	Response to LPS; morphology	No	McFarland et al., 2017
Human and Rat Blood monocytes & spleen macrophages	Co-culture with astrocytes	1-3 weeks	ICC (OX42, KiM6); morphology	Certain similarities when compared with primary mg for some characteristics	Sievers et al., 1994
Human & mouse Hematopoietic cells	Co-culture with astrocytes (+M-CSF and IL-34 for improvement)	1-2 weeks	Flow cytometry (CD45^{low}, Cd11b, F4/80); ICC (Iba1, TREM2, Cd11b); functional assay (LPS + phagocytosis assay); morphology	Certain similarities when compared with primary mg for some characteristics	Noto et al., 2014
Mouse non-adherent bone marrow stem cells/non-adherent MSC	Astrocyte conditioned medium (ACM) & granulocyte-monocyte colony stimulating factor (GM-CSF); activation with PMA	10 days	Flow cytometry (CD11b, CD45^{low}, F4/80, CD34); functional assay (phagocytosis and oxidative burst); tissue migration/invasion assay	Certain similarities when compared with primary mg for some characteristics, different to BM cells	Hinze & Stolzing, 2012
hiPSC	Multiple steps	>30 days	ICC (TMEM119, CD45, PU1, IBA1); functional assays (including LPS, IFNγ treatments); transcriptome analysis	Similar to primary microglia, with some differences in transcriptomes	Muffat et al., 2016
Mouse ESC	Multiple steps (63+characterisation)	60 days	Migration assay; Phagocytosis assay; Flow cytometry; ICC; qPCR (various genes)	No (however it is a protocol)	Beutner et al., 2010
Human peripheral blood monocytes	RPMI (monocyte medium) + M-CSF, GM-CSF, NGF-β, CCL2, IL-34	15 days	Gene (94 genes) & protein expression analyses; ICC; GWAS SNP analysis; functional assay (M1/M2 polarisation)	No, but comparison vs monocytes per individual	Ryan et al., 2017
Human peripheral blood monocytes	RPMI (monocyte medium) + GM-CSF ± IL-34	14 days	Morphology; Flow cytometry (CD11b, CD14, CD45, CD200R, CX3CR1, CCR2); ICC (CX3CR1, CCR2); qPCR for activation markers; functional assays	No, but comparison with monocytes	Ohgidani et al., 2014
Mouse BM cells	M-CSF	14 days	Functional assays; ICC (Iba1, Cd11b)	No, but comparison with monocytes	Toji et al., 2013
Mouse Mononuclear cells differentiated into Dendritic cells	Co-culture with CNS endothelial cells	14 days	Morphology; qPCR (iNOS); functional assays (migration, phagocytosis, cytokine secretion); Flow cytometry (CD-4, 7-AAD); ICC (cd11b)	Certain similarities when compared with primary mg for some characteristics	Bai et al., 2009
iPSC	Multiple steps	60 days	ICC(TMEM119, Iba1); Flow cytometry (cd11b, cd11c,CX3CR1, P2RY12); gene expression (including signature genes MerTK, P2RY12) functional assays	Significant similarities when compared with primary mg for many characteristics	Douvaras et al., 2017

Table 4.1 *Summary of some of the protocols used to produce microglia-like cells (or in some cases, titled microglia cells).* The table includes origin and type of the cells used as primary material, a summary of the methodology/materials used to differentiate the cells, time needed for differentiation, characterisation assays, and finally whether the microglia-like cells were compared to either the cells they originated from or to primary microglia.

The hypothesis here is that this supplemented medium will resemble the CNS milieu (which aids in the differentiation of both yolk-sac HSC and monocytes into microglia, and microglia-like cells respectively), and hence maintain the hematopoietic characteristics of the cells, while also pushing them into their CNS "counterpart" form: microglia-like cells. As this medium is not inflammatory in any way in the concentration indicated by the provider (as the same concentration is used to differentiate PSCs), any macrophage-like characteristics observed would not be due to the cells being primed into macrophages (as is done when using PMA (Auwerx, 1991)), but rather due to a shift towards a neural form. Although it has been claimed elsewhere that PMA treated THP-1 cells resemble microglia cells (McFarland et al., 2017), this is only based on the responsiveness of the produced cells to LPS, which is essentially a macrophage response (Park et al., 2007); nevertheless, to further investigate this, dTHP-1 cells (THP-1 cells treated with 25 ng/ml PMA as described in 2.2.1.2) will also be placed in the same conditions (NIS supplemented THP-1 medium) . Lastly, as several of the methods used to produce microglia-like cells (see table 4.1) use astrocyte conditioned medium (ACM), this will also be used in this case, both alongside NIS (to observe potential enhancement of the effect of the NIS medium), and also on its own; it is to be noted that ACM was also shown to differentiate ESC into NSC in addition to differentiating myeloid cells into microglia-like cells in some studies (Nakayama et al., 2004; Zhou et al., 2005; Nakayama & Inoue, 2006).

Finally, it is to be noted that the cells produced here should be referred to as microglia-like, and not CNS infiltrating monocyte-like, as CNS infiltration happens under specific conditions, such as inflammation, where the interaction of the infiltrating cells with the BBB occurs after the latter becomes "leaky" (Larochelle et al., 2011). Importantly, none of these conditions apply to this model. Nevertheless, upon entering the CNS, infiltrating monocytes also become microglia-like, potentially with some different characteristics but these cells are not the focus of this chapter.

<u>Aims</u>

The aims for this chapter are:

a)The 4 conditions mentioned above (which will be referred to as NIS/THP-1, NIS/dTHP-1, ACM+NIS/THP-1, ACM+NIS/dTHP-1) will be used, and the resulting differentiated cells will be examined for changes in morphology, and compared to both macrophage-like cells (in this case dTHP-1 cells cultured in our laboratory) and to microglia morphologies, as found in the literature (e.g. del Mar Fernández-Arjona et al., 2017, and a variety of other *in vitro*/in culture microglia studies-see discussion of this chapter, and section 1.1.5). Furthermore, the expression profile of microglia markers will be investigated, and there will be an attempt to clarify the ingredients that drive the differentiation (if any) from monocytes into microglia-like cells. qPCR will be employed for analysis of expression of a variety of microglia markers at mRNA level, on days when morphological "developmental" changes are observed in the cells.

b) Once the optimal conditions have been determined, a focus will be given on the expression at a protein level of different microglia-related and microglia-specific markers (using immunocytochemistry and Western blotting).

c) Last, this chapter will employ DNA methylation and ATP quantification assays in order to focus on the potential metabolic and epigenetic changes that the cells go through during their differentiation.

Thus, this chapter's overall objective is to describe the development of a protocol that differentiates THP-1 cells into a novel *in vitro* model for monocyte-derived microglia-like cells (entitled mgTHP-1 cells) using well defined, non-complex or time-consuming conditions, to characterise these cells, and to suggest ways in which this differentiation takes place.

4.2 Materials and Methods

4.2.1 Materials

The materials used for this chapter that were <u>not</u> previously mentioned in chapter 2 are the following:

Name	Used for	Provider
TBP probe (Hs00427620_m1)	qPCR-housekeeping gene	Thermofisher UK
PU.1/Spi1 probe (Hs02786711_m1)	qPCR-expression of PU.1	
Iba1/aif1 probe (Hs00610419_g1)	qPCR-expression of Iba1	
CD45/ptprc probe (Hs04189704_m1)	qPCR-expression of CD45	
GAPDH probe (Hs99999905_m1)	qPCR-expression of GAPDH	
Actin probe (Hs99999903_m1)	qPCR-expression of Actin	
P2Y12R (P2Yr12/P2Y12) probe (Hs01881698_s1)	qPCR-expression of P2Y12R	
CX3CR1 probe (Hs00365842_m1)	qPCR-expression of CX3CR1	
Cytokine Array – Human Cytokine Antibody Array (Membrane, 42 Targets) (ab133997)	Cytokine secretion analysis	Abcam, UK

Table 4.2 *Materials used for this chapter*

4.2.2. Methods

4.2.2.1 THP-1 cells culture and differentiation to mgTHP-1 cells

THP-1 cells were cultured as per chapter 2.2.1. In some cases, the cells were differentiated to dTHP-1 cells as per chapter 2.2.1.2. Also, as discussed in chapter 2, ACM was collected from astrocytic cultures in the lab. The four different combinations that were investigated with regard to promoting differentiation to mgTHP-1 cells in this chapter are: a)THP-1 cells+THP-1 medium+2%NIS (from now on NIS medium), b)THP-1 cells+ACM+2%NIS (from now on ACM/NIS medium), c)dTHP-1 cells+THP-1 medium+2%NIS, and d)dTHP-1

cells+ACM+2%NIS. Cells were incubated for up to 14 days for most of the experiments, although morphological observations continued until 28 days in some cases.

4.2.2.2 qPCR

In order to investigate the changes in microglia-related gene expression (namely PU.1, Iba1, CD45, P2y12R, CX3CR1) that may potentially accompanied the morphological changes observed, qPCR was performed as described in chapter 2.2.6; details of the probes are described in table 4.2.

4.2.2.3 Immunocytochemistry/Immunofluorescence

In order to investigate the protein expression patterns of microglia-related (Iba1, cd11b) and microglia-specific (TMEM119) proteins in different timepoints, ICC was used. The method is described in detail in chapter 2.2.5. Briefly, cells were grown on non-coated round glass coverslips, in 12-well plates, in the NIS medium. According to the timeframe provided above, cells were fixed, and antibodies for cell type-specific proteins were used; cell nuclei were counterstained with Hoechst 33342. Cells were then observed using a Nikon Eclipse 80i at 40x magnification and images were captured using Volocity 5.5.using appropriate filters for each secondary antibody. For the antibodies used here (primary vs myeloid and microglia proteins, and fluorescence-conjugated secondary antibodies), the concentrations and details are described in table 2.3. As the focus of ICC in this case was to provide images that show expression of the proteins in question, and their distribution (e,g, membrane, cytoplasm etc), the conditions of capturing the images were optimised per image to provide the best images possible (exposure time, contrast); however deconvolution was carried out to ensure that no background signal was influencing the images. As a result, the images cannot be used in order

to numerically quantify the protein expression, and hence to compare between days. (For that, Western blotting was used for the proteins of interest.)

4.2.2.4 Western Blotting

In order to determine the relative amount of protein expressed in the cells (i.e. densitometric comparison between different days), and compare it to the relative gene expression, Western blotting was used. The full method, as well as antibodies and concentrations used are described in 2.2.4.3. Relative protein quantification was determined using ImageJ and densitometric analysis as described in the appendix (Figure A2). The ImageJ value was adjusted vs control, and then normalised vs the housekeeping protein (actin); a summary of the full method followed can be found on this webpage: https://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. The adjusted/normalised results were then analysed using 1-way ANOVAS -with Bonferroni post hocs in significant results- between different conditions (i.e. THP-1, Day 3, Day 7, Day 14 cells protein content). In some cases, further bioinformatic analysis was conducted using the protein database Uniprot, in order to retrieve the protein sequences and identify potential isoforms. BLAST analysis was also conducted for investigation of the extracellular section of TMEM119, which the antibody used was raised against. Specifically, the extracellular sequence of the protein (residues 26-95 (Bennett et al., 2018)) was compared -both as a whole, and in segments- against the Uniprot protein sequence database for similarities. The proteins identified as scoring over an overall score of 25 in BLAST, were investigated for their molecular weight, and thus their potential to be the extra protein bands that appeared on the blot. The overall/total score shows the sum of alignment scores of all segments from the same subject sequence. As an example in figures 4.Ma,4.Mb the result of the full extracellular domain blast is presented (total score for TMEM119 139) vs one of the sub-sequences of the full extracellular domain (final 25 residues of the extracellular

137

domain, PITLGGPSPP TNFLDGIVDF FRQYV) (total score for TMEM119 84.2, with proteins S100 calcium-binding protein A12/Calcitermin, and p241/Drosha also showing similarities).

4.Ma

4.Mb
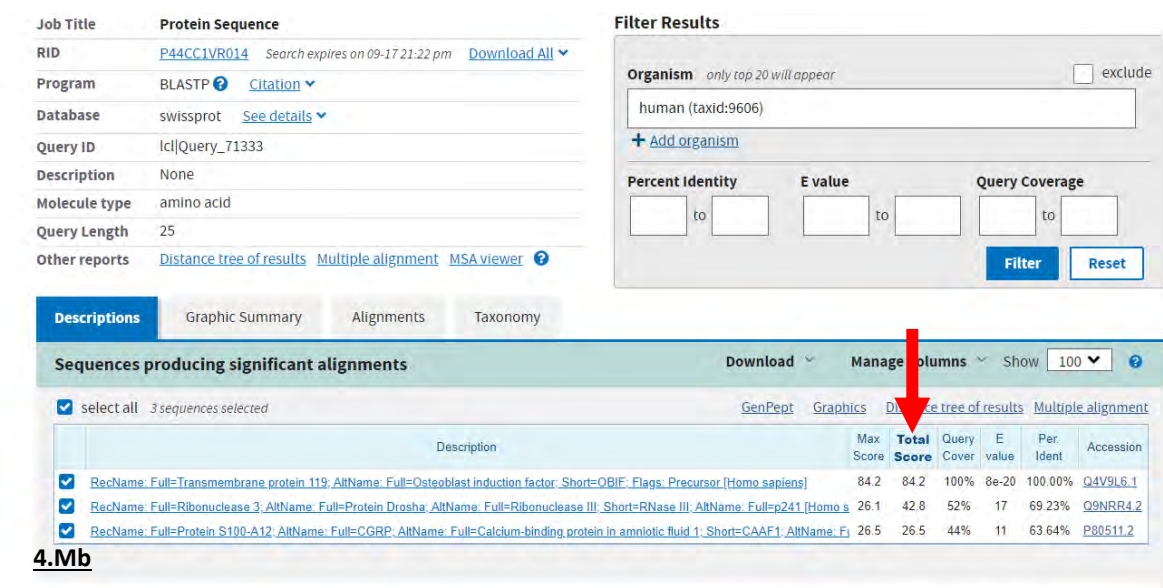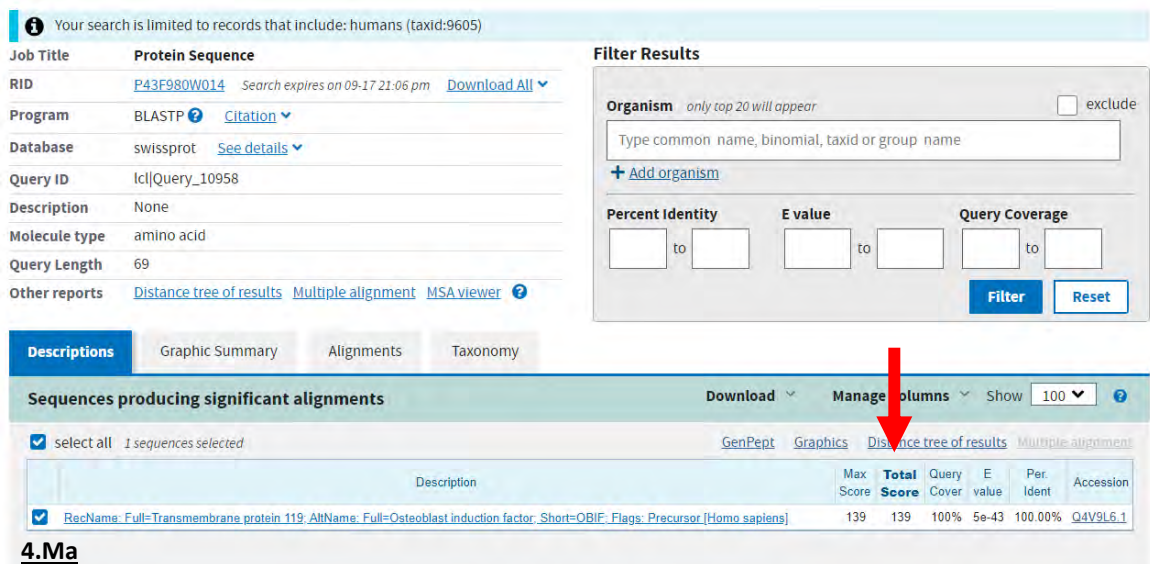
***Figures 4.Ma, 4.Mb** The results for BLAST analysis for the full extracellular domain of TMEM119, and the last 25 aa of the sequence.* Red arrows indicate Total score, where proteins with a value of over 25 were further investigated for their function and potential relevance to the differentiation of THP-1 cells to mgTHP-1 cells.

4.2.2.5 Metabolic analyses

4.2.2.5.1 [ATP]cell calculation

Cellular ATP content was determined using a commercial kit (CellTiterGlo; Promega, UK). The full method is described in 2.2.8. Results are represented as [ATP] nM per cell on each day of interest. Cell numbers per condition/sample were taken into account, and results were normalised accordingly.

4.2.2.5.2 Methylation analysis

Methylation analysis was conducted as described in 2.2.7. Briefly, DNA was collected from cells of all differentiation stages, as well as from untreated THP-1 cells. Using an ELISA-like method, the relative proportion of 5-mC per unit DNA was measured and compared between conditions.

4.2.2.6 Analysing the contents of the differentiation medium

4.2.2.6.1 Comparison of NIS with other supplements

In order to compare the (protein) ingredients of NIS, it was analysed along with 3 other supplements (B-27, B-27+, Stempro, and N-2). The proteins in the supplements were separated electrophoretically using the method described in 2.2.4.3.2 (up until the transfer, which did not occur in this case). Instead, after the gel was removed it was washed with deionised water 3 times, for 5 minutes each time. The gel was then stained with SimplyBlue™ SafeStain

(~20 mL) to cover the gel, by placing the gel in a tray with the Coommassie blue stain. This was left for 1 hour at RT, with gentle shaking. The gel was then washed overnight with 100ml of water, which was changed initially twice every hour, in order to remove background staining. Image was taken using a CANON PowerShot SX540 HS Bridge Camera.

4.2.2.6.2 Array analysis

In order to determine whether the NIS differentiation medium contained any of the growth factors or cytokines which can be detected with the array described in section 2.2.4, the method as described in 2.2.4.2 was used to analyse the NIS medium (THP-1 medium+2%NIS). As the question is essentially of a qualitative nature, no further analyses were done.

4.2.2.7 Statistical analysis

Statistical analysis was performed using SPSS, where 1-way ANOVAs were used in order to determine differences between control conditions (i.e. THP-1 cells) and samples from each of the key developmental days (day 3, 7, 14). Bonferroni post hoc tests were utilised in order to indicate differences between groups. Normalisation against housekeeping genes was performed (actin for WB and TBP for qPCR). Significance was set at $p<0.05$, and indicated by asterisks (* for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$ and **** for $p<0.0001$) on figures with significantly different values. Values are expressed as mean ± Standard deviation (SD). Unless otherwise mentioned, the n in the figures refers to biological replicates.

4.2.2.8 ImageJ cell measurements

ImageJ was used in order to measure the number (light microscopy images) or size of the cells of the 3 developmental days, as well as the nuclei sizes of the cells (ICC images). The analyse the number of cells/day, the particles function was used counting, and for the size, scale was set per image and then nuclei/cells were measured. For cell number, n=3 images were used per day. For size, measurements of n=12 cells/nuclei per day from n=3 images were used. Diameter for cells includes max and min values (in the same cell). Cells/clump number was measured manually, as imageJ was not able to perform that calculation in any of the settings tried, and added to the number calculated by the particles function of ImageJ.

4.3 Results

4.3.1 Morphology

THP-1/NIS: The THP-1 cells started exhibiting morphological changes after 3 days in the NIS medium. Sphere-like clumps started appearing, which were formed from >3 cells. In contrast, THP-1 cells are neither adherent, nor do they form clumps under normal conditions; they are classic monocytic cells, exhibiting sphere-like cellular morphology. From day 3 onwards, and especially after day 7, the cells as clumps started adhering to the plastic, and taking on microglia-like morphology, both amoeboid and ramified; the mg-like cells appeared to be originating from the spheres, and the areas where they adhered. As the days progressed, the clumps started to appear less often and mg-like cells were appearing more often. The mg-like morphology was predominant around day 14, when most of the experiments ended, however it continued to predominate up to day 30 when prolonged experiments were performed in an attempt to investigate for how long the day 14 morphology remains unchanged (data not shown). No observations were made after day 30. Figure 4.1 includes photomicrographs of the changes, as well as a summary sketch of the differentiation process and the morphologies observed.

From the observation of this differentiation process, it was determined that the days of interest, where significant changes occur to the morphology of the cells, were days 3, 7, and 14: in day 3 clumps started appearing, in day 7 mg-like cells started appearing to an appreciable degree, and in day 14, the highest amount of mg-like cells was detected. Notably the overall number of cells declined over time, reaching by day 14 approximately 20-50% of the cell number in day 0 (THP-1 cells: 300000/ml in day 0, mgTHP-1 cells counted in day 14: 60000-150000/ml). Table 4.3 includes approximate cell numbers/image (+/- SD for n=5 images), cell morphology and cell characteristics, as observed microscopically and counted from the light microscopy images. Although ICC images could also give this information, ICC has the disadvantage that

non adherent cells (e.g. day 3 clumps) do not "stick" to the coverslips in accurate numbers, and cells tend to detach during washes. Light microscopy images do not disturb the cells, and thus counting is more accurate hence why in this case it was preferred, however the measurements are approximate as the clumps are 3D so not all cells (including cells in the middle of the clump and cells adhering in the surface) can be counted. Characteristics given from the ICC (e.g. existence of cells with >1 nuclei) are also included. This variation (between 20% and 50%) could be due to the medium, as batches of two components varied between experiments and are of unknown composition: NIS and FBS (THP-1 medium ingredient); such differences have been shown in other studies to be caused by FBS, even in microglia survival (Bohlen et al., 2017). Moreover, reductions in the number of cells have also been observed in other microglia-like cell development studies (Table 4.1). Nevertheless, day 14 cells were microglia-like in all experiments. For those reasons, this and the next chapter will focus on those developmental days (i.e. 3, 7, and 14), and investigate the changes which take place on these days. Table 4.3 also mentions other characteristics of the cells, as those were measured from the ICC images, such as nucleus and cell diameter (with day 14 measurements for cell diameter containing min and max diameter as measured per cell

	Day 3	Day 7	Day 14
Cell numbers/image	420±85	189±32	25±12
Cell shape(s) (in order of most to least observed)	Clumps (3-70+ cells/clump), single non-adherent cells	Clumps (3-70+ cells/clump), microglia-like cells, single non-adherent cells	Microglia-like cells, few small clumps (<20 cells/clump), single non-adherent cells
Nuclei diameter (μm)	7.09±3.4	10.545±2.64	19.818±3.88
Cell diameter (μm)	11.2±3.18	19.2±12.38	82±14.6 (max) 26.6±17.7(min)
Clump diameter (μm)	51.16±29.71	69.16±34.65	28.95±12.87
Other characteristics		Multinucleated cells observed	Multinucleated cells observed

Table 4.3 Characteristics and numbers of cells per day of interest For cell shape, the different morphologies are mentioned in order of them being observed (e.g. in day 7, more clumps are observed, and a few microglia-like and few single non-adherent cells). In cell diameter, max signifies the maximum distance between the cytoplasmatic extensions of the cells, and min the minimum. Data from n=5 images

THP-1 **Day 3** **Day 7** **Day 14**
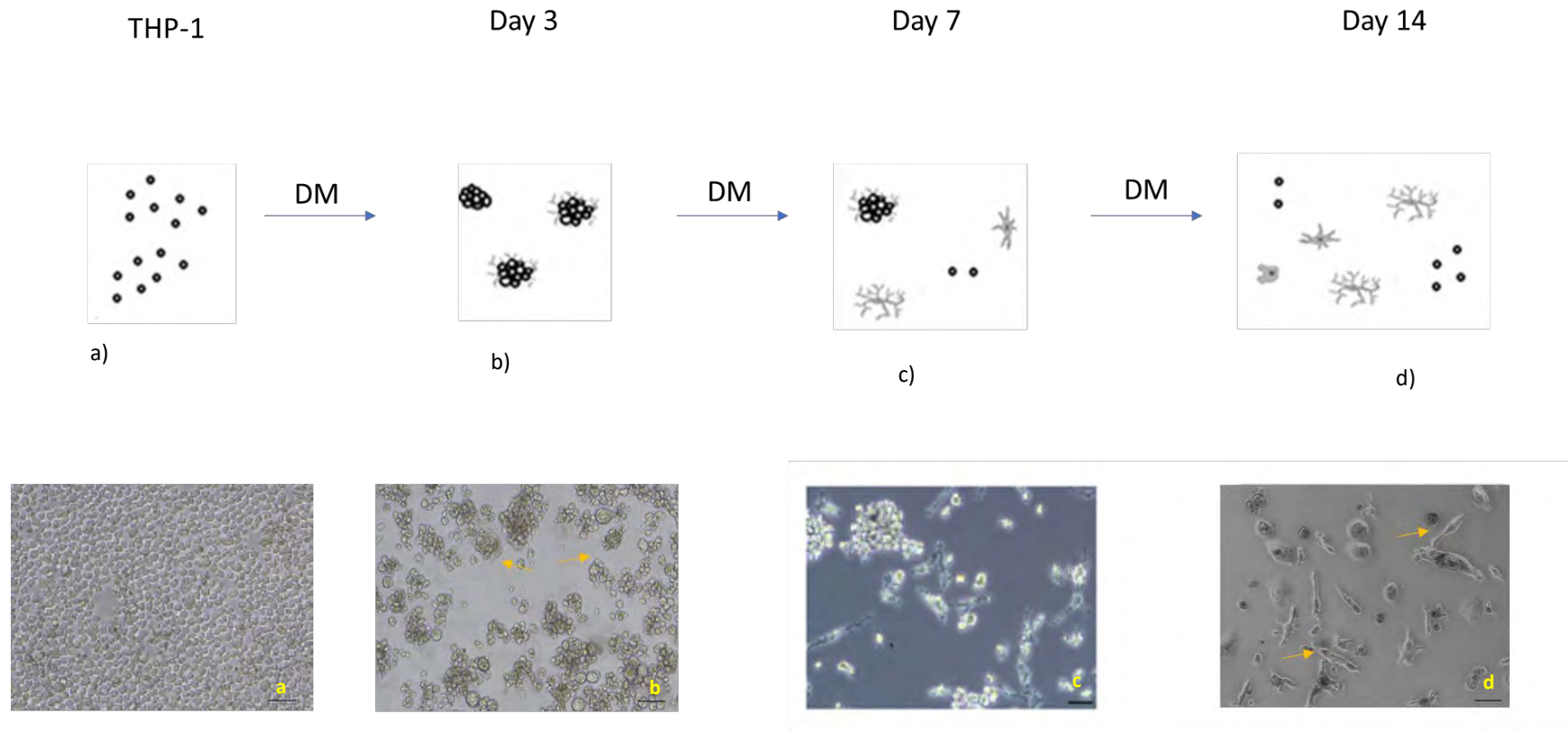
***Figure 4.1** **The different typical morphologies observed when THP-1 cells were exposed to NIS medium**_ THP-1 cells are spherical and do not form clumps usually (N.B. the THP-1 cells presented here are ~80% confluent, and had a count of ~700000cells/ml, not the freshly seeded 300000cells/ml used for the experiments)(a). After 3 days in the NIS differentiation medium, clumps started forming (b), which appeared to adhere to the plastic, and extensions seemed to appear in the place where they adhered (arrows in day 3 and higher resolution image in appendix, figure A5). Between days 3 and 14, but especially around day 7, the clumps started to disappear, and mg-like cells started appearing more often (c). In day 14, most of the cells were mg-like (arrows in day 14-d), however the number of the cells declined. Some non-adherent cells were also present. (Light microscopy images obtained using a Nikon U-200 attached to an inverted Leica (Leica Mocrosystems UK Ltd.) microscope. Scale bar 20μm). DM=NIS/RPMI Differentiation Medium.

4.3.2 Attempting optimisation of the differentiation protocol using ACM and dTHP-1 cells

Before proceeding to further experiments, it was crucial that the conditions were found where the most mg-like cells were generated, and therefore it was determined that the conditions of differentiation would be altered by one component at a time. (This was done after the initial experiments that showed differentiation, in order to potentially optimise the microglia-like nature of the cells produced-i.e. more microglia-like cells in day 14, and/or more cells exhibiting similar to microglial pattern of gene expression). For that reason in a set of comparator samples, dTHP-1 cells were employed instead of THP-1 cells in some cases, and ACM was also used alongside NIS in some cases. Microglia cells are macrophages, and dTHP-1 cells are macrophage-like, so it was possible that adding NIS or NIS+ACM, both of which in theory mimic CNS conditions, might guide these cells to be more CNS-macrophages-like.

Therefore, the conditions examined were the following:

- THP/NIS
- THP/NIS+ACM
- dTHP/NIS
- dTHP/NIS+ACM

ACM -as already discussed- is used in many protocols (Table 4.1), while dTHP-1 cells were dubbed as mg-like by one paper; indeed these cells are macrophage-like as they have already been primed to a "M0" state by PMA (Tedesco et al., 2018).

(N.B. The impact of ACM was also tested on the growth/differentiation; however it did not produce any noteworthy results which could be due to the fact that it was mostly deprived of nutrients, so it was not further examined as a condition (Fig. 4.2).)

The cells were observed for 14 days in all conditions in parallel. On the basis of the morphology alone, the other 3 conditions investigated did not exhibit an improvement in mg-like cell numbers when compared to the THP/NIS condition alone. Fig 4.2 shows a more detailed set of

illustrations in addition to what is presented in Fig. 4.1, including THP-1 cells, dTHP-1 cells (already adherent), and day 14 cells from all the other combinations of treatments, as mentioned above. The cells in all the other 3 conditions tested did not exhibit typical microglia morphology to the extent that was seen for the NIS/THP-1 condition; this suggests that the NIS/THP-1 condition may facilitate optimal differentiation. However, as microglia cells exhibit great plasticity, and as giant multinucleated microglia cells have been shown to exist in inflammatory conditions (PMA is inflammatory) (Fendrick et al., 2007; Hornik et al., 2014) in order to corroborate those morphological findings it was decided that all 4 conditions should be investigated at a molecular level, examining via qPCR the microglia "signature gene" expression, i.e. PU.1, Iba1, P2Y12R, CX3CR1 and CD45. TBP was used as the "housekeeping" gene, as it was found the most suitable between the genes examined (TBP, actin, and GAPDH). The results of these qPCR investigations are presented in section 4.3.3.
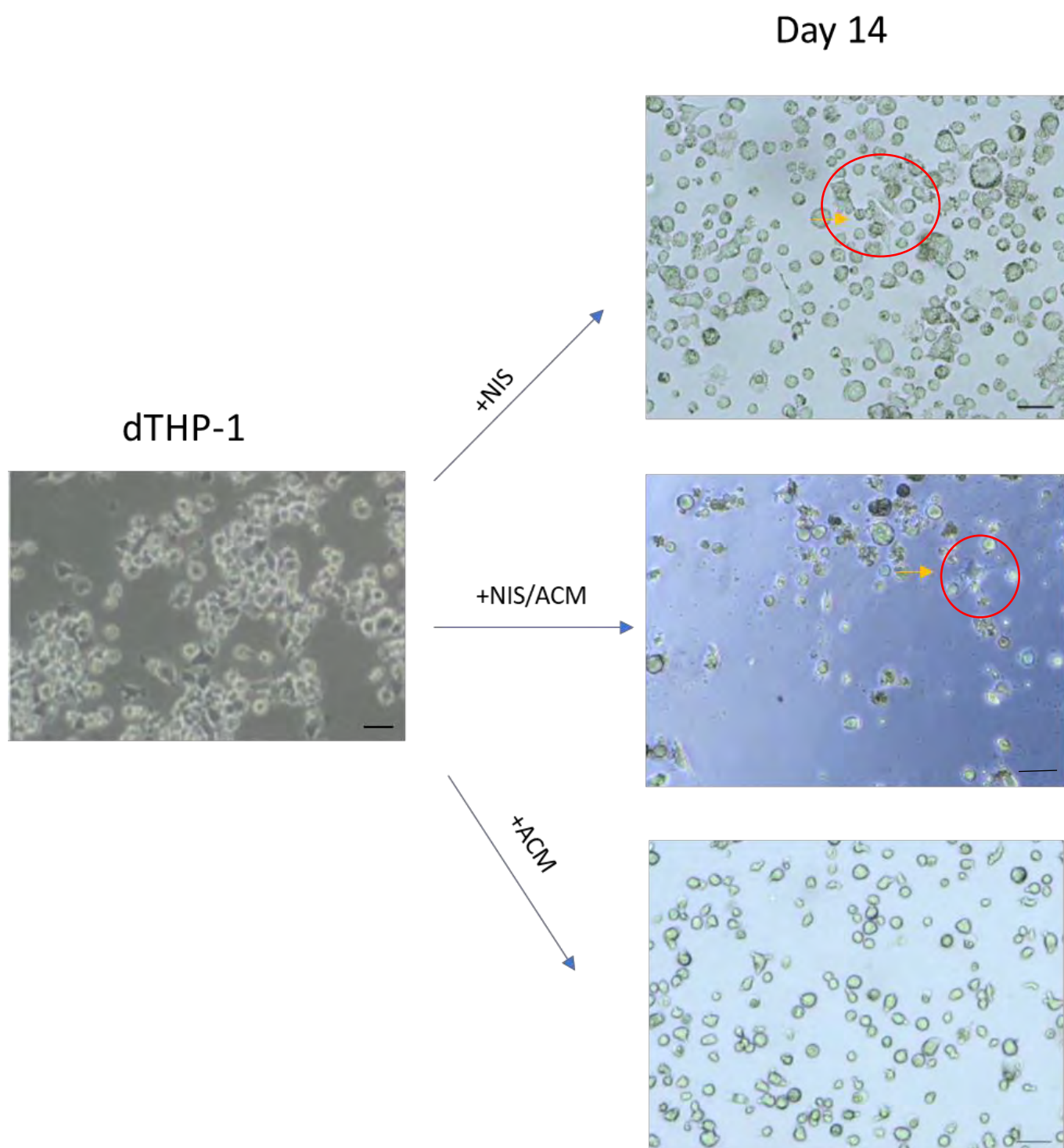
Figure 4.2a Typical findings of dTHP-1 cells being exposed in different conditions for 14 days Images with starting cell type dTHP-1 cells. Differentiation media used were NIS (RPMI THP-1 medium+NIS as described in 4.2), ACM with 2% NIS, and ACM. For the dTHP-1 cells, the cells were adherent and exhibited macrophage phenotypes, and during the treatment they lifted, some clumps appeared, but the final phenotype (d14) did not resemble mg-like cells overall; however there were some mg-like cells present (yellow arrows, inside red circles). (Light microscopy images obtained using a Nikon U-200 attached to an inverted Leica (Leica Mocrosystems UK Ltd.) microscope. Scale bar 20μm).
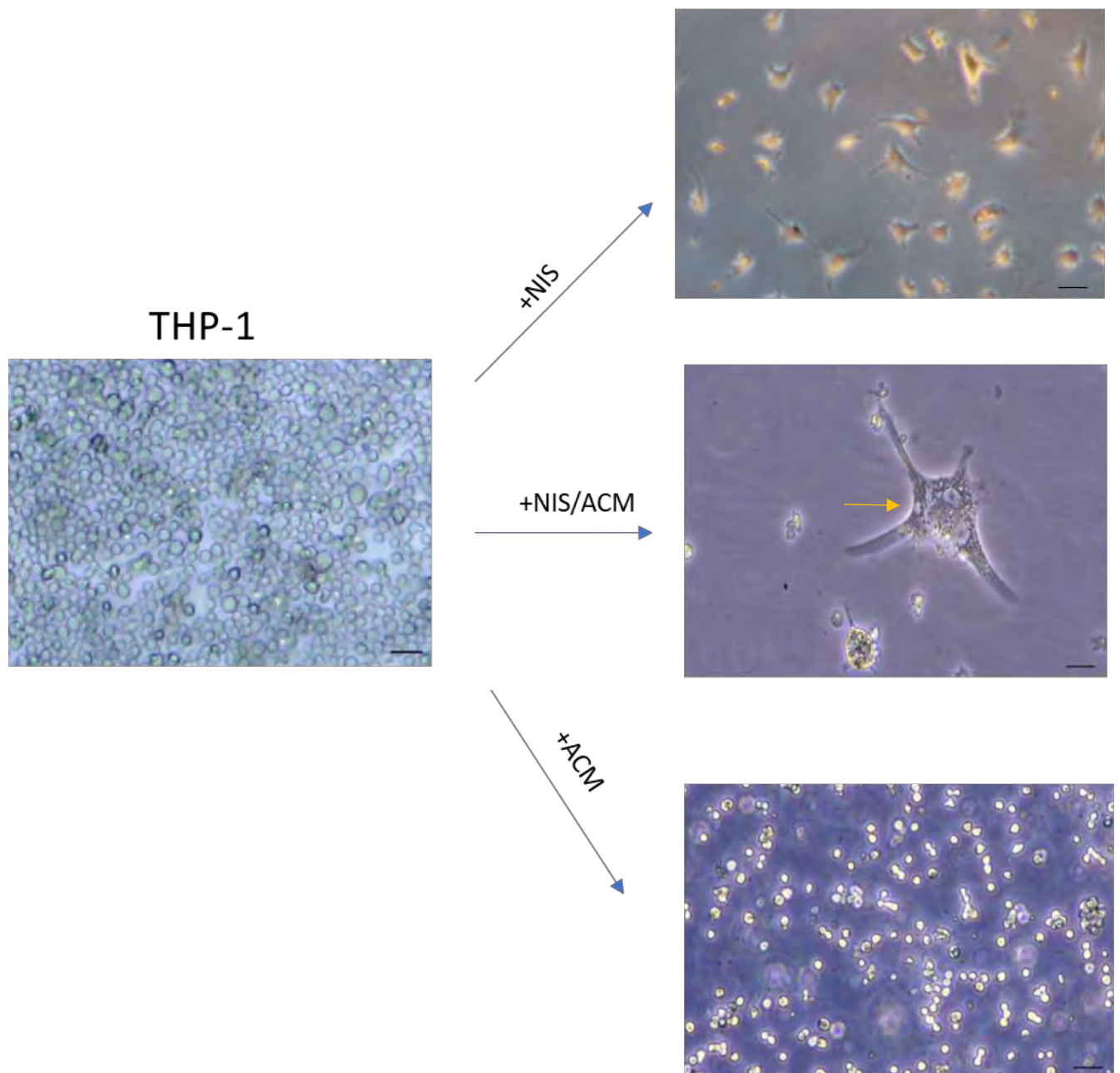
Figure 4.2b ***Typical findings of THP-1 cells being exposed in different conditions for 14 days*** Images with starting cell type THP-1 cells. Differentiation media used were NIS (RPMI THP-1 medium+NIS as described in 4.2), ACM with 2% NIS, and ACM. For the THP-1 cells, ACM alone did not seem to have any effect on the cells, with the exception of them shrinking. ACM/NIS produced some mg-like cells, however less than the NIS condition alone. In the ACM/NIS condition a plethora of "giant cells" (yellow arrow) were observed. (Light microscopy images obtained using a Nikon U-200 attached to an inverted Leica (Leica Mocrosystems UK Ltd.) microscope. Scale bar 20μm).

