Characterization of hiPSC-derived reactive astrocytes and co-cultures with neurons in microfluidic device

Master's thesis
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PRO GRADU-TUTKIELMAN TIIVISTELMÄ

Paikka: Tampereen yliopisto
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Otsikko: Indusoiduista monikykyisistä kantasoluista erilaistettujen astrosyyttien reaktiivisuuden karakterisointi ja niiden yhteisviljelmät hermosolujen kanssa mikrofluidisissa rakenteissa
Sivumäärä: 74
Ohjaajat: Dosentti Susanna Narkilahti, FT Sanna Hagman ja FM Tanja Hyvärinen
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Tutkimuksen tausta ja tavoitteet: Astrosyytit kuuluvat gliasoluihin ja ovat keskushermoston runsaslukuisin solutyyppi. Astrosyytit pitävät monin tavoin huolta keskushermoston tasapainosta, tukevat hermosoluja ja säätelevät synaptista aktiivisuutta. Näiden lisäksi astrosyytit reagoivat tulehdukselliseen ympäristöön erittämällä erilaisia tulehduksellisia aineita kuten sytokiineja ja kemokiineja. Keskushermoston vaurio- ja tautitiloissa astrosyytit reaktivoituvat ja niiden morfologia sekä geenien ilmentyminen muuttuvat. Astrosyyttien rooli keskushermoston traumatiloissa on kaksijakoinen. Ne voivat joko tukea hermosolujen toipumista tai toisaalta myös lisätä tulehdusta ja vauriota. Tuoreissa tutkimuksissa on löydetty kaksi reaktiivisten astrosyyttien alatyyppiä; vahingollinen A1 tyyppi ja suojaava A2 tyyppi. Tyypin A1 huomattiin indusoituvan hermoston tulehdustilasta, kun taas tyyppi A2 yhdistettiin iskemian aiheuttamaan vaurioon. Nämä löydökset viittaavat ympäristön vaikuttavan reaktiivisten astrosyyttien fenotyyppiin. Edistyneempiä *in vitro* tautimalleja on kehitettävä, jotta reaktiivisuutta ja sen vaikutuksia hermosoluihin voitaisiin tutkia tarkemmin. Tämän projektin tarkoitus oli tutkia kuinka ihmisen indusoiduista monikykyisistä kantasoluista (engl. induced pluripotent stem cells eli iPS-solut) erilaistetut astrosyytit reagoivat tulehdukselliseen ympäristöön. Lisäksi tarkoituksena oli muodostaa yhteisviljelmiä hermosoluista ja reaktiivisista astrosyyteistä mikrofluidisissa rakenteissa.

Menetelmät: iPS-soluista erilaistetut astrosyytit käsiteltiin tulehduksellisilla interleukiini 1 betalla (IL-1β) ja tuumorinekroositekijä alfalla (TNF-α), jotta saataisiin indusoitua reaktiivinen fenotyyppi astrosyyteissä. Astrosyyttien reaktiivisuutta karakterisoitiin western blot -menetelmällä, immunosytokemiallisilla värjäyksillä ja tutkimalla astrosyyttien erittämiä tulehduksellisia aineita. Yhteisviljelmät hermosoluilla ja astrosyyteillä suoritettiin ryhmässä kehitetyillä mikrofluidisilla rakenteilla. Näissä rakenteissa indusoitiin reaktiivinen astrosyytti fenotyyppi IL-1β and TNF-α käsittelyllä. Hermosolujen aksonien kasvua ja astrosyyttien reaktiivisuutta tarkkailtiin valomikroskoopilla sekä tutkittiin proteiinien ilmentymistä immunosytokemiallisilla värjäyksillä.

Tulokset: Sytokiinikäsittely aiheutti astrosyyttien reaktiivisuudelle tyypillisiä muutoksia morfologiassa, tulehduksellisten aineiden erityksessä ja välikokoisten säikeiden ilmentymisessä. Käsittelyn jälkeen gliaalisen fibrillaarisen happaman proteiinin (eng. glial fibrillary acidic protein eli GFAP) ilmentyminen väheni, mutta vimentiinin säilyi samalla tasolla. Sytokiinikäsittely myös laski solujen elinkykyä. Käsittelyn johdosta tulehduksellinen transkriptiotekijä tumatekijä kappa B (eng. nuclear factor kappa B eli NF-κB) aktivoitui ja tulehduksellisten aineiden kuten interleukiini 6:n (IL-6) eritys kasvoi. Astrosyyttien ja hermosolujen yhteisviljelmät onnistuivat hyvin mikrofluidisissa rakenteissa. Näissä sytokiinikäsittely aiheutti astrosyyteissä morfologian muutosta, joka pystyttiin immunosytokemiallisella värjäyksellä havaitsemaan. Sytokiinikäsittely ei myöskään vaikuttanut vahingollisesti aksonien kasvuun rakenteissa.

Johtopäätökset: IL-1β and TNF-α käsittelyllä saatiin indusoitua reaktiivisuudelle tyypillisiä piirteitä astrosyyteissä, joten ihmisen iPS-soluista erilaistetut astrosyytit tarjoavat lupaavan tutkimustyökalun neurologisten sairauksien tutkimukseen. Hermosolujen ja astrosyyttien yhteisviljelmiä voidaan tehdä mikrofluidisissa rakenteissa ja reaktiivisuutta voidaan mallintaa niissä.

ABSTRACT OF MASTER'S THESIS

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Background and aims: Astrocytes are heterogeneous glial cells and they are the most abundant cell type in the central nervous system (CNS). They participate in many homeostatic functions and provide support for neurons and regulate synaptic activity. Astrocytes also play a role in tissue repair after injury and respond to inflammatory environment by secreting many cytokines and chemokines. After CNS injury and disease, astrocytes transform into reactive phenotype and alter their gene expression profiles and morphology. Astrocytes are suggested to have a dual role in the CNS recovery meaning that they can have either protective or detrimental consequences for neuronal survival. Two distinct subtypes of reactive astrocytes were recently found; detrimental A1 type and protective A2 type. Type A1 was associated with neuroinflammation whereas type A2 was associated with ischemia-induced injury. This suggests that different subtypes of reactive astrocytes are induced with different inflammatory environment and astrocyte reactivation is a complex phenomenon. More advanced *in vitro* disease models are needed for studying the mechanisms of astrocyte reactivation and their effects on neurons. Aim of this project was to study how astrocytes derived from human induced pluripotent stem cells (hiPSCs) respond to inflammatory environment and establish co-cultures with neurons and astrocytes in microfluidic devices.

Methods: hiPSC-derived astrocytes were treated with interleukin (IL)-1β and tumor necrosis factor (TNF)-α to induce the reactive phenotype. Astrocyte reactivation was characterized with western blot -analysis, immunocytochemistry and analyzing secretion profiles. Co-cultures with hiPSC-derived astrocytes and neurons were performed with in-house developed multicompartment microfluidic devices. Astrocytes in these devices were treated with IL-1β and TNF-α to induce the reactive phenotype. Axonal growth and astrocyte reactivation were observed with phase-contrast microscope and immunocytochemistry was used for protein expression analysis.

Results: IL-1β and TNF-α treatment caused alterations in astrocyte morphology and secretion profile. Expression of glial fibrillary acidic protein (GFAP) was decreased and vimentin remained the same. Cytokine treatment decreased cell viability. In addition, IL-1β and TNF-α treatment induced activation of inflammatory nuclear factor kappa B (NF-κB) and upregulation of inflammatory cytokines, such as IL-6. Astrocyte and neuron co-cultures in microfluidic devices were successful and both cell types grew well in those. IL-1β and TNF-α treatment in devices induced morphological change in astrocytes, which could be detected with immunocytochemical staining. Reactive astrocytes did not, however, had detrimental effect on axonal growth.

Conclusion: Cytokines IL-1β and TNF-α could induce different cellular alterations typical for reactive astrocytes, thus hiPSC-derived astrocytes could offer a useful tool for studying astrocyte reactivation and neurological diseases. Co-cultures of neurons and astrocytes in microfluidic devices can be performed, and astrocytes respond to inflammatory cytokine treatment in those. Co-cultures in engineered microfluidic devices offer a promising platform for CNS disease modeling.

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Katariina Veijula

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Abbreviations

AA	Ascorbic acid
ARA	Arachidonic acid
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AQP4	Aquaporin 4
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
C-MYC	V-Myc avian myelocytomatosis viral oncogene homolog
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
EAAT1	Excitatory amino acid transporter 1
EAAT2	Excitatory amino acid transporter 2
EB	Embryoid body
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GABA	Gamma aminobutyric acid
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GS	Glutamine synthetase
hPSC	Human pluripotent stem cell
hiPSC	Human induced pluripotent stem cell
ICC	Immunocytochemical/immunocytochemistry
INF-γ	Interferon gamma
IL-1β	Interleukin 1β
KLF4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MS	Multiple sclerosis
NF-κB	Nuclear factor κB
NSC	Neural stem cell
NGF	Nerve growth factor
NPC	Neural progenitor cell
OCT3/4	Octamer-binding transcription factor ¾
OPC	Oligodendrocyte precursor cell
PB	Phosphate buffer
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PG	Prostaglandin
RA	Retinoic acid
Shh	Sonic hedgehog
SOX	SRY (Sex Determining Region Y)-Box
STAT3	Signal transducer and activator of transcription 3
S100β	S100 calcium-binding protein β
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α
WB	Western blot

1 Introduction

Astrocytes are specialized heterogeneous glial cells and they constitute approximately 30% of the cells in the central nervous system (CNS) which makes them the most abundant glial cell type (Liddelow & Barres 2017; Sofroniew & Vinters 2010). Astrocytes have many crucial functions in the healthy CNS maintaining the brain homeostasis and supporting the neurons (Allaman et al. 2011). More precisely, astrocytes participate in the supply of energy metabolites and neurotransmitter recycling for neurons and perform other functions related to glutamate, ion and water homeostasis (Allaman et al. 2011). In addition, astrocytes are involved in energy storage, formation and maintenance of the blood-brain barrier and formation of the synapses (Sofroniew & Vinters 2010). Besides these, astrocytes have been suggested to play a role in tissue repair after CNS injury and they can respond to inflammatory environment (Ben Haim et al. 2015).

It has been shown that astrocytes transform into reactive phenotype in response to inflammatory environment during CNS injury and disease (Liddelow et al. 2017; Ben Haim et al. 2015; Pekny & Nilsson 2005). Characteristics for reactive astrocytes are changed morphology, increased proliferation and alterations in the gene expression (Liddelow et al. 2017; Ben Haim et al. 2015). The role of reactive astrocytes in CNS injury and disease is not clear and astrocytes are thought to have dual effects on CNS recovery (Colombo & Farina 2016). Recently, two distinct phenotypes of reactive astrocytes were described in rodent studies; detrimental A1 type and protective A2 type (Liddelow & Barres 2017; Zamanian et al. 2012). Type A1 is classified as neurotoxic hindering the recovery of CNS after injury and disease (Liddelow et al. 2017). In addition, type A1 astrocytes were shown to lose the supporting abilities and they induced the death of neurons and oligodendrocytes (Liddelow et al. 2017). Type A2 was shown to upregulate many neurotrophic factors, which lead to conclusion that it might have beneficial roles in CNS recovery. Moreover, type A1 reactive astrocytes were associated with neuroinflammation whereas type A2 was ischemia-induced (Zamanian et al. 2012). Thus, the nature of astrocyte reactivation is suggested to be dependent on the type of injury and possibly more reactive astrocyte subtypes exist. Reactive astrocytes have been suggested to have a role in different neurodegenerative diseases (NDs) (Liddelow et al. 2017; Colombo & Farina 2016). Type A1 astrocytes have been found in human post-mortem tissues from patients with Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) (Liddelow et al. 2017). Rodents are widely used for modeling these different NDs and for studying reactivation of astrocytes (Sakakibara et al. 2019; Zamanian et

al. 2012; Sofroniew 2009). However, the results from rodent studies have limitations, because rodents and their astrocytes differ from human in many ways (Zhang et al. 2016). Advances in the stem cell field have made it possible to differentiate astrocytes from human pluripotent stem cells (hPSC) and more specifically human induced pluripotent stem cells (hiPSCs) (Lundin et al. 2018; Roybon et al. 2013; Shaltouki et al. 2013). hPSCs could provide new possibilities for studying the role of reactive astrocytes in CNS disorders. In addition, the expansion of the microfluidics in the field of neuroscience has offered possibilities to model the mechanisms of different NDs in more controlled way *in vitro* (Yi & Lin 2017; Jadhav et al. 2016). These disease models in a dish could clarify the complex nature of different cell-cell communications in CNS injuries and diseases.

This overall aim of this thesis project was to characterize the reactivation of hiPSC-derived astrocytes and to establish co-cultures with neurons utilizing in-house developed microfluidic devices.

2 Literature review

2.1 Central nervous system

Human nervous system can be divided into central and peripheral nervous systems. CNS can be further divided into brain and the spinal cord, which are the main regulators of different functions in the human body. CNS is formed by neurons and glial cells, which are derived from the neuroepithelial cells of the neural tube in the development of the CNS. Different morphogens such as fibroblast growth factors (FGFs), retinoic acid (RA), Sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) are involved in the patterning of the neural tube. (Guerout et al. 2014)

2.1.1 Cell types in central nervous system

Neurons are the most fundamental cell type in the CNS and their role is to receive, process and transmit information in the forms of electrical and chemical signals. The morphology of different neuron types varies in some respects, but they all share some similar features; the cell body, the dendrites, the axon and the axon terminals (Figure 1). Usually neurons have one single axon, which purpose is to conduct an electrical impulse called action potential outward to the axon terminals. Axon terminals branch in to several synapses, which are structures that permit the electrical impulse to be transferred in to chemical signal and passed on other neurons or to the target effector cells. These signal-passing neurons are also called pre-synaptic neurons and their axonal terminals are called pre-synaptic terminals. In neurons, dendrites are usually the ones that receive the chemical signal in the form of neurotransmitters. Dendrites receive the neurotransmitters in the post-synaptic structures, which are in close proximity with the pre-synaptic terminals of the signal-sending neuron. The chemical signal is further converted into electrical impulses and transmitted towards the cell soma. Dendrites and axons, so called neurites, along with neuron somas form complex neural circuits and create the functional core system of CNS. (Franze et al. 2013; Lodish et al. 2000) However, also other cell types are needed to perform the complex functions in CNS, like astrocytes, oligodendrocytes and microglia (Figure 1).