4.3.3 qPCR analysis of gene expression changes during mgTHP-1 differentiation

Differentiation from THP-1 cells
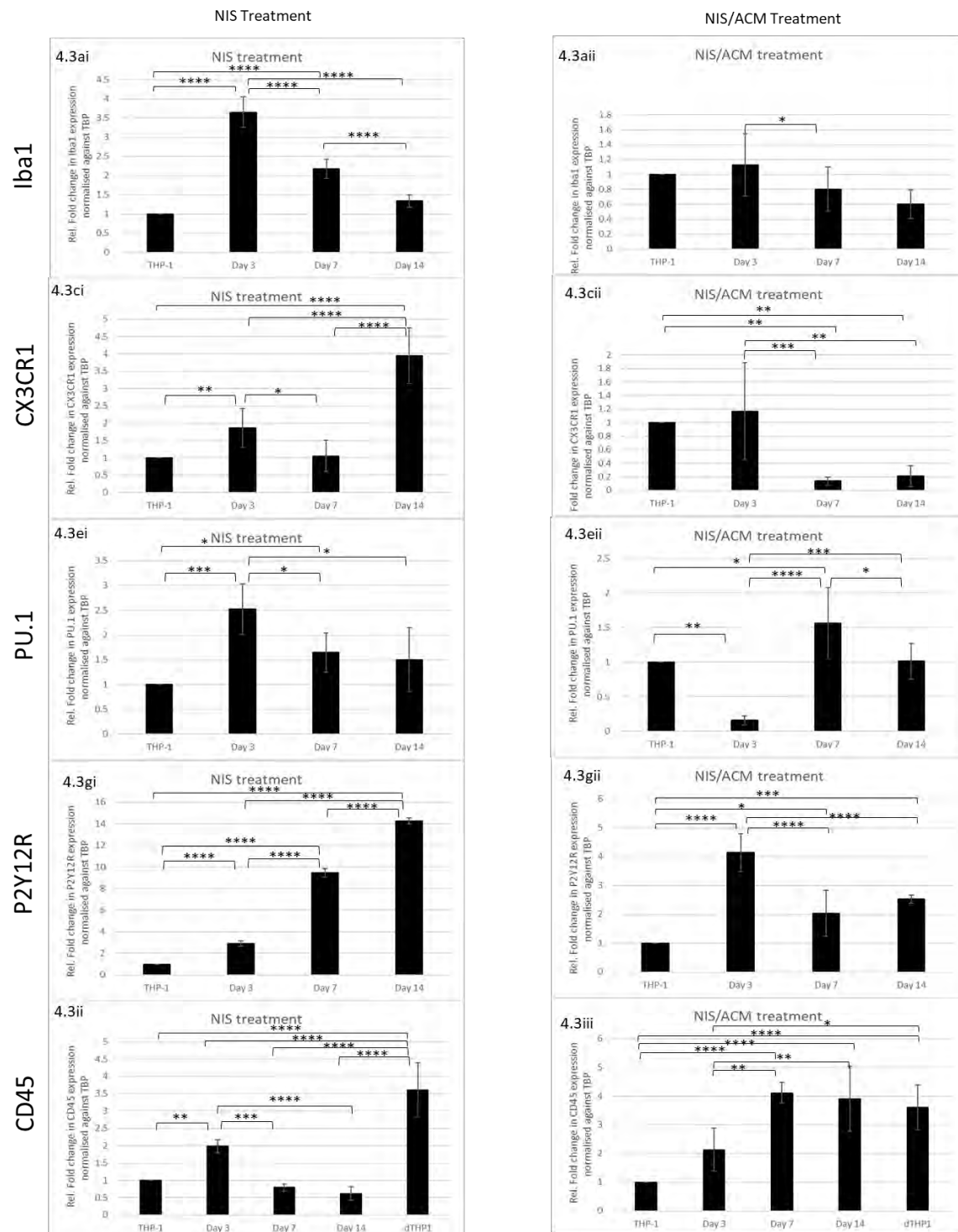
Figures 4.3a, c, e, g, i (i and ii)*: **The results of gene expression analysis in order to observe the changes in expression of 5 microglia-related genes (Iba1, CX3CR1, PU.1, P2Y12R, and CD45) during day 3, day 7 and day 14 of 2 of the 4 differentiation protocols under investigation.** Significance levels of $p<0.05$ are marked with an asterisk (*), $p<0.01$ with (**), $p<0.001$ with (***), and $p<0.0001$ with (****). Control/initial cell population are THP-1 cells, and NIS or NIS/ACM treatments are used. n= 6

For Iba1, and with THP-1 cells as initial cells with NIS treatment (Fig. 4.3ai), a 1 way ANOVA showed a significant difference between conditions ($F_{(3,20)}=179.1121$, $p<0.0001$). A Bonferroni post hoc correction revealed differences between THP-1 cells and Day 3 cells and Day 7 cells (both $p<0.0001$), but no difference between THP-1 cells and Day 14 cells. Further differences were found between day 3 cells Iba1 expression and Day 7 and Day 14, as well as between Day 7 and Day 14 Iba1 expression ($p<0.0001$ for all).

For Iba1, and with THP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3aii), a 1 way ANOVA showed a significant difference between conditions in the expression of the gene ($F_{(3,20)}=3.894$, $p<0.05$). A Bonferroni post hoc test showed a significant difference in the expression of the Iba1 gene between Day 3 and Day 7 of differentiation ($p<0.05$).

For CX3CR1, and with THP-1 cells as initial cells with NIS treatment (Fig 4.3ci), a 1 way ANOVA showed a significant difference between conditions ($F_{(3,20)}=34.51$, $p<0.0001$), with a Bonferroni post hoc revealing the differences to be between THP-1 and Day 14 cells ($p<0.0001$), as well as Day 3 and 14, and Day 7 and 14 cells as well ($p<0.0001$ for both), with Day 14 cells having the highest expression against all other conditions. Further differences were found between control and Day 3 ($p<0.01$), and Day 3 and 7 ($p<0.05$), with Day 3 having a higher expression of the gene against the other two.

For CX3CR1, and with THP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3cii), a 1 way ANOVA showed a significant difference between conditions in the expression of the gene ($F_{(3,20)}=12.49$, $p<0.0001$). A Bonferroni post hoc test showed differences between control (THP-1) cells and Day 7 cells ($p<0.01$), and control and Day 14 cells ($p<0.01$). Further differences in the expression of the gene were found between Day 3 cells and Day 7 cells ($p<0.001$), and Day 3 cells and Day 14 cells ($p<0.01$).

For PU.1, and with THP-1 cells as initial cells with NIS treatment (Fig 4.3ei), a 1 way ANOVA showed a significant difference between conditions ($F_{(3,20)}=10.0766$, $p<0.001$). A Bonferroni post hoc revealed a significant increase in the expression of the gene between control(THP-1 cells) and Day 3 (2.519 ± 0.515 fold increase) conditions ($p<0.001$), as well as significant decrease between Day 3, and Days 7 (1.646 ± 0.395 fold increase) and Day 3 and day 14 (1.502 ± 0.644) ($p<0.05$ for both).

For PU.1, and with THP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3eii), a 1 way ANOVA showed a significant difference between conditions ($F_{(3,20)}=19.910$, $p<0.0001$); a Bonferroni post hoc revealed these differences to be a significant decrease between control and Day 3 ($p<0.01$), and a significant increase between control and Day 7 cells in the expression of PU.1 ($p<0.05$). Further differences were shown between days 3 and 7 ($p<0.0001$), days 3 and 14 ($p<0.001$).

For P2Y12R, and with THP-1 cells as initial cells with NIS treatment (Fig 4.3gi), a 1 way ANOVA showed a significant difference between conditions ($F_{(3,20)}=1648.663$, $p<0.0001$). A Bonferroni post hoc showed differences between control (THP-1 cells) and Day 3, Day 7, and Day 14 gene expression ($p<0.0001$ for all), but also between days 3 and 7, 3 and 14, and 7 and 14 ($p<0.0001$ for all).

For P2Y12R, and with THP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3gii), a 1 way ANOVA showed a significant difference between conditions in the expression of the gene ($F_{(3,20)}=33.86$, $p<0.0001$). A Bonferroni post hoc correction showed that significant differences were between control (THP-1) cells and Day 3 cells ($p<0.0001$), Day 7 cells ($p<0.05$), and Day 14 cells ($p<0.001$). Further differences in the expression of P2Y12R were found between Day 3 and Day 7 cells ($p<0.0001$), and Day 3 and Day 14 cells ($p<0.001$).

For CD45, and with THP-1 cells as initial cells with NIS treatment (Fig 4.3ii), a 1 way ANOVA showed a significant difference between conditions ($F_{(4,25)}=53.77$, $p<0.0001$). A Bonferroni correction showed differences between THP-1 cells and Day 3 cells ($p<0.01$), Day 3 and Day 7 cells ($p<0.001$), and Day 3 and Day 14 cells ($p<0.0001$). Cells of all days as well as THP-1 cells had significantly lower expression of CD45 when compared to dTHP-1 cells ($p<0.0001$ for all).

For CD45, and with THP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3iii), a 1 way ANOVA showed a significant difference between conditions ($F_{(4,25)}=18.0344$, $p<0.0001$). A Bonferroni post hoc showed significant differences between control (THP-1) cells and Day 7 cells, as well between control and Day 14 cells ($p<0.0001$ for both comparisons). Further differences between THP-1 cells and dTHP-1 cells ($p<0.0001$), Day 3 cells and Day 7 cells ($p<0.01$), Day 3 cells and Day 14 cells ($p<0.01$) and Day 3 cells and dTHP-1 cells ($p<0.05$). No significant differences were found between dTHP-1 cells and Day 7 or Day 14 cells.

Differentiation from dTHP-1 cells
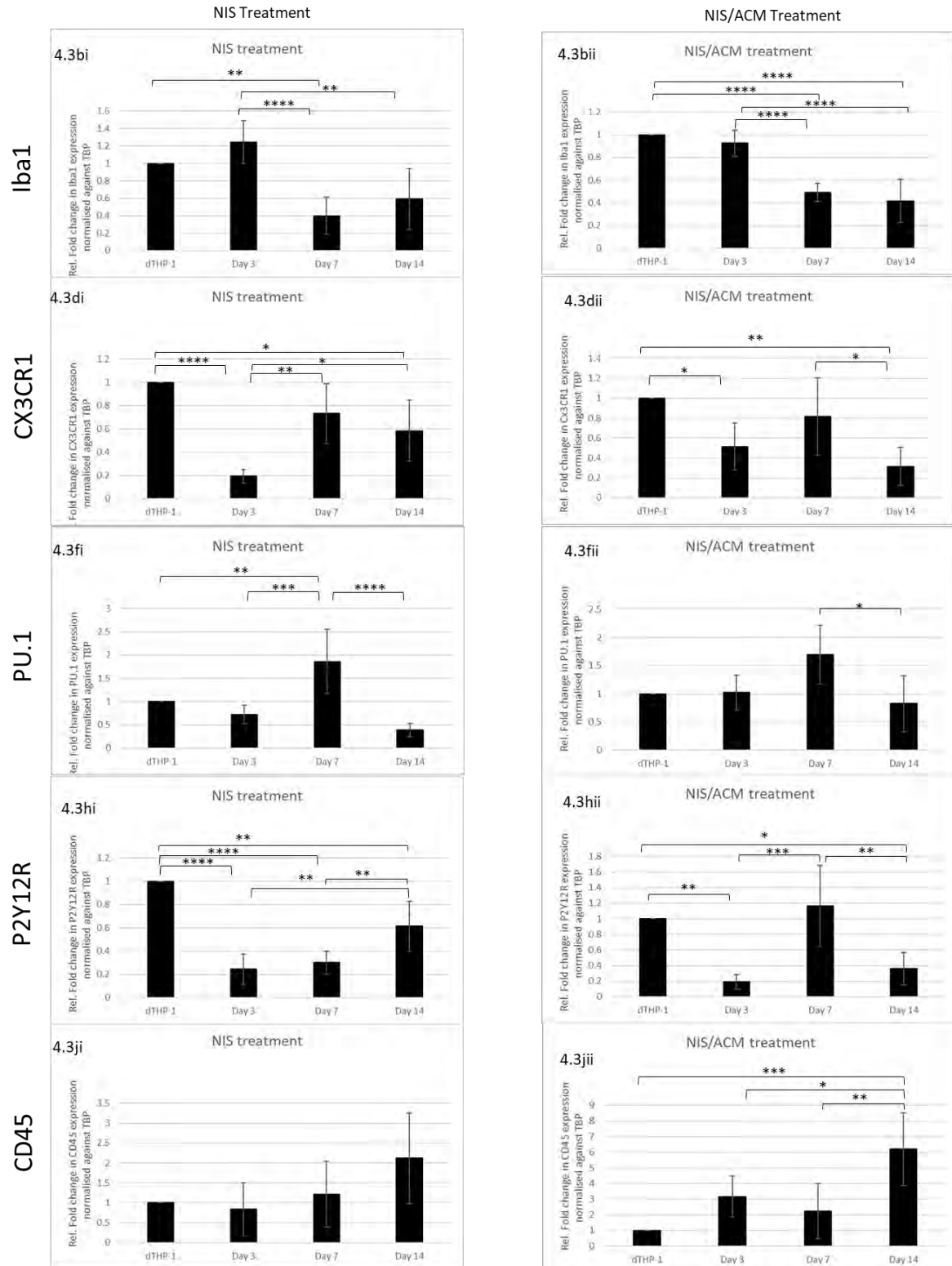
Figures 4.3b, d, f, h, j (i and ii): The results of gene expression analysis in order to observe the changes in expression of 5 microglia-related genes (Iba1, CX3CR1, PU.1, P2Y12R, and CD45) during day 3, day 7 and day 14 of 2 of the 4 differentiation protocols under investigation. Significance levels of $p<0.05$ are marked with an asterisk (*), $p<0.01$ with (**), $p<0.001$ with (***), and $p<0.0001$ with (****). Control/initial cell population are dTHP-1 cells, and NIS or NIS/ACM treatments are used. n=6

For Iba1, and with dTHP-1 cells as initial cells with NIS treatment (Fig 4.3bi), a 1 way ANOVA showed a significant difference between conditions ($F_{(3,20)}=13.476$, $p<0.0001$). A Bonferroni post hoc revealed differences in gene expression between control (dTHP-1 cells) and Day 7 cells ($p<0.01$), Day 3 cells and Day 7 cells ($p<0.0001$), and Day 3 cells and Day 14 cells ($p<0.01$).

For Iba1, and with dTHP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3bii), a 1 way ANOVA showed a significant difference in gene expression between conditions ($F_{(3,20)}=32.518$, $p<0.0001$). A Bonferroni post hoc showed differences between control (dTHP-1) cells and cells in the 7th day of differentiation ($p<0.0001$), as well as cells in the 14th day of differentiation ($p<0.0001$). Further differences were found in expression if the Iba1 gene between cells in Day 3 and Day 7 of differentiation ($p<0.0001$), and between Day 3 and Day 14 of differentiation ($p<0.0001$).

For CX3CR1, and with dTHP-1 cells as initial cells with NIS treatment (Fig 4.3di), a 1 way ANOVA showed a significant difference in gene expression between conditions ($F_{(3,20)}=16.595$, $p<0.0001$). A Bonferroni post hoc revealed the differences to be between control (dTHP-1) cells and Day 3 cells ($p<0.0001$), and control and Day 14 cells ($p<0.05$). Further differences in gene expression were found between Day 3 cells and Day 7 cells ($p=0.001$, it is signified as $p<0.01$ on the graph), and Day 3 and Day 14 cells ($p<0.05$).

For CX3CR1, and with dTHP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3dii), a 1 way ANOVA showed a significant difference in gene expression between conditions ($F_{(3,20)}=8.0757$, $p=0.001$). A Bonferroni post hoc indicated differences in gene expression between control (dTHP-1) cells and cells differentiated for 3 days ($p<0.05$), as well as differentiated for 14 days ($p<0.01$). Further differences were shown to exist between Day 7 and Day 14 cells ($p<0.05$).

For PU.1, and with dTHP-1 cells as initial cells with NIS treatment (Fig 4.3fi), a 1 way ANOVA showed a significant difference in gene expression between conditions $(F_{(3,20)}=13.830, p<0.0001)$. A Bonferroni post hoc correction revealed significant differences in gene expression between control (dTHP-1) cells and Day 7 of differentiation cells $(p<0.01)$, with further significant differences between Day 3 and Day 7 cells $(p<0.001)$, and Day 7 and Day 14 cells $(p<0.0001)$.

For PU.1, and with dTHP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3fii), a 1 way ANOVA showed a significant difference in gene expression between conditions $(F_{(3,20)}=4.882, p<0.05)$. A Bonferroni post hoc showed a significant difference in gene expression between Days 7 and 14 of differentiation $(p=0.0121)$.

For P2Y12R, and with dTHP-1 cells as initial cells with NIS treatment (Fig 4.3hi), a 1 way ANOVA showed a significant difference in gene expression between conditions $(F_{(3,20)}=34.91, p<0.0001)$. A Bonferroni post hoc confirmed significant differences in gene expression between control (dTHP-1) cells and Day 3 cells $(p<0.0001)$, Day 7 cells $(p<0.0001)$, and Day 14 cells $(p=0.001$, presented as $p<0.01$ on the figure). Further differences were found between Day 3 and Day 14 cells $(p<0.01)$, and Day 7 and Day 14 cells $(p<0.01)$.

For P2Y12R, and with dTHP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3hii), a 1 way ANOVA showed a significant difference in gene expression between conditions $(F_{(3,20)}=14.206, p<0.0001)$. A Bonferroni post hoc indicated significant differences between control (dTHP-1) cells and Day 3 differentiated cells $(p<0.01)$, and control and Day 14 differentiated cells $(p<0.05)$. Further differences were found between Day 3 and Day 7 cells $(p=0.0001$, signified on the figure as $p<0.001)$, and Day 7 and Day 14 cells $(p<0.01)$.

For CD45, and with dTHP-1 cells as initial cells with NIS treatment (Fig 4.3ji), a 1 way ANOVA showed no significant difference in gene expression between conditions $(F_{(3,20)}=2.806, p=0.0658)$.

For CD45, and with dTHP-1 cells as initial cells with NIS/ACM treatment (Fig 4.3jii), a 1 way ANOVA showed a significant difference in gene expression between conditions $(F_{(3,20)}=9.82, p<0.001)$. A Bonferroni post hoc showed differences in gene expression between dTHP-1 cells and Day 14 of differentiation cells $(p<0.001)$, as well as differences between Days 3 and 14 $(p<0.05)$, and Days 7 and 14 $(p<0.01)$.

With the exception of CD45, (whose expression is decreased in microglia when compared to macrophages (Rangaraju et al., 2018) -in our case dTHP-1 macrophage-like cells-), all other genes were expected to be expressed in a higher level in differentiated cells when compared to monocytes (in our case THP-1 cells), especially PU.1 and Iba1, which are involved in the development of microglia (Zöller et al., 2018; Hirasawa et al., 2005). Therefore, qPCR was used in order to investigate the changes in expression of microglia-related genes over time during the differentiation protocols in question, with measurements taken in the aforementioned 3 developmental days of significance (days 3, 7, and 14). Although on their own the changes in gene expression are not sufficient to indicate whether a cell is microglia or microglia-like, the combination of changes, and hence the overall pattern of expression gives valuable insights into the cells' identity. Even though having microglia cells to compare to would be useful, the lack of them does not make the results any less significant, as similar comparisons vs the origin cells (monocytes) (and taking into account literature data) have been used in other studies which focus on the development of mg-like cells (from Table 4.1 Ryan et al., 2017; Ohgidani et al., 2014; Toji et al., 2013 as examples).

Overall, the qPCR results for the panel of markers examined, indicate that the most reproducible and microglia-like cell producing condition is the combination of THP-1 cells with NIS containing medium, without the addition of ACM (therefore, the THP/NIS condition). In this condition, the error bars are narrower, and there are the most consistent statistically significant increases in the expression of the selected microglia marker genes, as well as a statistically significant decrease in CD45 expression that coincides with one or more of the differentiation stages of the cells (for example, please see the PU.1 elevation in day 3 and 7 where microglia-like cells are being developed is in agreement with its role in the development of microglia (Holtman et al., 2017), and P2Y12R's increasing expression in days 7 and 14 where more microglia-like cells appear, as this marker is expressed under normal conditions in microglia (Mildner et al., 2016) (see fig 4.3g))

As ACM is of unknown consistency and potentially differed between batches, and dTHP-1 cells are already differentiated almost terminally (dTHP-1 cells have been found to not to be proliferative, however they are in theory inflamed due to their activation with PMA, and thus potentially have elevated activation/inflammation markers e.g. Iba1 and CD45), the level of expression of microglia markers in the dTHP-1-derived and/or ACM supplemented samples was -as expected from observations of the phenotypes of the cells- not ideal. For figures 4.3i (i, ii) especially, a double comparison was done between the control cell type that the cells originated from (THP-1 cells) and its macrophagic counterpart (dTHP-1 cells). Day 14 CD45 expression in the THP/NIS condition is significantly lower than both controls, potentially showing the CD45^{low} marker observed in microglia phenotypes, at least in gene expression level. Therefore, it was decided that the cells developed using the NIS/THP-1 condition would be further investigated, and thus NIS/THP-1 differentiated cells are the ones that are being used in all the experiments from this point on.

4.3.4 Immunofluorescence

Immunofluorescence was carried out, in order to extend the previous microscopy findings with images (see figs 4.1, and 4.2) that exhibit that mgTHP-1 cells do not only look like microglia, but also they express proteins that have been found to be microglia-related (Cd11b, Iba1) or specific (TMEM119 a microglia-specific membrane protein marker (Satoh et al., 2016)). The images were taken under different settings, to ensure that protein localisation was apparent, and those included in figure 4.4 are representative of the morphology of cells in the days examined (although in day 14 amoeboid microglia-like cells were also present, see figure A4, Appendix 2). As can be seen in the images, staining of all 3 proteins of interest is evident at days 3, 7, and 14 stages of differentiation. For example, TMEM119 is expressed from day 3 onwards, and in day 7 even in the clump formations adhering to the glass coverslip and forming protruding membrane extensions (positive for all 3 markers). In day 7 photos were taken at two different depths, in order to investigate the 3-D structure of the adhering clumps. (N.B. Although this was consistently seen, the significance of those clumps adhering (in previous days) for the formation of mg-like cells in day 14 was not explored on this instance and could be explored in future studies).

Another observation that was made was that of difference in size of the cells. In day 3, the cells in clumps are smaller (both nuclear and overall size) than the cells in day 14 (table 4.3). Microglial cells show a great diversity in size, but are usually within the range of 30-120μm, including branches (Karperien et al., 2013), while THP-1 cells are usually <20 μm (Chitra et al., 2014). Thus, the fact that in the current study the cells in the clumps measure smaller than 20μm, and the size of the cells increases in days 7 and 14, places their morphological characteristics in line with what has been reported by others in previous studies.

Day 3
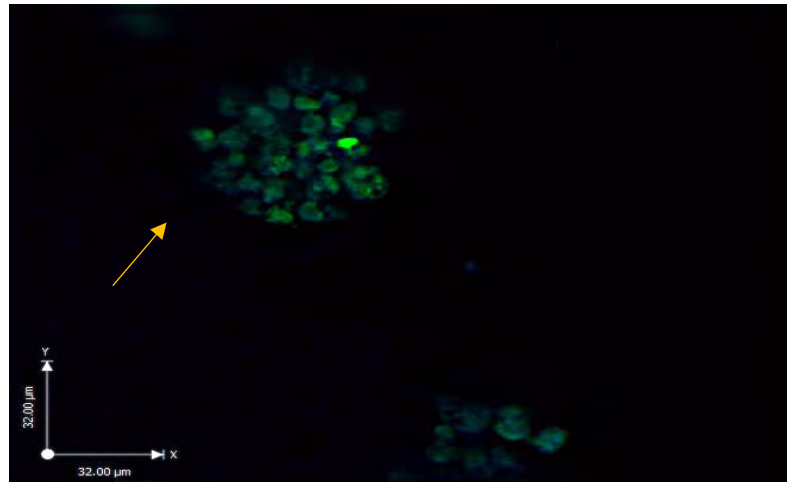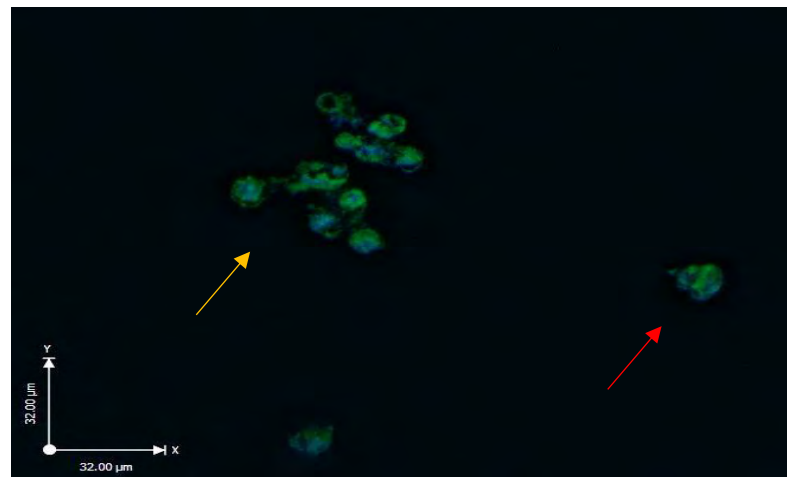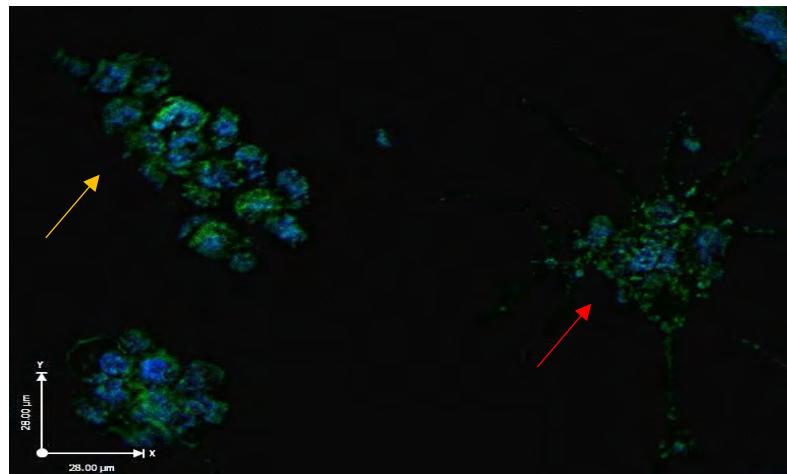
Figure 4.4a: _The results of the ICC for THP-1/NIS differentiated cells all three selected differentiation days for markers of interest (Cd11b, Iba1, TMEM119)_. For day 3, various multinucleated structures (red arrow Cd11b) and multicellular clumps (yellow arrows) as well as single cells (Iba1 stain red arrow) were apparent. Some of these structures adhered to the coverslip (cd11b stain), others were not (Iba1 and TMEM119 stains). The number of nuclei/cells per clump/structure varied. Green: protein of interest (TMEM119, Iba1, and Cd11b respectively), Blue: nucleus, scales as mentioned on figures. Images representative of at least n=3
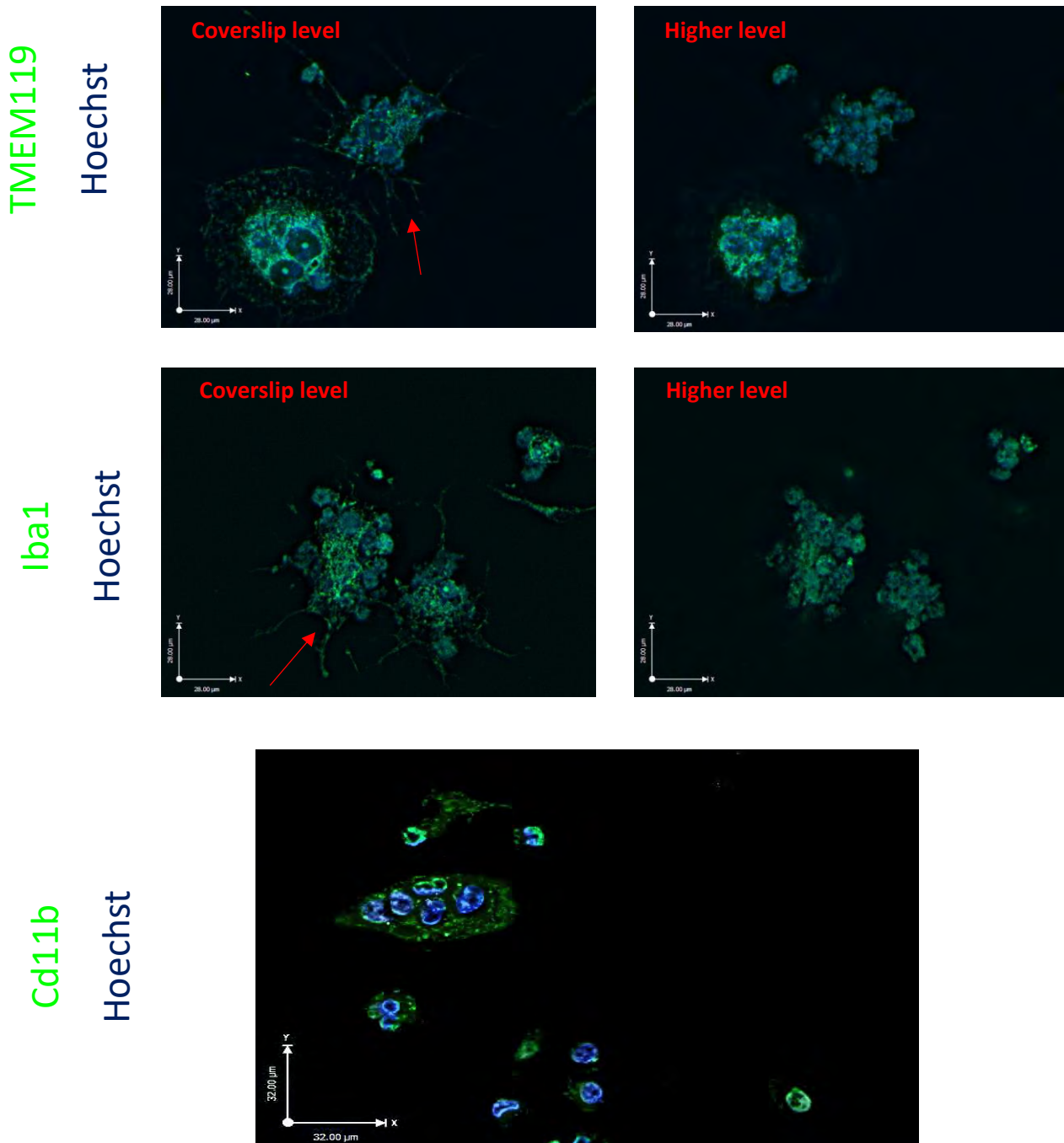
Figure 4.4b: The results of the ICC for THP-1/NIS differentiated cells all three selected differentiation days for markers of interest (Cd11b, Iba1, TMEM119). For day 7 the left and right images for Iba1 and TMEM119 detection are of different levels (coverslip level, where the clumps adhere to the coverslip, and higher levels, revealing a complex 3D multinucleated structure) for the same group of cells. Red arrows indicate ramified processes expanding from the clumps. One image shows attachment to the plastic surface (left) and the other the sphere-like clump of cells that begin to adhere (right). Green: protein of interest (TMEM119, Iba1, and Cd11b respectively), Blue: nucleus, scales as mentioned on figures. Images representative of at least n=3.

Day 14
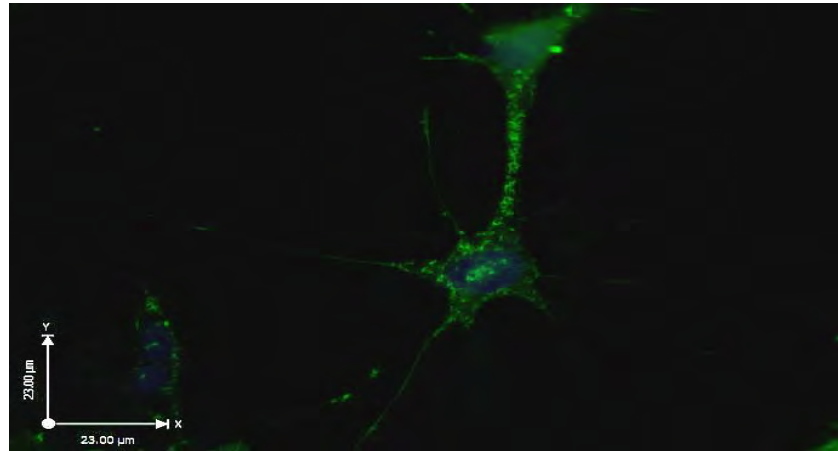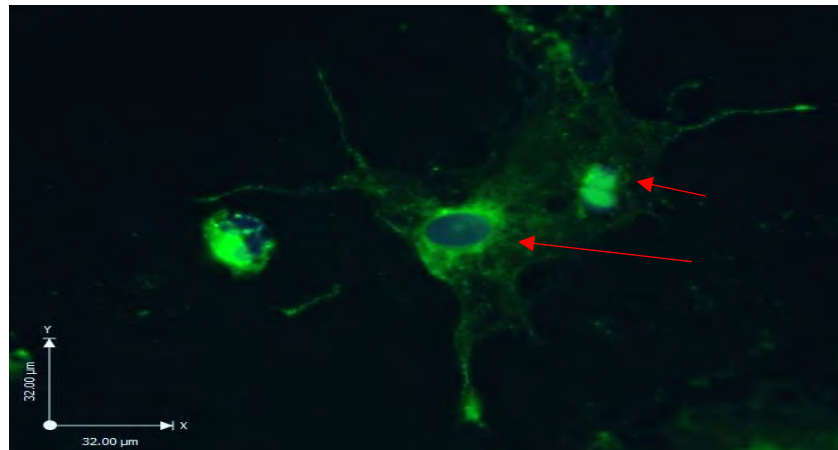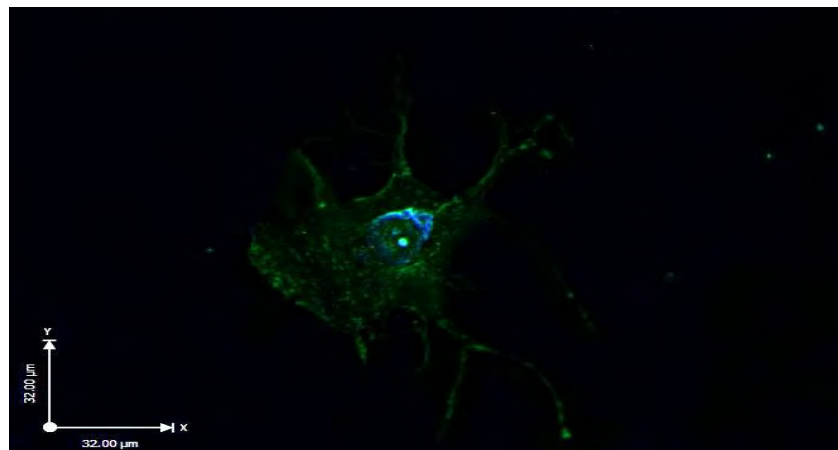
Figure 4.4c: The results of the ICC for THP-1/NIS differentiated cells all three selected differentiation days for markers of interest (Cd11b, Iba1, TMEM119). For day 14 different microglia-like morphologies were more apparent in the sample. Multinucleated cells were present (Iba1 stain), and both ramified (present pictures) and amoeboid cells (appendix, figure A4) were observed as well as multinucleated cells (red arrows, Iba1 stain) Green: protein of interest (TMEM119, Iba1, and Cd11b respectively), Blue: nucleus, scales as mentioned on figures. Images representative of at least n=3

4.3.5 Protein expression analysis via Western Blotting

<u>TMEM119</u>
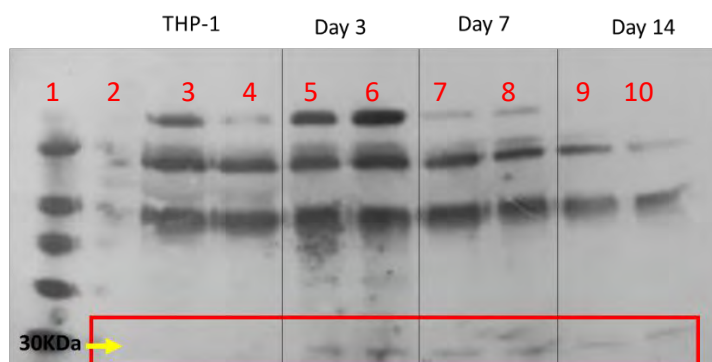
Figure 4.5**: **Western blotting results for TMEM119 detection in samples of days 3, 7, and 14 mgTHP-1 cells. TMEM119 was absent from THP-1 cells but present and detectable, albeit in low quantities in day 3, 7 and 14 mgTHP-1 cells at an apparent molecular weight of approximately 30KDa (see inset box and yellow arrow). More bands appear in higher molecular weights, which could signify unspecific binding, or binding due to similarities in sequence with TMEM119, or TMEM119 binding to either itself (dimers) or other proteins. Lane 1=ladder, 2=negative control, 3-10: samples as described on image, for this experiment n=2

As in the interest of this experiment is to determine presence/absence of TMEM119, no quantification was used. In all experiments, TMEM119 was not expressed by THP-1 cells as it was not detected in the samples. However, TMEM119 appears as a band of approximately 30KDa in day 3, 7, and 14 mgTHP-1 samples (see inset box in fig. 4.5). This confirms that the cells are microglia-like, even to the extent of exhibiting protein expression patterns that are microglia-specific, as the expected weight of TMEM119 is around 30KDa (Satoh et al., 2016). However in another TMEM119 microglia study, similar results have been found with a band appearing around 58 KDa, which represents TMEM119, and could possibly indicate a dimer (Satoh et al., 2016). As additional high-molecular weight bands appeared along with the expected TMEM119 band (see fig 4.5), bioinformatic analysis revealed that the area (the extracellular domain) against which the antibody was raised, could have affinity with other proteins, such as desmoplakin (involved in cell-cell adhesion, and is found at 250kDa in a western blot- which in our case would correspond to the top band in fig.4.5; also, please see Appendix Fig A6a for a sequence alignment between TMEM119 and desmoplakin) and hence

the antibody might be detecting a complex of proteins in addition to single TMEM119 monomers. Dimers (or n-mers) of the TMEM119 protein are also not excluded from being detected, nor are its interactions with other proteins. For example TMEM119 is known to bind to proteins such as SMAD1, SMAD6, and RUNX2 as indicated by Uniprot (entry Q4V9L6). A blocking peptide experiment was not performed; this could have acted as a quality control for the antibody, as it would indicate the ability of the antibody to recognize and bind the target protein (TMEM119). This however would not exclude cross-reacting with other high affinity sites of other proteins as discussed by Brownjohn &Ashton (2014).

Iba1
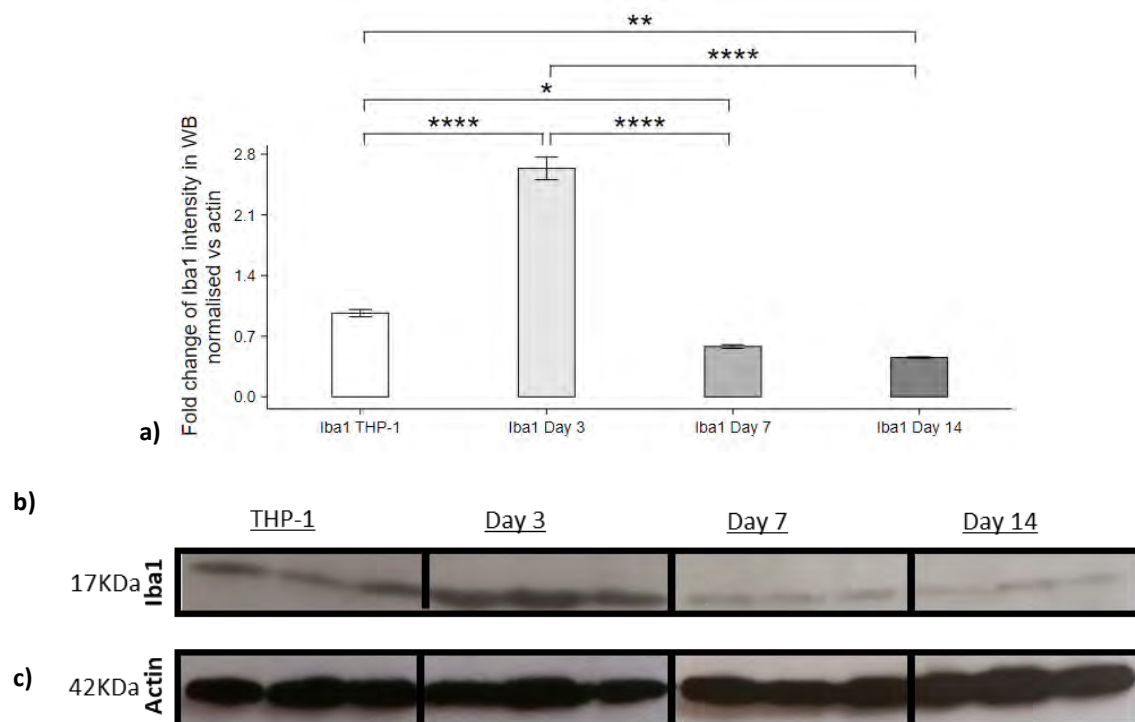
Figure 4.6*: **The results for densitometric analysis of western blotting for Iba-1 for the different days of differentiation of mgTHP-1 cells and the WB result with the actin band (c) presented as control.** The densitometry analysis was performed using ImageJ, and normalised against actin (a). The band was detected as expected at 17KDa (b) and actin was detected at 42KDa. L-R THP-1, Day3, Day 7, and Day 14 results. N=3 (N.B. 2 gels were used, gel 1 was THP-1, and Day3 samples, gel 2 was Day 7 and Day 14 samples). *, **, **** = p<0.05, p<0.01, and p<0.0001. Full membrane in the appendix, n=3*

The Iba1 Western blot results (figure 4.6) show a similar pattern with the qPCR results, in that Iba1 expression undergoes a marked increase in expression at day 3. A one way ANOVA between the different conditions showed a significant difference in fold change ($f(3,8)=210.552$, $p<0.00001$). A Bonferroni post hoc tests showed differences between Day 3 cells and all other conditions tested ($p<0.00001$), with day 3 samples showing the highest amount of protein expressed. Differences were also found between THP-1 and day 7 cells ($p<0.05$), THP-1 and Day 14 cells ($p<0.01$), with THP-1 cells expressing the protein in higher levels than days 7 and 14. However, these differences are not in total agreement with the qPCR results (Fig.4.3a), as the baseline THP-1 expression levels seen in the W.B. are higher than what is seen in the qPCR; as such the day 7 protein levels were detected at lower levels than the THP-1 as were the day 14. However, it is to be noted that after further research on the Iba1 protein sequence, it was found that Iba1 has 3 distinct isoforms (Uniprot ID P55008), with isoform 1(P55008-1) being the complete protein, while isoform 2 (P55008-2) is missing the first 54 amino acids, and isoform 3 (P55008-3) is missing the last ~30 amino acids and has changes in the sequence of the first part of the protein as well. The antibody used here (Wako, via alphalabs, UK, prod.code 019-19741) is created against the C-terminus of the protein. Therefore, the detection of the whole of the third isoform could be missed, which could provide an explanation for the difference between the qPCR and the W.Blotting results. Moreover, it is possible that DNA transcription into mRNA, mRNA processing, and translation into protein happen at different times, which often depends on the gene; indeed, in microglia cells, genes influenced by inflammation (such as Iba1), have been shown to have translation and transcription often done at different times, and therefore, changes in transcription are not necessarily shown in protein level (Boutej et al., 2017). So, although the present protein expression results seem to differ in some respects from the mRNA expression results observed in the previous section, this could be due to different translation/transcription regulatory

networks, rather than artefacts caused from the experimental procedure (e.g. sample loading, measuring the total protein etc.).