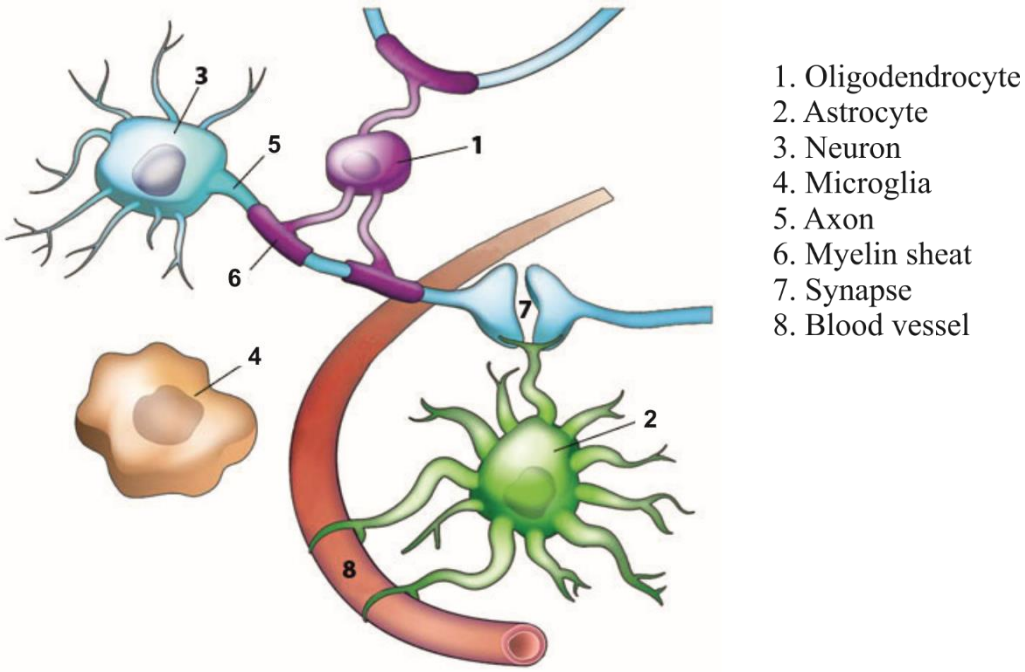


Figure 1. Different cell types in the central nervous system. Astrocytes are participating in the neuronal synapses and are connected to blood vessels. Oligodendrocytes envelop axons with myelin sheath. Figure modified from https://med.stanford.edu/news/all-news/2009/09/unsung-brain-cells-play-key-role-in-neurons-development.html.

Glial cells are the other cell group in CNS along with neurons and they can be divided into microglial and macroglial cells. Microglial cells act as primary immune cells in the CNS and they have phagocytic features. (Jäkel & Dimou 2017) Unlike other glial cells, which origin from embryonic tissue layer known as neuroectoderm, microglial cells are generated from the same embryonic layer than blood and immune cells; the mesoderm. To be more precise, microglia arise from yolk-sac fetal macrophages. Microglial cells provide protection against pathogens in CNS and can support CNS recovery after injury. (Rock et al. 2004)

Macroglial cells can be divided into oligodendrocytes and astrocytes. The main purpose of oligodendrocytes is to provide support for neuronal axons in CNS by generating a myelin sheath as an insulator. The insulative myelin sheath allows rapid and precise electrical signaling between neurons. This allows controlled motoric functions and higher cognition. One oligodendrocyte can wrap as much as 50 axons with its processes. (Guerout et al. 2014; Araque & Navarrete 2011) Before being fully mature, oligodendrocytes are called as oligodendrocyte precursor cells (OPC). They are derived from specialized domain of the ventral ventricular zone. From there, they migrate through the spinal cord and differentiate to mature oligodendrocytes. Later, an additional source of OPCs arises in the dorsal spinal cord. (Bradl & Lassmann 2010)

Other types of macroglial cells are astrocytes, which are star-shaped cells that outnumber neurons over fivefold in CNS and play a major role in the normal physiology and function of the brain (Sofroniew & Vinters 2010). Astrocytes rise from the neuroepithelial cells in the developing CNS and differentiate from neural progenitor cells via glial precursor cell state to mature astrocytes (Chandrasekaran et al. 2016). Astrocytes are relatively heterogenous with respect to their origin of development, morphology and functional properties (Shaltouki et al. 2013). Several astrocyte subtypes have been defined and the major subtypes are fibrous and protoplasmic astrocytes (Chandrasekaran et al. 2016; Sofroniew & Vinters 2010). Fibrous astrocytes are located in the white matter of CNS and they exhibit long fiber-like processes. Protoplasmic astrocytes in turn are found in the grey matter and have very branched morphology. (Sofroniew & Vinters 2010) Other astrocyte subtypes are also recognized; the Bergmann glia of the molecular layer of the cerebellum, the Muller cells of the retina, polarized and interlaminar astrocytes in the human brain cortex (Oberheim et al. 2012; Kimelberg 2010). Due to the diversity of astrocyte subtypes and their heterogeneity, full characterization methods are challenging to develop. Glial fibrillary acidic protein (GFAP), an intermediate filament protein, is one of the most used identification marker for astrocytes during differentiation (Chandrasekaran et al. 2016; Krencik & Zhang 2011). However, some rodent and human astrocyte studies have shown that some subpopulations of resting astrocytes do not express GFAP, thus better identification markers are needed (Zhang et al. 2016; Kuegler et al. 2012). Furthermore, astrocyte maturation is a complex and poorly understood event containing series of stages in which expression of different protein markers is overlapping considerably (Chandrasekaran et al. 2016). Nevertheless, some specific protein markers in addition to GFAP are commonly in use. Vimentin, S100 calcium-binding protein beta (S100β) and cell-surface glycoprotein CD44 expression can be used as astrocyte progenitor markers for developing astrocytes (Chandrasekaran et al. 2016; Krencik & Zhang 2011). For more mature astrocyte identification, excitatory amino acid 1 and 2 (EAAT1 and EAAT2), aquaporin 4 (AQP4) and glutamine synthetase (GS) can be used (Chandrasekaran et al. 2016; Roybon et al. 2013). In addition, aldehyde dehydrogenase 1 family member (Aldh1L1) is also found to be co-expressed in mature astrocytes with GFAP and S100β (Adam et al. 2012). However, more studies are needed to clarify the complex development and maturation of astrocytes.

Astrocytes are involved in many different homeostatic functions and to put simply, their main goal is to help functionality of neurons. In addition to trophic support for neurons, astrocytes are in close contact with neuronal synapses forming tripartite synapses including pre- and postsynaptic terminal and astrocytic process enveloping them (Figure 2) (Allaman et al. 2011). Astrocytes express many of the same receptors as neurons do, and therefore neuron-derived neurotransmitters can induce effects also in astrocytes. Activation of these neurotransmitter receptors induce calcium-based signaling pathways in astrocytes, which in turn leads secretion of substances that can alter the neuronal activity. (Allen & Barres 2009) In addition, astrocytes promote the formation and function of synapses by releasing different gliotransmitters and proteins that alter the neuronal excitability (Allen & Barres 2009). Furthermore, astrocytes remove excess neurotransmitter molecules, for example glutamate, from the synaptic cleft with glutamate transporters EAAT1 and EAAT2 allowing precise neurotransmission (Zagami et al. 2005). Astrocytes have enzymes like glutamate synthase, which can convert glutamate taken from the synaptic cleft into precursor form glutamine. The converted glutamine can be then recycled back to neurons. (Allama et al. 2011) Astrocytes processes are connected to the parenchymal blood vessels, which helps astrocytes to regulate the blood flow in response to increased neuronal activity. Astrocytes do the regulation by secreting different molecular mediators, such as prostaglandins (PGs), nitric oxide and arachidonic acid (ARA). These molecules either decrease or increase the CNS fluid flow by altering the blood vessel diameter. (Sofroniew & Vinters 2010) Related to this blood flow regulation, astrocytes ferry glucose and oxygen from blood to the neurons. It is hypothesized that they also convert glucose to lactate, which is further converted into pyruvate and used as adenosine triphosphate (ATP) in neurons. (Allen 2014) In addition, astrocytes sustain neuronal function and viability by releasing several mediators, such as trophic factors and cytokines. It is studied that astrocytes play a role in scar formation and tissue repair after injury in CNS. (Liddelow & Barres 2017; Allaman et al. 2011)