Actin and GAPDH

To verify equal loading, actin was used as a "housekeeper" as indicated by WB and by densitometric measurements of the protein level using imageJ (Figure 4.7aa), where a one way ANOVA between days showed no significant differences ($f(3,8)=0.03480$, $p=0.993$). Figure 4.7b represents a synopsis of the results for all the proteins investigated, normalised against actin (except TMEM119-normalisation was not done as only presence/absence was in the focus of the current investigation). Interestingly GAPDH expression increased in day 3, as shown by a 1 way ANOVA with Bonferroni post hoc test ($f(3,8)=65.8719$, $p<0.0001$, with Day 3 protein expression being significantly higher than those of THP-1 cells, Day 7, and Day 14, all $p<0.0001$), while actin expression was stable in all days. In order to further investigate this, we explored the GAPDH expression at the mRNA level (see figure 4.8), and performed additional metabolism-related experiments (see fig 4.9) as differences in metabolism could be due to differentiation of the cells and could give hints towards what fuels the differentiation-see section 4.3.6.

a)

b)

42KDa

THP-1   Day 3   Day 7   Day 14

c)

d)

42KDa
37KDa

THP-1   Day 3   Day 7   Day 14
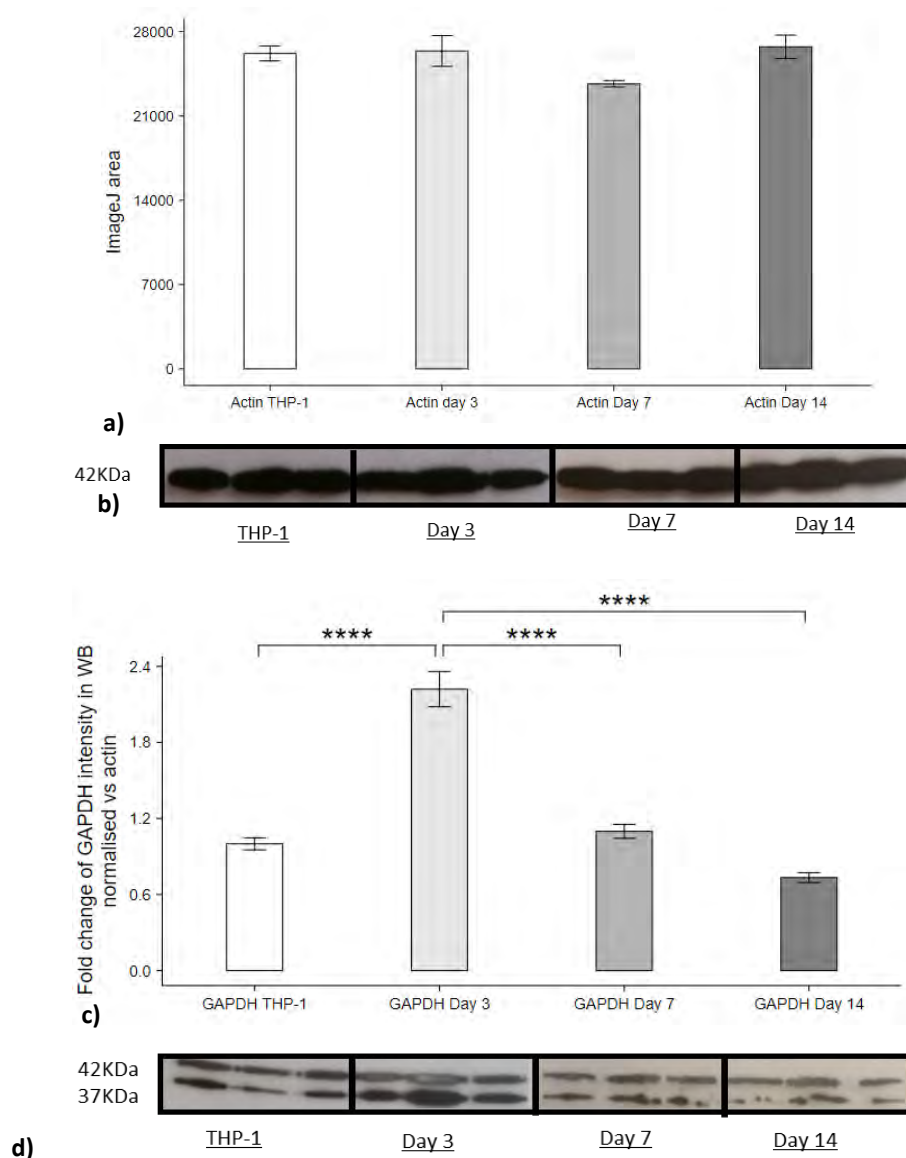
Figure 4.7a ***A synopsis of the Western results per day for actin and GAPDH:*** The results of densitometry for actin, as quantified using ImageJ (a) and for GAPDH normalised against actin (c), and the blots (b,d) N=3, for full gels please see appendix, figure A3. N.B. In d both actin and GAPDH can be seen, due to the fact that due to its intensity, actin was not possible to wash off the membrane, so it remained for the subsequent experiments; nevertheless, Imagej analyses were per protein, and did not include both actin and GAPDH. N=3 for this series of experiments
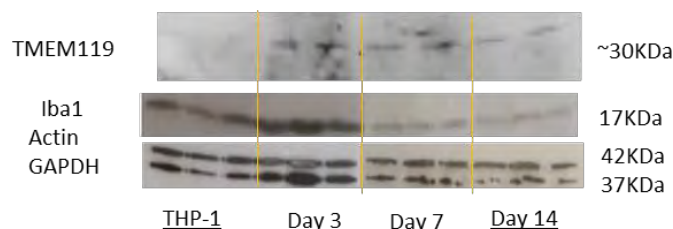
Figure 4.7b ***A synopsis of the Western results per day and per protein investigated.*** N=3 per condition were used except for TMEM119 (full membranes presented in appendix, figure A3 for Iba1, actin and GAPDH, and figure 4.5 for TMEM119)

4.3.6 Investigating the metabolism changes during the development of mgTHP-1 cells

In order to investigate the potential mechanism that drives these cells from a monocytic to a microglia-like state, we examined two metabolism-related elements: GAPDH expression in both protein and gene level, and cellular ATP content. As the cells are going through a differentiation process, their metabolism is expected to change to accommodate the differentiation.

As shown in Figures 4.8 and 4.9, GAPDH (unlike actin) undergoes a transient increase in expression at day 3 of the differentiation process ($p < 0.001$ vs control and days 7 and 14, as indicated by a one way ANOVA with Bonferroni corrections), and this is mirrored by a marked transient increase in cellular ATP content ($p < 0.001$ vs control and days 7 and 14, as indicated by a 1 way ANOVA with Bonferroni post hoc). As GAPDH encodes an enzyme involved in glycolysis, this indicates that glycolytic energy generation transiently increases at day 3, while it may suggest a role for glycolysis providing metabolic energy to drive the differentiation.
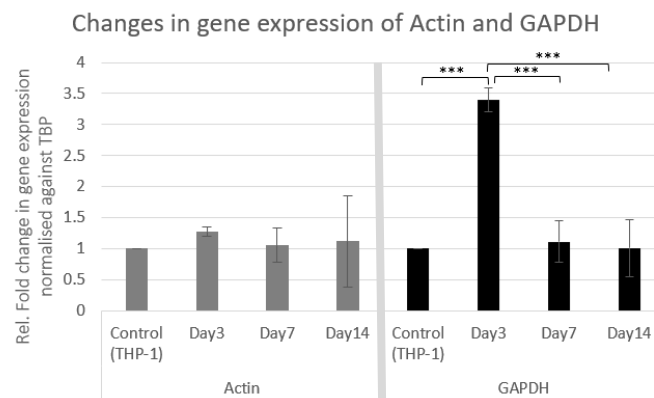
Figure 4.8 ***Actin and GAPDH gene expression per day, as examined by qPCR***. qPCR for GAPDH and actin expression during mgTHP-1 differentiation (normalised against TBP). Control cells were THP-1 cells. A 1 way ANOVA found no differences between any conditions for the actin expression ($F(3,8)=0.6520$, ns). There is a significant increase in the expression of GAPDH in day 3 vs control or Days 7 and 14 (signified with ***, $p<0.001$ for all comparisons, as indicated by a 1-way ANOVA with Bonferroni corrections). N=3
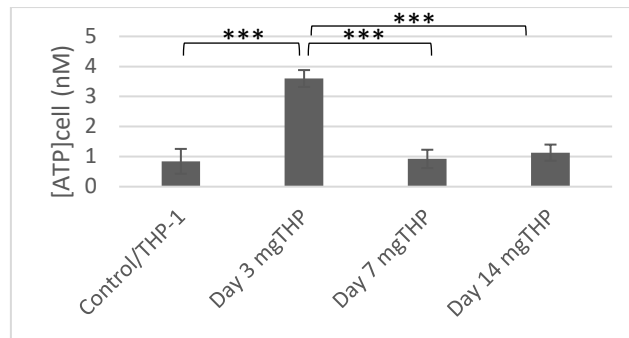
***Figure 4.9** **Results of the CellTiterGlo experiment investigating the amount of [ATP]cell per day**. There is a significant increase of ATP cell content in day 3 (3.598 ± 0.28 nM) when compared to the control/THP-1 cells but also vs days 7 and 14 (signified with ***, for p<0.001 for all comparisons, as indicated by a 1-way ANOVA with Bonferroni corrections) n=3

4.3.7 Investigating the epigenetic changes during the development of mgTHP-1 cells
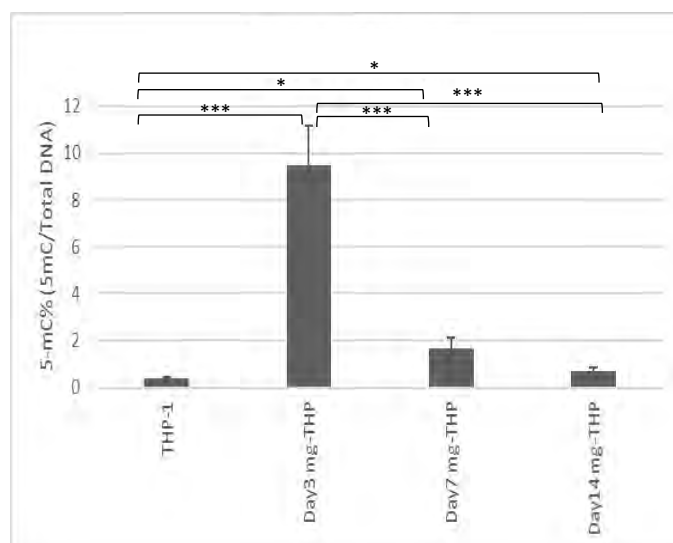
***Figure 4.10** Quantification of 5-mC content of DNA samples of different developmental days during mgTHP-1 differentiation and also in THP-1 cells*. The mgTHP-1 cells in all the days examined seem to have a higher amount of 5-mC than the control/THP-1 cells. Asterisk signifies significant difference, * for $p<0.05$, *** for $p<0.001$, n=3

As shown in figure 4.10, and as indicated by a one-way ANOVA with Bonferroni post hoc, there is a significant increase of methylation in day 3 compared to that of untreated THP-1 cells (0.373 ± 0.067 vs 9.47 ± 1.7, $p<0.001$); this is reduced in day 7 ($p<0.001$), but is still significantly increased when compared to THP-1 cells (1.646 ± 0.465 vs 0.373 ± 0.067 for THP-1 cells, $p<0.05$)), and declines further by day 14 ($p<0.001$) -however there is still a small but significant increase above control levels at day 14 (0.709 ± 0.13, $p<0.05$ vs THP-1 cells). This methylation/demethylation pattern over time could signify silencing of monocyte-related genes around day 3, where the first changes are observed. Later, and until day 14, a different set of genes could be becoming unmethylated, and thus expressed, which may correlate with the new morphology and function of the cells.

4.3.8 An investigation of the ingredients of the differentiation medium (NIS)

In an attempt to investigate which ingredients produce the changes observed, we first contacted Gibco/Thermofisher UK. Unfortunately, as NIS is trademarked, the company declined the request to provide the full ingredient list and the concentrations. However, they allowed questions regarding suspected ingredients. As in other iPSC to NSC differentiation media the following are included, a list was sent to the company, which came back positive only for the latter: Retinoic Acid, EGF, bFGF, Noggin, TGF-β and LIF. To examine the protein nature of the supplement, it was compared to two other supplements of known ingredients, namely B-27 and N-2 (ingredients list can be found for both in the appendix 1) as well as B-27+ (an improved form of B-27 that has all the ingredients of the normal B-27 however formulated differently) and Stempro (unknown ingredients). A coomassie blue staining protocol was employed, in order to find similarities between the supplements; the results can be found in Fig. 4.11.

Figure 4.11 ***Coomassie blue staining after SDS-page electrophoresis*** *(l-r: protein ladder, N-2 supplement, B-27+ supplement, empty, NIS, B-27, Stempro).* A band around or below 10kDa (10KDa indicated by blue arrow in 1st lane of the ladder) is apparent in all supplements tested except Stempro (red arrows). As insulin, which has a molecular weight of ~6kDa is found in all the supplements of known composition (i.e. N-2, B-27, B-27+), and the band is detected in all of them, as well as NIS it could be deducted that this band could potentially represent insulin. N=1

A band under 10 kDa was detected in N-2, B-27+, NIS, and B-27. By looking at the ingredients of the known composition supplements, it was found that they contain insulin, which in

Western blotting and Coomassie blue staining is found around or under the 10 kDa mark. Therefore, it could be hypothesised that NIS might contain insulin among other ingredients.

Cytokine array analysis

In order to further investigate the potential ingredients, we employed cytokine arrays (ab133997, Abcam, UK used as described in 4.2) for the whole medium (RPMI/THP-1 medium+NIS). These arrays have the potential to detect 42 targets in samples, most of which are cytokines, chemokines, and growth factors.

a)

Pos	Pos	Neg	Neg	ENA-78	GCSF	GRO	GRO-α	I-309	IL-1α	IL-1β
Pos	Pos	Neg	Neg	ENA-78	GCSF	GRO	GRO-α	I-309	IL-1α	IL-1β
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-12	IL-13	IL-15	IFN-γ
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-12	IL-13	IL-15	IFN-γ
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	RANTES	SCF	SDF-1	TARC	TGF-β1
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	RANTES	SCF	SDF-1	TARC	TGF-β1
TNFα	TNFβ	EGF	IGF-I	Angiogenin	Oncostatin-M	VEGF	PDGF	Leptin	Neg	Pos
TNFα	TNFβ	EGF	IGF-I	Angiogenin	Oncostatin-M	VEGF	PDGF	Leptin	Neg	Pos

b)

Figure 4.12 a and b*: Cytokine array analysis of the differentiation medium used (n=1 membrane used, the one presented)*. A)table of the cytokines/chemokines/growth factors, antibodies for which are included in the membrane (Pos=positive control, neg=negative control).In red boxes are the positive signals as found in B. B) the result of the analysis. Immunoreactive areas in coloured/dashed boxes in b correspond to cytokines boxed in a.

The only undefined ingredients are NIS and FBS, so the dots appearing on the array belong to either of these ingredients. Figure 4.12a shows the position of each cytokine/chemokine on the array, and figure 4.12b is the image of the array itself. Unlike chapter 5, where comparison between conditions was performed, here we only wanted to investigate whether some of the ingredients could be uncovered. be. Therefore no quantitative analysis was performed, rather the results of positive signal are determined visually, in a qualitative manner. The strong positive signals are those of: GRO-α, IL-7, MCP-3, TARC, VEGF, Oncostatin-M, and Angiogenin. There are also traces of Leptin, PDGF, TGF-β , SDF-1, SCF, MSCF and possibly RANTES. Although it is not possible to be certain which ingredients belong to NIS or FBS, it can tentatively concluded that some of these may potentially drive the differentiation of THP-1 cells to mgTHP-1 cells.

4.4 Discussion

4.4.1 Main findings

In the current chapter we used a monocytic cell line (THP-1), which upon exposure to a neural induction supplement (NIS), was guided to obtaining a microglia-like phenotype (referred to as mgTHP-1), strong evidence of which started appearing in day 7 post incubation, and peaked at around day 14. Between days 7 and 14 there was a shift of the cell morphology observed, from mainly ameboid in day 7 to a more branched/ramified morphology in day 14 (see Fig. 4.1 and 4.2). However only a relatively small fraction (approximately 20-50%) of the initial cell number remained and became microglia-like; the precise extent of cell loss differed between experiments, but overall less than half of the original cell number was counted in day 14.

After establishment of the timeline of events via preliminary observation of the changes in the cells morphology, there was an attempt to optimise the method, by incorporating ACM, which has been shown elsewhere to guide monocytic cells to microglia-like cells (e.g. Hinze & Stolzing, 2012). Another change which was attempted was to drive the monocytic cells to macrophage-like cells (dTHP-1 cells) before introducing them to the neural induction medium. These changes, alone and in combination did not produce better results, as measured in final mg-like cell number, however, different microglia-like morphologies were present, which could not exclude them from producing mg-like cells and so 4 protocols (THP-1/NIS, THP-1/NIS+ACM, dTHP-1/NIS and dTHP-1/NIS+ACM) were included in subsequent optimisation experiments. (The only exception was the effect that ACM had on the cells alone, which unfortunately did not produce any mg-like cells, and appeared to stress the cells, as evaluated by changes in their morphology (cells appeared to be shrinking, and in the dTHP-1 cells case,

detaching from the flasks or wells); therefore, culturing in ACM alone was not included in the subsequent experiments.)

Although the other 3 protocols did not achieve any improvement on the morphological changes seen with NIS/THP-1, to provide an alternative method of determining which condition produced the most microglia-like cells, qPCR was used in order to observe the changes in gene expression for 5 microglia-related genes in the four conditions tested (i.e. THP/NIS, THP/NIS+ACM, dTHP/NIS and dTHP/NIS+ACM), and at the three developmental days, where changes in morphology were most apparent (i.e.days 3, 7, and 14). The condition which produced the most reproductible results, and results which were closer to a gene profile which resembled microglia cells according to the literature (Iba1+, CX3CR1+, P2Y12R+, PU.1+, CD45+[low] (Denker et al., 2008; Haage et al., 2019; Patir et al., 2019; Bonham et al., 2019)- that is, with all the genes except CD45 being expressed higher in the mg-like cells when compared to the initial cells) was the NIS/THP-1 combination. To confirm expression of microglia markers at the protein level, and their localisation, ICC and Western Blotting were used, and showed that the cells were cd11b+ and TMEM119+ in addition to Iba1+ (which was also shown at a gene level). TMEM119 is a microglia marker which is not expressed in monocytes, and its expression was shown via Western blotting as well as ICC. Further densitometric protein analysis of Western blot images showed slightly smaller differences in Iba1 expression when compared to gene expression, which could potentially be explained either due to the existence of Iba1 isoforms or due to different times of transcription and translation in the cell.

As differences had been observed when testing housekeeping gene expression (specifically determination of GAPDH and actin expression via qPCR), this was further explored. GAPDH expression changed especially in day 3, where it increased significantly both in gene and protein level. This lead to the conclusion that possibly differentiation-related glycolysis takes place, as in day 3 the first significant changes in cell morphology take place: the cells become

sphere-like clumps, and join in groups of 4+. To investigate this, we tested the ATP content of the cells, and indeed in day 3 cellular ATP levels were higher than either the initial THP-1 condition, or the days 7 and 14 mgTHP-1 cells.

Last, to investigate a potential mechanism to underpin the above observed changes, DNA 5-mC methylation analysis was conducted; interestingly, in day 3 there was a high and significant change in methylation, followed by a decrease in the subsequent days which suggests an epigenetic mechanism might contribute to the observed THP-1 to mgTHP-1 differentiation. The contents of the differentiation medium were also investigated in protein and growth factor/cytokine/chemokine level, and although some interesting results were shown in a qualitative way, this cannot be confirmed in a quantitative way at the present time.

4.4.2 Discussion of findings

Microglia, as described in chapter 1 are the tissue resident macrophages of the brain. They originate from myeloid precursors, and are established during embryogenesis. After their establishment they have low proliferative rates, as they are terminally differentiated (Askew et al., 2017; Spalding et al., 2005). Moreover, the cells change during development from an amoeboid phenotype in the embryonic development, and until birth, to a more ramified phenotype after birth with few amoeboid cells remaining in general, but with high plasticity as a cell type, in which shift between forms is often observed (Kostović & Judaš, 2002; Hutchins et al. 1990; Fujimoto et al., 1989). This indicates that the amoeboid phenotype is the most immature one, and that the ramified one is the most mature one. Our findings agree with this, as in days 3 and 7 more amoeboid cells were present than ramified ones, and the ramified phenotype was more obvious towards day 14. Phenotypes observed in the model described here have been previously reported both in primary microglia cell cultures (Levtova et al.,

2017), and in immortalized human microglia (e.g. figures one and two from Garcia-Mesa et al., 2017). However, there is a constant shift between the two phenotypes (with intermediates also presenting) which is observed in physiological conditions (e.g. activated microglia are amoeboid) as well as differences in phenotypes between tissue and cell culture. Ramification is more obvious in tissue examination of microglia, as the extensions essentially scavenge and explore their environment, in which other cells exist, and they communicate with them. Moreover, ramification is associated with the downregulation of markers associated with activation of microglia, such as Iba1, which is downregulated in day 14, when compared to day 3 (see figure 4.3a, and also Imai et al., 1996). In cell culture conditions, many factors influence morphology, including the matrix which cells adhere on, and compounds they are exposed to, and whether they are primary or cell line microglia; in addition, ramification is less obvious in cultured cells, as the cells are not tightly packed within the tissue, so they have no reason to have as many branches (Milner & Campbell, 2003; Choi et al., 2010).

Additional matters that need addressing are a) the sphere-like clumps observed in the beginning of the development of mg-like cells and b) essentially the loss of a proportion of the cells between days 0 (or the beginning of the experimental procedure) and 14. The first can be addressed by observing the microglia repopulation timeline as described by Yao et al., (2016), where in the beginning Iba1+ cell clusters appear, which however are not present in day 14 post repopulation; this agrees with the observations made in the beginning of this chapter, as clumps are observed intensely in day 3, and between days 7 and 14 the number of clusters dramatically reduced, with mg-like phenotype replacing them. Interestingly, an intermediate phenotype was observed in our case, with clusters adhering to the plastic of the wells/flasks, via ramified extensions. Whether this is of physiological significance, is unknown.

The loss of cells on day 14 (when compared to previous days) could lead to the hypothesis that the cells stopped proliferating and thus they reached a terminal differentiation point in their

microglia-like form. In order to proliferate, ramified microglia must be reactivated, and that such activation can be achieved for example by addition of macrophage colony-stimulating factor (MCSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-3 (IL3), IL-1, or by adding the ramified cells on an astrocytic monolayer. Microglia proliferation could be inhibited by the cells' growth medium containing IL-2, IL-4, IL10, TNFα, and IFNγ, which inhibit microglial proliferation (Kloss et al., 1998). In chapter 5 it will be shown that such "inhibitory" cytokines are secreted by the cells in days 7 and 14. Moreover, the differentiation supplement was shown to contain low concentrations of such cytokines (figure 4.12) and FBS which is included in the differentiation medium might contain cytokines or cytokine inhibitors (McKenzie et al., 1990) which could also be a contributing factor to the lack of proliferation of the cells during this period. This could be addressed in the future by adding the factors that have been shown to induce microglia proliferation, while changing the medium into one that does not contain FBS or differentiation supplement.

Regarding gene expression profiles, PU.1 is one of the essential transcription factors for the development and maintenance of microglia. This transcription factor is involved in the expression of other microglia-related genes, such as TMEM119, and is also involved in other epigenetic and transcriptional changes (Yeh & Ikezu, 2018; Satoh et al., 2015; Holtman et al., 2017). As shown in figure 4.3ei, PU.1 expression is elevated throughout the observation timeframe (when compared to THP-1/control expression), with other genes that are microglia-related either having similar patterns of expression, or being upregulated following its elevation in a delayed way (Fig. 4.3a-i). PU.1 increase in day 3 coincides with the appearance of clump formations. PU.1 plays an important role in the development of microglia (Holtman et al., 2017), as it is a transcription factor, which activates other microglia genes, such as TMEM 119 (Satoh et al., 2015) and Iba1 (Zhou et al., 2018). Once the cells started obtaining a more microglia-like morphology, the amount of PU.1 the cells expressed started to drop (as seen by

comparing days 3 (developing cells), 7 (some developing, some developed), and 14 (developed cells)), which coincides with changes in morphonology. In order to further investigate the role of PU.1 in the development of the mgTHP-1 cells, further analyses could include reducing the amount of PU.1 the cells express (e.g. through RNAi), investigating whether PU.1⁻ cells can differentiate into mgTHP-1 cells, and using protein-based assays such as WB to investigate the protein level of PU.1. Moreover, ICC, and flow cytometry could be used to investigate the exact amount of cells expressing PU.1 in high levels per developmental day (FACS), and correlate the expression with the morphology of the cells (ICC), with the addition of additional days investigated if needed, as well as to compare it with PU.1 expression in primary microglia or microglia cell lines, in order to investigate potential differences. In addition it would of interest to investigate whether upregulating PU.1 might lead to changes in the number of Day 14 mgTHP-1 cells with the rationale that lowering the expression of PU.1 leads to issues with microglia function and viability (Smith et al., 2013).

The gene expression patterns were confirmed in a protein level for Iba1 via WB and ICC. Iba1 along with TMEM119 and cd11b, were all found via ICC to be expressed in the cells, starting as early as day 3. TMEM119 is, as discussed already a microglia-specific protein, which is not found in infiltrating macrophages, and is specific for microglia with high plasticity (Segal & Giger, 2016). Although the expression pattern was confirmed by Western blotting (see figure 4.5), additional bands appear in the TMEM119 WB (fig 4.5). This could indicate that the antibody used attaches to other targets, so using a different antibody or a blocking peptide experiment could give more clear results. A bioinformatic analysis indicated one such potential target as be desmoplakin (250kDa protein, the function of which is cell-cell adhesion); this could be confirmed in the future doing a WB targeting desmoplakin, and also an ICC experiment in order to determine the potential localisation. This 250kDa band seems to disappear in day 14, which coincides with changes in the appearance of the clumps/clusters.

Therefore, although the fluorescence observed in the ICC could be partially due to other proteins which are involved in different ways in the development of these cells, at least part of it is due to specific TMEM119 binding, which did not appear in THP-1 cells in the WB. An additional issue with the antibodies used (as already mentioned) is that Iba1 has 3 isoforms due to alternate splicing, and the antibody targets an area which could potentially be missing one of the isoforms, which in turn could explain the differences observed between qPCR and WB results.

Moving on to the metabolic investigation, day 3 seems to exhibit 3 metabolic (and/or epigenetic) adaptations: heightened expression of GAPDH, increased cellular ATP content, and greater proportions of 5-mC. It has been shown that glycolysis accompanies the differentiation of monocytes into active macrophages (Suzuki et al., 2016). While methylation is often associated with gene silencing, it can also be associated with upregulation and modulation of gene expression, when it comes to differentiating cells, showing its dynamic nature as a process (Michalowsky & Jones, 1989; Ehrlich & Lacey, 2013). It can be thus tentatively suggested that part of the changes observed in gene and protein expression may be due to methylation changes, as in day 3 gene expression of many of the marker genes under investigation either drops or is elevated when compared to controls. A suggestion could be that due to epigenetic changes at around day 3, monocyte-specific genes are silenced, and mg-specific genes are more accessible to be transcribed due to changes to either their regulatory elements, or their coding regions. Similarly, this epigenetic mechanism may underpin the GAPDH upregulation, (and possibly increased glycolytic ATP generation) observed on day 3 of mgTHP-1 differentiation. Hence, the differentiation process under investigation may be underpinned by a rather complex mechanism of transcriptional control, including epigenetic modifications, where a network of genes are involved and interact with each other. However,

due to its complexity (and also the time constraints of this PhD study) the details of this mechanism was not fully examined in this case.

Finally, in the investigation of the components of the NIS medium, several growth factors were found; however which of these, and in which way, they affect the differentiation of the cells towards a microglia-like phenotype, is again, unfortunately, beyond the scope of this thesis. Nevertheless, it would be useful to mention that both MCSF and VEGF (which were detected using the cytokine arrays, fig.4.12) have been used elsewhere in order to differentiate haematopoietic progenitors and bone-marrow derived stem cells into microglia-like cells (McQuade et al., 2018; Avraham-Lubin et al., 2012). In addition, Oncostatin M has been shown to control the expansion of haematopoietic stem cells in zebrafish (Mahony et al., 2018). However, as the full list of NIS components is not known, it would not be wise to make the claim that the compounds mentioned alone are responsible for THP-1 to mgTHP-1 differentiation.

Overall, it can be concluded that the mgTHP-1 model described here is a relatively accurate and potentially useful novel model of microglia-like cells, as the cells produced have gene signatures and morphologies that closely resemble microglia cells. It is based on an easily performed protocol, with relatively reproducible results (some differences were found in number of cells in day 14 when using different batches of FBS and/or NIS, however this was not explored further), low development time, and initial use of cells (THP-1) which are easy to culture and maintain. Importantly, the overall protocol is easy to use, as it is essentially a series of changes of medium and incubation over a 14 day period.

4.4.3 Limitations

1. THP-1 cells only were used, which are a cancer patient-derived cell line (Tsuchiya et al., 1980). Both aspects (i.e. it being cancer derived and an immortalised cell line) have advantages and disadvantages. Accordingly, this method should be replicated in PBMC derived CD14+ cells in order to confirm that the results shown are due to the differentiation medium, and not to an idiosyncrasy of the cell line. Moreover, using the THP-1 (supplemented RPMI) medium without serum could be tried, as the serum seemed to produce some fluctuations in the results depending on the batch.

2. There were no primary mg cells or mg cell line cells to compare to in our laboratory studies (although comparison with reports in the literature was performed). However, different mg cell lines have different characteristics, and other papers mentioned in table 4.1 validated the mg-like models they generated and described, by comparing to the monocytes the cells of the respective studies came from instead of comparing with mg cells. Therefore, further investigations could include primary or immortalised microglia, but also different methodologies such as flow cytometry in order to easier quantify and confirm the differences observed.

3. The model has a low yield of resulting mg-like cells, the reason for which has already been discussed. Using up to 4% NIS instead of 2% did not produce a significantly increased amount of cells (data not shown). However the cost of increasing the NIS to concentrations beyond would not be advisable. Also some of the ingredients that at 2% are non-inflammatory, could be inflammatory in higher concentrations (as an example LIF is mentioned (Alaaeddine et al., 1999)), and thus give false positive results (i.e. produce macrophages instead of mg-like cells). Instead, in order to further optimise the model, different approaches could be used, such as using GM-CSF in order to make the

cells proliferative again (Suzumura et al. 1991), or change the medium around day 14 from the differentiation medium to a microglia-specific medium.

4. Although morphologically and from a gene expression point of view these cells are mg-like, function wise this has not yet been investigated. Such an attempt of functional characterisation will be made in chapter 5, by testing their response to pro and anti-inflammatory stimuli, however other functional assays (e.g. phagocytosis) should also be employed in the future.

Chapter 5

Investigating the pro-inflammatory potential and response of CNS cells, part 2: CNS macrophage-like cells

5.1 Introduction and aims

CNS macrophages, including microglia, have been shown to follow the M1/M2 paradigm that has already been discussed. Therefore, in addition to the microglia-like morphology and expression patterns the mgTHP-1 cells from chapter 4 were found to have, it would be useful to examine in the current chapter whether these cells are functionally similar to microglia and CNS macrophages. As in chapter 3, we will use a specific set of pro-inflammatory stimuli at different timepoints; this will allow a better comparison between different cell types. Moreover, it will give us insights into how mgTHP-1 cells respond to inflammation by assuming a M1 phenotype in different stages of their differentiation (i.e. days 3, 7, and 14) which could hint towards their function as microglia-like after assuming microglia-like phenotypical characteristics. As was discussed by Yao et al. (2016), CNS infiltrating monocytes seem to disappear around 2 weeks after they've entered the brain. As shown in chapter 4, mgTHP-1 cells appear to partially conform to this pattern in that the overall cell number lessens within 14 days, and exhibit characteristics that were also discussed by Yao et al., (2016). One would therefore assume that around day 14 their pro-inflammatory activity would reduce. On the other hand, in the microglia models tested and mentioned in table 4.1, microglia-like cells are considered fully differentiated in day 14, and reduced numbers when compared to the initial cell population are also observed, which can be attributed to their end-point differentiation; their activity in day 14 when it comes to cytokine secretion is confirmed in the studies that focus on that aspect of their function (i.e. day 14 cells of the models secrete M1-related cytokines upon inflammatory challenge).

It has also been shown that CNS macrophages can have an anti-inflammatory effect (Shechter et al., 2009), by adopting an M2 phenotype and secreting anti-inflammatory cytokines upon entering the brain. The ability of mgTHP-1 cells to exert anti-inflammatory effects will also be

tested, as understanding how the cells behave in a CNS-resembling environment would give us further indications about whether mgTHP-1 cells are a good model for all the functions of microglia-like cells. In order to examine the M2 phenotype, we will employ the methodology previously used in our laboratory (Hassan et al., (2018)), which showed that a flavonoid, namely Vicenin-2 (V-2) can shift dTHP-1 cells towards an M2 phenotype, reducing their pro-inflammatory activity (as measured -among other markers- via cytokine secretion). More about V-2, its structure, and a brief discussion about its properties relating to the CNS can be found in section 1.2.4, subsection Flavonoids. The rationale behind using V-2 in order to investigate its potential to shift mgTHP-1 cells towards an M2-like phenotype, was that this has already been shown in macrophages (dTHP-1 cells), in addition to the fact that V-2 has been shown to have potentially beneficial effects on the CNS as it can cross the BBB, so using the specific flavonoid on a model of microglia-like cells (mgTHP-1) it could give an indication of its effects on microglia and CNS macrophages.

Aims

Therefore, the aims for this chapter are:

a) To investigate the inflammatory response (as measured by secretion of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-18 with ELISA) of mgTHP-1 cells to different inflammatory stimuli (LPS and IFNγ) and at different timepoints (similar to those used in chapter 3). Moreover, the expression of LPS and IFNγ receptors will be investigated and compared to any observed differences in cytokine secretion, in order to explore different regulatory mechanisms.

b) To investigate the potential M2/anti-inflammatory effect of V-2 on these cells, by employing similar methodology to Hassan et al., (2018), and investigating the impact on

secretion of additional cytokines (e.g. IL-10) (and potentially the underpinning mechanistic pathways).

c) To investigate the inflammatory profile of the cells at different differentiation stages (i.e. days 3, 7, and 14), as well as the influence of V-2 on this profile in the absence of any pro-inflammatory stimuli. To do so, cytokine arrays will be used, which will give us semi-quantitative results on changes in secretion of 42 different cytokines/chemokines/proteins during the development of mgTHP-1 cells, and how V-2 influences this.

5.2 Materials and Methods

5.2.1 Materials

The materials used for this chapter that were <u>not</u> previously mentioned in chapter 2 are the following

Name	Used for	Provider
Human TNF alpha ELISA Kit (ab181421)	ELISA detection of TNFα	Abcam, UK
Human IL-6 ELISA Kit (ab178013)	ELISA detection of IL-6	
Human IL-18 ELISA Kit (ab215539)	ELISA detection of IL-18	
Human IL-1 beta ELISA Kit (ab214025)	ELISA detection of IL-1β	
Human RAGE ELISA Kit (ab190807) And Human RAGE DuoSet ELISA DY1145	ELISA detection of RAGEs (RAGE isoform sRAGE-delta for DY1145, unknown isoform for ab190807)	Abcam, UK and R&D Systems/Biotechne Ltd
TBP probe (Hs00427620_m1)	qPCR-housekeeping gene	Thermofisher UK
TLR2 probe (Hs01014511_m1)	qPCR-expression of TLR2	
TLR4 probe (Hs00152939_m1)	qPCR-expression of TLR4	
IFNgR1 probe (Hs00988304_m1)	qPCR-expression of IFNγR1	
Cytokine Array – Human Cytokine Antibody Array (Membrane, 42 Targets) (ab133997)	Cytokine secretion analysis	Abcam, UK

Table 5.1 _Materials used for this chapter_

5.2.2. Methods

5.2.2.1 THP-1 cells culture and differentiation to mgTHP-1 cells

THP-1 cells were cultured as per chapter 2.2.1. The cells were differentiated as per chapter 2.2.1.2; as discussed in chapter 2, and selected in the previous chapter (chapter 4) the condition

that produced cells with the most stable, microglia-like and reproducible phenotypical characteristics was the one that used only NIS at the concentration suggested by the manufacturer (50x dilution, or 2% concentration).

5.2.2.2 Treatment of cells with pro-inflammatory stimuli and pre-treatment with V-2

Pre-treatment with V-2 (Sigma, product code 03980585-10MG, reconstituted in dimethyl sulfoxide (DMSO); please see section 1.2.4, subsection Flavonoids for information relating to its structure and properties) was performed as described in 2.2.3.1. The concentration of V-2 used was 50ng/ml (≈80nM, as per Hassan et al., 2018). After 1 hour of this pre-treatment with V-2, cells were treated with IFNγ or LPS as described in 2.2.3, with the V-2 not being removed from the incubation medium. For IFNγ the concentration used was 500U/ml for 24 hours, as these conditions have been used in a variety of microglia/mixed cell culture studies (e.g. Rozenfeld et al., 2003; Goodwin et al., 1995; Beutner et al., 2010), and in these studies (unlike the non-microglial cells in chapter 3 of the present study), this concentration was enough to elicit a strong response from the mgTHP-1 cells due to their microglia/macrophage-like nature. For the same reason, LPS was used in the minimum concentration of those used in chapter 3 (100ng/ml) for 4 different time points (1h, 3h, 6h, and 24h), in order to have the opportunity to study a variety of cytokines that are produced at different timepoints (i.e. early and late response cytokines, as explained by Hildebrand et al., 2005). Although THP-1 cells are a cell line, and thus biological replicates and technical replicates might -in theory- be an issue to determine, in this thesis the Lazic et al., (2018) definition of biological and technical replicates in cell lines was followed. Therefore, N=3 biological replicates with 3 technical replicates per biological replicate were used per condition and per developmental stage (days 3, 7, and 14). To exclude the possibility that V-2 on its own can lead to the secretion of the pro-inflammatory cytokines investigated, a 24h V-2 only control was used.

5.2.2.3 qPCR

As the cells were to be exposed to different stimuli, it was essential to investigate whether the cells that were used were expressing the receptors for these stimuli, namely TLR2, TLR4, and IFNγR1. To do so, qPCR was performed as described in chapter 2.2.6; details of the probes are described in table 5.1.

5.2.2.4 Cytokine array analysis

Cytokine arrays (ab133997, Abcam, UK) were employed in order to explore the differential secretion of 42 cytokine targets during the different developmental stages (i.e. days 3, 7, and 14), and to investigate whether V-2 changed that secretion. The method is described in section 2.2.4.2, and the full protocol can be found in the methods-related appendix (Appendix 1).

After the membranes were exposed, ImageJ (http://rsb.info.nih.gov/ij/) was employed, in order to process and densitometrically analyse the images obtained from these experiments. For that, the membranes were scanned, and uploaded on imageJ. There, the protein array analyser for ImageJ by Gilles Carpentier (Carpentier, 2017) was used as per guidelines. The images were analysed per array, and were standardized against the positive and negative controls on the array in question in each case. The images were then compared between conditions (i.e. +V-2 vs -V-2). The programme gave as an output the relative density of the dots per cytokine for each condition. The focus was on 11 cytokines (namely MCP-1, TNFα, ENA-78, GM-CSF, RANTES, TGF-β1, IL-4, IL-10, IL-12, IL-13, IL-6, and IL-1β) that either showed changes between conditions, belonged in an anti-inflammatory or pro-inflammatory category, and/or could be correlated to the ELISA results (see section 5.2.2.5). For each condition a heatmap was generated from the densitometric conversion of the cytokine array scanned image into semi-quantitative data, and for each group of conditions, a graph was produced comparing the secretion of 11 cytokines, in the presence or absence of V-2. As the method is semi-quantitative,

and most references in the literature do not use it for statistical analysis (and as n=1 (pooled from 3 biological replicates) sample was tested/condition), such analysis was not possible.