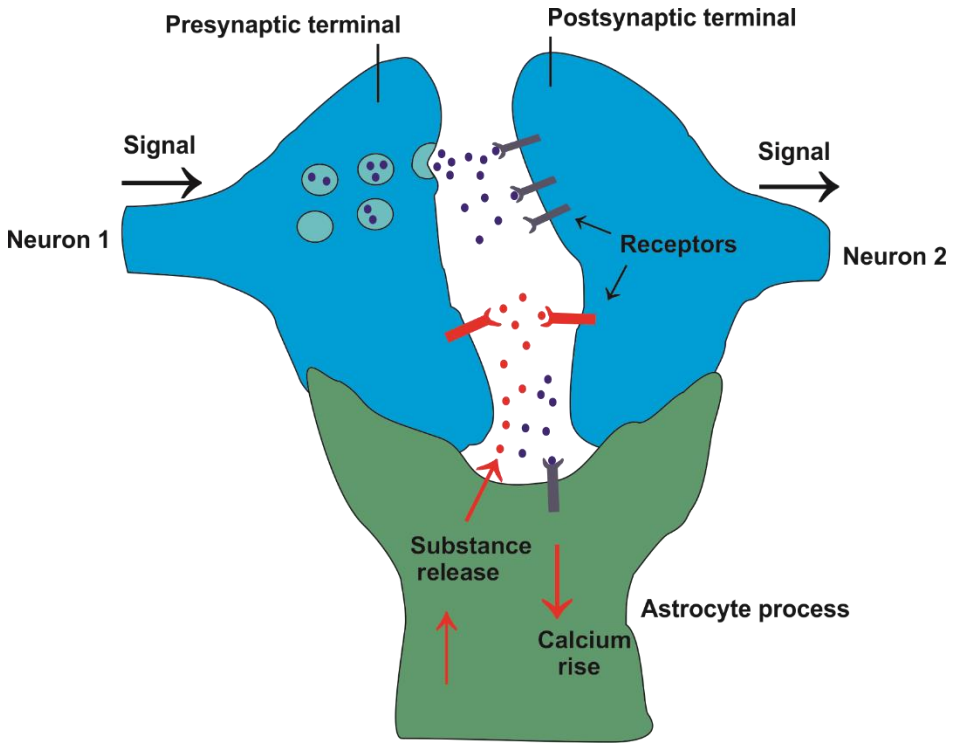


Figure 2. Astrocytic processes envelop pre- and postsynaptic terminals in neurons and together they form tripartite synapses. Neurotransmitters secreted by neurons activate calcium-based signaling cascades in astrocytes. Astrocytes also release substances that regulate the electrical activity in the synapses. Figure is redrawn based on figure from Allen & Barres 2009.

2.1.2 Reactivation of astrocytes

In response to inflammatory environment induced by CNS injury or disease, resting astrocytes can transform into reactive astrocyte phenotype (Liddelow & Barres 2017; Roybon et al. 2013). Reactivation can be initiated by different cytokines and growth factors secreted in the injury site, such as interleukin (IL)-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), IL-1, IL-10, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α and interferon (INF)- γ. In addition, reactivation can be a result of oxidative stress resulted from reactive oxygen species (ROS), hypoxic conditions or some modulators related to neurogenerative diseases, like amyloid-beta (Aβ) in AD. (Sofroniew 2009) All of these molecular mediators of reactivation can be released from all the cell types in the CNS including neurons, microglial cells, other astrocytes, endothelial cells, oligodendrocytes and leukocytes (Sofroniew & Vinters 2010). Reactivation of astrocytes is not all-or-nothing action but rather a wide spectrum of changes in astrocytes. These changes can vary in nature and severity depending on the insult in CNS. (Anderson et al. 2014; Sofroniew & Vinters 2010) In general, different studies have shown that astrocyte reactivation leads to secretion of inflammatory factors like chemokines, cytokines and growth factors, changes in the transcription of certain genes, upregulation of cytoskeletal proteins like GFAP and morphological changes (Figure 3) (Roybon et al. 2013; Allaman et al. 2011).

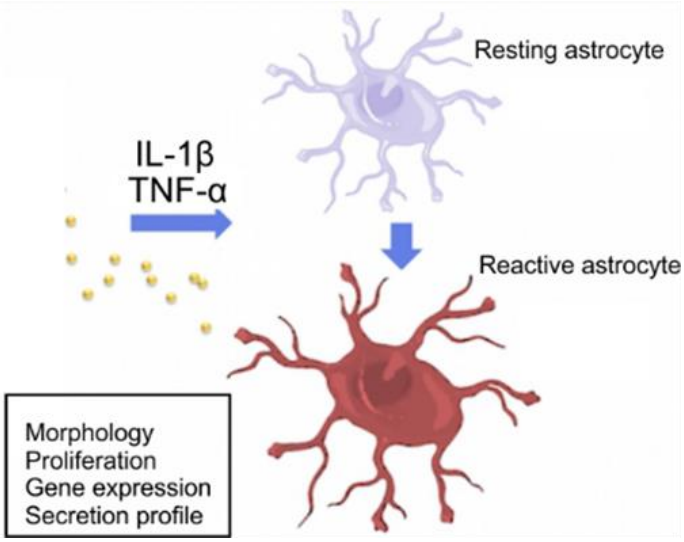


Figure 3. Illustration of resting astrocytes transforming into reactive astrocytes when encountering inflammatory cytokines, for example, IL-1β and TNF-α. Reactive astrocytes undergo different changes including morphological changes, increased proliferation and alterations in gene expression and secretion profile. Figure modified from https://www.brainpost.co/weekly-brainpost/2018/2/26/0q9qv2xnfvchnl47qjdy2un02l0h98.

Astrocytes undergo many functional and morphological alterations when transforming from resting quiescent state to the reactive phenotype. One of the characteristics for reactivation has been the morphological change, in which the cell body is hypertrophied (Ben Haim et al. 2015; Anderson et al. 2004). Related to this, the expression of cytoskeletal proteins, GFAP and vimentin, increases and astrocyte processes turn thicker and reach the injury site depending of the severity of the damage (Sofroniew & Vinters 2010). According to earlier study, this hypertrophy of cell bodies and astrocytic processes still does not increase their overlap over other astrocytic domains. Thus, the hypertrophied astrocytes still preserve their individual non-overlapping domain and do not occupy a greater tissue volume than non-reactive astrocytes. (Wilhelmsson et al. 2006) In addition to cellular hypertrophy, it is stated that reactive astrocytes increase their proliferation in the case of severe CNS trauma or disease (Robel et al. 2011; Sofroniew 2009). In mild or moderate astrocyte reactivation, proliferation is minimal or absent (Sofroniew 2009). It is suggested that the misinterpretation that reactive astrocytes would always be highly proliferative is explained with the increase of GFAP positive astrocytes after CNS injury (Liddelow & Barres 2017). In addition, the amount of GFAP+ astrocytes vary in different brain areas under normal conditions (Liddelow & Barres 2017). According to rodent studies modeling AD and ALS, the proliferative cell counts in reactive astrocytes were limited (0-7%) (Sirko et al. 2013; Lepore et al. 2008). The cells were stained with the cell proliferation marker Ki67. However, considerable proliferation in reactive astrocytes can be seen after CNS trauma

when the purpose is to generate a protective scar around the injury site (Anderson et al. 2016). Thus, there are some different interpretations related to proliferation in reactive astrocytes and it should be noted that more relevant human models are needed to study proliferation of reactive astrocytes in CNS injury.