5.2.2.5 ELISA

ELISAs were performed in a different way than described in chapter 2.2.4.1. The kits used are described in table 3.1, and the detailed protocols can be found in the method protocol related appendix (Appendix 1). Briefly, supernatants of the treated cells and controls were collected at the different timepoints following exposure to stimuli (1h, 3h, 6h, and 24h). These supernatant samples were frozen at -20ºC until use. Samples or standards (standards were prepared using serial dilutions of a stock standard cytokine solution) were added to the wells of the 96-plate provided, and antibody cocktail was then added (this cocktail consisted of the capture and detector antibodies, diluted in antibody diluent). After a 1hour incubation at RT on a plate shaker (400RPM), followed by a wash step, the substrate to the enzyme linked to the detector antibody (TMB) was added, and after a short incubation (different per cytokine examined) the stop solution was added; all incubations were done with the plate on a plate shaker at 400RPM. The concentration of the cytokines was determined by measuring the optical density at a wavelength instructed by the manufacturer, and by using the appropriate standards to produce a standard curve.

NB: For RAGEs, because initial experiments using the Abcam kit gave no detection of RAGEs in any of the samples (however the standards worked, so the assay overall worked), the samples were re-tested using a different ELISA kit (from R&D Systems), which had given positive results for the neural cells examined in chapter 3. The methodology for that ELISA can be found in section 3.2.2.5 as well as 2.2.4.1; please note that it tests for a specific RAGEs isoform (see table 5.1 for details), whereas the ELISA initially used for this chapter does not indicate any specific isoform.

5.2.2.6 Cell viability/cell counting

In order to ensure that any apparent effects within the results observed were not artefact due to cell death, the viability of the cells was measured under the appropriate conditions (control vs 24 h highest concentration for both stimuli, and in either the presence or absence of V-2). The methodology was as described in 2.2.2.

5.2.2.7 Statistical analysis

Statistical analysis was performed using SPSS, where ANOVAs with Bonferroni post hoc corrections or t-tests were used in order to determine differences between control conditions and treatments. Significance was set at <0.05. Unless otherwise stated, n refers to biological replicates.

5.3 Results

5.3.1 qPCR analysis of receptor gene expression

In order to investigate whether the mgTHP-1 cells could respond to the pro-inflammatory mediators used in chapter 3 (i.e. LPS, and IFNγ), and thus examine their ability to assume an M1 phenotype, qPCR was used comparing the in expression of the genes for IFNγR1, TLR-2, and TLR-4. Cells at all developmental stages were found to be expressing the receptors of interest. For IFNγR1, a one way ANOVA showed significant differences between conditions $(F(3,20)=16.512, p<0.0001)$ with a Bonferroni post hoc revealing significant differences between control (THP-1) cells and day 7 cells $(p<0.001)$, control and day 14 cells $(p<0.05)$, as well as that the gene is expressed significantly higher in days 7 $(p<0.0001)$ and 14 $(p<0.05)$ compared to day 3 (black bars). For TLR-2, a one way ANOVA $(F(3,20)=21.25, p<0.0001)$ with Bonferroni correction found that expression significantly lower in all days when compared to the control/THP-1 condition $(p<0.0001$ for day 3, $p<0.01$ for day 7, $p<0.001$ for day 14), with day 3 cells expressing the gene at significantly lower amounts than day 7 $(p<0.01)$ and day 14 $(p<0.05)$ (dark grey bars). For TLR-4, a one way ANOVA $(F(3,20)=54.357, p<0.0001)$ with Bonferroni corrections showed that while day 14 cells are significantly higher in expression than day 7 cells $(p<0.01)$, they show no significant difference when compared to day 3 $(p=0.582, ns)$, but all three days show expression significantly lower than the THP-1 control cells $(p<0.0001$ for all).
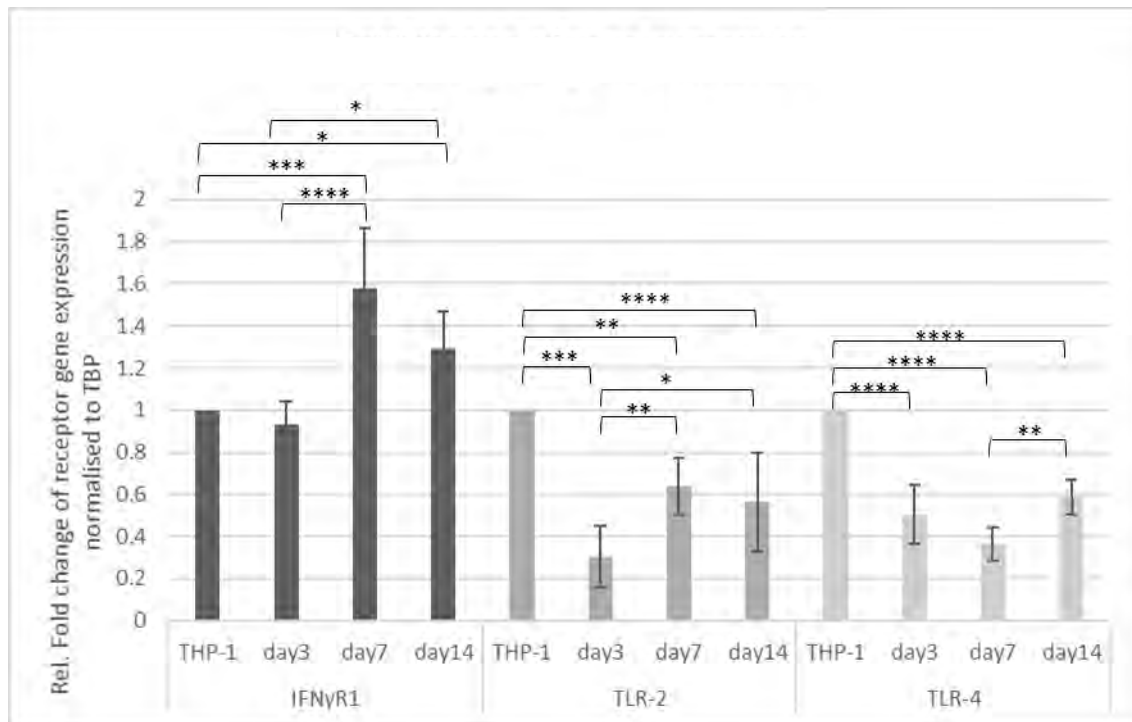
Figure 5.1** **The fold-change of the gene expression for the genes IFNγR1, TLR-2, and TLR-4 during mgTHP-1 differentiation. (* signifies $p<0.05$, ** signifies $p<0.01$, *** signifies $p<0.001$, and **** signifies $p<0.0001$) Cells from all three developmental stages and control cells all express the three genes investigated, however in different degree. Results normalised against THP-1 cells (control) and TBP (housekeeping gene). N=6

These results indicate that the cells have the potential to respond to both stimuli used in this chapter, however, as the responses depend on more than simply the expression of the receptor, there can be no conclusions made about the magnitude of the response, and whether it will follow the trends shown here. For example, see chapter 3, where the neural cells receptors are expressed in different levels, however the inflammatory response (or lack thereof) does not seem to be influenced in a great degree by this -at least in mRNA expression levels-, which indicates a rather complex mechanism of response to stimuli, which depends on a variety of factors, such as cell type and active cell processes (e.g. differentiation/development) in addition to receptor expression levels.

5.3.2 Viability

None of the treatments (LPS, IFNγ, or V-2, vs control, as measured after 24 hours incubation) caused any significant change in the viability of cells between pre- and post-treatment conditions, although viability was not measured after each combination of treatments (e.g. LPS+V2 or IFNγ+V2) and/or timepoint. However, the number of cells per well decreased in all cases between days 0 and 14 (from ~300000 to ~100000 for this series of experiments) (as previously discussed in chapter 4). Please also note, that in days 3 and 7, due to the appearance of clumps of different sizes (and cell number content) as well as the adherent nature of some clumps/cells, cell counting was approximate, and not as accurate as in day 14. Nevertheless, the overall viability results comparing control condition vs treatments were consistent. For Day 3 cells a one way ANOVA showed no significant differences between conditions($F_{(3,8)}=0.88$, p=0.489, ns). For Day 7 cells, a one way ANOVA showed no significant differences between conditions ($F_{(3,8)}=0.2357$, p=0.868, ns). For Day 14 cells, a one way ANOVA showed no significant differences between conditions ($F_{(3,8)}=0.1802$, p=0.906, ns).
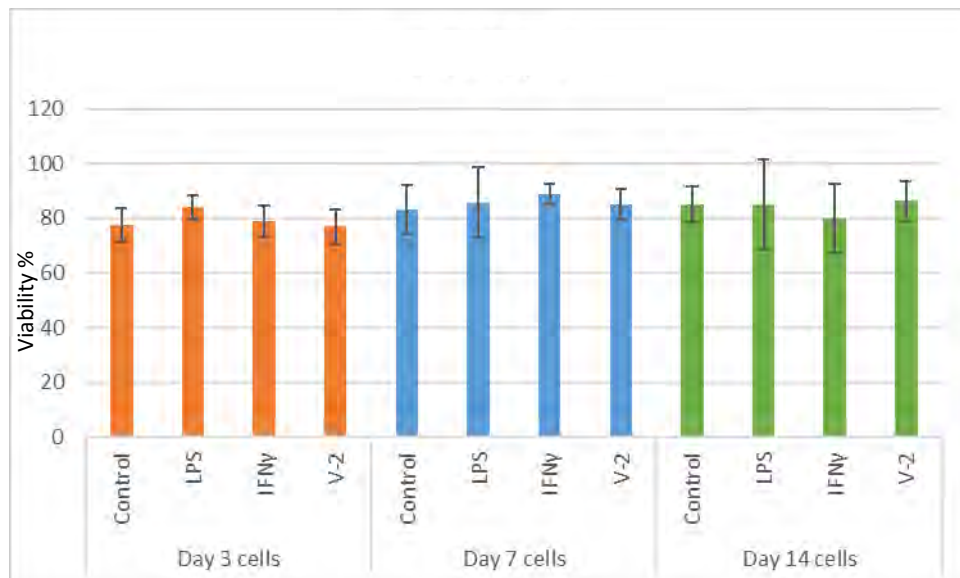
***Figure 5.2** Presentation of viability data for the cells of all 3 developmental days, under different condition treatments.* (n=3)

5.3.3 ELISA results

After cells were treated as described in section 5.2, secretion of cytokines was measured using ELISA. The aim was to investigate whether the cells were able to adopt M1 and M2 phenotypes during their differentiation; all analyses was carried out on cells at 3 developmental stages: Day 3, 7 and 14. The ELISA results are separated to the two responses of interest, M1 and M2. For M1 response, it was examined whether the cells respond to LPS and/or IFNγ by increasing the amounts of proinflammatory cytokines secreted. 1-way and 2-way ANOVAS as well as t-tests (in the case of +/- V2 for 24h IFNγ treatments) were used. For the M2 response the focus was on whether pre-treatment with V-2 could lower the amount of secreted pro-inflammatory cytokine, when compared to the amount of cytokine secreted with the inflammatory stimulus (LPS or IFNγ) without pre-treatment.

M1 response

In order to investigate the potential of mgTHP-1 cells to produce a M1-like response, the cells were treated with LPS for various timepoints, and IFNγ for 24 hours. The responses to these inflammatory compounds -which are known to elicit a M1 response- were compared to the appropriate controls. Further, a comparison between treatments for 24 hours of treatment was done, as well as vs V-2. The latter was done in order to exclude the possibility V-2 (which was in the next section investigating the M2 response) to elicit an inflammatory response to the cells. The results are presented per day and per cytokine in Figures 5.3.1-5.3.24, and the statistical analyses are described in the figure legends.

Day 3

TNFα

Figure 5.3.1 TNFα secretion from Day 3 LPS treated mg-THP1 cells in different timepoints vs controls Day 3 cells LPS treatment in different timepoints, a 2 way ANOVA revealed a significant effect of treatment $(f(1,37)=1494.15, p<0.05)$ and time $(f(3,37)=239.57, p<0.05)$ and interaction $(f(3,37)=238.19, p<0.05)$. A post hoc Bonferroni's test showed significant differences $(p<0.0001)$ between Control 3 h vs LPS 3h (nd vs 5.19 ± 0.65 pg/ml), 6h control and LPS 6h (nd vs 11.71 ± 1.37 pg/ml), 24 h control and LPS 24 h (nd vs 6.53 ± 0.91 pg/ml) (where nd=not detected, as negative value was found in the ELISA results). Additional significant differences (all $p<0.0001$) were found between different timepoints in the LPS treatment, with differences between LPS 1 hour treatment being found significantly lower than all others (nd vs 5.19 ± 0.65 pg/ml for 3 h, 11.71 ± 1.37 pg/ml for 6h, and 6.53 ± 0.91 pg/ml for 24 hours) and the LPS 6h treatment being significantly higher $(p<0.0001)$ than all other treatments. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

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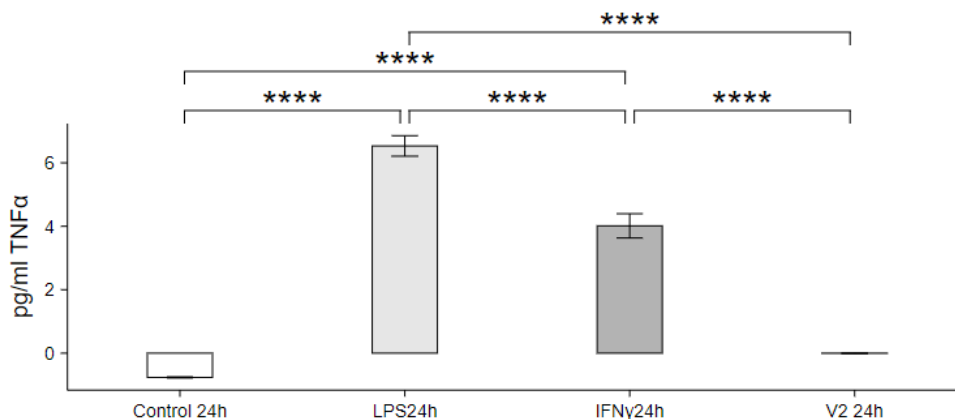
Figure 5.3.2 TNFα secretion in 24h from Day 3 mg-THP1 cells under different conditions *TNFα secretion from Day 3 cells under different treatments* In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment $(f(3, 26)=117.71, p<0.0001)$. A post-hoc Bonferroni test revealed no differences between Control and 24h V2 treatment, however differences were found between control and IFNγ treatment (nd vs 4.01 ± 1.08 pg/ml, $p<0.0001$). Another significant difference that was found was between 24 h treatment with LPS vs that with IFNγ $(6.53\pm0.91$ pg/ml vs 4.01 ± 1.08 pg/ml, $p<0.0001)$, with the cells producing less TNFα under IFNγ treatment. Both LPS and IFNγ treatments were also found to be significantly different to the V2 (24 h) treatment. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)N=9 (3 biological replicates *3 technical replicates each)

IL-6

Figure 5.3.3 IL-6 secretion from Day 3 LPS treated mg-THP1 cells in different timepoints vs controls For Day 3 cells LPS treatment in different timepoints, a 2 way ANOVA revealed a significant effect of treatment ($f(1,37)=667.87$, $p<0.0001$) and time ($f(3,37)=11.44$, $p<0.0001$) and interaction ($f(3,37)=8.26$, $p<0.0001$). A post hoc Bonferroni's test showed significant differences between all controls and treatments in different timepoints ($p<0.001$ for all) (Controls 1h: 6.37±0.32 pg/ml, 3h 6.35±0.11 pg/ml, 6h 7.10±0.14 pg/ml, 24 h 6.37±0.3 pg/ml vs LPS 1h 8.41±0.12 pg/ml ($p<0.001$), 3h 9.6±0.25 pg/ml ($p<0.0001$), 6h 9.28±0.53 pg/ml ($p<0.001$), 24h 9.25± 0.84 pg/ml ($p<0.0001$). Further differences were shown between the difference timepoints in the LPS treatments, with 1hr treatment with LPS eliciting a significantly lower response than 3h ($p<0.001$) and 6h response ($p<0.05$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
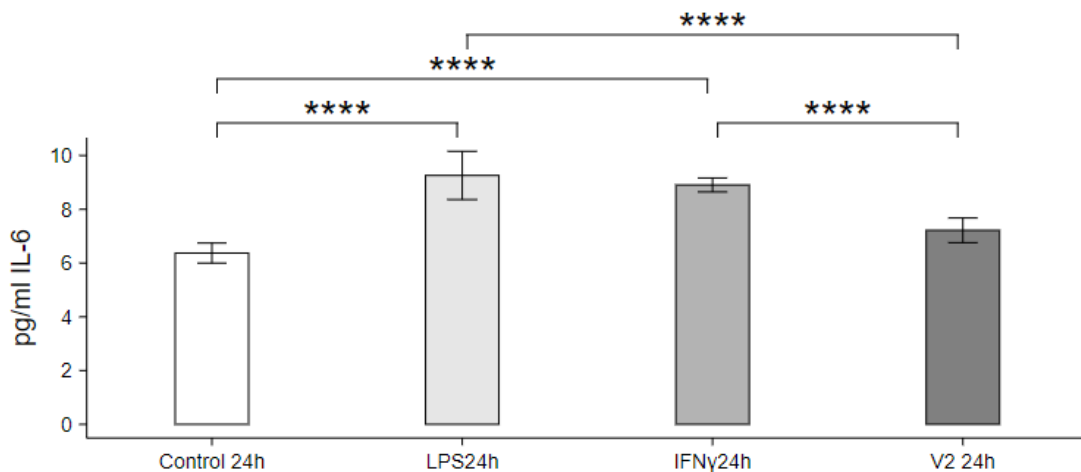
Figure 5.3.4 IL-6 secretion in 24h from Day 3 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3, 26)=32.15$, $p<0.0001$). A post-hoc Bonferroni test revealed no differences between 24h Control (6.37±0.30pg/ml) and 24h V2 (7.21±0.43pg/ml) treatment, however differences were found between control and IFNγ treatment (6.32±0.30pg/ml vs 8.91±0.24pg/ml, $p<0.0001$) and both LPS and IFNγ treatments and V2 treatment ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

IL-1β

Figure 5.3.5 IL-1β secretion from Day 3 LPS treated mg-THP1 cells in different timepoints vs controls For Day 3 cells LPS treatment in different timepoints, a 2 way ANOVA revealed a significant effect of treatment ($f(1,37)=22.94$, $p<0.05$) and time ($f(3,37)=9.89$, $p<0.001$) and interaction ($f(3,37)=16.14$, $p<0.001$). A post hoc Bonferroni's test showed no significant differences between controls and LPS treated samples, however differences within the LPS samples were found at different timepoints with the cells treated for 3 hours secreting the least amount of IL-1β (16.43 ± 0.79pg/ml) than the cells treated for 1 (19.38 ± 0.29pg/ml), 6 (19.98 ± 0.175pg/ml), or 24h (19.34 ± 2.08pg/ml). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

Figure 5.3.6 IL-1β secretion in 24h from Day 3 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3, 26)=37.89$, $p<0.0001$). A Bonferroni post-hoc test revealed no differences between control and treatment with V2 or LPS for 24h, but the amount of IL-1β secreted by the cells when treated with IFNγ for 24h (23.69 ± 0.08pg/ml) was significantly higher than all control ($17.21.1$pg/ml), LPS (19.34 ± 2.088pg/ml), and V2 (17.79 ± 0.43pg/ml) treatments for 24 hours ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

IL-18

Figure 5.3.7 IL-18 secretion from Day 3 LPS treated mg-THP1 cells in different timepoints vs controls For Day 3 cells LPS treatment in different timepoints, a 2 way ANOVA revealed a significant effect of treatment ($f(1,37)=6725.86$, $p<0.05$) and time ($f(3,37)=1942.2$, $p<0.001$) and interaction ($f(3,37)=1925.16$, $p<0.001$). A post hoc Bonferroni's test showed significant increase in the secretion of IL-18 between controls and LPS treated cells in all timepoints (Controls: 1h: 113.92±1.0pg/ml, 3h: 102.2± 1.95pg/ml, 6h 107.32± 3.1pg/ml, 24h108.74± 6.3pg/ml vs LPS 1h 165.65± 8.9pg/ml, 3h 171.53± 9.3pg/ml, 6h 178.34± 0.5pg/ml, 24h 456.7± 11.8pg/ml)($p<0.00001$ for all). The analysis further showed that the cells secrete significantly more IL-18 after 24h treatment compared to the other 3 timepoints for LPS treatment. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

Figure 5.3.8 IL-18 secretion in 24h from Day 3 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3,26)=2446.6$, $p<0.0001$). A post hoc Bonferroni correction showed no significant difference between Control and V2 treated cells for 24h. The analysis further revealed that the amount of IL-1β secreted by the cells increased when they were treated with not only LPS (already shown), but also with IFNγ for 24 hours (304.73±6.1pg/ml), $p<0.0001$, with LPS producing a more profound effect in IL-18 secretion when compared to IFNγ treatment. Both LPS and IFNγ treated cells produced significantly higher amounts of IL-1β when compared to V2 ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)N=9 (3 biological replicates *3 technical replicates each)

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Day 7

TNFα

Figure 5.3.9 TNFα secretion from Day 7 LPS treated mg-THP1 cells in different timepoints vs controls For day 7 cells LPS treatment in different timepoints, a 2 way ANOVA revealed significant effect of treatment ($f(1,37)=63181.92$, $p<0.0001$), of time ($f(3,37)=18110.53$, $p<0.0001$), and interaction ($f(3,37)=18010.38$, $p<0.0001$). A post hoc Bonferroni test revealed that all controls except the 1h control were significantly lower ($p<0.0001$) than the LPS treated cells of the same timepoint (3h control 3.38pg/ml±0.4, 6h control 2.13±1.29, 24h control 2.89±1.003 vs LPS treated cells 3 hours 120.3±3.19, 6hours 290.2±2.69, and 24hours 60.41±1.09). More differences were found between the different timepoints in the treated cells, with all other timepoints being significantly higher ($p<0.0001$) than the 1h timepoint (2.68±0.52), the 6h timepoint being the overall highest timepoint, and the 3h timepoint being also higher than the 24h timepoint. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

Figure 5.3.10 TNFα secretion in 24h from Day 7 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3,26)=3248.77$, $p<0.0001$). A post hoc Bonferroni correction revealed no differences between the 24 h control (2.89±1.003pg/ml) and the 24h V2 (2.085±0.21pg/ml) treatment, however significant differences were found between control and 24h treatment with IFNγ (2.89±1.003pg/ml for control vs 80.05±2.96pg/ml for IFNγ, $p<0.0001$) in addition to the already mentioned difference between control and LPS 24h. Further differences were found between 24h treatments of LPS and IFNγ, with IFNγ eliciting a stronger response (60.41±1.09pg/ml vs 80.05±2.96pg/ml, $p<0.0001$), and both treatments also being significantly higher than treatment with V2 for 24h. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)N=9 (3 biological replicates *3 technical replicates each)
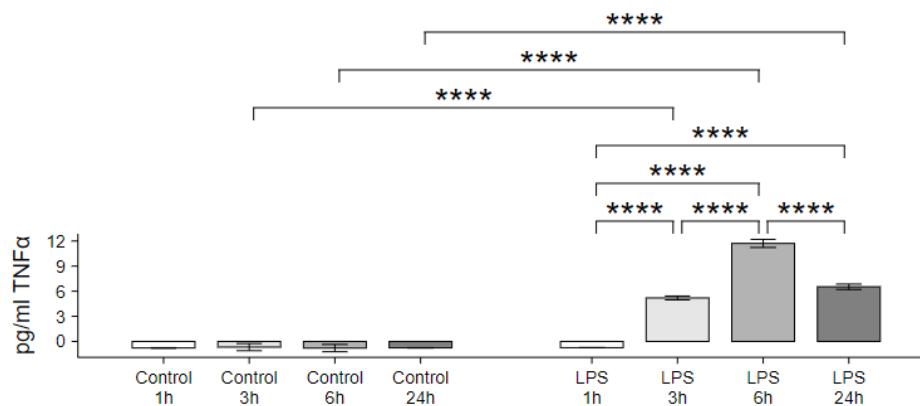
198

IL-6

Figure 5.3.11 IL-6 secretion from Day 7 LPS treated mg-THP1 cells in different timepoints vs controls For Day 7 cells LPS treatment in different timepoints, a 2 way ANOVA revealed a significant effect of treatment ($f_{(1,37)}=2537.42$, $p<0.0001$) and time ($f_{(3,37)}=738.97$, $p<0.0001$) and interaction ($f_{(3,37)}=747.45$, $p<0.0001$). A post hoc Bonferroni's test showed significant differences between controls and treatments for 3h, 6h and 24 hours timepoints ($p<0.001$ for all) (Controls 1h: 6.37±0.32 pg/ml, 3h 6.35±0.11 pg/ml, 6h 7.10±0.14 pg/ml, 24h 7.93±0.89pg/ml, LPS 1h 7.74±0.75pg/ml, 3h 18.89±0.69pg/ml, 6h 175.22±10.77pg/ml, 24h 156.87±14.85pg/ml). Further differences were found between the different timepoints for the LPS treated cells, with 6h of treatment with LPS eliciting the strongest response. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
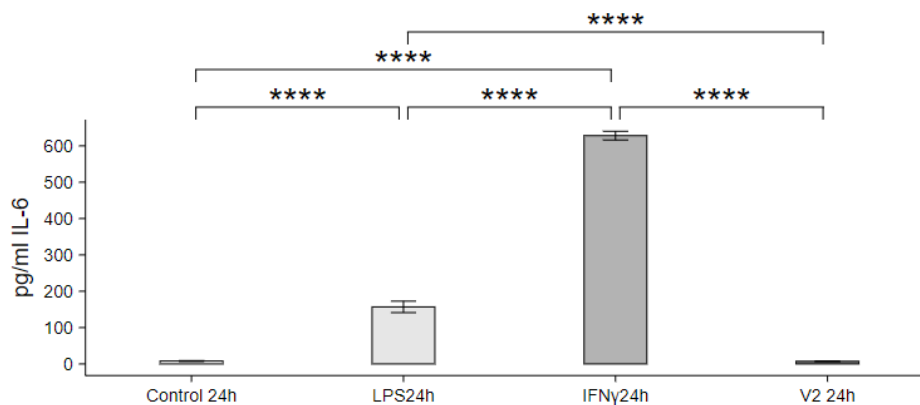
Figure 5.3.12 IL-6 secretion in 24h from Day 7 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f_{(3,26)}=5704.99$, $p<0.0001$). A post hoc Bonferroni correction revealed no differences between the 24 h control (7.93±0.88pg/ml) and the V2 24h treatment (7.20±0.82pg/ml), but revealed a significant increase in secretion of IL-6 vs control after 24 h treatment with IFNγ (627.98±11.2pg/ml, $p<0.0001$). The response to IFNγ was higher than the response to LPS, when it comes to secretion of IL-6 from the cells. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
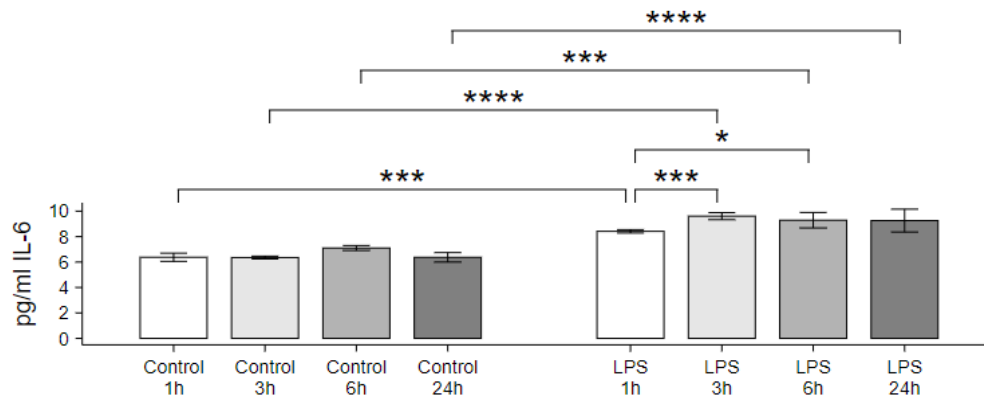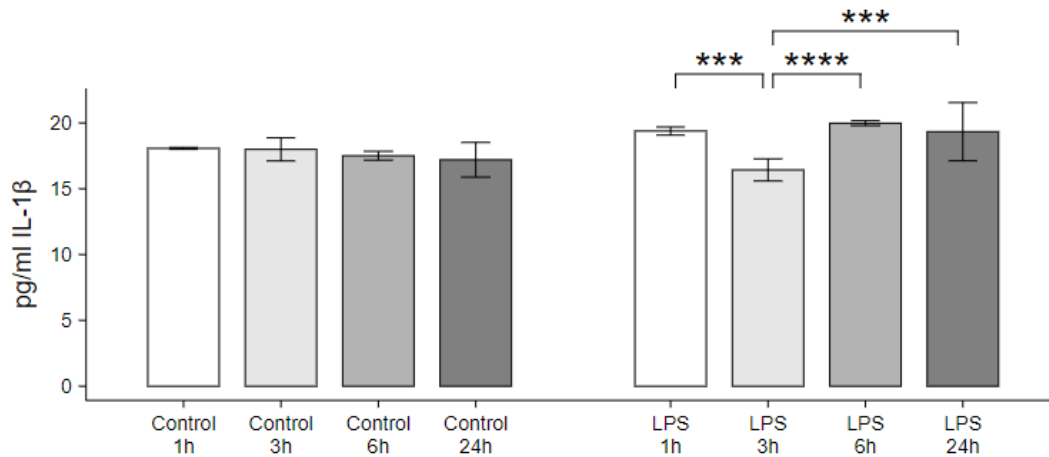
IL-1β
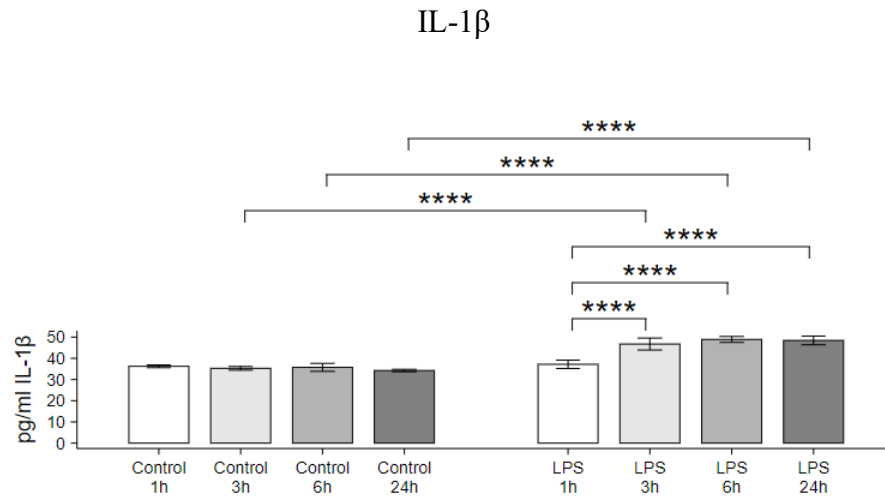
Figure 5.3.13 IL-1β secretion from Day 7 LPS treated mg-THP1 cells in different timepoints vs controls For day 7 cells LPS treatment in different timepoints, a 2 way ANOVA revealed significant effect of treatment ($f(1,37)=714.47$, $p<0.0001$), of time ($f(3,37)=48.03$, $p<0.0001$), and interaction ($f(3,37)=60.97$, $p<0.0001$). A post hoc Bonferroni test revealed that all controls except the 1h control were significantly lower in their secretion of IL-1β ($p<0.0001$) than the LPS treated cells for the timepoints investigated (Controls: 3h 35.29±0.61pg/ml, 6h 35.74±1.32pg/ml, 24h 34.24±0.5pg/ml vs LPS 3h 16.49±0.79pg/ml, 6h 19.98±0.18pg/ml, and 24 h 19.34±2.08pg/ml). The analysis also revealed that the 1h response to LPS was the lowest observed within all LPS treated samples ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
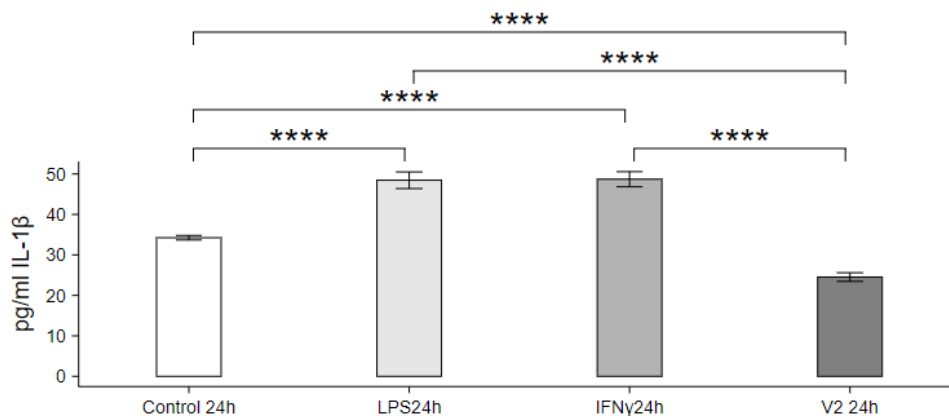
Figure 5.3.14 IL-1β secretion in 24h from Day 7 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3,26)=432.04$, $p<0.0001$). A post hoc Bonferroni correction showed a significant difference between Control and V2 treated cells for 24h, with the V2 treated cells (24.53±1.01pg/ml) secreting lower amounts of IL-1β when compared to the untreated cells (34.24±0.46pg/ml) ($p<0.0001$). The analysis further revealed that the amount of IL-1β secreted by the cells increased when they were treated with not only LPS (already shown), but also with IFNγ for 24 hours (48.7±1.8pg/ml), $p<0.0001$. Both LPS and IFNγ treated cells produced significantly higher amounts of IL-1β when compared to V2 ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
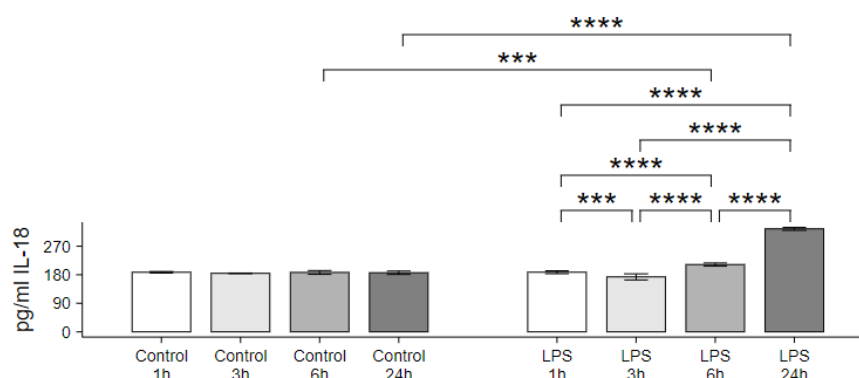
Figure 5.3.15 IL-18 secretion from Day 7 LPS treated mg-THP1 cells in different timepoints vs controls For day 7 cells LPS treatment in different timepoints, a 2 way ANOVA revealed significant effect of treatment $(f(1,37)=362.01, p<0.0001)$, of time $(f(3,37)=25.10, p<0.0001)$, and interaction $(f(3,37)=264.4, p<0.0001)$. A post hoc Bonferroni test revealed that cells treated with LPS for 6h and 24 h secreted significantly higher amounts of IL-18 than the untreated cells for the same timepoints (Control 6h 187.25±4.3pg/ml vs LPS 6h 212.12±4.7pg/ml, and control 24h 186.327±4.4pg/ml vs 324.76±5.4pg/ml for the LPS treatment) $(p<0.0001$ for both), and it further revealed that the 24h LPS treated cells secreted significantly higher amounts of IL-18 when compared to the amounts secreted by LPS treated cells in all other timepoints. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
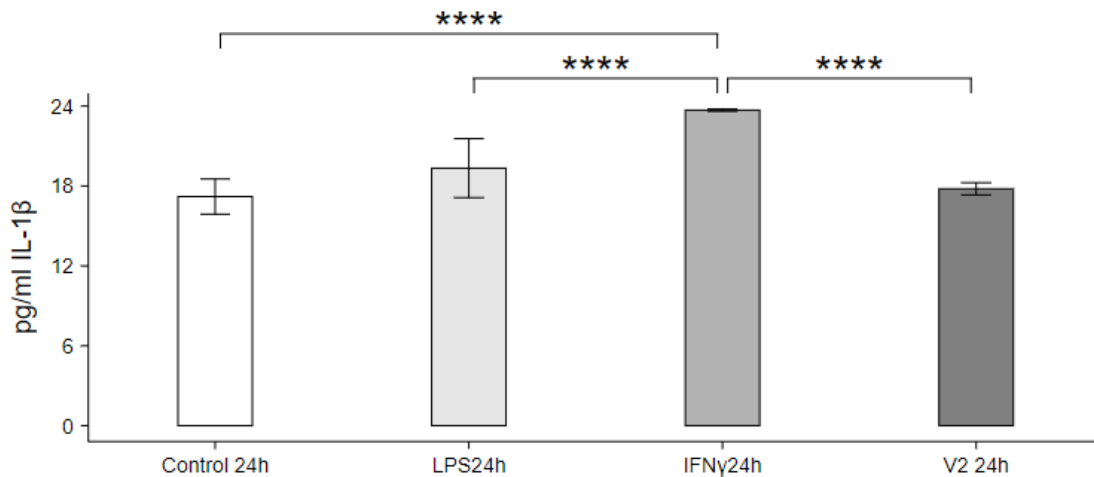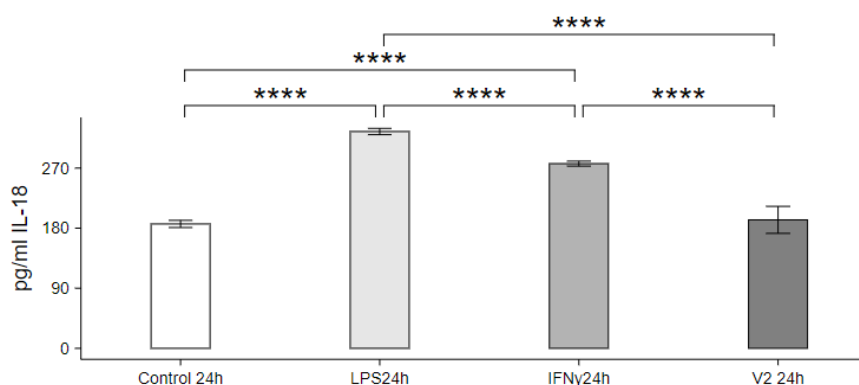

Figure 5.3.16 IL-18 secretion in 24h from Day 7 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment $(f(3,26)=233.07, p<0.0001)$. A post hoc Bonferroni correction showed that when treated with IFNγ for 24h the cells secrete a significantly higher amount of IL-18 (276.67±3.53pg/ml) when compared to untreated cells (186.32±5.4pg/ml) as well as V2 treated cells (192.43±19.1pg/ml), but a lower amount when compared to LPS treated cells (324.76±5.4pg/ml) (all differences $p<0.0001$). No difference was found between untreated cells and cells treated with V2 for 24h. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
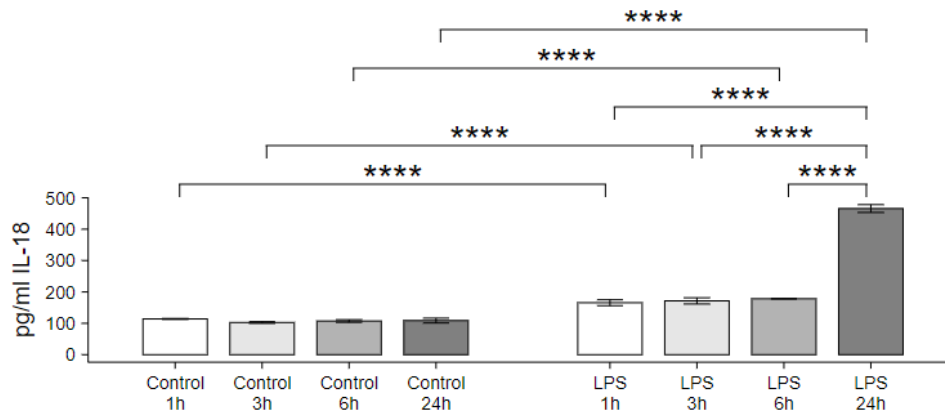
Day 14