Other commonly used hallmark for astrocyte reactivation has been the upregulation of intermediate filament proteins GFAP and in less emphasis, vimentin (Ben Haim et al. 2015; Pekny & Pekna 2014; Wilhelmsson et al. 2006). The concept of astrocyte reactivation truly emerged after the discovery of GFAP in 1971 (Eng et al. 1971) and since then, the use of upregulated GFAP as a marker for reactivation has strengthened. However, connection between GFAP and reactive astrocytes are based mainly on rodent studies and human pathological specimens after injury or disease in CNS (Liddelow & Barres 2017; Zamanian et al. 2012). Thus, GFAP is not the most straightforward marker for reactive astrocytes in humans since there are many morphological and functional differences between rodent and human astrocytes (Liddelow & Barres 2017; Zhang et al. 2016). In addition, astrocytes are very heterogeneous cells and express GFAP in different amounts (Anderson et al. 2004). Related to this, reactivation of astrocytes is heterogeneous phenomenon and the degree of severity varies along with the GFAP expression (Sofroniew 2009).

In normal conditions, astrocytes participate in many functions of CNS by secreting vast number of molecules and these secretion profiles are altered in reactive astrocytes (Sofroniew & Vinters 2017; Eddleston & Mucke 1993). Altered secretion includes changed production of gliotransmitters, trophic factors, antioxidants, ROS and inflammatory cytokines and chemokines (Ben Haim et al. 2015). Major inhibitory gliotransmitter in the mammalian brain is the gamma-aminobutyric acid (GABA), which has been shown to be produced aberrantly and abundantly by reactive astrocytes in mouse models of AD (Jo et al. 2014). Production of other gliotransmitters glutamate and ATP by reactive astrocytes has also been reported. Studies suggest that reactive astrocytes release more ATP than resting astrocytes and alter their glutamate release, which may induce neuronal hyperexcitability and excitotoxicity (Ben Haim et al. 2015; Agulhon et al. 2012). In addition, one of the main functions of astrocytes is to provide trophic support for neurons. Astrocytes do this by secreting trophic factors including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and FGFs (Haim et al. 2015). However, abnormal secretion of these trophic factors can be detrimental to neurons (Haim et al. 2015). According to rodent study modelling ALS, increased amounts of NGF released by reactive astrocytes had neurotoxic effects (Pehar et al. 2004). Normally astrocytes fight against oxidative stress and release many

antioxidants for neurons, such as glutathione and ascorbic acid (AA) (Allaman et al. 2011). However, it has suggested that reactive astrocytes may produce less AA and glutathione and increase oxidative stress to neurons by releasing ROS (Ben Haim et al. 2015). Inflammatory molecule secretion profile of reactive astrocytes is vastly altered (Choi et al. 2014; Zamanian et al. 2012; Sofroniew & Vinters 2010). Different studies have shown that different inflammatory molecules, such as TNF-α/β, IL-1β, IL-6, IL-8 (CXCL8), interferon gamma-induced protein 10 (IP-10, also known as CXCL10), macrophage inflammatory protein 1 α (MIP-1α, also known as CCL3) and Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES, also known as CCL5) are either newly produced or upregulated in reactive astrocytes (Choi et al. 2014; Dowell et al. 2009). These altered secretion profiles can either increase inflammatory reactions and tissue damage or promote immunosuppression and tissue repair (Colombo & Farina 2016). In addition, a study done with two mouse models, stroke and neuroinflammation, suggested that the altered gene expression profile in reactive astrocytes was dependent on the type of injury (Zamanian et al. 2012). Thus, the role of reactive astrocytes after CNS injury is still not clear and astrocytes can have both protective and detrimental roles.

2.1.3 The role of reactive astrocytes in neuroinflammation

In most if not all cases of CNS injuries and diseases there is inflammation in the CNS (Sofroniew 2015). Cells in the CNS can generate different inflammatory mediators, which in turn can recruit immune cells and activate glial cells (Lucas et al. 2006). Astrocytes are regarded as immunocompetent cells and they control immune cell activation and respond to danger signals by secreting different cytokines and chemokines (Colombo & Farina 2016; Sofroniew 2015). Thus, astrocytes have a key role in the activation of innate and adaptive immunity response after CNS injury or disease. Reactive astrocytes have been related to many CNS diseases, in which neuroinflammation plays a key role (Ben Haim et al. 2015; Sofroniew & Vinters 2010).

It has been discussed, whether the role of reactive astrocytes is protective or detrimental to the neural tissues after CNS injury (Liddelow & Barres 2017). It is suggested that the nature of astrocyte reactivation is dependent on the inflammatory milieu and different signaling pathways in astrocytes are activated by different stimuli (Colombo & Farina 2016; Zamanian et al. 2012). In a recent article, different signaling pathways in reactive astrocytes were categorized as protective or detrimental (Colombo & Farina 2016). Different supportive and detrimental factors were listed in the article, and couple of them are presented in the Figure 4. Supportive factors in the protective pathway included glycoprotein 130 (gp130) and signal transducer and activator of transcription 3 (STAT3), whereas IL-17 and nuclear factor κB (NF-κB) triggered the detrimental pathways (Figure 4). Both

transcription factors STAT3 and NF-κB are strongly related to reactivation of astrocytes (Colombo & Farina 2016; Ben Haim et al. 2015). STAT3 predominantly mediates cytokine signaling in cells and is involved in many functions including cell growth, proliferation, differentiation and inflammation (Ben Haim et al. 2015). STAT3 is activated by different growth factors and cytokines, including IL-6, IL-10, epidermal growth factor (EGF), TGF-α, LIF and CNTF. It is suggested that those are important in intercellular signaling after CNS injury in astrocytes. (Herrmann et al. 2008) For example, IL-6 has been shown to be upregulated in reactive astrocytes (Choi et al. 2014). IL-6 activates STAT3 by binding to the gp130-IL6 receptor (gp130-IL6R) (Figure 4), which is shown to be crucial for the glial cell survival and control of disease expression in rodent study (Ben Haim et al. 2015; Haroon et al. 2011). Another study with rodents suggests that STAT3 activation is implicated in axon regeneration after injury (Dominguez et al. 2010). These support the role of STAT3 as a protective pathway in astrocytes.

The other key transcription factor related to reactive astrocytes is NF-κB, which is commonly activated in any inflammation reaction (Colombo & Farina 2016; Shih et al. 2015). In addition, NF-κB is involved in many processes including immune response, cell division and apoptosis in almost all cell types (Mattson & Meffert 2006). NF-κB is ubiquitously present in the cell cytoplasm but localizes in the nucleus when activated (Shih et al. 2015). After activation, NF-κB can modulate the transcription of various genes and thereby regulate inflammation (Liu et al. 2017). Various agents can activate NF-κB, including lipopolysaccharides (LPS), IL-1β and TNF-α (Kaltschmidt et al. 2005). In addition, NF-κB pathway in reactive astrocytes can be induced by the binding of IL-17 to the IL-17 receptor (IL-17R) and it is regarded as detrimental pathway (Figure 4) (Colombo & Farina 2016; Meares et al. 2012). Binding of the IL-17 results recruitment of adaptor protein Act1, which is known as an activator of NF-κB (Qian et al. 2007). Recruitment of Act1 induces a downstream activation cascade which triggers the production of cytokines and chemokines (Colombo & Farina 2016). The activation of NF-κB is also related to many NDs, such as HD and PD, but the mechanisms underlying those are not totally clear (Ben Haim et al. 2015). According to studies, NF-κB was found activated in CNS cells including dopaminergic neurons of PD patients and peripheral immune cells in HD patients but not in glial cells (Trager et al. 2014; Hunot et al. 1997; Kaltschmidt et al. 1997). Thus, it is suggested that NF-κB activation is not essential for reactivation and more studies are needed to solve the link between NF-κB and reactive astrocytes (Ben Haim. 2015).