TNFα
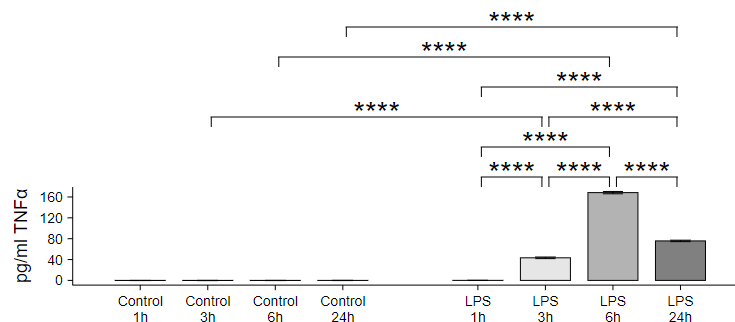
Figure 5.3.17 ***TNFα secretion from Day 14 LPS treated mg-THP1 cells in different timepoints vs controls*** For day 14 cells LPS treatment in different timepoints, a 2 way ANOVA revealed significant effect of treatment ($f(1,37)=93345.26$, $p<0.0001$), of time ($f(3,37)=19492.03$, $p<0.0001$), and interaction ($f(3,37)=19479$, $p<0.0001$). A post hoc Bonferroni test revealed differences between the control and LPS conditions for 3, 6, and 24h of treatment (nd for all control conditions vs 43.31 ± 1.45 pg/ml for 3h LPS treatment, 168.36 ± 2.22pg/ml for 6h LPS treatment, and 75.73 ± 1.32pg/ml for 24hLPS treatment, $p<0.0001$ for all). Within the LPS treatments, 1h treatment secreted TNFα (0.071 ± 0.053pg/ml) was significantly lower than the TNFα secreted by all other treatments ($p<0.0001$), 3h treatment produced significantly lower amounts of TNFα when compared to 6h and 24h, and treatment of the cells for 6h produced the highest amount of TNFα ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
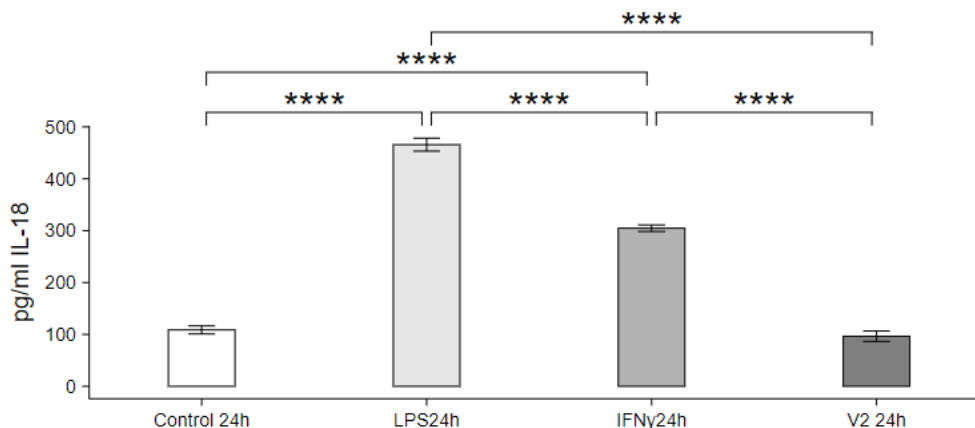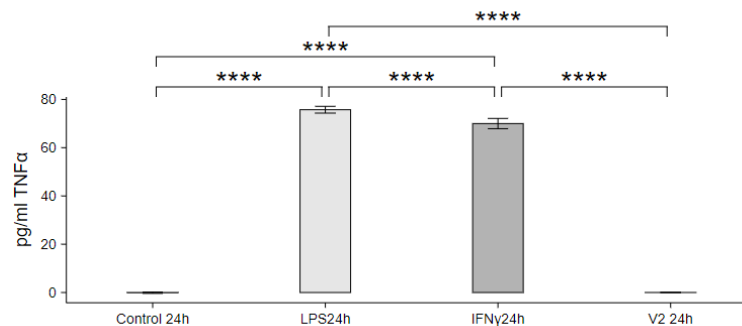

Figure 5.3.18 ***TNFα secretion in 24h from Day 14 mg-THP1 cells under different conditions*** In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3,26)=6415.5$, $p<0.0001$). A post hoc Bonferroni correction revealed no differences between the 24 h control (nd (-0.0921 ± 0.2764pg/ml) and the 24h V2 (0.014 ± 0.06)), while in addition to the difference between the control condition and 24h treatment with LPS which was already shown, an additional difference between control and 24h treatment with IFNγ was shown (75.73 ± 1.32pg/ml for LPS vs 70 ± 1.99pg/ml for IFNγ). Last, treatment with IFNγ produced significantly lower amounts of TNFα when compared to that with LPS ($p<0.0001$), and both treatments with inflammatory compounds produced a higher amount of TNFα when compared to treatment with V2. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
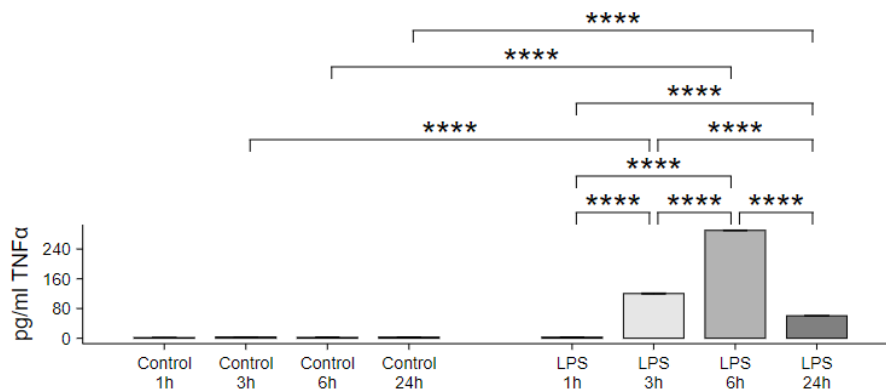
IL-6
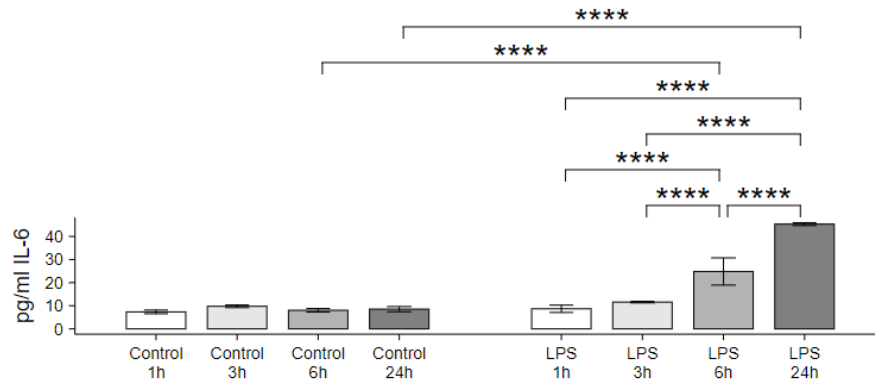
Figure 5.3.19 IL-6 secretion from Day 14 LPS treated mg-THP1 cells in different timepoints vs controls For day 14 cells LPS treatment in different timepoints, a 2 way ANOVA revealed significant effect of treatment $(f(1,37)=256.83, p<0.0001)$, of time $(f(3,37)=735.5, p<0.0001)$, and interaction $(f(3,37)=254.36, p<0.0001)$. A post hoc Bonferroni test revealed differences between the control and LPS conditions for 6 and 24h of treatment $(p<0.0001$ in both cases) (Controls: 1h: 7.34±0.56 pg/ml, 3h: 9.76±0.4pg/ml, 6h: 8.04±0.54pg/ml, 24h: 8.53±0.92pg/ml, LPS treated cells: 1h:8.68±1.5pg/ml, 3h: 11.58±0.32pg/ml, 6h: 24.84±5.54pg/ml, and 24h: 45.33±0.58pg/ml). Further differences were found between the LPS treated samples at different timepoints, with cells treated with LPS for 24h secreting the highest amount of IL-6 compared to all the other timepoints. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)N=9 (3 biological replicates *3 technical replicates each)
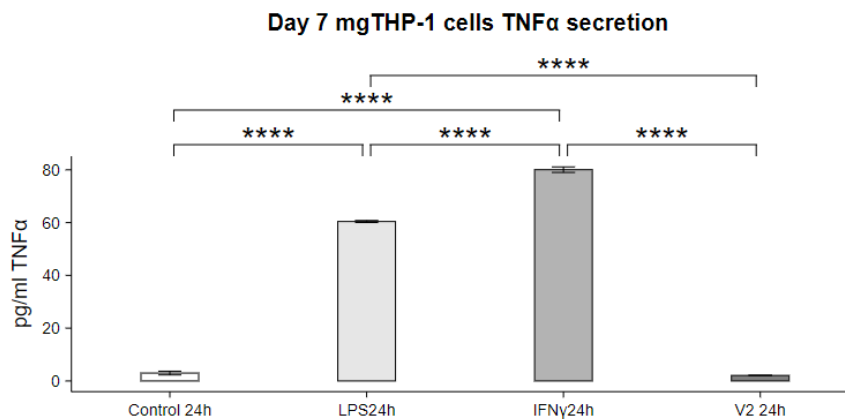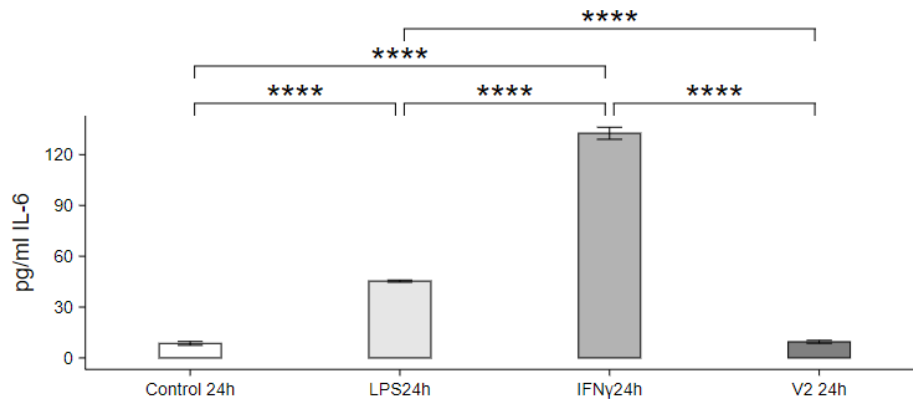

Figure 5.3.20 IL-6 secretion in 24h from Day 14 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment $(f(3,26)=6170.7, p<0.0001)$. A post hoc Bonferroni correction revealed no differences between the 24 h control (8.53±0.922pg/ml) and the V2 24h treatment (9.49±0.81pg/ml), but treatment with IFNγ for 24h (132.6±3.34pg/ml) elicited a strong response in the amount of IL-6 secreted $(p<0.0001)$, which was even stronger than the response to treatment with LPS for 24h (45.33±0.58pg/ml). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
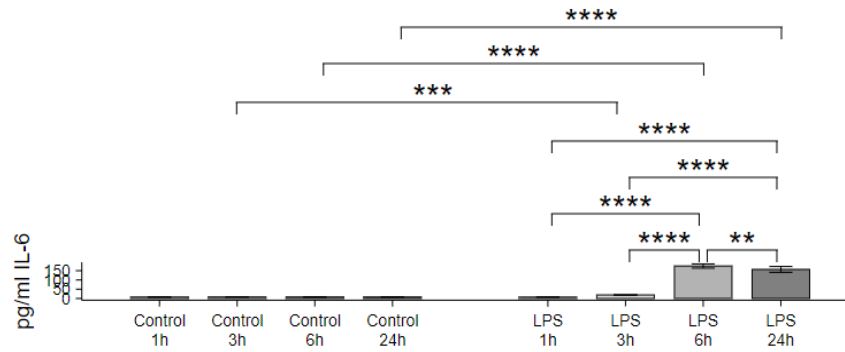
IL-1β

Figure 5.3.21 IL-1β secretion from Day 14 LPS treated mg-THP1 cells in different timepoints vs controls For day 14 cells LPS treatment in different timepoints, a 2 way ANOVA revealed significant effect of treatment ($f(1,37)=1552.76$, $p<0.0001$), of time ($f(3,37)=54.76$, $p<0.0001$), and interaction ($f(3,37)=65.22$, $p<0.0001$). A post hoc Bonferroni test revealed significant differences (1h $p<0.001$, 3, 6, 24h $p<0.0001$) between controls and the LPS treated samples of the same timepoints (Controls: 1h:19.89±0.207pg/ml, 3h:20.28±0.79pg/ml. 6h 19.78±0.26pg/ml, 24h 19.53±0.1pg/ml LPS 1h:23.82±0.1pg/ml, 3h 228.01±1.01pg/ml, 6h 27.93±0.5pg/ml, 24h 31.41±1.8pg/ml). Further differences were shown between the LPS treated samples, with the 24h treated sample secreting the most IL-1β when compared to the other timepoints ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
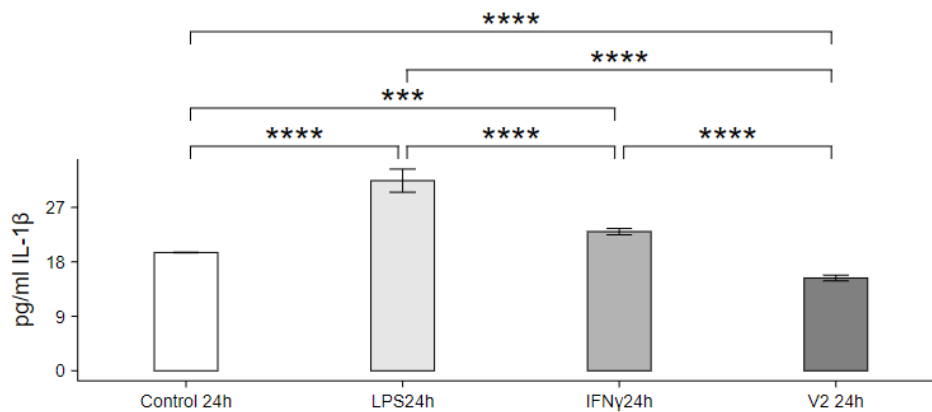
Figure 5.3.22 IL-1β secretion in 24h from Day 14 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3,26)=314.01$, $p<0.0001$). A post hoc Bonferroni test revealed differences between control (19.53±0.1pg/ml) and all other treatments investigated, with the amount of IL-1β secreted by the cells increasing with treatments of LPS (31.41±1.802pg/ml) or IFNγ (22.99±0.5pg/ml) and decreasing with V2 treatment for 24h (15.32±0.44pg/ml) ($p<0.001$ for all). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
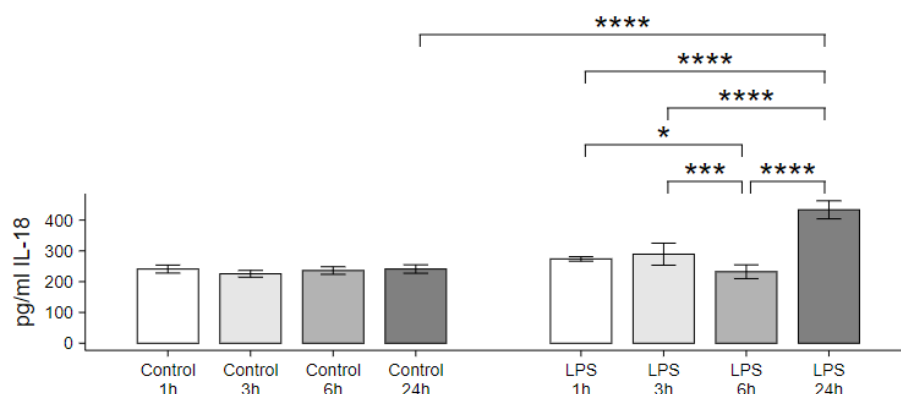
IL-18

Figure 5.3.23 IL-18 secretion from Day 14 LPS treated mg-THP1 cells in different timepoints vs controls For day 14 cells LPS treatment in different timepoints, a 2 way ANOVA revealed significant effect of treatment ($f(1,37)=232.14$, $p<0.0001$), of time ($f(3,37)=93.79$, $p<0.0001$), and interaction ($f(3,37)=86.1535$, $p<0.0001$). A post hoc Bonferroni test revealed differences between untreated cells and LPS treated cells only in the 24h timepoint (241.0 ± 11.21pg/ml vs LPS 433.38 ± 27.6pg/ml, $p<0.0001$); other differences were found between the amounts of IL-18 secreted by LPS treated cells in different timepoints, with the 24h treated cells secreting the highest amount of IL-18, and the 6h treated cells secreting the lowest. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)


Figure 5.3.24 IL-18 secretion in 24h from Day 14 mg-THP1 cells under different conditions In order to determine the effect of IFNγ treatment, as well as compare between treatments for 24h for LPS, IFNγ, and V2, a one way ANOVA was performed, which revealed a significant effect of treatment ($f(3,26)=188.5$, $p<0.0001$). A post hoc Bonferroni test revealed differences between untreated cells and IFNγ 24h treated cells (241.0 ± 11.21pg/ml vs IFNγ 300.6 ± 8.1pg/ml, $p<0.001$); further differences were found between LPS and IFNγ treatments and V2 treatment, with the latter having the lowest secretion of the three (in addition to having no significant difference when compared to untreated cells), and LPS was shown to cause a higher secretion of the cytokine when compared to all other conditions. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

Overall, it can be concluded that mgTHP-1 cells can take on an M1 phenotype, as increased secretion of pro-inflammatory cytokines was detected in the supernatants of almost all of the treated cell samples (with the exception of TNFα detection in the1h treated samples). The cells secretion patterns differed between treatment with LPS and IFNγ for 24h, with instances where the response for one inflammatory mediator was higher than the other for some cytokines, but the differences were not constant, so it cannot be concluded whether a specific cytokine's increase was constantly higher with one mediator rather than the other (except maybe the case of IL-18, where 24h IFNγ response was lower than 24h LPS response in all days).

M2 response

In order to investigate whether the cells produce an M2-like response, there was a comparison between LPS and IFNγ treated cells with and without pre-treatment with V-2 using 2-Way ANOVAs with Bonferroni corrections when appropriate (LPS treatments) or t-tests (IFNγ treatments). The results are presented in figures 5.4.1-5.4.12, with the statistical analyses results described in the legends. For all charts, a) includes LPS +/-V-2 treatment for 1,3,6, 24h LPS, whereas (2-way ANOVA)b)includes 24h IFNγ treatment +/-V-2 (t-test).

Day 3

TNFα
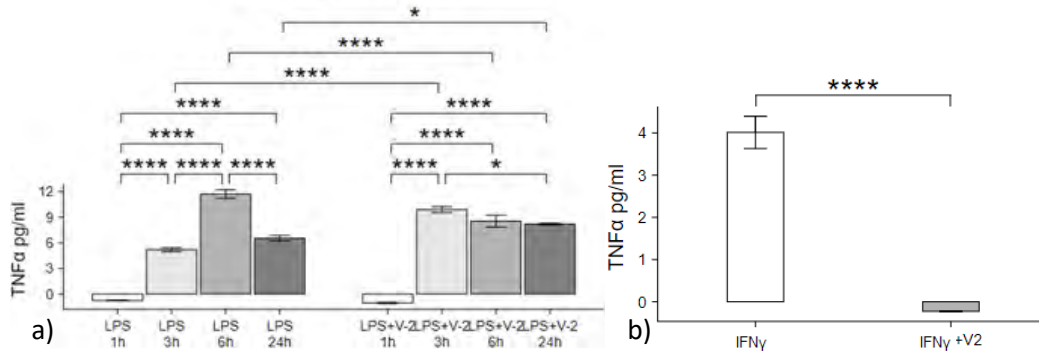
***Figure 5.4.1a,b** Examining the effect of V-2 treatment on TNFa secretion in Day 3 mgTHP-1 cells* a) A two way ANOVA with Bonferroni post hoc showed a significant effect of treatment $(F_{(1,64)}=8.34, p<0.001)$, time $(F_{(3,64)}=364.45, p<0.0001)$ and interaction $(F_{(3,64)}=43.577, p<0.0001)$, where pre-treatment with V-2 increased the secretion of TNFα in a statistically significant way after 3 $(5.19\pm0.65$ vs 9.89 ± 0.99 pg/ml, $p<0.0001)$ and 24 hours (b)$(6.53\pm0.91$ vs 8.20 ± 0.26 pg/ml) incubation with LPS $(p<0.001)$, while V-2 reduced the TNFα secretion in response to 6 hours LPS treatment when compared to LPS treatment alone $(11.71\pm1.37$ vs 8.54 ± 1.95 pg/ml, $p<0.0001)$. b) Interestingly, pre-treatment with V-2 reduced the response to IFNγ to non-detectable amounts $(p<0.0001)$ (*NB*. the differences, even though statistically significant, are those of a few pg/ml, so not necessarily translate to biologically significant. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). N=9 (3 biological replicates *3 technical replicates each)

IL-6
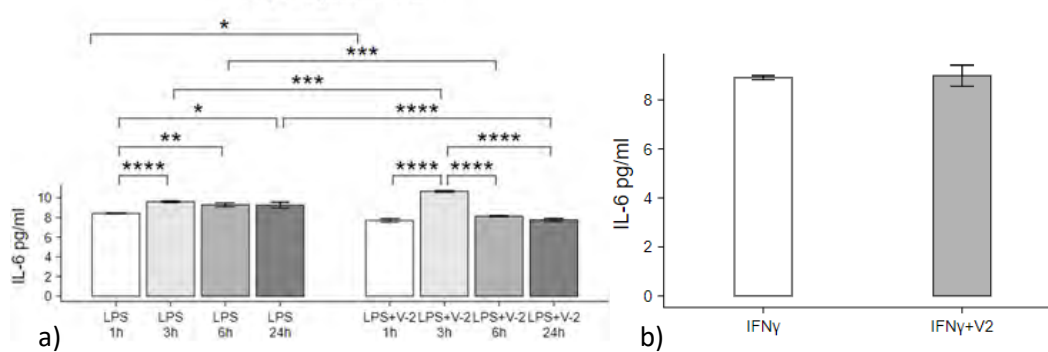
***Figure 5.4.2a,b** Examining the effect of V-2 treatment on IL- 6 secretion in Day 3 mgTHP-1 cells* a) A two way ANOVA with Bonferroni post hoc showed a significant effect of treatment $(F_{(1,64)}=26.022, p<0.0001)$, time $(F_{(3,64)}=61.70, p<0.0001)$, and interaction $(F_{(3,64)}=24.6188, p<0.0001)$, where V-2 significantly decrease the LPS-induced IL-6secretion after 1 hour $(8.44\pm0.12$ vs 7.69 ± 0.5pg/ml, $p<0.05)$ but increased it after 3 hours $(p<0.001$, 9.60 ± 0.25 vs 10.65 ± 0.29 pg/ml), while after 6 $(9.28\pm0.57$ vs 8.31 ± 0.16 pg/ml, $p<0.001)$ and 24hours $(9.25\pm0.84$ vs 7.75 ± 0.42 pg/ml, $p<0.0001)$ it significantly blunted LPS-induced IL-6 secretion. No significant difference was found for the V-2/IFNγ treated cells when compared to IFNγ alone $(8.91\pm0.24$ vs 8.98 ± 1.21 pg/ml, ns). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). N=9 (3 biological replicates *3 technical replicates each)

IL-1β

a)   b)
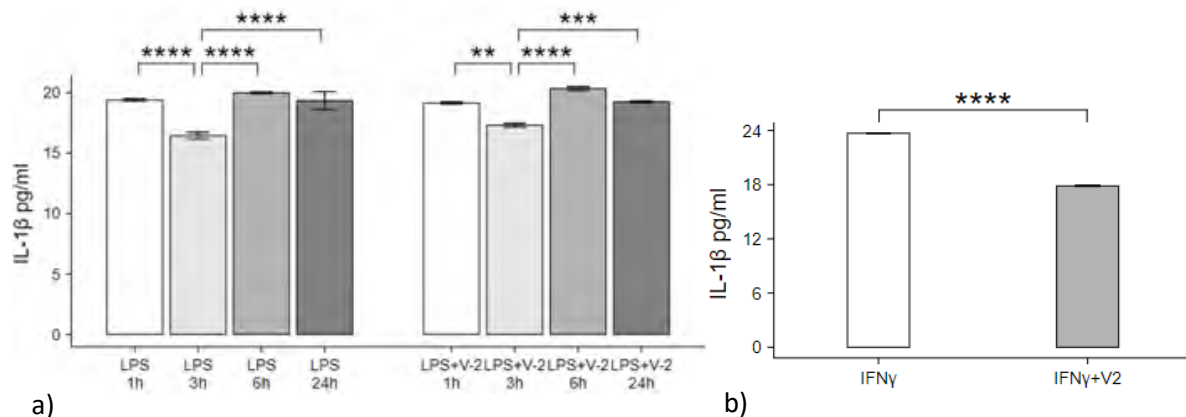
Figure 5.4.3 Examining the effect of V-2 treatment on IL-1β secretion in Day 3 mgTHP-1 cells a) A 2 way ANOVA with post hoc corrections showed a significant effect of time ($F_{(3,64)}=45.56$, $p<0.0001$), which shows that time has an effect when the cells are being treated with LPS (M1 response already explored) or LPS/V-2, but V-2 pre-treatment was not associated with a significant change in LPS-induced cytokine secretion .b) V-2 significantly decreased IFNγ-induced IL-1β secretion (23.69 ± 0.1 vs 17.85 ± 0.17 pg/ml, $p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). N=9 (3 biological replicates *3 technical replicates each)

IL-18

a)   b)
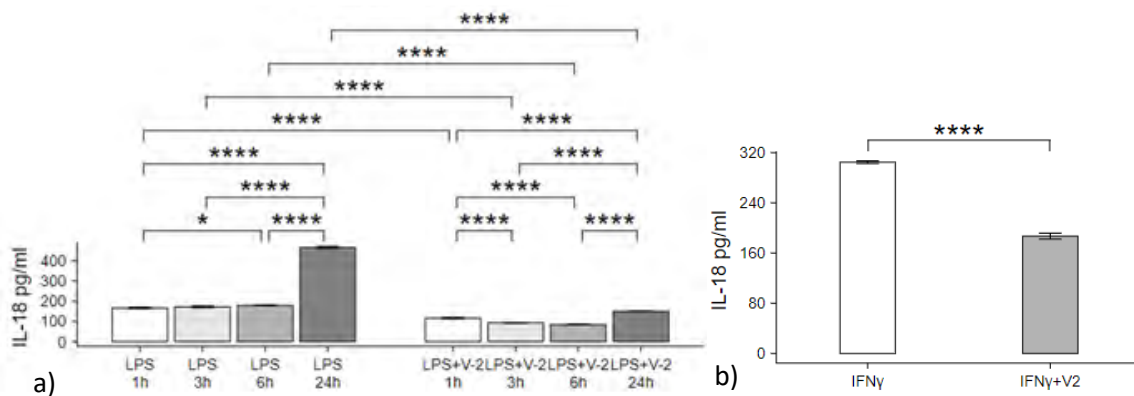
Figure 5.4.4 Examining the effect of V-2 treatment on IL-18 secretion in Day 3 mgTHP-1 cells A 2 way ANOVA with Bonferroni corrections showed a significant effect of treatment ($F_{(1,64)}=5806.82$, $p<0.0001$), time ($F_{(3,64)}=2389.48$, $p<0.0001$) and interaction ($F_{(3,64)}=1187.3454$, $p<0.0001$). a)V-2 significantly decreased the secretion of cytokines in all timepoints examined ($p<0.0001$ in every case). b) V-2 significantly decreased the amount of IL-18 secreted after IFNγ treatment. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). N=9 (3 biological replicates *3 technical replicates each)

Day 7

TNFα
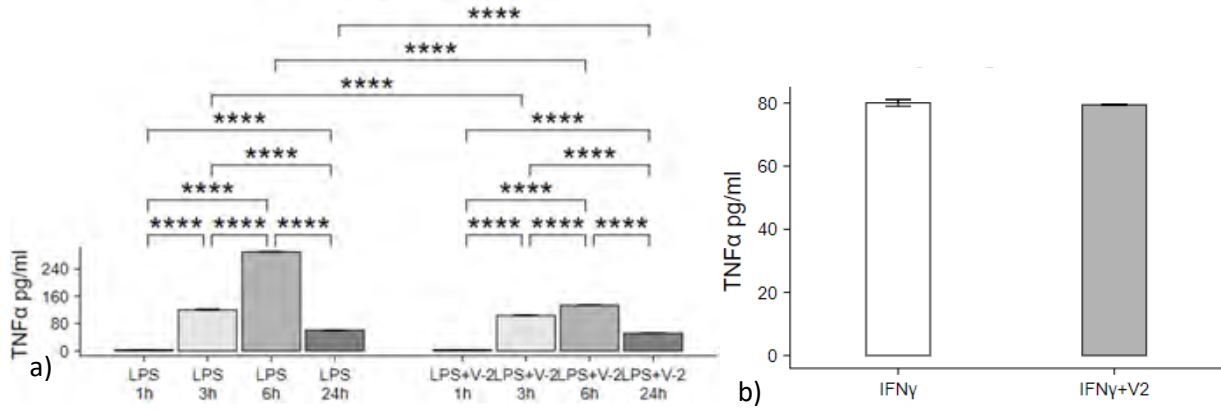
Figure 5.4.5a,b Examining the effect of V-2 treatment on TNFα secretion in Day 7mgTHP-1 cells A 2 way ANOVA with Bonferroni corrections showed a significant effect of treatment ($F_{(1,64)}=4916.15$, $p<0.0001$), time ($F_{(3,64)}=28290.52$, $p<0.0001$), and interaction ($F_{(3,64)}=4916.14$, $p<0.0001$), where pre-treatment with V-2 significantly decreased the secretion of TNFα at all but the 1h incubation times with LPS ($p<0.0001$ for all) b)V-2 pre-treatment showed no significant difference in the case of IFNγ (80.04 ± 2.96 vs 79.4 ± 0.55 pg/ml, ns). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

IL-6
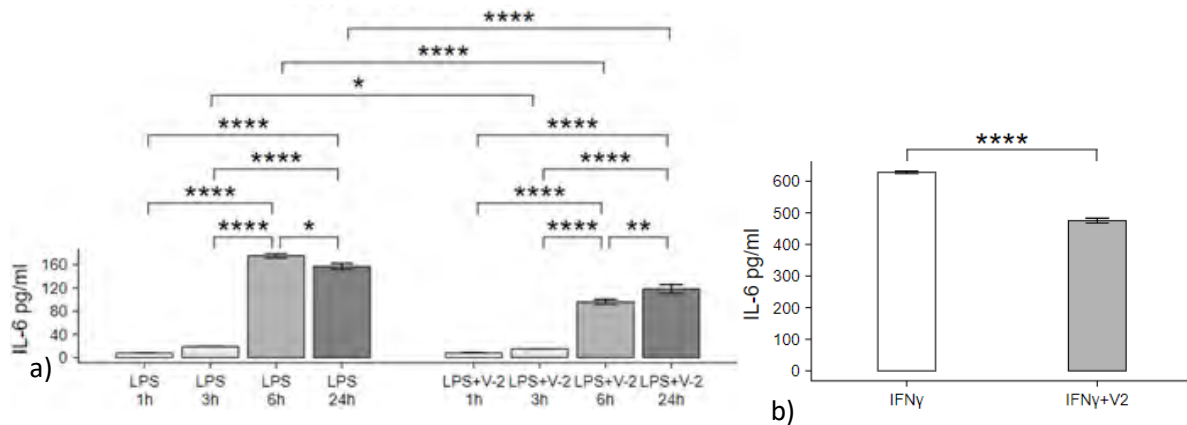
Figure 5.4.6a,bExamining the effect of V-2 treatment on IL-6 secretion in Day 7mgTHP-1 cells A 2-way ANOVA with Bonferroni corrections showed a significant effect of treatment ($F_{(1,64)}=125.93$, $p<0.0001$), time ($F_{(3,64)}=699.199$, $p<0.0001$), and interaction ($F_{(3,64)}=46.51$, $p<0.0001$), whereV-2 significantly blunted LPS-induced IL-6 secretion after 3 ($p<0.05$), 6 ($p<0.0001$) and 24hours ($p<0.0001$) of treatment. b) V-2 also blunted IFNγ-induced IL-6 secretion (627.98 ± 11.28 pg/ml vs 475.35 ± 21.7 pg/ml, $p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

IL-1β

a)   b)
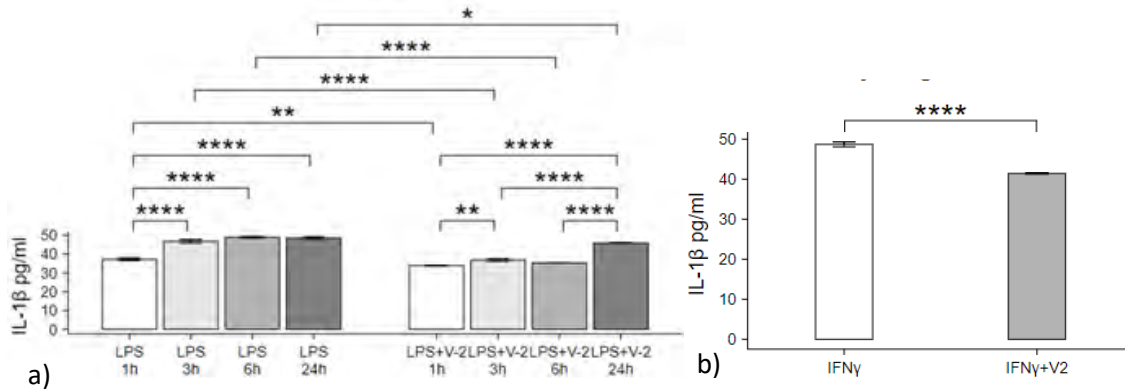
Figure 5.4.7a,b Examining the effect of V-2 treatment on IL-1β secretion in Day 7mgTHP-1 cells a) A two way ANOVA with Bonferroni correction showed a significant effect of treatment $(F(1,64)=348.8, p<0.0001)$, time $(F(3,64)=146.724, p<0.0001)$, and interaction $(F(3,64)=45.70, p<0.0001)$ where V-2 was found to decrease the secretion of the cytokine in a statistically significant way in all conditions (for 1h $p<0.01$, for 3h $p<0.0001$, for 6h $p<0.0001$, for 24h $p<0.05$). b)V-2 significantly decreased the amount of cytokine secreted after treatment with IFNγ alone $(p<0.0001)$. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). N=9 (3 biological replicates *3 technical replicates each)

IL-18

a)   b)
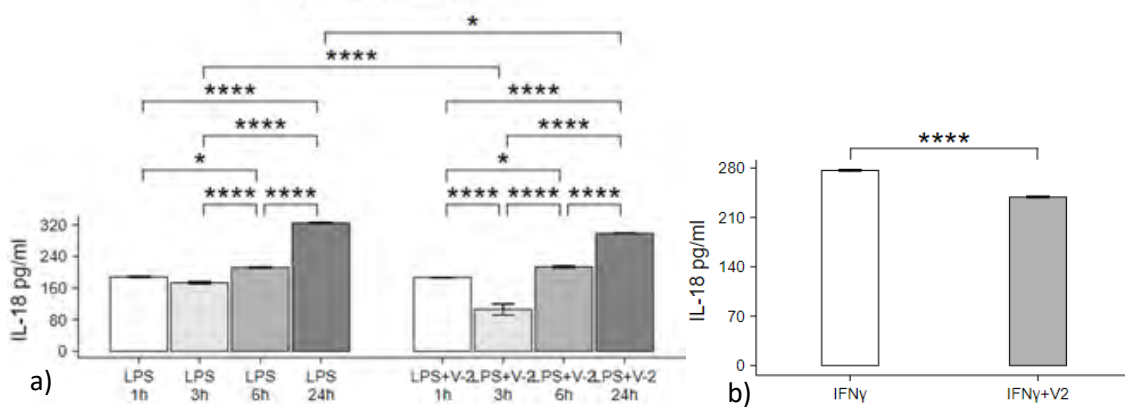
Figure 5.4.8a,bExamining the effect of V-2 treatment on IL-18 secretion in Day 7mgTHP-1 cells a) A two way ANOVA with Bonferroni corrections showed a significant effect of treatment $(F(1,64)=40.23, p<0.0001)$, time $(F(3,64)=386.45, p<0.0001)$ and interaction $(F(3,64)=18.74, p<0.0001)$ whereV-2 significantly blunted LPS-induced secretion of cytokines in the 3h treatment samples $(173.23\pm9.07$ vs 105.76 ± 39.36 pg/ml, $p<0.0001$). and in the 24h timepoint $(p<0.05)$. b)V-2 significantly decreased the amount of cytokine secreted after treatment with IFNγ $(p<0.0001)$. Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
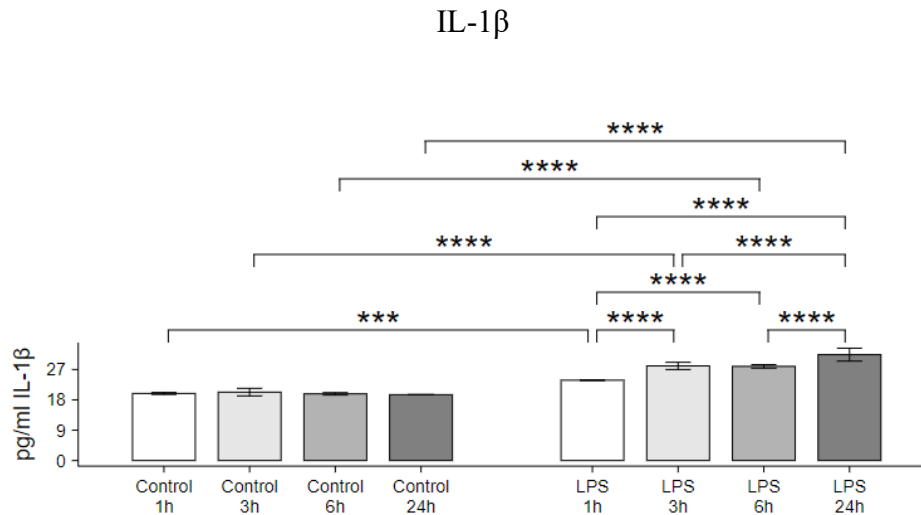
Day 14

TNFα
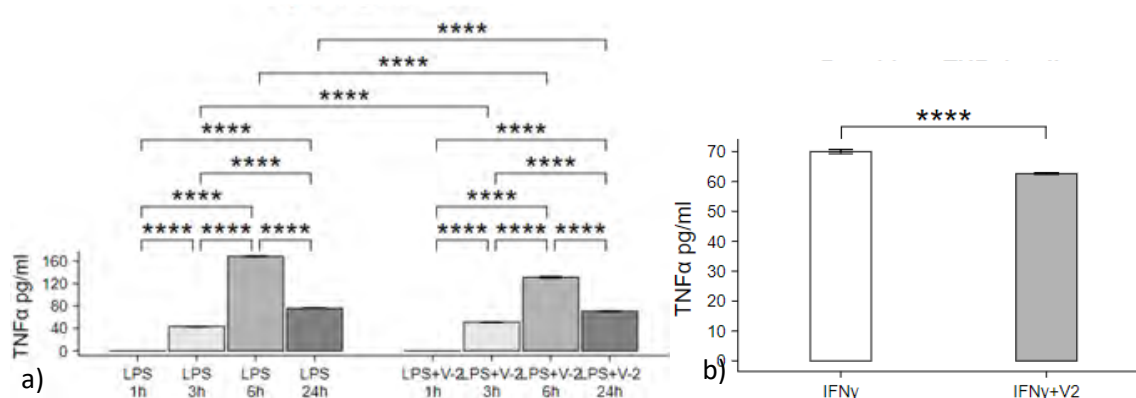
Figure 5.4.9a,b Examining the effect of V-2 treatment on TNFa secretion in Day 14mgTHP-1 cells A 2 way ANOVA with Bonferroni corrections showed a significant effect of treatment ($F_{(1,64)}=543.03$, $p<0.0001$), time ($F_{(3,64)}=23399.93$, $p<0.0001$) and interaction ($F_{(3,64)}=586.6$, $p<0.0001$), where pre-treatment with V-2 significantly increased LPS-induced secretion of TNFα after 3 hours (51.03 ± 0.77 for V-2 vs 43.31 ± 1.45 LPS only treatment pg/ml, $p<0.0001$), while it significantly reduced the secretion after 6 hours ($p<0.0001$) and 24 hours ($p<0.0001$). b)Similarly a significant blunting of secretion was observed for the IFNγ condition (V-2/IFNγ 62.64 ± 0.78 pg/ml, $p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****)N=9 (3 biological replicates *3 technical replicates each)

IL-6
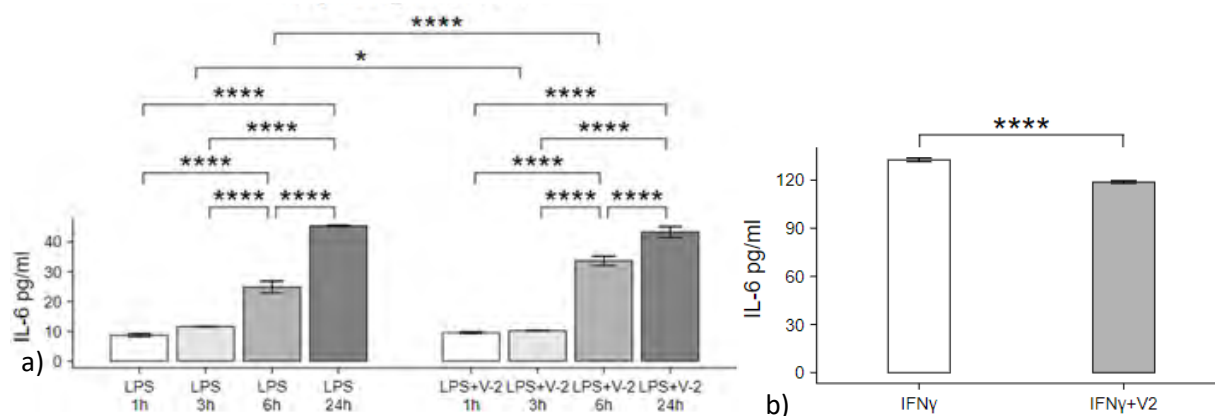
Figure 5.4.10 a,bExamining the effect of V-2 treatment on IL-6 secretion in Day 14mgTHP-1 cells a) A 2 way ANOVA with Bonferroni corrections showed a significant effect of treatment ($F_{(1,64)}=7.774$, $p<0.05$), time ($F_{(3,64)}=440.08$, $p<0.0001$), and interaction ($F_{(3,63)}=9.894$, $p<0.0001$) where V-2 significantly increased IL-6 secretion after 6h (24.83 ± 5.54 pg/ml LPS alone vs V2+LPS 33.63 ± 4.3 pg/ml, $p<0.05$) in response to LPS while it blunted LPS-induced IL-6 secretion at the 3h (10.19 ± 0.16 pg/ml, $p<0.01$) timepoint, and had no significant effect at the 24h timepoint (43.25 ± 5.2 pg/ml, ns vs LPS 24h).b) V-2 pre-incubation blunted IFNγ-induced secretion (118.74 ± 2.21 pg/ml) when compared to IFNγ alone ($p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)

IL-1β
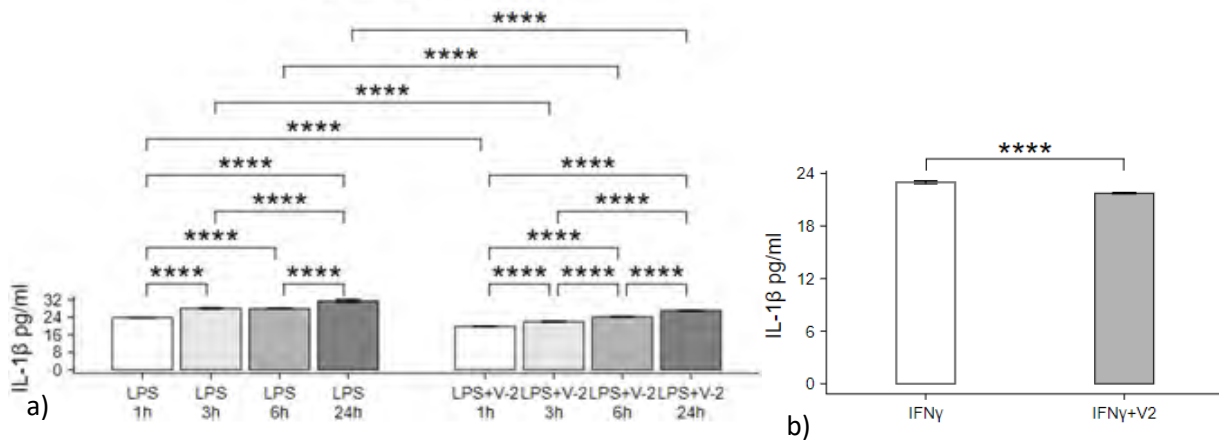
***Figure 5.4.11a,b*Examining the effect of V-2 treatment on IL-1β secretion in Day 14mgTHP-1 cells** A 2 way ANOVA with Bonferroni post hoc revealed a significant effect of treatment ($F_{(1,64)}=508.16$, $p<0.0001$), time ($F_{(3,64)}=232.1013$, $p<0.0001$) and interaction ($F_{(3,64)}=6.73$, $p<0.001$) where V-2 pre-treatment significantly blunted LPS-induced IL-1β secretion (LPS 1h:23.82±0.1pg/ml, 3h 228.01±1.01pg/ml, 6h 27.93±0.5pg/ml, 24h 31.41±1.8pg/ml and LPS/V-2 for 1h 19.78±0.21 pg/ml, for 3h 22.01±0.22 pg/ml, for 6h 24.23±0.26 pg/ml, for 24h 22.98±0.49 pg/ml, $p<0.0001$ for all when compared to corresponding LPS only conditions), b) V-2 elicited a significant blunting of IFNγ-induced IL-1β secretion (20.73±0.22 pg/ml, $p<0.0001$ vs IFNγ only condition (22.98±0.49 pg/ml)). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****) N=9 (3 biological replicates *3 technical replicates each)
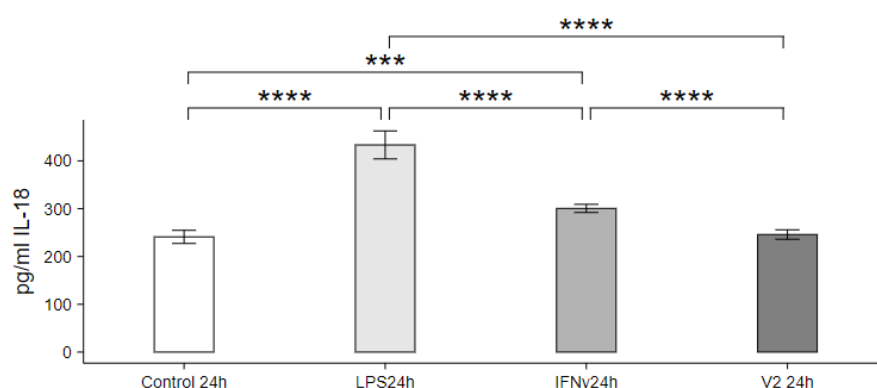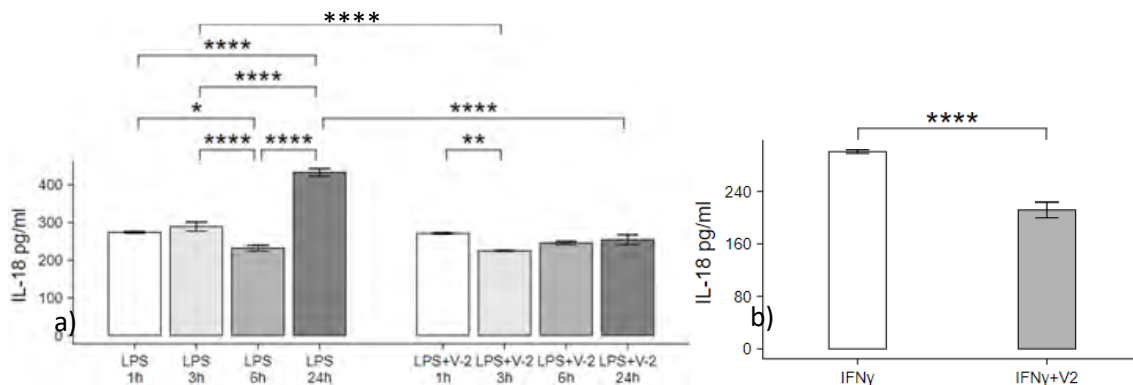
IL-18

***Figure 5.4.12a,b*Examining the effect of V-2 treatment on IL-18 secretion in Day 14mgTHP-1 cells.** A two way ANOVA with Bonferroni corrections revealed a significant effect of treatment ($F_{(1,64)}=108.73$, $p<0.0001$), time ($F_{(3,64)}=67.53$, $p<0.0001$), and interaction ($F_{(3,64)}=60.8$, $p<0.0001$) where V-2 significantly blunted LPS-induced secretion of the cytokine at 3h ($p<0.0001$ vs LPS alone) and 24h timepoints of treatment ($p<0.0001$), b) V-2-associated blunting was seen for the IFNγ condition (211.71±33.74 pg/ml, $p<0.0001$). Significant results are marked with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****). N=9 (3 biological replicates *3 technical replicates each)

The ability of V-2 (in some cases at least) to reduce the magnitude of those pro-inflammatory responses, suggests that M1 polarisation can be blunted-if not fully reversed- by (pre-) treatment with anti-inflammatory mediators, such as V-2, indicating an M2 response (similar to the one shown by Hassan et al., (2018)). Table 5.2 summarises the findings of the ELISA results and this section with the addition of information on the receptors of the inflammatory stimuli used.

	M1-like response to LPS/IFNγ	V-2 induced M2-like response	IL-6 response range (cytokine secreted)	TNFα response range (cytokine secreted)	IL-18 response range (cytokine secreted)	IL-1β response range (cytokine secreted)	Expression of IFNγR/TLR2/TLR4
Day 3	+/+	+	6-10pg/ml	0-11pg/ml	100-495pg/ml	16-25pg/ml	+/+/+
Day 7	+/+	+	7-627pg/ml	2.5-290pg/ml	100-330pg/ml	24-49pg/ml	+/+/+
Day 14	+/+	+	9-130pg/ml	0-168pg/ml	230-430pg/ml	15-31 pg/ml	+/+/+

Table 5.2 _The summary of the findings for section 5.3.3_. The table summarises the M1-like and M2-like responses per day, and gives a summary of the response range per day and cytokine investigated, as well as whether the cells express the receptors for the inflammatory stimuli used..

NB: Please note that RAGEs was not detected in any of the conditions, so it is not included in the graphs shown in this chapter.

5.3.4 Cytokine arrays

As V-2 appeared to have anti-inflammatory effects were able to blunt responses to pro-inflammatory stimuli (see figures 5.3.1-5.3.24, and 5.4.1-5.4.12), the investigation of the effects of V-2 alone on the cells was essential to confirm its ability to polarise cells towards an M2 phenotype. This would also reveal whether the flavonoid has other anti-inflammatory effects (i.e. increase of the secretion of anti-inflammatory molecules) on its own, and hence provide insights regards what are its effects overall on the cells. To investigate this, cytokine arrays were used to which a variety of (neuro)inflammation related cytokine antibodies are adhered, and

which can indicate potential changes in the secretome of the cells; the intention was to treat with V-2 alone and to identify changes that could be attributed to V-2.

The results of the cytokine array analysis are presented below. First, a table of all 42 of the cytokines analysed is presented (table 5.2), which can be used as a guide for interpreting the heat maps generated via analysis of the membranes with ImageJ (Protein Array Analyser tool by Gilles Carpentier). In order to do the analysis, each array had the background noise removed with the help of negative controls/dots, and the signal from each sample was then normalised (in pairs) against the positive controls, and then compared between conditions (+ V-2 or - V-2). The following cytokines/proteins were selected as being most interest, due to their changes between conditions, or because they have been found to play important roles to neuroinflammation were then analysed further (i.e. densitometric analysis was followed by generation of graphs after subsequent analysis with ImageJ): MCP-1, TNFα, ENA-78, GM-CSF, RANTES, TGF-β1, IL-4, IL-10, IL-12, IL-13, IL-6, and IL-1β. In some cases, the results from the ELISAs above can be used as corroborative "controls" in order to confirm the results from the membranes (specifically, ELISA controls vs 24h V2 incubation at all stages of the differentiation process were used as comparators when interpreting array data for TNFα, IL-6, IL-1β).

Pos	Pos	Neg	Neg	ENA-78	GCSF	GRO	GRO-α	I-309	IL-1α	IL-1β
Pos	Pos	Neg	Neg	ENA-78	GCSF	GRO	GRO-α	I-309	IL-1α	IL-1β
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-12	IL-13	IL-15	IFN-γ
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-12	IL-13	IL-15	IFN-γ
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	RANTES	SCF	SDF-1	TARC	TGF-β1
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	RANTES	SCF	SDF-1	TARC	TGF-β1
TNFα	TNFβ	EGF	IGF-I	Angiogenin	Oncostatin-M	VEGF	PDGF	Leptin	Neg	Pos
TNFα	TNFβ	EGF	IGF-I	Angiogenin	Oncostatin-M	VEGF	PDGF	Leptin	Neg	Pos

Table 5.3 _A representation of the layout of the cytokine array used. Pos=positive control, Neg=negative/background control._

DAY 3
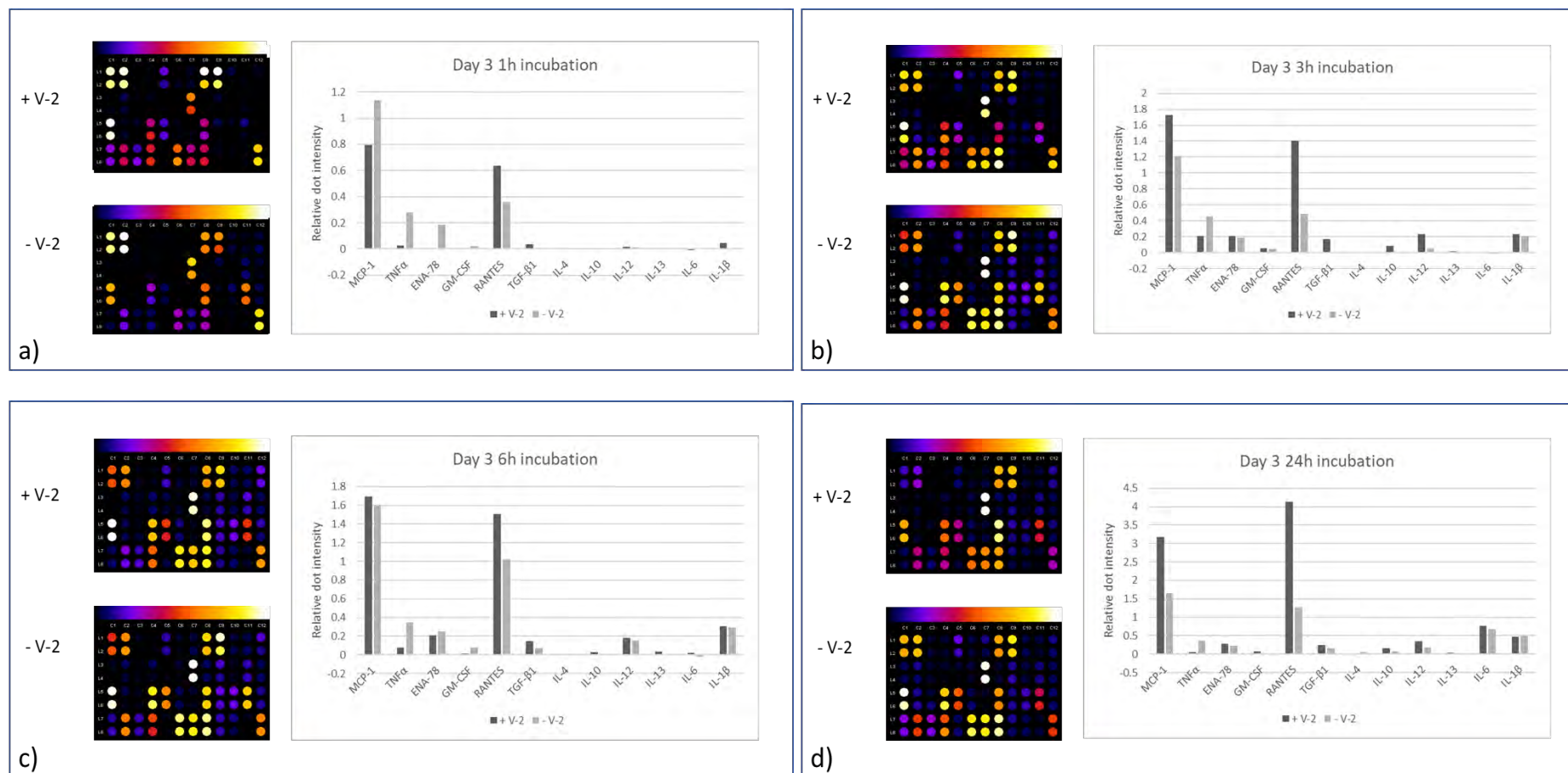
Figure 5.5a, b,c and d. *Results of the cytokine arrays for mgTHP-1 cells developmental day 3, after 1, 3, 6 and 24 hours of incubation with V-2 vs control* Results appear as heatmaps, from which the cytokines/proteins of interest are further analysed and compared in the graphs for each case. N=1 (from n=3 biological replicates pooled)

DAY 7
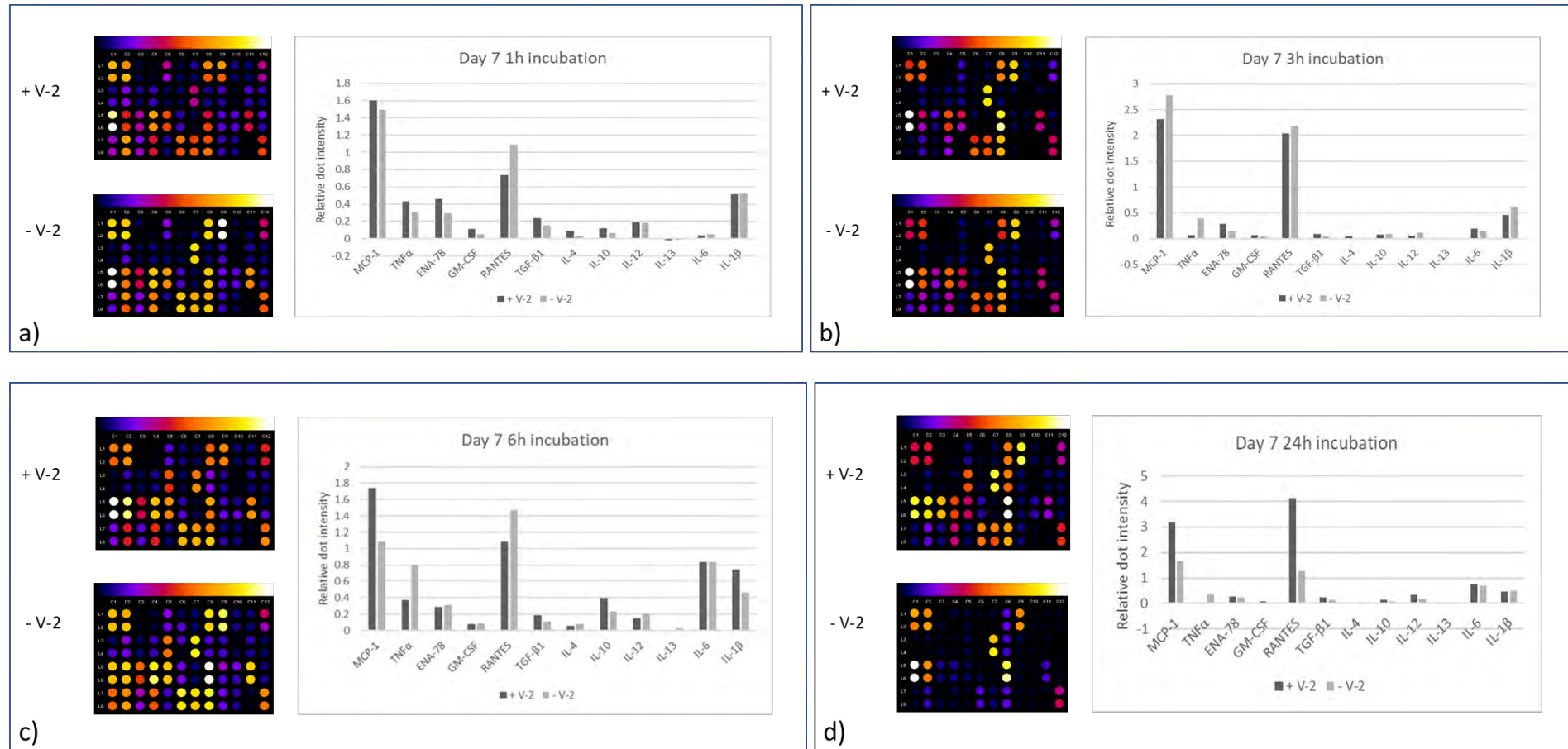
Figure 5.6a, b, c and d Results of the cytokine arrays for mgTHP-1 cells developmental day 7, after 1 and 3 hours of incubation with V-2 vs control Results appear as heatmaps, from which the cytokines/proteins of interest are further analysed and compared in the graphs for each case. N=1 (from n=3 biological replicates pooled)

DAY 14
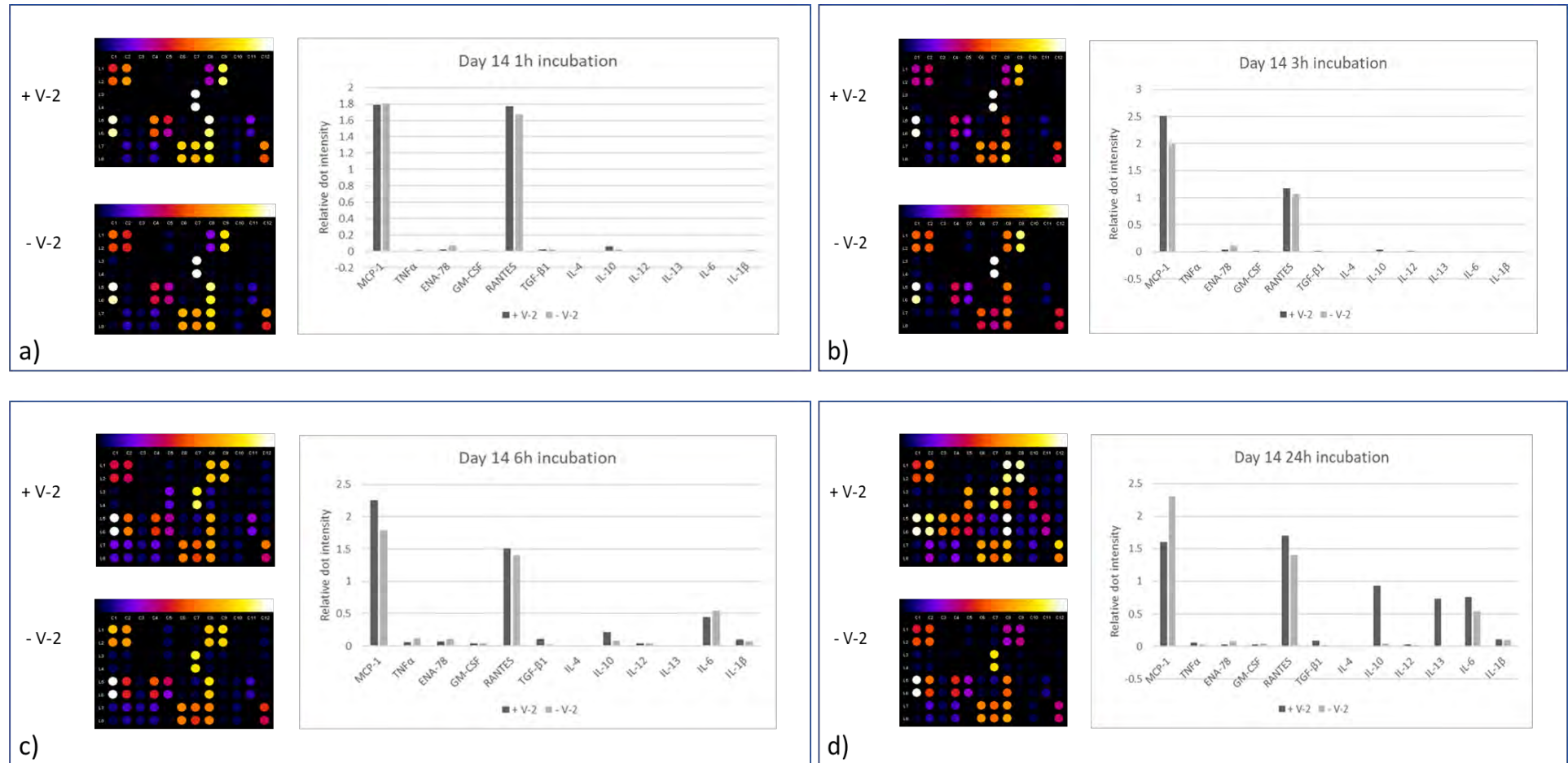
Figure 5.7a, b, c and d Results of the cytokine arrays for mgTHP-1 cells developmental day 14, after 1 and 3 hours of incubation with V-2 vs control Results appear as heatmaps, from which the cytokines/proteins of interest are further analysed and compared in the graphs for each case. N=1 (from n=3 biological replicates pooled)

Results of the cytokine arrays for mgTHP-1 cells developmental day 3 are shown in figures 5.5(a-d): a) after 1 hour, V-2 reduced the amount of MCP-1, TNFα, ENA-78, and GM-CSF secreted from the cells, while it had the opposite effects on RANTES, IL-1B, and TGF-β1. IL-12 was unaffected by V-2, in that it was secreted in low amounts in the presence or absence of V-2. b) after 3 hours, V-2 increased MCP-1, RANTES, TGF-β1, IL-1B, ENA-78, IL-10, IL-13, and IL-12 while having the opposite effect on TNFα. Overall, the secretion seen at the 3h timepoint was higher than that at 1h. c) After 6 hours, V-2 reduced the amount of TNFα, ENA-78, and GM-CSF secreted from the cells, while it had the opposite effects on MCP-1, RANTES, IL-10, IL-12 and TGF-β1; it slightly increased secretion of IL-1β, and IL-6 . d) after 24 hours, V-2 increased the secretion of MCP-1, ENA-78, RANTES, GM-CSF, TGF-β1, IL-6, IL-10, IL-13 (marginally), and IL-12 while having the opposite effect on TNFα and IL-4 (to some extent). Overall in the 24h timepoint, secretion of most cytokines was higher than 6h.

Results of the cytokine arrays for mgTHP-1 cells developmental day 7 are shown in figures 5.6(a-d): a) after 1 hour, V-2 reduced the amount of RANTES and marginally of IL-6 and IL-1β secreted from the cells, while it had the opposite effects on MCP-1, TNFα, ENA-78, GM-CSF, TGF-β1, IL-4, IL-10, while IL-12 secretion was unaffected. b) after 3 hours, V-2 increased ENA-78, GM-CSF, TGF-β1, IL-4, and IL-6 while having the opposite effect on MCP-1, TNFα, RANTES, IL-12, and IL-1β. IL-10 secretion was unaffected; overall secretion was higher for most cytokines at the 3h timepoint than it was at 1h. c) after 6 hours, V-2 reduced the amount of TNFα, ENA-78, RANTES, IL-12 and GM-CSF (marginally) secreted from the cells, while it had the opposite effects on MCP-1, TGF-β1, IL-10, and IL-1β; it slightly reduced the amounts of GM-CSF and IL-6 . d) after 24 hours, V-2 increased MCP-1, ENA-78, RANTES, GM-CSF, TGF-β1, IL-6, IL-10, and IL-12, while having the opposite effect on TNFα and IL-1β (slightly). Overall at the 24h timepoint, secretion of most cytokines was detected as higher than after 1, 3, or 6h of V-2 treatment.

Results of the cytokine arrays for mgTHP-1 cells developmental day 14 are shown in figures 5.7(a-d): a) after 1 hour, V-2 reduced the amount of MCP-1, TNFα, ENA-78, and IL-1β secreted from the cells, while having the opposite effects on RANTES, and IL-10, while TGF-β1 secretion was unaffected. b) after 3 hours, V-2 increased MCP-1, RANTES, TGF-β1, IL-10, while having the opposite effect on TNFα, IL-12. Overall secretion was higher for most cytokines at the 3h timepoint than at the 1h one. c) after 6 hours, V-2 reduced the amount of TNFα, ENA-78, and IL-6 secreted from the cells, while having the opposite effects on MCP-1, RANTES, TGF-β1, IL-10, and IL-1β; it had no effect on the secretion of GM-CSF or IL-12 . d) after 24 hours, V-2 increased secretion of TNFα (marginally), RANTES, TGF-β1, IL-6, IL-10, IL-13, and slightly IL-12, and TNFα, with IL-1β seemingly unaffected. It had the opposite effect on MCP-1, ENA-78, and GM-CSF. Overall at the 24h timepoint, secretion of anti-inflammatory cytokines was higher than at 1, 3, or 6h.

Thus, overall, V-2 appears to have an anti-inflammatory effect on the cells, by not only reducing the amount of pro-inflammatory cytokines secreted after treatment with a pro-inflammatory stimulus (ELISA data), but also promoting the secretion of anti-inflammatory cytokines (such as IL-10, and IL-13) (cytokine array data especially Day 14 24h samples). Moreover, it has additional effects on molecules such as RANTES, and MCP-1 (which have been shown to have pleiotropic effects when it comes to neuroinflammation, and will be discussed further later on this chapter).

N.B. Although most cytokine array data are in agreement with the ELISA data, there are some exceptions (e.g. IL-1β day 14, 24h ELISA shows that V-2 decreases the amount of cytokine secreted, while the array does not show such a result). This could be due to a variety of factors, such as sensitivity of the assays, data handling (an amount of error is expected with imageJ analysis, as the analysis depends on how well the membrane has developed, how in agreement the controls are, choice of the dots on the dot plot while processing the membrane etc.).

5.4 Discussion

5.4.1 Main findings

For this chapter, the microglia/macrophage-like functional qualities of mgTHP-1 cells (as were generated and characterised in the previous chapter) were examined by focusing on the M1/M2 paradigm of macrophage activation (which also applies to microglia and other CNS macrophages) (Murray et al., 2014; Tang & Le, 2016). Through using pro-inflammatory stimuli, namely LPS, and IFNγ, and investigating the secretion of 5 pro-inflammatory cytokines (namely TNFα, IL-1β, IL-18, IL-6, and RAGEs), it was shown that: a) mgTHP-1 cells can be activated and polarised to an M1 phenotype(classically activated macrophages/pro-inflammatory), and b) that the exact nature of this activation differed for the different developmental stages (developmental days 3, 7, and 14), and for different exposure times to the stimuli.

Through using Vicenin-2 -which Hassan et al., (2018) showed to polarise macrophage like cells (dTHP-1) in an M2 phenotype- the potential of the mgTHP-1 cells to polarise to M2 was also investigated. To achieve this we examined the effects of V-2 on cells treated with pro-inflammatory stimuli (via ELISA), as well as the effects of V-2 on mgTHP-1 cells without any other stimulus)bia both ELISA and cytokine arrays). The secretion of a variety of targets (both pro- and anti-inflammatory cytokines) was examined, and it was shown that in the majority of cases, V-2 decreased the release of pro-inflammatory cytokines (e.g. TNFα, IL-1β) and enhanced the release of anti-inflammatory cytokines.

Last, we examined whether the cells secreted a variety of cytokines in the absence of a classical stimulus, by focusing on the differential secretion of cytokines from non-stimulated cells at different timepoints and developmental days. The cells were shown to secrete both pro- (e.g. TNFα) and anti-inflammatory cytokines (e.g. IL-10) without any stimulus present; for example the

secretion of IL-10 was observed in most cases, as was the secretion of IL-1β, and IL-6. The latter cytokines, as discussed in chapter 1 have pleiotropic effects, while IL-10 has been shown to elicit only anti-inflammatory effects, therefore the potential anti-inflammatory role of the cells overall should be investigated in the future, especially as it has been shown elsewhere that CNS macrophages (e.g. Shechter et al., 2009; Andreou et al., 2017; Unger et al., 2018) can have anti-inflammatory properties.

Finally, it should be noted that some values after the cytokine array analysis seem to be negative, when it comes to intensity. That is an artefact of the methodology, as sometimes negative controls exhibit some intensity that surpasses that of certain cytokines (the software used for the analysis does not have the option to remove such values, and even though that could easily be done in excel, I preferred to keep them, in order to show the fact that although the method is sensitive, sometimes that sensitivity can be a disadvantage). As already noted, some differences observed in the ELISA data did not appear in the array data. Nevertheless, as the amount in question here is ~5pg/ml this could give an indication of the sensitivity of the array as a method, as the manufacturer does not give an indication of difference in sensitivity per cytokine (i.e. if the same intensity of a dot equals to same amount of cytokine when comparing two dots). Furthermore, for the cytokine arrays, only one overall sample was used (pooled together n=3), and only one membrane per condition, due to cost of the assay. Further studies focusing on the cytokine amounts detected from the arrays could elucidate such differences.

5.4.2 Discussion of findings

M1 macrophage activation is followed by the release of pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα (Martinez et al., 2008). Increase in IL-18 and IL-1β secretion is also induced via the activation of the NLRP-3 inflammasome (Vandanmagsar et al., 2011). NLRP3 has been

shown to be highly expressed in M1 phenotypes (Awad et al., 2017), and has been elsewhere found to have a regulatory role with regard to M2 polarisation (Liu et al., 2018). LPS and IFNγ both polarise macrophages into an M1 state (Juhas et al., 2015); the data in figures 5.3-5.4 confirms the hypothesis that these cells are indeed macrophage-like as LPS and IFNγ upregulate the secretion of pro-inflammatory cytokines. However unlike other methods of differentiating monocytes into M0 macrophages (e.g. PMA treatment of THP-1 monocytes -with PMA being inflammatory- leads to THP-1 macrophage-like cells, resembling the M0/resting state (Genin et al., 2015)), in this case the differentiation medium used was not inflammatory. Therefore, the changes, as mentioned in the previous chapter, are not attributed to an inflammatory environment-triggered change (which is found in most tissues where monocytes are attracted to due to an inflammatory event), but rather the cells assuming a CNS macrophage/microglia-like phenotype (including morphology and expression pattern of CNS macrophages/microglia) due to changes of a different nature (including epigenetic and metabolic changes, as shown in chapter 4).

The finding that the two major inflammatory cytokines from those mentioned above, namely TNFα, and IL-6, are secreted in miniscule amounts by the control cells from each developmental stage is in agreement with findings where microglia cells have a low baseline secretion of both cytokines (Kaushik et al., 2013; Njie et al., 2014), while IL-1β has been shown to be produced at a similar baseline level in microglia (Kim et al., 2006); this is an additional finding (i.e. in addition to the ones from chapter 4) which supports the view that the mgTHP-1 cells are microglia-like. In infiltrating monocytes, IL-1β is expected to be secreted at higher amounts, as the cells entering the brain essentially enter an inflamed environment, and therefore could theoretically exhibit a high secretion of IL-1β, as a response to their environment (Lévesque et al., 2016); this is in contrast to our findings where IL-1β is secreted in relatively low amounts. IL-18 has also been shown to be continuously secreted in microglia as well as MDM, with LPS enhancing the secretion in microglia cell cultures (Conti et al., 1999).

Notably, as shown in figure 5.1, cells of all developmental stages were shown to express the LPS receptor TLR-4, and also the potential LPS receptor (although debated by some e.g. Hirschfeld et al., 2000) TLR-2 (Takeuchi et al., 1999; Good et al., 2012). LPS induces an M1 phenotype, and thus a pro-inflammatory signalling cascade via its receptor TLR-4, and possibly TLR-2 (Yang et al., 1999; Hirschfeld et al., 2000; Burgueño et al., 2016). The cytokine secretion level patterns do not necessarily follow the TLR expression levels, however, which could be explained by the fact that the LPS TLR-4 activation can initiate either MyD88 (Myeloid differentiation factor 88) dependent or independent pathways (Pålsson-McDermott & O'Neill, 2004; Kawai et al., 2001). MyD88 is a cytoplasmatic protein that is essentially an adaptor for TLR-4, but also TLR-2 (Pålsson-McDermott & O'Neill, 2004; Quigley et al., 2009).

Such responses to LPS by following a MyD88 dependent (inflammatory) or independent (regulatory) route, can lead to different downstream responses (Kawai et al., 2001). The different levels of activation could thus be explained due to factors relating the cells' different differentiation stage, but further experiments that focus on the role of MyD88 on the differentiation and activation of such cells would be needed in order to confirm such a claim. Nevertheless, it is worthy to mention here, that MyD88 has been shown to be essential for the differentiation of monocytes into macrophages, and thus the majority of the protein in cells that are differentiating (days 3, and 7) could be used for that purpose, rather than an inflammatory-response pathway (Gelman & Kreisel, 2007), which could perhaps suggest that a MyD88-independent route is more relevant in this instance.

IL-18 was activated later than the IL-6 and TNFα (the former peaking at 24h post LPS stimulation in most cases compared to a 6 hour peak observed for the latter in most cases of the results), while IL-1β is the least inducible of the so called early release cytokines (for an explanation behind "early" and "late" stage cytokines, see Hildebrand et al., 2005). IL-18 has been known to be expressed after other cytokines in microglia, especially in CNS inflammation (Jander et al., 2002).

This could be because its processing and release are a rather complicated procedure, which involves the NLRP3 inflammasome, as well as the NF-κB pathway. Although the maturation of IL-1β can involve the same pathway, it involves not one, but rather a "spectrum of release" mechanisms with both early and delayed release potential of the cytokine (Lopez-Castejon & Brough, 2011). As shown in the ELISA data shown in figures 5.3-5.4, as well as from the cytokine array findings (see figures 5.5-5.7) the secretion of IL-1β does increase, however not to as great an extent as IL-6 or TNFα; thus, IL-1β secretion essentially remains relatively low and within the same levels between conditions, no matter the incubation timepoint examined. Further investigations to elucidate the mechanisms underpinning the cells' responses to LPS could, therefore, include examination of the NLRP3 inflammasome and its related genes, both at a protein, as well as at a gene level, and correlation of the resulting findings with the developmental stage of the mgTHP-1 cells.

For the response to IFNγ, the inflammatory response overall follows the IFNγR1 expression patterns, with day 3 having lower response, and days 7 and 14 higher. IFNγ has been known to be an inducer of TNFα (Vila-del Sol et al., 2008) as well as IL-1β (Masters et al., 2010) something which agrees with the ELISA data presented in this chapter. On the other hand, IFNγ has not been shown to directly affect the secretion of IL-18 (which in contrast induces the secretion of IFNγ), and has a rather complicated relationship with IL-6, however, it has been shown to induce its secretion in some circumstances (Yi et al., 1996). The findings of the present chapter show an increase of the secretion of IL-6, IL-1β, TNFα and IL-18 (fig. 5.3-5.4) after IFNγ treatment for 24hours. This may be not solely due to effect of IFNγ on the cells, but rather to a synergistic effect of IFNγ and TNFα (N.B. TNFα as shown in figures 5.3-5.4 is secreted in high amounts even 3 hours after LPS incubation, and as mentioned is an early stage cytokine). Indeed, such a combination has previously been shown to trigger the expression of IL-6 in monocytic cells (Sancéau et al., 1995), and elsewhere TNFα alone has been shown to trigger the secretion of IL-6 (De Cesaris et al., 1998), while IFNγ has been reported to be an enhancer of IL-6 production in

inflamed monocytic cells (Biondillo et al., 1994). Similarly, an enhancing effect of IFNγ on IL-18 production from already inflamed cells, has also been reported (Suk et al., 2001). In addition to TNFα alone triggering the secretion of IL-18 via the activation of NF-κB pathway (Chandrasekar et al., 2003) it is also known that IFNγ activates downstream both NF-κB and the Jak/Stat pathways (Deb et al., 2001; Horvath, 2004), and hence a mechanism can be envisaged for IFNγ-triggered IL-18 secretion. Moreover, TNFα has been shown to influence the transcriptional regulation of the NLRP3 inflammasome, -in addition to the activation of NF-κB that was mentioned above- and this inflammasome complex is important for the maturation and secretion of IL-18 (McGeough et al., 2017; Schutze et al., 1995). Therefore the increases observed could be the effect of indirect NLRP3/NF-κB IFNγ induced activation via TNFα as shown in Figure 5.8. Notably, in Figure 5.8,, IL-18BP is mentioned, which is an inhibitor of the action of IL-18. It acts as a decoy receptor, and stops the cytokine from binding to the IL-18 receptors of the target cells. This is discussed further in section 6.2, subsection " On cytokines, receptors, and binding proteins".
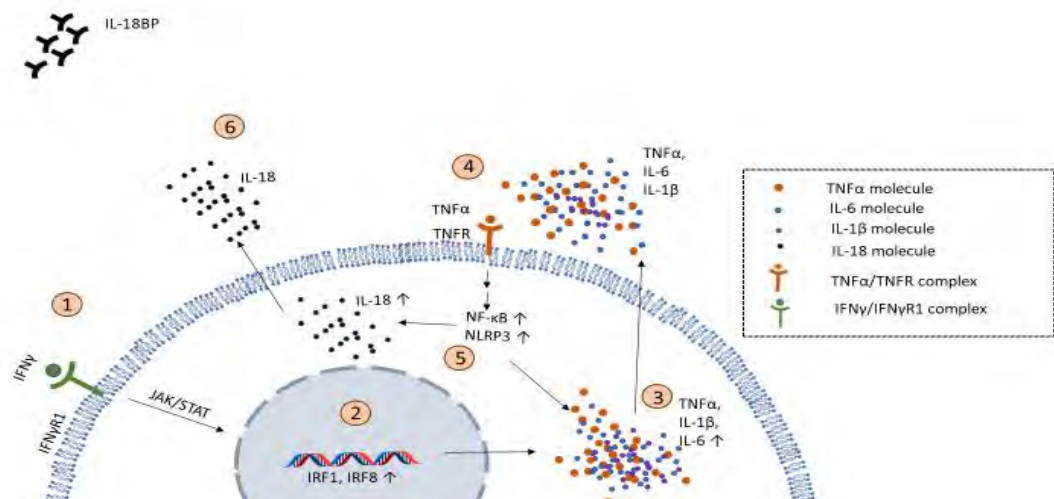
Figure 5.8. Proposed mechanism of IFNγ indirectly induced IL-18 production When IFNγ binds to the IFNγR1 (1), the JAK/STAT pathway is instigated. That, activates the expression of specific genes via the IRF1 and IRF8 regulatory elements (2) which in this case leads eventually to the upregulation and production of TNFα, IL-1β and IL-6 (3), and their early observed secretion. The secreted (early effect) TNFα binds to its ligand, TNFR (4), which activates the NLRP3 inflammasome and NF-κB pathway (5), which leads to the production and secretion of IL-18 (6)(late effect). IL-18BP represents the IL-18 binding protein, as an example of post-secretion control of the cytokines. More details and references in text.