The dual role of reactive astrocytes has recently been studied *in vivo*. Rodent studies have shown that reactive astrocytes can be divided into two distinct subtypes termed A1 and A2 (Liddelow et al. 2017; Zamanian et al. 2012). Type A1 reactive astrocytes have detrimental effects on neurons and oligodendrocytes. These neurotoxic A1 astrocytes were shown to upregulate classical complement cascade genes which are shown to be destructive to synapses. By contrast, type A2 reactive astrocytes are shown to have protective features and promote CNS recovery after injury by producing neurotrophic factors. (Liddelow et al. 2017) It is strongly suggested that detrimental type A1 astrocytes are induced by NF-κB pathway and are linked to neuroinflammation (Liddelow & Barres 2017; Zamanian et al. 2012). Protective type A2 astrocytes in turn are suggested to be mediated by STAT3 pathway, which supports neuronal regeneration after acute trauma (Liddelow & Barres 2017; Anderson et al. 2016). This division into two reactive astrocyte subtypes raises questions like are there other reactive astrocyte subtypes and what are the signaling pathways leading to them. It is likely, that the newly found subtypes are part of a continuous spectrum of reactive profiles.

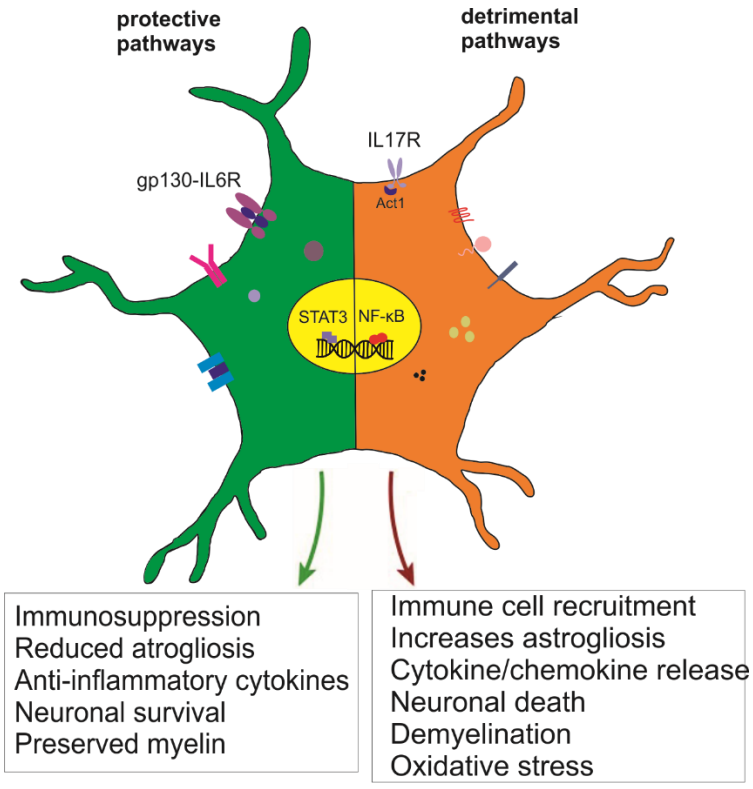


Figure 4. Impact of astrocyte reactivation on neuroinflammation. Two possible sides of reactive astrocytes in CNS recovery are presented; protective and detrimental. The figure shows couple of key transmembrane proteins, cytoplasmic proteins and transcription factors involved in astrocyte reactivation. Activation of STAT3 is related to protective pathway and NF-κB to detrimental. In the boxes are listed different protective and detrimental effects of the astrocyte reactivation. Abbreviations: Act1, adaptor protein 1; gp130-IL6R, glycoprotein 130/interleukin 6 receptor; IL17R, interleukin 17 receptor; NF-κB, nuclear factor κB; 3; STAT3, signal transducer and activator of transcription 3. Figure is redrawn based on figure from Colombo & Farina 2016.

2.2 Disease modelling with stem cells

Cells from CNS have been cultured *in vitro* for decades and these cultures have included cells from animals and primary human tissues from fetal or post-mortem origin. However, differences between species in molecular and cellular level hinders the value of studies done (Schnerch et al. 2010). In addition, the use of primary human tissues is not totally trouble-free due to limitations in the availability of tissues along with the ethical problems related to fetal material utilization (Quadrato et al. 2016; Cefalo et al. 1994). Over the past few decades the progression in the stem cell field have offered many new possibilities for neuronal studies. hPSCs can be used for differentiation of astrocytes and neurons and they provide abundant cell source (Shaltouki et al. 2013; Shi et al. 2012; Fortier 2005). This advance in the neural differentiation possibly enables the development of more defined model systems to study the mechanisms of different CNS diseases and injuries.

2.2.1 Human pluripotent stem cells

hPSC are defined by their capability to renew themselves infinitely and they can form all three germ layers; endoderm, ectoderm and mesoderm. In that sense, they can differentiate into almost any cell type of the human body, excluding the extra-embryonic tissues such as the placenta. (Wilhelmsson et al. 2006) hPSCs can be divided into human embryonic stem cells (hESCs) and hiPSCs. hESCs are pluripotent stem cells derived from the inner cell mass of a blastocyst, an early stage embryo before implantation. Usually hESCs are derived from donated blastocysts, which could not be used in infertility treatments (Thomson et al. 1998). hiPSCs were first introduced in 2007 by Shinya Yamanaka's research group and since then their utilization and potential for different research areas has been growing. hiPSCs are usually derived from human adult dermal fibroblast, but in theory, they could be generated basically from any somatic cell type (Takahashi et al. 2007). The stem cells are transduced with four transcription factors: octamer-binding transcription factor 3/4 *(Oct3/4),* SRY-box 2 *(Sox2),* Kruppel-like factor 4 *(Klf4),* and *V-Myc* avian myelocytomatosis viral oncogene homolog *(c-Myc)* (Takahashi et al. 2007). These are also called as "Yamanaka factors" and are extensively used nowadays (Gonzalez et al. 2009). However, several new combinations of reprogramming factors and methods have been established since the finding of "Yamanaka factors" (Singh et al 2015). Compared to hESCs, hiPSCs are more ethical cell source since they can be generated from the patient's own somatic cells and reprogram them into embryonic/pluripotent state and there is no need for embryos as in with hESCs.

2.2.2 Neuronal differentiation from human pluripotent stem cells

Neuronal differentiation from hPSCs was first described in 2001 and since then, several differentiation protocols deriving neurons from hPSCs have emerged, utlizing both hESCs and hiPSCs as a source (Pasca et al. 2015; Salimi et al. 2014; Shi et al. 2012; Chambers et al. 2009; Carpenter et al. 2001; Reubinoff et al. 2001; Zhang et al. 2001). Some of the first protocols utilized animal-derived substances such as mouse embryonic fibroblast feeder layer or conditioned medium (Carpenter et al. 2001; Reubinoff et al. 2001; Zhang et al. 2001). In addition, many of these first differentiation protocols used the spontaneous embryoid body (EB) formation followed by adherent monolayer cell culturing with neural differentiation medium. EBs are 3D aggregates of pluripotent stem cells and can differentiate to all cell lineages, including neural lineages (Liyang et al. 2014). After the EB formation step, neural lineage cells form rosette structures. This formation resembles the in *vivo* development of the neural tube. (Mertens et al. 2016; Lappalainen at el. 2010) Rosette structures are radially arranged and comprise neural stem cells (NCS), which usually express early neuroectodermal markers Pax-6 and Sox1 (Elkabetz et al. 2008). Rosettes can be selectively isolated and cultured for further differentiation (Muratore et al. 2014). Differentiation protocols based on the adherent monolayer cultures usually comprise homogeneous composition of NSCs (Conti & Cattaneo 2010).

Besides the adherent monolayer culture, 3D cell aggregates can be cultured in free floating suspension cultures called neurosphere systems (Figure 5) (Conti & Cattaneo 2010). Neurosphere method relies on the dissociation of hPSCs and exposure to mitogens (Nat et al. 2007). The NCSs should respond to the mitogens, divide and form floating aggregates called primary neurospheres. These neurospheres can be further dissociated and re-plated to expand the NCS population. (Conti & Cattaneo 2010) Neurosphere culturing method results more heterogenous cell populations with only a portion of the cells exhibiting NSC properties (Conti & Cattaneo 2010). Both adherent monolayer and 3D neurosphere method are highly variated and new protocols have emerged based on them. In 2009, new neuron differentiation method based on the adherent monolayer culture was presented (Chambers et al. 2009). It was called dual SMAD inhibition method and it was based on the inhibition of BMP and TGF-β signaling pathways with Noggin and small molecule SB43154 (Muratore et al. 2014). After introduction on dual SMAD inhibition, many new protocols based on that have been developed and it has been combined with EB formation (Pasca et al. 2015; Yuan et al. 2015; Muratore et al. 2014; Patani et al. 2012). Moreover, efficient differentiation protocols using xeno-free mediums and defined conditions have emerged (Yuan et al. 2015; Swistowski et al. 2009).

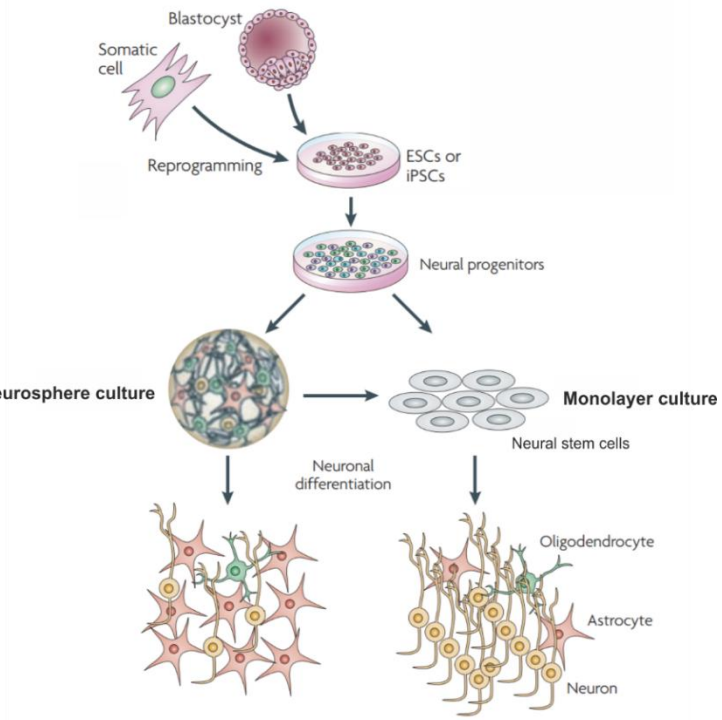


Figure 5. Simplified illustration of differentiation of ESCs and iPSCs towards neural lineage cells via neurosphere and monolayer cultures. As a result, mixtures of neurons, oligodendrocytes and astrocytes are formed. Figure modified from Conti & Cattaneo 2010.

2.2.3 Astrocyte differentiation from human pluripotent stem cells

Differentiation of hPSCs to astrocytic lineage cells is relatively new field. In the first differentiation protocols, generated astrocytes were mainly side products of neuronal differentiation (Ruiz et al. 2010; Johnson et al. 2007; Itsykson et al. 2005; Tabar et al. 2005; Zhang et al. 2001). The results were not pure astrocyte populations and the methods in differentiation protocols varied (Krencik & Zhang 2011). Directed differentiation of hPCSs to astrocytes had not been presented until 2011 by Krencik and colleagues (Krencik et al. 2011). Their differentiation protocol resulted immature astrocytes strongly positive for astrocyte markers GFAP and S100β approximately in 180 days. In their protocol, hPSCs were induced into neuroepithelial cells, which further were patterned to regional progenitors by morphogens such as RA, FGF and SHH. The formed regional neural progenitors in rosettes were further expanded in suspension culture with EGF and FGF2. Neural progenitors in the suspension cultures formed spheres, which were disaggregated to reduce the cell contact and to promote gliogenesis instead of neurogenesis. Gliogenesis is the developmental process by which glial cells are formed whereas neurogenesis creates neurons (Sugimori et al. 2007). The final astrocyte differentiation in Krenrick and colleagues' protocol was performed by exposing the progenitor spheres to CNTF (Krencik et al. 2011). Later astrocyte differentiation protocols have partly utilized the same steps, but with some additional components like AA, BDNF and BMP (Chandrasekaran et al. 2016).

In 2013, more rapid astrocyte differentiation protocol was introduced using hPSC-derived NSCs (Shaltouki et al. 2013). Their differentiation protocol used defined xeno-free medium and strongly GFAP positive astrocyte population was generated in 5-6 weeks. The protocol was based on EBs, and formed neural rosettes were isolated and further cultured to get homogenous population of NSCs (Chandrasekaran et al. 2016). Astrocytes were differentiated from NSCs via intermediate precursors that expressed CD44 (Shaltouki et al. 2013). Intermediate precursor cells expressing CD44 are suggested to be restricted to mature into astrocytes (Liu et al. 2004). Shaltouki and colleagues showed that they can direct the differentiation of CD44+ intermediate precursors to astrocytes with BMP and CNTF. They also used FGF2 for differentiation (Shaltouki et al. 2013). Generated astrocytes showed morphological characteristics and functional properties similar to primary astrocytes. In year 2013, another new astrocyte differentiation article using hiPSCs was published, and instead of EB formation they used monolayer method with dual SMAD inhibition (Roybon et al. 2013). They showed that short exposures of FGF1 or FGF2 after early neuralization can induce a mature, quiescent astrocyte phenotype. However, earlier studies have shown different effects of FGFs to astrocyte development. Some studies have shown that FGFs induced mitosis, gap junction coupling or even de-differentiation, but not maturation (Goldshmit et al. 2012; Garre et al. 2010; Lin & Goldman 2009). Thus, the exact role of FGFs in astrocyte differentiation is not clear (Chandrasekaran et al. 2016).

In the past few years, new astrocyte differentiation protocols using hiPSCs have emerged, indicating the growing popularity of utilizing hiPSCs in research (Lundin et al. 2018; Perriot et al. 2018; Santos et al. 2017; TCW et al. 2017). Despite of the many existing astrocyte differentiation protocols, there are no golden standard for generating pure astrocyte population robustly. Generating an efficient differentiation protocol is challenged by the insufficient knowledge of astrocyte specification during development and in adult CNS (Santos et al. 2017). In addition, the heterogeneous nature of astrocytes makes it more complex to evaluate the differentiation results.