However, to confirm the above hypothesis a more detailed timepoint analysis would be required, as well as experiments involving treatment with both IFNγ and TNFα, and also an examination of the NLRP3 inflammasome pathway and regulation during IFNγ exposure; unfortunately due to time constraints and their potential complexity these were not included as part of this study.

Notably, sRAGE was not detected in any of the conditions tested, and therefore further studies are needed to focus on the expression of RAGE at a gene level, and potentially detection via alternate antibodies for different sRAGE isoforms. However, it should be taken into account that the gene for RAGE is found on the chromosome 6 (6p21.32) (Sugaya et al., 1994). This area of the chromosome could be included in the del6p21 karyotypic anomaly which has previously been described for THP-1 cells (Adati et al., 2009), without further information on the magnitude of the deletion. The authors found several deletions in the 6p area, however although the same cell line as used here, variations are expected to exist between labs; it is well known however that studies on RAGE have used THP-1 cells in the past (e.g. Hasegawa et al., 2003; Eggers et al., 2011; Mahajan et al., 2010) . N.B.: the gene for Iba1 is also located in the same chromosome in an adjacent area (6p21.33) (Mungall et al., 2003), however its expression in both gene and protein level was detected. Therefore, the existence of the RAGE gene in a DNA level in the batches of THP-1 cells used for this thesis should also be confirmed (for example using gDNA-based PCR, or other methods, such as fluorescent in situ hybridization (FISH)) or more sensitive protein detection (e.g. high sensitivity ELISA or SDS PAGE or WB) or detection of different sRAGE isoform methods can be used as well in order to make further conclusions.

To summarise, overall findings show that cells at day 7 and 14 are more easily and successfully triggered by pro-inflammatory stimuli than cells at day 3. This is demonstrated by the secretion of inflammatory cytokines, which is likely to be accompanied by inflammasome activation (although

this is not directly tested in the current study), as exhibited by the increase in both IL-18 and IL-1β. It could be speculated that day 3 cells are using their resources towards differentiation, rather than response to inflammation, and their nature resembles more THP-1 monocytes rather than macrophages. The differences in response between cells of days 7 and 14 could be attributed to the number of cells (cell number approximately halved by day 14; however more cells were adherent in day 14 than in day 7, making comparison of the overall counts quite difficult as in order to lift the cells these were exposed to trypsin prior to gentle mechanical dissociation, and this could have resulted to cell death), while cell numbers in days 7 and 3 were relatively similar, therefore the differences cannot be attributed to experiment-related errors and factors. Nevertheless, the findings suggest that over days 3-14 the cells shift towards a macrophage/microglia-like functional phenotype, which is development-dependent, and coincides with the changes in morphology and gene expression as seen in chapter 4.

Microglia cells, as well as other macrophages, are known to have the ability to polarise into an M2 state, although differences between the results of such polarisation by classical M2 activators such as IL-13 have been shown for microglia vs macrophages (Stenzel et al., 2009). Most notably, the effect of IL-13 on *activated* microglia has been shown to be cell death (Yang et al., 2002; Liu et al., 2010), which is confirmed in vivo, as resident activated microglia died, while alternatively activated macrophages were increased (Dooley et al., 2016). In contrast, in macrophages IL-13 has been shown to reduce the amount of inflammatory cytokines secreted (Doherty et al., 1993), where a shift was observed towards the M2 state. Such a shift was also observed in a microglia/CNS macrophage study (Taj et al., 2018), however this study did not focus on microglia/macrophage cell death, but instead on the overall state of the cells.

Recently, in our research group, Hassan et al., (2018), showed that a flavonoid, namely Vicenin-2 (V-2) has the potential to shift macrophage-like cells towards an M2 phenotype; accordingly, V-2 was selected for use in the present study as a stimulus which may polarise mgTHP-1 cells to an M2

phenotype. In the majority of cases, the findings of the present study show a decrease in pro-inflammatory cytokine secretion from cells that were pre-treated with V-2 before being exposed to pro-inflammatory stimuli (fig. 5.3-5.4). It was also shown that in most cases, V-2 alone promotes the secretion of anti-inflammatory cytokines, namely IL-10 (at least in the 24h timepoint for all developmental days tested see fig.5.5d, 5.6d, 5.7d), and IL-13 in some cases (e.g. fig. 5.7d). The IL-10 finding, along with the lower inflammatory response observed in the V-2 alone treated cells (e.g. regarding TNFα secretion-see figures 5.5-5.7) were in agreement with the Hassan et al. (2018) paper, which proposed the mechanistic explanation that such changes are due to a decrease in NLRP3 expression, and an increase in TET-2 expression. However, the present study also showed a decrease in inflammasome unrelated cytokines (IL-6, and TNFα) at certain timepoints (i.e. in addition to concurrent increases in IL-13), which could indicate that V-2 interferes with more than one pro-inflammatory pathway. Nevertheless, V-2 overall has a suppressive effect to the LPS or IFNγ induced secretion of all the cytokines examined in the majority of cases, and this can be considered to be an indicator of M2-like polarisation of these mgTHP-1 cells.

In addition to the above, by observing the patterns of secretion of the cells in control (i.e. V-2 untreated) conditions, a difference can be seen in the secretion of IL-10, as well as IL-13 between the different developmental stages/days. There seems to be a constitutive secretion of IL-10, starting at day 3, which seems to get increased in further developmental stages (fig. 5.5-5.7). This indicates that the cells themselves can have an anti-inflammatory role, which has also been shown by Shechter et al., (2009), who showed that such a role was IL-10 based; microglia has also been shown to have a baseline IL-10 secretion (Wu et al., 2017). IL-13, was detected in V-2 untreated cells in days 7 and 14, albeit at low levels. As mentioned above, IL-13 potentially kills activated microglia, thus lowering the inflammatory response that comes from these cells; this could be another way in which infiltrating monocyte derived microglia-like cells can have an anti-inflammatory effect. One more interesting molecule that has been shown to be upregulated from V-

2 during most 6 and 24h incubations (fig.5.5c,d-5.7c,d) is TGF-β1. Some recent papers suggest that if this regulatory factor is silenced, or downregulated, microglia assume an activated and even primed phenotype, making them more reactive, whereas treatment with TGF-β1 can have anti-inflammatory effects (Taylor et al., 2017; Zoller et al., 2018). These two findings are suggestive of V-2 driven TGF-β1 regulation, which could correlate with polarisation towards a M2 phenotype. Following, however the brief discussion in section 5.3 regarding relative level of cytokines appearing in the arrays vs actual level of cytokines this needs further examination, for example using a quantitative method for measuring these cytokines.

Three more chemokines that seem to be influenced both by the developmental stage of the cells, and by V-2 treatment are RANTES, ENA-78 and MCP-1. All of these molecules are considered pro-inflammatory in nature in most cases; they are also chemotactic molecules, especially when it comes to neutrophils (ENA-78), macrophages and microglia (RANTES, MCP-1), which could be the reason they are considered pro-inflammatory (Yang et al., 2011; Guedes et al., 2018). To add to the aforementioned, Ji et al., (2007) showed that in neuroinflammatory events, resident microglia die, and infiltrating cells (including neutrophils and monocytes) become the major inflammatory cells. All of these chemokines not only can attract monocytes and macrophages, but can also activate them, and have been found to be secreted by neural cells, such as neurons and astrocytes under inflammatory conditions (Pittaluga, 2017; Che et al., 2001). V-2 seems to either attenuate or enhance their secretion by mgTHP-1 in the various differentiation stages investigated, but there was no consistent pattern of upregulation or downregulation. The V-2 regulation of these chemokines could have implications regarding V-2's anti-inflammatory action via modulating the amount of cells attracted depending on the role of the cells that are attracted. This could be an interesting area of research in the future, as investigating the chemotaxis and distribution of different cells in the progression of an inflammatory condition or disease could help in better understanding the inflammatory disease in question, and thus possibly intervening in its progression. At this time,

however, and from the results of this study, the roles of V-2-associated changes in these chemokines cannot be easily explained.

As shown in our research group and elsewhere, flavonoids such as V-2 can shift cells from an M1 to an M2 state and hence regulate the polarisation of macrophages and microglia (Feng et al., 2016; Hassan et al., 2018). Interestingly, flavonoids have been shown to essentially rescue activated microglia from cell death under certain conditions of activation (Suk et al., 2003) similar to the ones that IL-13 promotes (as discussed previously). This could have interesting implications when moving from an *in vitro* to an *in vivo* setting. As IL-13 is constitutively produced by mgTHP-1 even without a trigger, this could lead to the death of all activated microglia; however if a flavonoid can rescue some microglia cells, depending on characteristics assumed by their way of activation (e.g. cells activated by LPS exhibit different characteristics than those activated by IFNγ (fig. 5.3-5.4 show such differential activation)), this could mean salvaging cells that could be more readily polarised towards an M2 phenotype, for example via the influence of IL-10, which is also produced in higher amounts because of V-2 (see figures 5.5-5.7).

Microglia that can be shifted towards an M2 phenotype has been shown to be extremely beneficial during events that follow inflammation, for example remyelination (Miron et al., 2014). Lloyd et al., (2019) recently showed that M2 polarised microglia alone are not sufficient for an anti-inflammatory environment and thus CNS healing/regeneration, but that cell death of activated microglia is also required. If V-2 has differential polarisation effects on microglia and macrophages, and if it leads towards secretion of IL-13 which can kill activated microglia that cannot shift towards an M2 phenotype, this would mean that more cells with an M2 phenotype would be present in the presence of V-2, suggesting that healing/CNS regeneration would then be faster and even more effective. However, once again, this could not be claimed by our findings alone, and further studies are needed in order to confirm or reject these speculative proposals.

Another interesting observation that can be made from the array results (fig.5.5-5.7), is that V-2 seems to start acting on many cytokines/proteins/chemokines as early as after 1 hour of incubation (including the pro-inflammatory cytokines that were also examined via ELISAs, i.e. IL-1β, IL-6, TNFα), but for others (e.g. TGF-β1) it needs longer times of incubation. This suggests that more than one different pathway is activated/altered because of V-2, which could have implications on investigating its effectiveness. In this study, V-2 was used as a pre-treatment for 1 hour, and remained in the culture medium as the treatment with pro-inflammatory stimuli took place. The reduction of cytokine secretion observed when comparing controls vs V-2 treatment can be seen as similar patterns of results in the ELISA and array data (see fig. 5.3-5.4 vs 5.5d-5.7d). However in some cases, more noticeable reductions are shown with the arrays than in the ELISAs, while in one case a reduction which appears to happen (e.g. in the case of IL-1β) in the ELISAs and already discussed does not seem to occur in the array data. This could be because the arrays are more sensitive as a method for some cytokines, but not for others, as no data is given about the range of detection per cytokine. Therefore, the arrays' sensitivity cannot be quantified as it can be with the ELISAs, due to the semi-quantitative nature of the data and other limitations (e.g. 1 array per condition reduces the robustness of the data). Nevertheless, despite these occasional discrepancies, the array results broadly confirm the ELISA results, and support findings of previous studies (e.g. Hassan et al., 2018) of the anti-inflammatory effect of V-2 on the cells as already discussed.

As a final point of this discussion of the results, as mentioned previously (see chapter 4), the cell number changes during the differentiation process. As previously discussed, cells in days 3 and 7 are an heterogenous population consisting of cells in clumps of different sizes, some clumps adhering (see fig.4.1 arrow in day 3, and fig 4.4b day 7 different levels of adhering clumps), whereas others have obtained microglia-like morphology. Thus, we reported a decrease to approximately 20-50% of the original cell number by day 14. Although a statistical analysis regarding the precise extent of loss of the cells in different days would provide potentially valuable

information (including information regarding the cells' responses to pro- and anti-inflammatory stimuli), this was not attempted in this instance due to the heterogeneity of the cell morphologies. Although a quantification of the cells per day was attempted in chapter 4, this was not very accurate for a variety of reasons, including: the method that was used (imageJ could not count the amount of cells within clumps) and the nature of the cells (i.e. in 3D clumps although the cells that were apparent were measured, the ones beyond the surface of the clump and inside the clump (as shown in image 4.4b) were not). Therefore, and due to the above obstacles, providing such a precise analysis is not is the focus of the current chapter. However, taking into account that the cell number decreases in time (and especially due to the dramatic decrease between days 7 and 14 where the morphology of clumps essentially disappears), and the fact that the amount of cytokines released upon pro-inflammatory challenge increases over the same time-period (see section 5.3.3), it could be claimed that cells become more macrophage/microglia-like towards the later stages of differentiation. Focusing on the differences between days and how reactive to stimuli the cells are, and not on when the cells can obtain an M1/M2 phenotype (which, again, was the focus of this study) should thus be the aim of future studies.

While it could be claimed that as the cells are microglia-like, the reduced secretion of cytokines observed when the cells were treated with V-2 + inflammatory stimuli is due to death of the cells. However, if that was the case, then the reduction would be similar for all cytokines secreted; in fact there were different effects in the reduction of pro-inflammatory cytokines secreted. Importantly, therefore, as flavonoids have been shown to not only change the polarisation of macrophages towards an M2 phenotype, but also rescue activated microglia, that would essentially die otherwise (e.g. due to the enhanced effect of IL-13 that the cells secrete under the influence of V-2), it can be concluded that the present study provides evidence for V-2 inducing an anti-inflammatory M-2 phenotype in polarisation which is not due to cell death. (Nevertheless, in this study, viability was not measured under all conditions, only after treatment for 24 hours for LPS, IFNγ, and V-2, but not

the combinations of LPS/V-2 and IFNγ/V-2. Therefore, we cannot completely exclude the possibility that part of the ELISA results observed to be due to cell death.)

5.4.3 Limitations

1. Although the cells were treated with different pro-inflammatory stimuli and for different timepoints, a single stimulus dose was employed, rather than a variety of concentrations (which would be more relevant to physiological/*in vivo* conditions). Therefore, a dose-response approach should be employed in future experiments. Nevertheless, the cells responded in a way that demonstrated that they have macrophage/microglia-like functional properties.

2. As already mentioned in the previous chapter, these cells originate from the THP-1 monocytic cell line (Tsuchiya et al., 1980), so future research could focus on whether a)cells with mgTHP-1-like characteristics could be produced with the same method, but using different monocytes (e.g. PBMCs), and b) whether these cells respond in a similar way to both pro-inflammatory stimuli, and V-2.

3. Although V-2, as well as other flavonoids, have been shown to influence the inflammatory response of macrophages and microglia, we do not know whether our results could be generalised and therefore whether the increase in anti-inflammatory cytokines observed, would be similarly observed *in vivo* or in other cells. Therefore, more studies focusing on other CNS cells would be useful, in order to elucidate the full potential of V-2 and flavonoids.

4. This study focused on secretion of certain cytokines, and not on the influence of either the pro-inflammatory mediators or V-2 on the expression of their genes. In the light of the results indicating that V-2 affects more than one pathways due to the different molecules it differentially influences the secretion of, so investigating the genes encoding the component proteins of these pathways (e.g.

Jak/Stat, NLRP3, NF-κB and Jnk which are downstream of upstream of the cytokines examined) would be an interesting area for further research.

5. As mentioned above, cell viability was not measured under combined LPS/V-2 or IFNγ/V-2 conditions, so the possibility cannot be ruled out that part of the reduced cytokine secretion observed could be due to cell death. Therefore, future experiments will need to explicitly rule out this possibility. Nevertheless, if it was due to cell death, the degree of reduction would be similar in all the cytokines, and that was not the case.

6. Although broadly there was confirmation of the array results from the ELISA results, this cannot be claimed for all cytokines examined in the arrays. As discussed, V-2 seems to influence different cytokines in different timepoints and different developmental stages. Moreover, cytokine arrays give only a semi-quantitative result, so further focus could be given to more quantitative methods (e.g. ELISA) in future studies that focus on cytokines of interest, such as IL-10, and IL-13.

7. As in chapter 3, the element of different cell type cell interactions is missing from here. Communication between neural and myeloid cells is important and it has been found that this communication could have modulatory effects as discussed already. Therefore, future studies could include co-culture of different cell types under optimised conditions.

Chapter 6

General discussion

6.1 A brief synopsis of the current study's findings

Neuroinflammation consists of all the inflammatory responses of the CNS cells, as measured in a variety of ways (e.g. pro-inflammatory cytokines secreted from the CNS cells, cellular phenotypes such as the M1/M2 paradigm in microglia, and other changes such as NOS activation, ROS, etc.). As already discussed in Chapter 1, neuroinflammation is the cause and/or a consistent finding in a plethora of CNS-related pathologies. So far, the focus of neuroinflammatory research, in the aspect of contribution, has been on microglia cells. Here, we investigated how and in what degree CNS cells contribute to the pro-inflamamtory environment which exists in an inflamed brain. In other words, the focus was on the proinflammatory potential of CNS cells.

In Chapter 3 we focused initially on elucidating the role that non-microglial cells had with regard to CNS inflammatory responses (as measured by inflammatory cytokine secretion). Specifically, we investigated whether non-microglial CNS cells secrete pro-inflammatory cytokines if they are exposed to a simulated inflammatory environment. The short answer to that, was -at least for the conditions tested here- not that much. Although detectable baseline secretion was found in all three cell types (NSC, neurons, and astrocytes) for all the cytokines tested (IL-6, IL-18, TNFα, IL-1β, and sRAGE), secretion did not consistently change in a scale that would allow a robust conclusion to be drawn (especially when their responses are compared to that of the microglia-like mgTHP-1 cells in chapter 5 or to microglia secreted cytokine levels as found in the literature). Nevertheless, some interesting changes were observed; for example with regard to NSCs responses a reduction (instead of an increase) of pro-inflammatory cytokine secretion was observed in some cases. NSCs have been found in some contexts to have an anti-inflammatory role as already discussed in chapters 1 and 3 (e.g. reducing the pro-inflammatory response of macrophages as shown by Peruzzotti-Jametti et al., (2018) as well as by Cheng et al., (2016) in a transplantation study); therefore, this reduction

could be a way in which they express this role. Non-microglial cells are not considered by nature to be inflammatory, and even though they do have the ability to secrete cytokines as already discussed, in the present study the secretion levels did not change to a sufficient extent to prove the opposite.

In chapter 4, a novel model for microglia-like cells was developed, using the THP-1 monocytic cell line as the initial starting point for differentiation into these cells (hence "mgTHP-1" cells). Initially, the generated cells were thought to resemble CNS infiltrating monocytes due to the initial cells used (THP-1) being monocytic in nature. Infiltrating monocytes -in their turn- are microglia-like cells (Karlen et al., 2018; Bennett et al., 2018; Lund et al., 2018); indeed, some older studies, call such cells "indistinguishable from microglia" (e.g. Kaur et al., 1987; Mietto et al., 2015). However, in more recent years, microglia-specific markers, such as TMEM119, and a more general microglia specific marker panel (Bennett et al., 2016) has been developed in order to help in distinguishing between the two types of cells. Moreover, infiltrating monocytes need -as suggested by their name- to infiltrate the BBB, and such event occurs usually under inflammatory conditions, both of which elements were absent in this case. An important finding was that the mgTHP-1 cells were found to express TMEM119, while THP-1 cells did not. This, in addition to the results of investigation of other microglia-related markers (e.g. Iba1, CX3CR1, PU1), and the levels of their expression, allowed us to conclude that our model was more microglia-like, and less infiltrating monocyte-like (for example, in such cells expression of TMEM119 has not been found; also because our cells did not interact with the BBB or similar structure, and were not in an inflammatory environment, they did not justify the term "infiltrating monocyte-like").

The investigation on these cells went further, by examining potential mechanisms which facilitate their differentiation. Differences in DNA methylation, ATP content, and GAPDH expression were found, all of which peaked in day 3 of the 14-day differentiation protocol

which we describe. Interestingly, this is the same day in which morphological changes started to be observed in the cells.

This, in conclusion, these findings demonstrate that using a CNS-like environment (i.e. the differentiation medium which was used here and is in part used to differentiate iPSC/ESC into NSC) and a monocytic cell line (THP-1 cells), microglia-like cells can be generated in 14 days, without the need for more complex processes, such as genetic manipulation.

In chapter 5, mgTHP-1 cells were functionally characterised, by exposing them to inflammatory conditions similar to those to which NSCs, neurons, and astrocytes had been exposed in Chapter 3. The results varied (depending on day of differentiation), however cells of days 7 and 14 produced a significant amount of cytokines (IL-1β, TNFα, IL-6, and IL-18) in response to stimulation with LPS or INFγ. This suggested that the cells are of a macrophage-like nature (and microglia are the resident CNS macrophages (Sevenich 2018; Norris & Kipnis, 2019)), as did the subsequent observation that they were able to polarise into an M1 phenotype. Further work then included investigating if these cells could adapt an M2 phenotype, which is usually done after being exposed to an anti-inflammatory mediator. V-2 was used in this instance, as previous work in our lab showed that V-2 can polarise dTHP-1 cells (macrophage-like cells differentiated from THP-1 cells upon exposure to PMA, an inflammatory substance) to an M2-like phenotype (Hassan et al., 2018). Indeed the cells responded to pre-treatment with V-2 by reducing the amount of the pro-inflammatory cytokines IL-6, IL-1β, IL-18, and TNFα secreted upon inflammatory challenge in the majority of cases. In addition, the secretome of the cells was investigated with and without exposure to V-2 in the absence of an inflammatory challenge. V-2 induced the cells to secrete a greater amount of anti-inflammatory cytokines (e.g. IL-10, although it should be noted that the cells were secreting these constitutively in lower amounts at a baseline level as well). Therefore, it can be concluded that mgTHP-1 cells can differentiate into either an M1 or an M2 phenotype, with proinflammatory mediators

guiding the differentiation towards M1, and agents such as V-2 towards M2. Such findings for the V-2 are in agreement with Hassan et al., 2018, as well as with other studies which have shown flavonoids modulating microglia cells (da Silva et al., 2019; Velagapudi et al., 2018; Kim, 2015).

Taken together, the results of this PhD study show differential roles of the different CNS cells in response to neuroinflammation, and indicate that their responses can be modulated by certain compounds, which may have implications in future targeted treatments for neuroinflammatory disorders. Nevertheless more work is needed on this area, and below are analysed some factors which are relevant to the findings of this study, and the interpretation of its impact.

6.2 Study-specific factors, and their influence

Reasoning for timepoints selection

As briefly discussed in section 3.2.2.4, but also in the discussion of the appropriate chapters the timepoints as well as concentrations used in this study were determined based on previous studies (in cases of cytokine secretion), or morphological changes (in the case of the mRNA and protein timepoints for mgTHP-1 cells). As such, in the first case, the objective was to determine whether non-microglial cells contributed to an inflamed CNS in the same way and timeframe microglia has been shown to do so. In previous studies focusing either on microglia or mixed cultures timepoints of 1, 3, 6, and 24h have been used (e.g. Goodwin et al., 1995; Rozenfeld et al., 2003; Mangus et al., 2005; McMillian et al., 1997; Gao et al., 2002; Olajide et al., 2013; Jung et al., 2005). In addition, the cytokines/proteins in focus have been shown previously to be either early-response (e.g. TNFα) or late-response (e.g. IL-18) as discussed in chapter 5. The fact that the cells investigated in chapter 3 did not respond to inflammatory stimuli in the selected timepoints does not mean they don't respond at all; however on this

instance the focus was to investigate whether they potentially have an inflammatory response similar to the one microglia and/or mixed cultures have been found to have in aspects of timeframe. The pros of the approach to do as others have done is that there is a basis on timepoints in which to expect changes, as these have already been shown. The cons are that at other timepoints interesting things might be happening as well, which are being missed. However a full timepoint analysis over 24h (or even longer than that, in the case of mgTHP-1 cells for example, a 14 day analysis) although would be interesting, it would also be rather difficult in time of work needed, resources for the experiments, and analysis of the amount of data that would be generated.

In the differentiation process of mgTHP-1 cells the days selected were the days where robust morphological changes appeared in the cells. Although a more thorough investigation of days 2, 6, and 13 could give more information on the genes expression that guides the change that is seen in days 3, 7, and 14, this was beyond the scope of this study and was not investigated, however it is an area that future studies could focus on.

Evaluation of the differentiation process of H9 derived NSCs

As analysed in chapter 3, H9 derived NSCs were used as were protocols to differentiate into astrocytes and neurons as supplied by the provider (Gibco) and widely used by other investigators in this field (see Appendix 1). Therefore -and in order to remove optimisation steps which would require time, and reagents- we chose to follow those. Although optimisation steps did happen in the aspect of timeframe in which the cells were considered as fully differentiated, as well as matrixes used for the growth of different cell types, the results were consistent when it comes to the resulting cells. However it is not known how the NSC cells were derived from H9-ESC; one simple answer would be the PSC neural induction medium (cat no A1647801), which includes the neural induction supplement (as also used for the

differentiation of THP-1 cells in mgTHP-1 cells in chapters 4 and 5), and neurobasal medium. Neural induction medium has been manufactured in order to differentiate PSCs and ESCs into NSC within 7 days, without the step of embryoid bodies which is found in most protocols (Gibco NIM manual and description). Although the NSCs used for this PhD study exhibited the expected multipotency (as in they could differentiate into either neurons or astrocytes), further characteristics of the cells provided are not known (although they were nestin+ (as shown in chapter 3), Pax6+ (a NSC proliferative and multipotency marker-data not shown), and Sox2+ (a multipotency marker- data not shown)). Therefore, some of the results observed here could be due to their differentiation progress, which might have influenced their (and their derivatives) immunologic profile. Nevertheless, as already discussed in depth in chapter 3, neither NSCs nor neurons are cells of the immune system. As such, they are not expected to react to inflammatory stimuli by further enhancing the inflammation. Astrocytes, although they are regulatory cells, are also not expected to secrete a major amount of pro-inflammatory cytokines. Also, especially as the H9 cells are female derived, it is worth mentioning that estrogen-related signalling has been found to lead astrocytes to have an anti-inflammatory and neuroprotective role, rather than a pro-inflammatory one (Spence et al., 2013; Santos-Galindo et al., 2011). Therefore, even if chapter 3 does answer the question: 'Do non-microglial CNS cells contribute to an inflamed CNS with additional pro-inflammatory cytokines', it does not fully uncover the depth and breadth of the ways that these cells might respond to inflammation.

Evaluation of the use of THP-1 cells

THP-1 cells were established as a cell line with monocytic properties from Thuchiya et al., (1980), and were derived from a 1 year old boy with Acute Monocytic Leukemia. They have been since used as a model for human monocytes and macrophages (Qin, 2012; Bosshart et al., 2016). Although gene expression profiles of dTHP-1 macrophage-like cells (PMA treated THP-1 cells) have been shown to be different from other macrophages (in addition to THP-1

cells having a different expression profile from other monocytes (Kohro et al., 2004)), both THP-1 and dTHP-1 do express genes related to their functions as monocytes and macrophages in a similar way as primary monocytes and macrophages do. These cells, however, either due to their identity as cancer-derived, or due to the multiple passages they have been through, are known to have several chromosomic and therefore gene anomalies including: deletions (6p, 12p, 17p), trisomy in the chromosome 8, breakpoints in methylation-related (MLL), and tumour suppressor genes (e.g. PTEN, TP73, CDKN2A) (Adati et al., 2009); interestingly, one of the molecules examined with ELISA, and was not detected in the Chapter 5 experiments, namely RAGEs, is located in 6p (6p21.32), and therefore deletion of the locus could provide a reason why our attempts to detect RAGEs secretion from mgTHP-1 cells were unsuccessful. However this was not investigated further. Nevertheless, differentiated dTHP-1 cells seem to resemble native macrophages better than other monocytic cell lines (Auwerx, 1991). In addition, THP-1 cells have further been shown to express stem cell markers, such as Sox-2 (Picot et al., 2017), as well as monocyte related markers, as shown in chapter 4 (THP-1 cells were used as controls for qPCR assays for Iba1, CD45, CX3CR1 etc., and so they are appropriate models for monocytes prior to any differentiation having taken place).

THP-1 cells have been successfully differentiated as already mentioned into macrophage-like cells (Auwerx, 1991), but also into dendritic cells (Berges et al., 2005); in both cases, differentiated THP-1 cells cease proliferation, indicating their differentiated form should be considered as a terminally differentiated form (Schwende et al., 1996). The fact that they can differentiate, and further shift towards polarised forms (M1/M2) (Tarique et al., 2015) shows that these cells demonstrate high plasticity. In addition to the fact that they also express pluripotent stem cell markers (and taking into account the fact that a subset of monocytes does exhibit stem cell-like properties (Zhao et al., 2003)), this could indicate an even greater level of plasticity, especially as these cells are a)cancer-derived and b)essentially infant-derived.

Therefore, there is a reasonable justification for using this cell line for the purpose of validating a novel model in which THP-1 cells differentiate into microglia-like cells. However, in order to fully validate the method, a use of other either monocytic cell lines, or stem cell derived monocytes, or primary monocytes (from donors of different ages) could clarify whether the method described in this thesis is cell-line specific, cell age specific (functional differences have been found between adult and both foetal and children monocytes (Krow-Lucal et al., 2014; Mandron et al., 2008), or cell-type specific, or alternatively whether it provides a more generally applicable template for studying differentiation into microglia.

On cytokines, receptors, and binding proteins

Although the cytokines secreted by cells might give an indication of how this can affect an inflamed CNS environment, this is often more complicated, due to the variety of binding proteins, inhibitors, and receptors cells might express and/or secrete. Although various examples could be used (e.g. the differential roles of TNFα receptors TNFR1 and TNFR2, where when TNFα binds can results in different outcomes (Wajant &Siegmund, 2019)) the case of IL-18 will be discussed. IL-18 is a member of the IL-1 family of cytokines, and is increased in pathological conditions, such as Alzheimer's disease (Ojala et al., 2009), and Traumatic brain injury (Yatsiv et al., 2002), and is considered by some a key player in neuroinflammation and neurodegeneration (Felderhoff-Mueser et al., 2005). Unlike the other NLRP3-related cytokine investigated in this thesis (namely IL-1β), the precursor of IL-18 is expressed continuously in all cells. Its secretion is affected by factors already discussed in the discussion of chapter 5, however, after it is secreted, and in order to act upon other cells it needs to bind to the appropriate receptors as a free cytokine. However, circulating with the free cytokine, are also binding proteins for the cytokine, which resemble the aforementioned receptors, and thus acting as a decoy, in order to reduce the effect of the cytokine on other cells, by preventing it from getting attached to the receptor. The primary receptor for IL-18, is the

IL-18Rα, which is a low affinity receptor; in cells where the β chain of the receptor (which acts as a co-receptor) is expressed, the affinity is higher, and so the intracellular signalling initiated by IL-18 is initiated (Medina et al., 2014). However, in order for IL-18 to bind to the cell surface receptors, it needs to be in its free form, which is often not the case due to the IL-18BP, a constitutively secreted protein, with high affinity for IL-18. The binding protein is often not considered a soluble receptor, as it is encoded by different genes than the α and β chains of the cell receptors, and in humans 4 isoforms of the binding protein exist (Dinarello & Fantuzzi., 2003). Therefore, it is a balance of the free IL-18 and IL-18 which is bound to the IL-18BP that determine its functions, rather than only how much the cells secrete. Although in this thesis IL-18BP was not investigated, this (along with other soluble receptors, cell-surface receptors, and cytokine inhibitors) could be the focus of future studies, in order to investigate the true impact of secreted cytokines on surrounding cells and tissues.

Are all microglia-like models the same?

Although several models for the generation of microglia-like cells have been described (some of which are found in table 4.1), would it be fair to say that they all produce the same type of cells? Microglia in the brain show both a great heterogeneity and a great plasticity, as already discussed in chapter 1. Under physiological conditions, microglia are established from myeloid cells originating from the yolk sac, and can be detected in humans at the early age of 4.5 weeks in gestation (Ginhoux et al., 2010), while there is an influx of macrophages at around 9-11 weeks, and a proliferation of microglia at around 10.5 weeks (Monier et al., 2007). Embryonic microglia are critical for the formation of the BBB (Fantin et al., 2010) and express proteins which are macrophage chemoattractants, such as MCP-1 (MCSF). Also, as shown in the cytokine array work (see chapter 5), MCSF was secreted abundantly by mgTHP-1 cells especially in day 14 (see Fig.5.5-5.7), however this is also true for most peripheral macrophages (Oster et al., 1989). In addition to potential differences between microglia with

regard to age, and development (for example as mentioned in chapter 4, newly developed microglia are amoeboid, while more "established" cells are ramified, while usually activated microglia are also amoeboid), more recent papers have shown that adult microglia (and potentially embryonic as well) not only differ in phenotype and morphology, but also in function, and response to stimuli. For example, Zhan et al., (2019) showed that a specific microglial subset does not respond to inhibition of Csf1r signalling, which is otherwise essential for microglia viability; these cells are Mac2+ and do not originate from peripheral monocytes. Similar findings were shown by Elmore et al., (2015), who showed that cells of this microglia subtype are nestin+.

Interestingly, certain monocytes cells have been found to be both Mac2+ (e.g. Lee et al., 2014 showed this for THP-1 cells), and nestin+ (Seta et al., 2010 who focused on CD14+ circulating monocytes, while Ha et al., (2003) showed the same for human cord blood monocytes, and data from our lab (not included in this thesis) shows the same for THP-1 cells). Therefore, microglia-like cells generated from such monocytes could be mirroring the functions of the cells which, after microglia depletion, remain in the CNS, and with the help of infiltrating cells give rise to the microglia cells that repopulate the brain. This repopulation, as found by Yao et al., (2016), in mice takes around 14 days, which is the timeframe around which most microglia-like model studies find it takes to generate microglia-like cells. No study has been done thus far to follow the further development and characteristics of these cells after the 14 days mark, and how they end up giving rise to the different subpopulations of microglia cells (N.B. This is briefly described in 1.1.5 subsection 'The many faces of microglia'). Indeed, the different models generated could represent different states and phenotypes of microglia, and investigating them further (in phenotypic characteristics and functions) would be not only interesting, but also useful, especially as it has been shown that microglia can change their

functions and characteristics depending on factors such as age (Koellhoffer et al., 2017 for a review), and sex (Rahimian et al., 2019; Villa et al., 2018; Lenz et al., 2015).

Evaluation of the markers used for microglia and macrophage research

Microglia are essentially the CNS resident macrophages, and therefore express macrophage markers, as already discussed in section 1.5, as well as chapter 4. Nevertheless some markers have been found that can help discriminate between the two cell types, which when used with caution, and in combination with other markers whose expression -although it exists in both- differs between macrophages and microglia, can help us identify these cells. Two of those markers are -as discussed in chapter 1, and investigated in chapter 4- the microglia specific marker TMEM119, which has also been shown to be expressed in certain peripheral macrophages in mice some cases (Li et al., 2018), and Glut5, which is also found to be expressed in peripheral macrophages, and monocyte-derived macrophages (Malide et al., 1995; Fu et al., 2004) (however, Glut5 was not investigated in this study as Fu et al., 2004 found expression of this protein in THP-1 cells as well, which would complicate our findings).

Importantly, although other markers, such as TREM2, CD11c, CD14 (Kamphuis et al., 2016) could give further information on the characteristics of the mgTHP-1 cells, the current study's characterisation of the cells as Iba1+, TMEM119+, CX3CR1+, P2Y12R+, CD45^{low}, CD11b+, PU.1+, and taking into account their overall phenotype and response to inflammatory stimuli, allows us to establish the cells generated through our protocol, i.e. the mgTHP-1 cells, as a novel model specifically for microglia-like cells.

Flavonoids and how they can affect the brain

V-2 is a flavonoid and C-glycoside, which has been reported to show great pharmacological potential (due to its anti-inflammatory, antioxidant, and anti-tumour effects (Satyamitra et al., 2014; Nagaprashantha et al., 2011)). It is rapidly absorbed by the small intestine, and due to its

resistance to enzymatic degradation *in vivo* (Buqui et al., 2015) like many flavonoids, it can travel through the BBB (Figueira et al., 2017; Faria et al., 2014; Yang et al., 2014), both intact, but also as its metabolites (Gasperotti et al., 2015; Youdim et al., 2003). It is not yet clear how this transportation takes place but it has been shown that during that transport, flavonoids might also be metabolised towards other compounds (characterised as novel by Figueira et al., 2017). Moreover, V-2 has been patented as having a "beneficial effect on neurological and/or cognitive function" (Buchwald-Werner & Fujii, 2014). The above, in addition to findings from previous work done at our lab (Hassan et al., 2018) made V-2 a good target to investigate the functions of mgTHP-1 cells in a CNS (-macrophage) related environment.

Our findings (see figs 5.3-5.7) support findings of previous studies (see chapter 1), which showed V-2 (and flavonoids overall) to have an anti-inflammatory effect on microglia, microglia-like cells, or macrophages *in vitro*. *In vivo,* it has been shown that flavonoids reduce the cognitive decline observed with aging (Letenneur et al., 2007), as well as incidences of neurodegenerative disorders, including AD (Williams & Spencer, 2012; Orhan et al., 2015). Taking into account both the results from the *in vitro* and the *in vivo* studies, it can be concluded that flavonoids -possibly mostly by modulating microglial responses- can have an overall neuroprotective and anti-inflammatory effect on the CNS, which could potentially be of clinical use in the reduction of neuroinflammatory and neurodegenerative disorders.

6.3 Suggestions for future work

In addition to future work in order to address the limitations of each chapter already briefly discussed in the end of each chapter, here is suggested further experimental work that would in a more general sense enhance the impact of the findings of this thesis, and hence could use the current findings as stepping stones towards a future greater understanding and applicability of the work presented in this thesis:

- Given that most probably the differences found in secretion of cytokines in neurons, astrocytes and NSCs (Chapter 1) was not physiologically significant, future work should include employing different pro-inflammatory mediators (e.g. TNFα or IL-6), and measurements (e.g. measuring NOS expression, ROS, metabolic changes, or even anti-inflammatory mediators and prostaglandins, all of which are implicated in neuroinflammation, and have been discussed before) in order to explore whether these cells have any pro-inflammatory contribution to the inflamed CNS.

- Using different differentiation protocols to produce subpopulations of these cells (e.g. different types of neurons (e.g. dopaminergic, GABAergic etc), or NSCs. As mentioned in chapter 3, the NSCs used here were considered multipotent NSCs, and could differentiate in all three subsequent types of cells (i.e. neurons, astrocytes, and oligodendrocytes); therefore differentiating them could be into neuronal or oligodendrocytic progenitors, or even obtaining radial glia-like cells could provide valuable information on the different roles these cells can play under neuroinflammatory conditions.

- Additionally, using neurodegenerative/neuroinflammatory disease derived cells, could give insights on how these cells behave differently to "healthy" cells, both in function, and in viability.

- Finally, if iPSCs or ESC were used, these could be differentiated into monocytes, and then microglia-like cells, or -using one of the established protocols as mentioned in table 4.1- directly into microglia(-like) cells. These cells could be used for co-cultures, and investigations of functional interactions under inflammatory conditions with the other CNS cells. One other use of these cells would be comparing them with mgTHP-1 cells, as well as primary microglia, or microglia cell lines, in order to further confirm our model as a well-established microglia model.

- In the same line, if the protocol described in chapters 4 and 5 can be applied to other monocytes (e.g. PBMC-derived monocytes), this could be beneficial for studies of microglia-like cells from patients, without having to go through the iPSC route. As generating iPSC from somatic cells alters their epigenetic profile (with the alterations depending on a variety of factors (Liang & Zhang, 2013; de Boni et al., 2018; Samoylova et al., 2020)), it would be worthy to examine whether the age and desease-specific epigenetic profile of the resulting mgTHP-1 cells is similar to the one of the initial cells. If that is the case (i.e. the epigenetic profile is not erased or altered), and the model can be applied directly to patient-derived monocytes, this would allow studying disease-specific and patient-specific microglia and its roles and responses more accurately.

- Other future work could employ experiments to further characterise the functional phenotype of the mgTHP-1 cells. These could include -as already suggested in chapter 4-, phagocytic assays (either bacteria, beads, apoptotic cells (via co-culture) or Aβ peptides have all been shown to be engulfed by microglia), or electrophysiology (Kettenmann et al., 1993; Boucsein et al., 2000), and direct comparison with other microglia models, or primary microglia. Further, successfully applying the same methodology in either other types of monocytes (e.g. CD14+ PBMC derived monocytes, or iPSC generated monocytes) and differentiating these cells into microglia(-like) cells using the method described in chapter 4, would essentially mean that through an easy to use and relatively simple protocol, scientists could generate -thus far- difficult to obtain cells.

- Moreover, as microglia transplantation studies have been done in rats (Akhmetzyanova et al., 2018) and mice (Shimizu et al., 2018) with positive results, and as our model showed that cells pre-conditioned with V-2 have a more anti-inflammatory character

(Chapter 5), studies could focus on V-2 pre-conditioned or non-treated mgTHP-1 cells being transplanted and thus studying a)the function of these cells alone and in combination with V-2, and b)interactions between mgTHP-1 cells and other cells *in vivo.*

- One other important aspect of our system is the insights it yields on developmental process for the generation of microglia-like cells, and the efficiency of this process. As only 20-50% of the initial cell number remains after the 14 day differentiation process, it would be interesting to determine if that number could be increased. Other models presented in table 4.1 have reported similar or lower outputs, therefore it appears that this is a phenomenon that is often seen in such methods. As suggested in chapters 4 and 5, this could be due to the fact that the microglia-like phenotype observed is a final differentiation stage, and from then on the cells will not proliferate, and only die.

However as is also discussed in chapter 4, GM-CSF and a microglia-specific medium might allow the cells to become proliferative again, allowing the cultures to remain proliferating for longer periods of time, and potentially enhance the output of the method. It should be also mentioned that as the ingredients of NIS are not known, while higher concentrations might give some results (as the ingredients which guide the differentiation are there), some of the observed results might be due to inflammation (as discussed briefly in chapter 4, some ingredients like LIF can be inflammatory in higher concentrations) and differentiation of the cells into macrophage-like cells as well as microglia-like cells. This would also make the cost of producing these cells higher, and the modified differentiation medium could no longer be described as a "CNS-like environment which guides the differentiation of iPSCs into NSCs" which is the reasoning that underpinned this study designed employed in this thesis.

- Development-wise, the focus was on three developmental days, as selected by morphological observations (i.e. Day 3, Day 7, and Day 14). In the future, this initial approach could be extended in order to lead to uncovering different genetic profiling and functions of these cells in their respective days. Further characterisation of these days, and especially day 3, when major methylation and metabolism changes occur (see Fig. 4.8, 4.9, and 4.10) might provide information on the mechanism(s) by which these processes happen *in vivo*, which in turn it would allow us to understand better the development of CNS monocytes. Experiments focusing on genes of interest when it comes to microglia development and their methylation status should be the first ones investigated (e.g. PU.1 and its target genes).

- As a last point, V-2 should be the focus of future experiments, both in non-microglial CNS cells and in microglia(-like) cells. V-2 has been shown in the current study, as well as by Hassan et al., (2018), to shift cells towards an anti-inflammatory phenotype. For mgTHP-1 cells, future experiments should follow the exact methodology that Hassan et al followed, and investigate the exact molecular mechanisms through which V-2 achieves its actions. For example, V-2 acting directly on TET-2 was already shown in the aforementioned study, however Hassan et al., (2018) focused only on NLRP3-regulated cytokines, i.e. IL-1β, and IL-18. Here we showed action of the flavonoid on other pro-inflammatory cytokines as well (IL-6, and TNFα), which indicates that V-2 acts on more than one inflammatory pathway. However, its actions may not be as beneficial for non-microglial CNS cells, as it has been shown that in rat neurons, exposure to flavonoids leads to both necrosis and apoptosis (Jakubowicz-Gil et al., 2008) but on the other hand, Frandsen & Narayanasamy (2017) showed the exact opposite, as flavonoid treatment enhanced viability. Thus if V-2 -or even other flavonoids- are to be used as therapeutic or preventative agents in the future, more

research on their functions on different cell types on their own, but also on the cell-cell interactions is crucial.

6.4 Final conclusions

In conclusion, the current study has demonstrated that different CNS cells respond in different ways when exposed to inflammatory stimuli. Moreover, a novel model for microglia-like cells was developed, optimised, and characterised in a molecular, developmental, and functional way. Last, it was shown that by using a compound, namely V-2, which has been shown to have the ability to cross the BBB, the microglia-like cells which were generated via our protocol were shown to exhibit an M2 phenotype; in contrast an M1 phenotype was exhibited when the cells were exposed to pro-inflammatory stimuli.

This study has, therefore, generated evidence for further studies which may focus on other ways in which CNS cells might respond to inflammation, and has provided means by which that response can be modulated. In addition, by using the mgTHP-1 model generated within this study, it will be possible that aspects of the development of microglia(-like) cells can be studied further, but also the role of microglia-like cells in neuroinflammatory disorders (including neurodegenerative ones) to be investigated more thoroughly.

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Appendices

1 Methodology-related appendix

Links to the exact methodology used, when an already available protocol was used are found below. The links are provided as, in different versions of the same experiment (e.g. ELISAs), different volumes may have been used for different cytokines. For all links below, the protocols were followed without any changes, unless otherwise stated in chapter 2.

- Gibco® H-9 derived NSC handbook: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/GIBCO_hNSC_man.pdf

- Differentiation of NSCs into astrocytes and neurons (differentiation media): https://www.thermofisher.com/uk/en/home/references/protocols/neurobiology/neurobiology-protocols/differentiating-neural-stem-cells-into-neurons-and-glial-cells.html

- RNA extraction, using TRIzol and chloroform: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/trizol_reagent.pdf

- cDNA conversion: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017977_highcap_cDNA_RT_UG.pdf

- DNA extraction: https://www.abcam.com/ps/products/156/ab156900/documents/ab156900%20Genomic%20DNA%20Extraction%20Kit%20protocol%20v2%20(website).pdf

- Methylation analysis: https://www.epigentek.com/docs/P-1030.pdf

- Cytokine arrays:

  https://www.abcam.com/ps/products/133/ab133997/documents/ab133997%20Human%20Cytokine%20Antibody%20Array%20-%20Membrane%20(42%20targets)%20v4a%20(website).pdf

- ELISAs (R&D):

  TNFα: https://www.rndsystems.com/products/human-tnf-alpha-duoset-elisa_dy210#assay-procedure

  IL-1β: https://www.rndsystems.com/products/human-il-1-beta-il-1f2-duoset-elisa_dy201#assay-procedure

  IL-6: https://www.rndsystems.com/products/human-il-6-duoset-elisa_dy206#assay-procedure

  IL-18: https://www.rndsystems.com/products/human-total-il-18-duoset-elisa_dy318-05#assay-procedure

  RAGEs: https://www.rndsystems.com/products/human-rage-duoset-elisa_dy1145#assay-procedure

  ELISAs (Abcam):

  TNFα:

  https://www.abcam.com/ps/products/181/ab181421/documents/Human%20TNF%20alpha-protocol-book-ab181421-20190625%20(website).pdf

  IL-1β:

  https://www.abcam.com/ps/products/214/ab214025/documents/ab214025_Human%20IL-1beta_20190912_ACW%20(website).pdf

  IL-6: https://www.abcam.com/ps/products/178/ab178013/documents/Human-IL-6-ELISA-kit-protocol-book-ab178013%20(website).pdf

IL-18:

https://www.abcam.com/ps/products/215/ab215539/documents/ab215539_Hu%20

IL-18_20%20Dec%202016a%20(website).pdf

RAGEs:

https://www.abcam.com/ps/products/190/ab190807/documents/ab190807_Hu%20

RAGE_14%20Aug%2015a%20(website).pdf

For Chapter 4, figure 4.11 the components of the two defined supplements used can be found below:

- B-27 components: as described by Brewer et al., 1993

- N-2 components: as described by Bottenstein & Sato, 1979

2 Additional data

Chapter 3: co-expression of Tuj-1 and GFAP in neurons
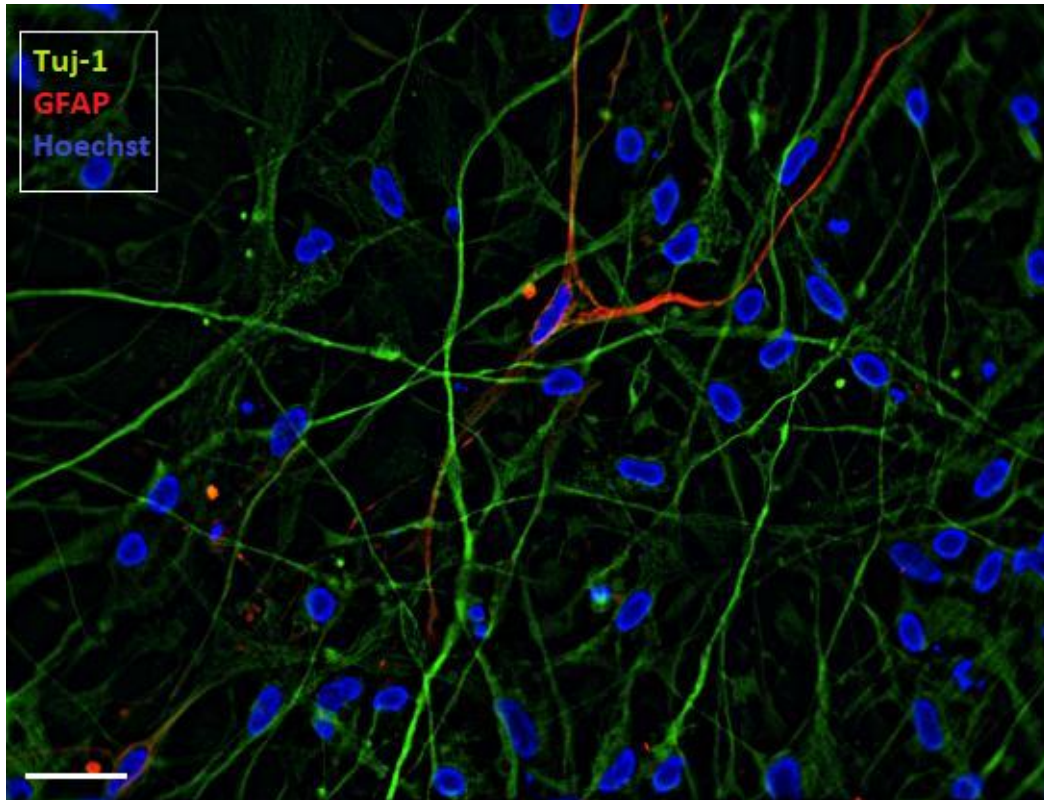
Figure A1: ICC results for neurons, showing co-expression of GFAP (Red), and Tuj-1 (Green) in neurons. Less than 1% of neurons were found to express both markers. For more information, please see chapter 1. Scale bar: 28μm.
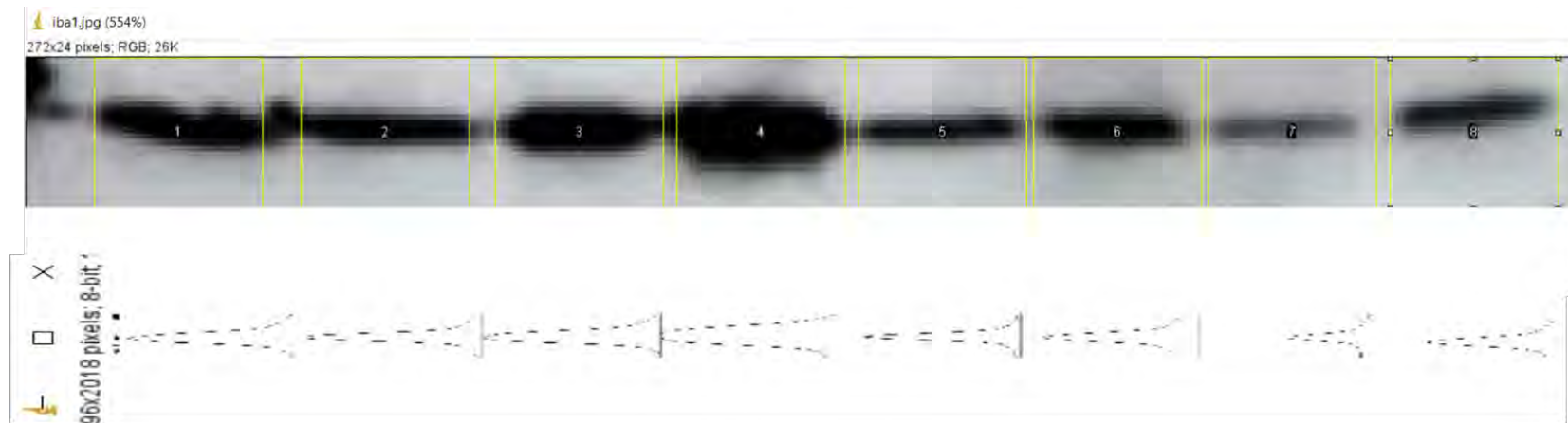
THP-1	THP-1	Day3	Day3	Day7	Day7	Day14	Day14
4908.598	5577.426	6908.205	9279.276	3504.82	3961.82	1623.456	2521.163

	THP-1	Day3	Day7	Day14
Average/condition	5243.012	8093.741	3733.149	2072.31

Figure A2: ***An example of ImageJ use for densitometry for relative quantification of Western Blotting, using Iba1 as an example*** First, the bands are selected, and densitometric analysis gives a histogram for each band. Then, using the gel analysis option, measurements for the histograms are produced. For the purpose of the present study, the quantifications were averaged per condition, adjusted, and then normalised vs actin (housekeeping protein), in order to produce the charts as presented in figures 4.6 and 4.7. Full instructions for normalisation and adjustment can be found here: : https://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/
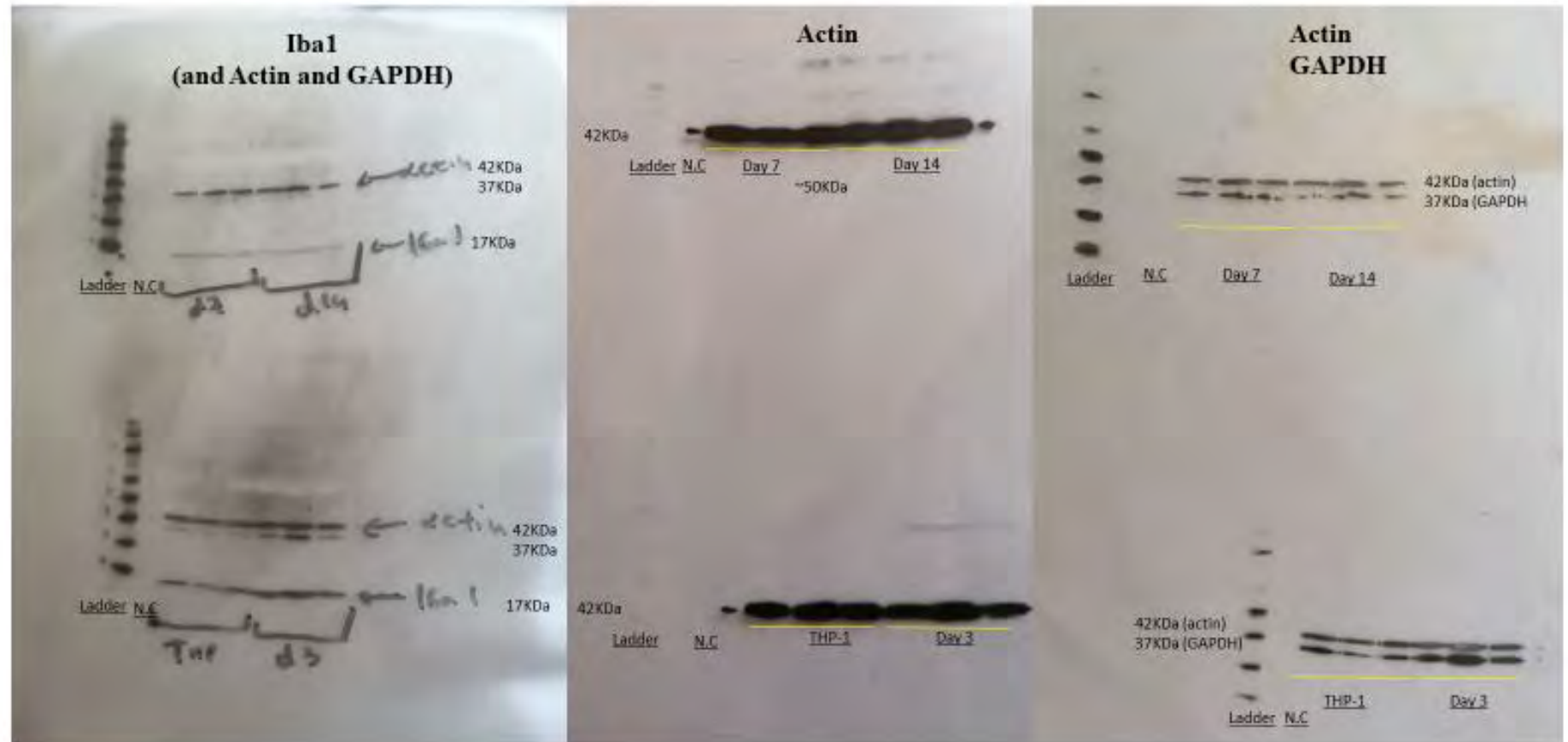
*Figure A3: **The full images of the WB membranes**.* Due to actin bands intensity, the bands remained in the next analyses for GAPDH and Iba1. N=3, NC=negative control.
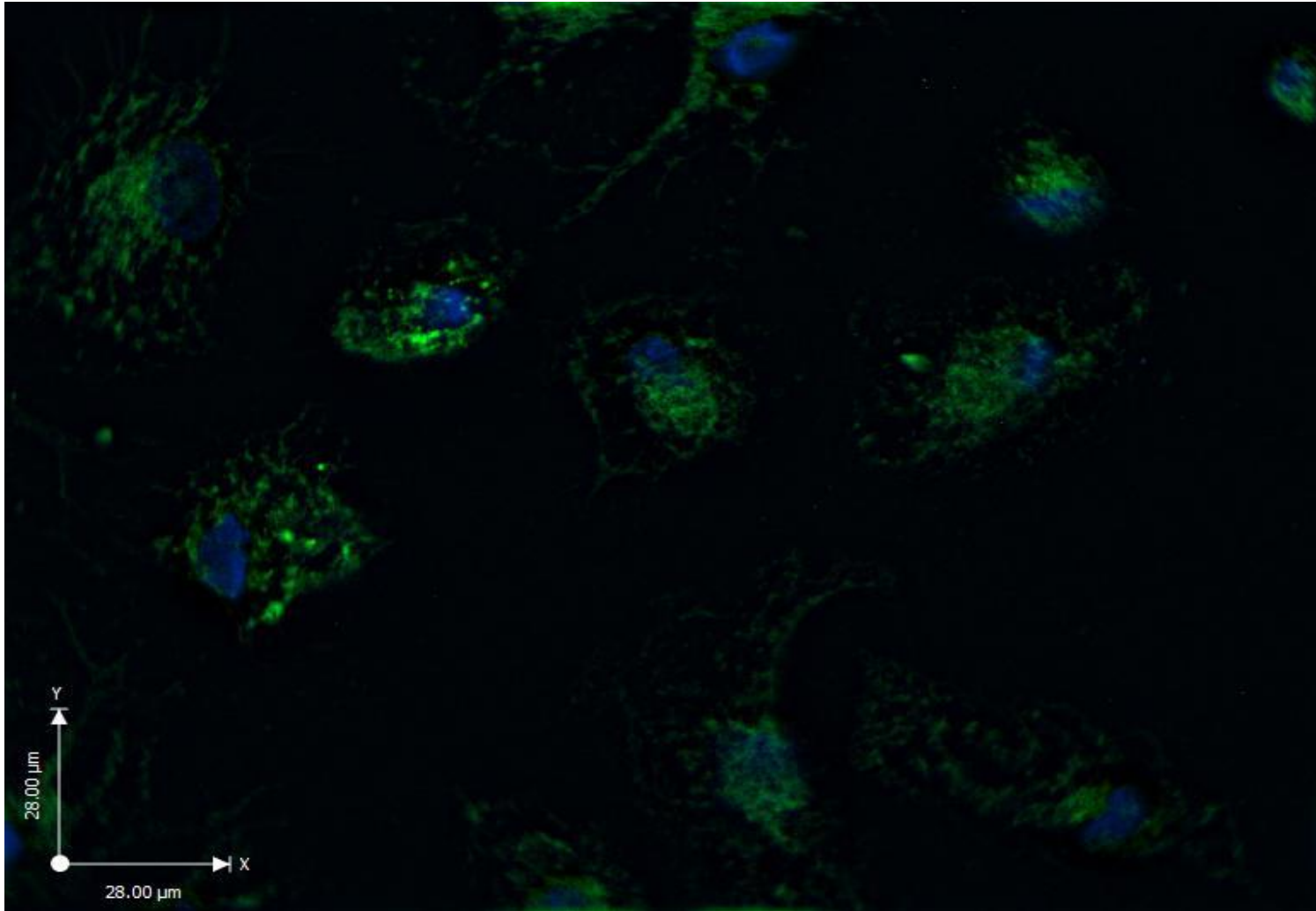
Figure A4** **Ameboid microglia-like cell morphology in day 14 mgTHP-1 cells, as observed using ICC for TMEM119 (green) and Hoechst (blue-nuclei) Green: microglia marker TMEM119, blue: nuclei, scale as mentioned in picture

High resolution image 4.1b

Figure A5 High resolution/magnification of image 4.1b

a)

```
sp|P15924|DESP_HUMAN    ILQITKCIDVHLKENAAYFQFFEEAQSTEAYLKGLQDSIRKKYPCDKNMPLQHLLEQIKE 420
sp|Q4V9L6|TM119_HUMAN   ------------------------------------------------------------ 0


sp|P15924|DESP_HUMAN    LEKEREKILEYKRQVQNLVNKSKKIVQLKPRNPDYRSNKPIILRALCDYKQDQKIVHKGD 480
sp|Q4V9L6|TM119_HUMAN   ----------------MV---------SAAAPSLLILLLLLLGSVPA--TDARSVPL-K 31
                                        :*      *.       ::* ::      * : *   .

sp|P15924|DESP_HUMAN    ECILKDNN---ERSKWYVTGPGGVDMLVPSVGLIIPPPNPLAVDLSCKIEQYYEAILALW 537
sp|Q4V9L6|TM119_HUMAN   ATFLEDVAGSGEAEGSSASSPSLPPPWTPALSPTSMGPQPITLGGPSPPTNFLDGIVDFF 91
                         :*:*    * .  .:.*.   .*::      *:*:::.  .  :: :.*: ::

sp|P15924|DESP_HUMAN    NQLYINMKSL------VSWHYCMIDIEKIRAMTIAKLKTMRQEDYMKTIADLELHYQEFI 591
sp|Q4V9L6|TM119_HUMAN   RQYVMLIAVVGSLAFLLMFIVCAAVITRQKQK--------ASAYYPSSFP--KKKYVDQS 141
                         .*  :: ::   :: *  *  ::       .  * .::  : :*:

sp|P15924|DESP_HUMAN    RNSQGSEMFGDDDKRKIQSQFTDAQKHYQTLVIQLPGYPQHQTVTTTEITHHGTCQDVNH 651
sp|Q4V9L6|TM119_HUMAN   DRAGGPRAFSEVPDRAPDSRPEEALDSSRQLQADILAATQNLKSPTRAALGGGDGARMVE 201
                         .: * . *.: .* :*: :*. . * :: . *: . *     *   :.

sp|P15924|DESP_HUMAN    NKVIET----NRENDKQETWMLMELQKIRRQIEHCEGRMTLKNLPLADQGSSHHITVKIN 707
sp|Q4V9L6|TM119_HUMAN   GRGAEEEEKGSQEGDQEVQGHGVPVETPEAQEEPCSGVLEGAVVAGEGQGELEGS----- 256
                         .: *     .:*.*::   : ::. . * * *.* :   :   .**. .
```
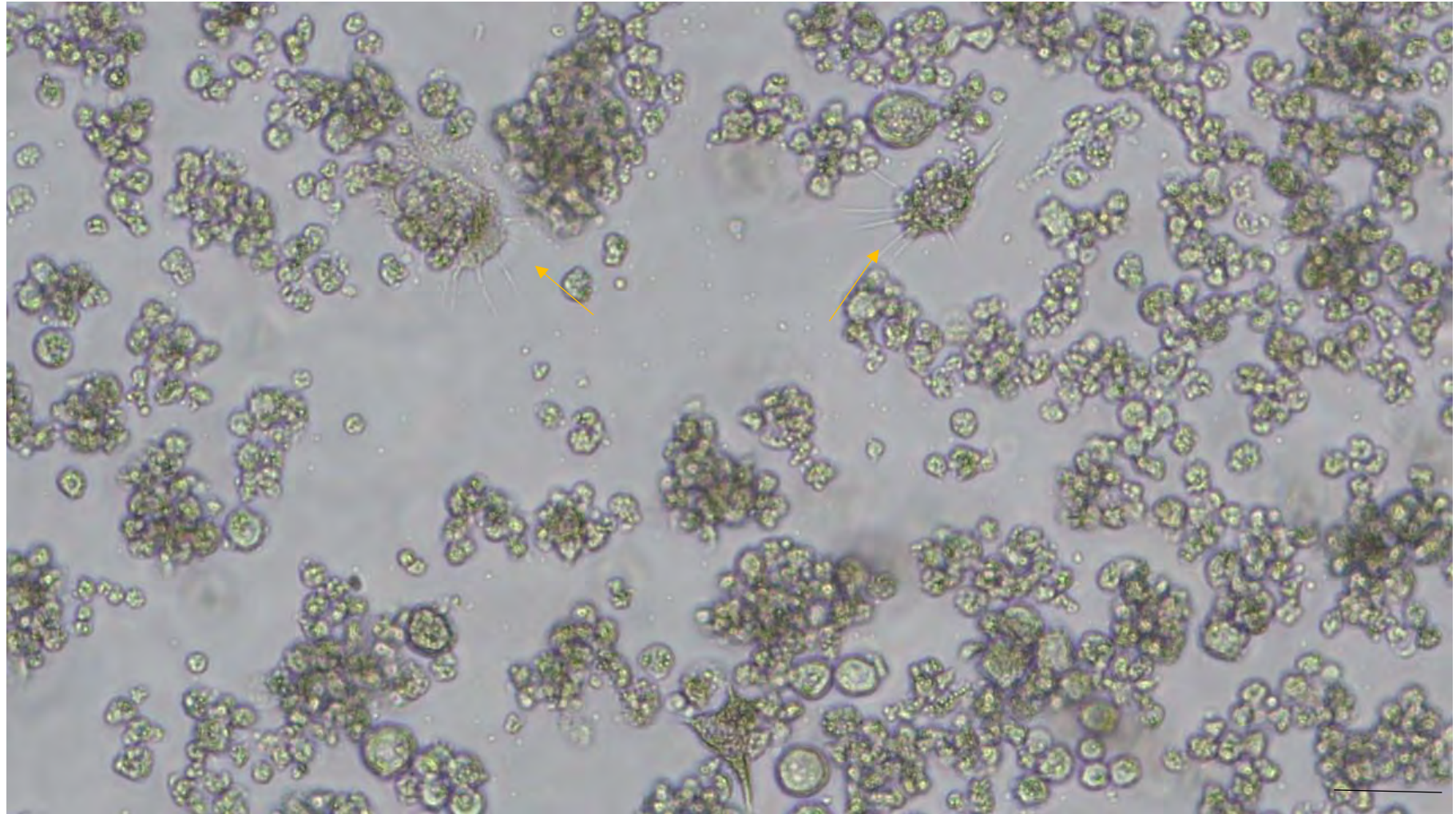
b)

```
        10         20         30         40         50
MVSAAAPSLL ILLLLLLGSV PATDARSVPL KATFLEDVAG SGEAEGSSAS
        60         70         80         90        100
SPSLPPPWTP ALSPTSMGPQ PITLGGPSPP TNFLDGIVDF FRQYVMLIAV
       110        120        130        140        150
VGSLAFLLMF IVCAAVITRQ KQKASAYYPS SFPKKKYVDQ SDRAGGPRAF
       160        170        180        190        200
SEVPDRAPDS RPEEALDSSR QLQADILAAT QNLKSPTRAA LGGGDGARMV
       210        220        230        240        250
EGRGAEEEEK GSQEGDQEVQ GHGVPVETPE AQEEPCSGVL EGAVVAGEGQ
       260        270        280
GELEGSLLLA QEAQGPVGPP ESPCACSSVH PSV
```
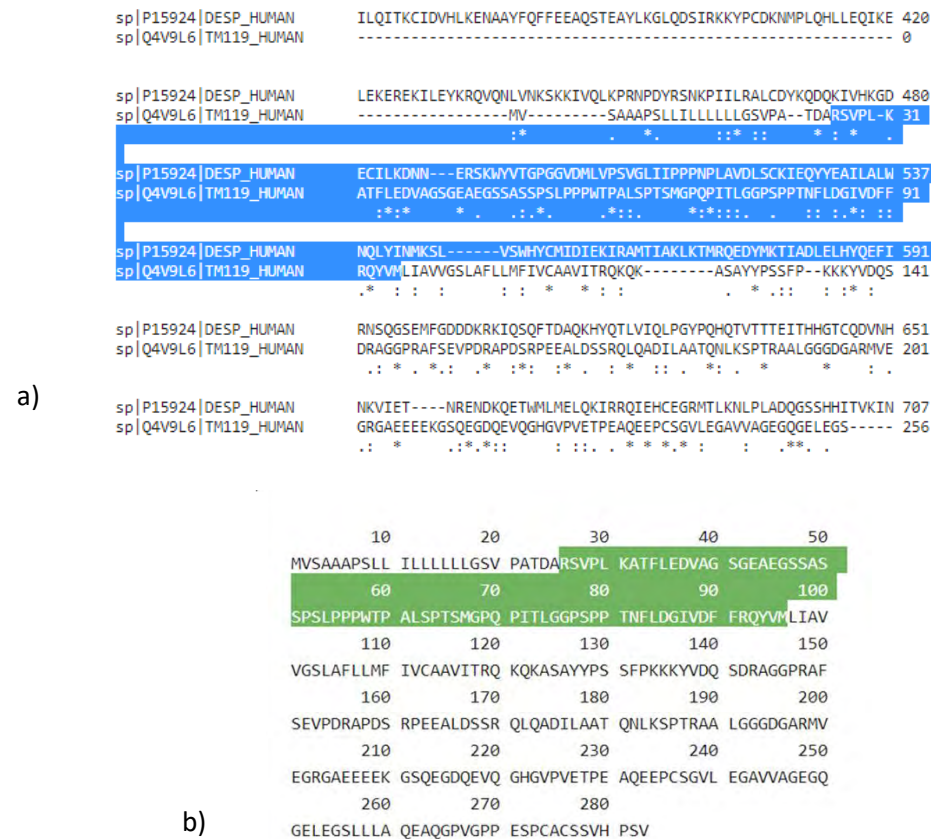
Figure A6a and b: a) ClustalW sequencing alignment between the extracellular domain of TMEM119 (highlighted) and desmoplakin. b) the sequence of the extracellular domain of TMEM119 (highlighted).

3 Additional introduction section

On oligodendrocytes, their development, and role and response to CNS inflammation

While oligodendrocytes are important for the CNS, their importance can be shown only when other CNS cells are present (i.e. neurons), and as this study focuses on single cell type investigations, coverage within this section will primarily focus on the results of the communication between oligodendrocytes and other CNS cells under inflammatory conditions. As such, we will attempt to explain why oligodendrocytes were not directly investigated in the present thesis, but also discuss the current findings that link them indirectly to the current study. Developmentally, oligodendrocytes have the same origin as astrocytes, as both have been shown to come from Oligodendrocyte-type II astrocyte progenitor cells (OAP), which can be isolated from neonatal rats (Raff et al., 1983). The main role of oligodendrocytes is to produce myelin, which insulates neurons, and thus keeps them intact and functioning properly. Secondary roles often include providing energy sources and neurotrophic factors for the neurons (Lee et al., 2012). The importance of their role can be shown if one observes the effects their loss has on disorders such as multiple sclerosis (MS): demyelination of the neurons leads essentially to axonal degeneration and neuronal loss. In that aspect, they are extremely important cells, however they have been shown to be one of the most difficult cells to study due to many factors, one of which being their vulnerability. Despite the fact that these cells have been found to have precursors (Oligodendrocyte precursor cells-OPC) in the adult CNS, which could replace the lost cells, in pathological conditions the turnover of the cells is not always sufficient to prevent or repair the damage caused by other cells (e.g. microglia, astrocytes, or infiltrating monocytes). Remyelination after demyelination is an active process, highly regulated, that needs to be rapid. It is not clear whether this process is triggered by demyelination itself, or inflammation. Myelination starts quite late in the embryonic life, and

maintenance of the myelin membrane is a constant event in the healthy CNS. The myelin-related genes are expressed long after the completion of myelination (Lajtha et al., 1977), a process which is instigated, and maintained by other CNS cells, such as neurons (Demerens et al., 1996) and astrocytes (Ishibashi et al., 2006) and mediated by cytokines (and other mediators such as ATP), as well as cell/cell interactions.

Focusing on cell/cell interactions, and how important they are in order to reveal the potential functions of oligodendrocytes, microglia, and infiltrating monocyte derived macrophages (MDM) have been shown to have a positive effect on remyelination, and survival of oligodendrocytes (Kotter et al., 2005). Last but not least, more recently CNS macrophages (including resident microglia and infiltrating monocyte derived macrophages) have been shown to have a positive effect on remyelination, but essentially being the driving force behind it. Miron et al.,(2013) showed that a change from M1 to M2 CNS macrophage polarization coincided temporally with the beginning of remyelination, and that the timing of that change is essential for the process of OPC differentiation to mature oligodendrocytes. It was further shown that activin-A, which is secreted from M2 macrophages is one of the molecules responsible for the differentiation and maturation of the OPCs.

In regards to inflammation, and response to inflammatory events, oligodendrocytes are, as already mentioned, quite vulnerable. Oligodendrocytes do express the receptors for inflammatory mediators such as pro and anti-inflammatory cytokines (including IL-10, IL-6, and IL-18), as well as molecules such as IFNγ, however they do not secrete nearly any cytokines of the ones examined and mentioned above (Cannella, & Raine, 2003); for this reason, these cells were not included in the present study. These cells also express receptors such as TLR4, however their response to TLR4 ligands, such as LPS binding to the receptor is to undergo apoptosis, rather than react by secreting pro-inflammatory mediators, unlike the other glial cells (Lehnardt et al., 2002; Yao et al., 2010). The cell death caused by LPS was

found to be neuronal nitric oxide synthase related (Yao et al., 2010). Additionally, it has also been shown that factors secreted from microglia and astrocytes, which have been LPS conditioned, promoted oligodendrocyte injury, and cell death (Pang et al., 2000).

When it comes to the response of the cells to IFNγ, there have been indications of interferon inducing oligodendrocyte cell death, as well as having a protective role against oxidative stress. Firstly, direct exposure of oligodendrocytes to IFNγ resulted in an increase in apoptosis; that type of cell death was not nitric oxide related, and could be avoided using growth factors that belong in the leukemia inhibitory factor (LIF) family, as well as anti-IFNγ antibodies; the cell death observed in this study was also not microglia related (Vartanian et al., 1995). In a more recent *in vivo* study in mice, however, it was shown that mice that have reduced responsiveness to IFNγ, had increased symptoms, oligodendrocyte cell death, and onset of experimental autoimmune encephalomyelitis (EAE), and that in vitro treatment of oligodendrocytes with IFNγ had more of a protective rather than a deleterious effect on the cells (Balabanov et al., 2007). For the *in vitro* part of the study, pre-treatment of oligodendrocytes with IFNγ resulted to them being less sensitive to cell death caused by H_2O_2, as well as by lactacystin (a proteasome inhibitor), which has been shown to induce cell death to oligodendrocytes in a dose-dependent manner (Balabanov et al., 2007). This study also found IFNγ induced secretion of cytokines of the CCL family (including MCP1/CCL2, and RANTES/CCL5), as well as CXCL10; no IL-1 family, IL6, or TNFα cytokines were detected. As both studies used rat in vitro systems, as well as similar levels of IFNγ, the differences could be attributed to the protocols, where using the first protocol they observed an expected 10% of astrocytes present, and in the second, the cultures were found to be >95% oligodendrocytic.

From the above, it can be deduced that oligodendrocytes can be studied better via their interactions and communication with other cells, as their main function is the myelination of neurons, rather than alone. Especially of interest are oligodendrocytes and oligodendrocytic

progenitors (OPCs) originating in pathological brains, and how differently these work together with other cells. Oligodendrocytes derived from induced pluripotent stem cells (iPSC), NSC, or OAP can be generated relatively easy as an extended amount of protocols exist.

Dysfunction of oligodendrocytes is the focus of a review by Bankston et al,. (2013). Bankston concludes that changes in oligodendrocyte function as a result of acute or chronic inflammation and degeneration have a great impact on the integrity of the CNS, and better understanding the relationships between the aforementioned is a big challenge. Focusing on studies that aim to uncover the differences between a healthy and diseased brain, several studies show the change of oligodendrocytes from myelinating cells, to non-myelinating ones, especially in chronic demyelinating disorders, such as MS (Chang et al., 2002) and even ALS (Kang et al., 2013); studies have shown further implication of oligodendrocyte and OPC dysfunction in disorders such as schizophrenia (de Vrij et al., 2018; Cassoli et al., 2015) and even in normal processes such as aging (Bagi et al., 2018). Another major focus has been on cell/cell interaction, how oligodendrocytes shift from myelination to energy production for the neurons, and neuron/oligodendrocyte coupling and metabolism. An interesting study by Minchenberg & Massa (2016) focuses on the metabolism of oligodendrocytes and how the immune system influences the metabolic processes. More specifically, they found that a negative immunoregulatory protein, namely the tyrosine phosphatase SHP-1 influences regulatory transcriptional modulatory factors such as STAT genes, which in their turn are involved in lipid metabolism. As lipid synthesis is essential for the construction and preservation of myelin, and as STAT genes have also been shown to be regulated via cytokines such as IFNγ and IL-10, this study demonstrates how tightly regulated and how complex the role of oligodendrocytes is in the CNS.

From the above, it can be deduced that oligodendrocytes are not expected to be major influencers in neuroinflammatory conditions on their own. However, they are one of the cell

types majorly affected in a negative way by inflammation. As discussed, these cells do not produce the cytokines that are the focus of this study in response to inflammatory stimuli, so they don't communicate with nearby cells via secreting cytokines. As they have been shown to have receptors for a variety of cytokines (Cannella & Raine, 2004), these cells are affected by molecules secreted from cells they communicate with during neuroinflammation. Therefore, although not a direct focus for investigation our study, (as at least the first part focuses on the contribution of non-microglial CNS cells on neuroinflammation) oligodendrocytes can be thought as one of the reasons this study is conducted: they are the recipients of the messages sent by other cells during neuroinflammation.

Kodosaki, Eleftheria
MPhil

Dear Applicant

Re: Application for Amendment to Existing Ethical Approval

Project Title: The effects of dietary flavonoids on human neural cell lines

Project Ref. Number: 7181

Amendment Number: 0

Your application to amend your existing ethics approval, as shown above, was considered by the Biomedical Sciences Ethics Panel on 24-01-18.

I am pleased to inform you that the requested **Amendment was APPROVED,** subject to the conditions listed below – *please read carefully.*

Standard Conditions of Approval

1. Please continue to quote the original Project Reference number on all documentation relating to work undertaken on the project (e.g. consent forms).

2. A revised **Risk Assessment** must be undertaken for this proposal if the amendment involves a change in the protocol, and be made available to the Committee if requested.

3. Any further changes in connection to the proposal or amendments as approved, must be referred to the Panel/Committee for consideration *without delay quoting your Project Reference Number*. Changes to the proposed project may have ethical implications that require further consideration.

4. Any untoward incident which occurs in connection with this proposal must be reported back to the Panel *without delay*.

5. If your project involves the use of **human samples**, your approval is given on the condition that you or your supervisor **notify the HTA Designated Individual** of your intention to work with such material by **completing** the form entitled *"Notification of Intention to Work with Human Samples"*. The form must be submitted to the PD (Sean Duggan), **BEFORE** any activity on this project is undertaken .

This amendment of approval expires on 24-01-19. Please set a reminder on your Outlook calendar or equivalent if you need to continue beyond this extended date.

Yours sincerely

PLEASE RETAIN THIS LETTER FOR REFERENCE

Dr Rachel Adams
Chair of BMS Ethics Panel
Cardiff School of Health Sciences

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