2.3 Inducing reactive astrocyte phenotype *in vitro*

In recent years, reactive astrocytes and their role in different CNS injuries and diseases has evoked researchers' interest. Studies with reactive astrocytes have mainly been done with rodents whereas reactivation in human astrocytes has been studied less (Liddelow et al. 2017; Roybon et al 2013; Zamanian et al. 2012). However, many structural and functional differences between rodent and human astrocytes have been discovered (Zhang et al. 2016; Oberheim et al. 2009). This emphasizes the importance of gathering more knowledge with human astrocytes to characterize their reactivation. Most of the information about human astrocyte reactivation is based on studies with human fetal or primary adult tissues and astrocytoma cell lines (Fan et al. 2016; Zhang et al. 2016; Malik et al. 2014). However, ethical issues related to human fetal tissue utilization and availability in primary adult tissues limit the studies using the mentioned (Quadrato et al. 2016; Cefalo et al. 1994). Protocols using hiPSC-derived astrocytes have recently been established and their potential in reactive astrocyte studies has been evaluated (Lundin et al. 2018; Perriot et al. 2018; Santos et al. 2017).

Table 1 collects together some of the studies, in which astrocyte reactivation is induced with inflammatory environment in rodent and human astrocytes from different sources including hESCs, hiPSCs, immortalized cell lines and primary tissues. In different studies, reactivation is mainly induced with IL-1α/β, TNF-α, and LPS (Lundin et al. 2018; Ronco et al. 2014; Roybon et al. 2013), but also other inflammatory inducers have been used, such as Aβ, IL-6, and complement component 1q (C1q) (Perriot et al. 2018; Liddelow et al. 2017). There are differences in the used concentrations and combinations of these pro-inflammatory mediators, thus there are no clear consensus in the method for reactivation. In addition, some of the reactivation studies used fetal bovine serum (FBS) in the astrocyte culture (Santos et al. 2017; Ronco et al. 2014; Choi et al. 2013; Roybon et al. 2013) and some did not (Lundin et al. 2018; Perriot et al. 2018; Liddelow at el. 2017). The use of FBS in astrocyte medium *in vitro* has shown to induce irreversible reactive changes in astrocytes, thus it might bias the results. Perriot and colleagues tested the treatment of hiPSC-derived astrocytes with FBS and indeed find out that it can cause the same effects than pro-inflammatory cytokines, such as TNF-α (Perriot et al. 2018).

Despite the versatile protocols, the results for reactive astrocyte characterization between the studies share many similarities. Gene expression analysis was often performed to detect the possibly altered expression of inflammatory cytokines, chemokines and growth factors. To name a few, upregulation of IL-6 and IL-8 were seen in many studies (Table 1) (Lundin et al. 2018; Santos et al. 2017; Roybon et al. 2013) and expression of IP-10 was also highlighted (Choi et al. 2013; van Kralingen et al. 2013). The common results usually stated that the stimulated astrocytes were immunocompetent, thus

suggesting their possible reactivation. In addition, upregulation of GFAP has been one of the hallmarks for astrocyte reactivation, but its upregulation has not been shown in hPSC-derived astrocytes after treating them with inflammatory cytokines. Roybon and colleagues showed that treatment with TNF-α in fact decreased GFAP expression compared to FBS control in hPSC-derived astrocytes (Roybon et al. 2013). Furthermore, reactivation study with rodents showed that treatment with IL-1β, TNF-α and LPS also decreased GFAP expression (Table 1) (Ronco et al. 2014). Studies using hiPSC-derived astrocytes did not study GFAP upregulation after treatment with inflammatory cytokines (Lundin et al. 2018; Perriot et al. 2018; Santos et al. 2017). There were some differences when characterizing the astrocytes after exposing them to inflammatory environment. In general, studies did not show damaging effects on astrocytes after inflammatory cytokine treatments. However, one study showed detrimental effects on astrocytes after 96h treatment with IL-1β and TNF-α (Table 1) (van Kralingen et al. 2013). Astrocytes were compromised and finally cytokine treatments resulted death of astrocytes. These results regarding astrocyte reactivation characterization imply that astrocytes are heterogeneous cells and the reactive phenotype is mediated by variety of factors. The reactivation is dependent on different things including the source and subtypes of the astrocytes, the cytokine exposure time and the dose of inflammatory stimulus.

Table 1. The most relevant and recent astrocyte reactivation studies collected together.

Reference	Origin	Stimulants	Stimulation time	Reactive astrocytes characterization
Lundin et al. 2018	Human primary astrocytes Astrocytoma cell line hiPSC-derived astrocytes	IL-1β (10 and 50 ng/ml) or TNF-α (10 and 50 ng/ml)	24h & 48h	IL-1β and TNF-α stimulation induced IL-6 and IL-8 production in all cell models, especially hiPCS-derived astrocytes were immunocompetent
Perriot et al. 2018	hiPSC-derived astrocytes	IL-1β (10 ng/ml and/or TNF-α (10 ng/ml) or IL-6 (100 ng/mL)	5 days	TNF-α induces transcriptomic changes, IL-6 secretion and expression of MHCI molecules; TNF-α/IL-1β may have a dual effect on astrocytes; pro-inflammatory and pro-remyelination
Santos et al. 2017	hiPSC-derived astrocytes hESC-derived astrocytes Human primary astrocytes	IL-1β (10 ng/ml) or TNF-α (50 ng/ml)	5h & 24h	hiPSC-derived astrocytes were immunocompetent after cytokine stimulation; IL-6 and IL-8 upregulated in treated astrocytes; IL-1β stimulated astrocytes reduce on neuron viability and dendrite length in co-cultures; Modified transcriptomic inflammatory signature
Liddelow et al. 2017	Rodent	IL-1α (3 ng /ml); TNF-α (30 ng /ml); C1q (400 ng/ml)	24h	Neurotoxic A1 reactive phenotype is induced with Il-1α, TNFα, and C1q secreted by activated microglia; Neurotoxic A1 phenotype lose normal astrocyte functions
Ronco et al. 2014	Rodent	IL-1β (10 ng/ml) or TNF-α (20 ng/ml) or LPS (100 ng/ml) or amyloid-β 100 nM	24h & 72h	IL-1β, TNF-α and LPS decreased GFAP and vimentin expression; Proinflammatory cytokines altered calcium signaling
Roybon et al. 2013	Human and rodent ESCs and hiPSCs	IL-1β (10 ng/ml) or TNF-α (50 ng/ml)	7 days	Upregulation of IL8, CCL5, IL-6 and Lcn2 after stimulations; TNF-α induces astrocyte phenotype with high GFAP and production of inflammatory chemokines and cytokines
Choi et al. 2013	Human fetal astrocytes	IL-1β (10 ng/ml); TNF-α (10 ng/ml)	24h	Altered expression of many cytokines/chemokines, which could induce neurotoxic and neuroprotective responses in the CNS
van Kralingen et al. 2013	Ntera2/D1 cell line from primary embryonic carcinoma, differentiated to astrocytes	IL-1β (5 ng/ml) or TNF-α (5 ng/ml)	96h	IL-1β and TNF-α induced production of inflammatory mediators including IL-6 and IP-10; IL-1β and TNFα induced apoptosis and finally, astrocyte death

CCL5 = Chemokine (C-C motif) ligand 5, C1q = Complement component 1q, GFAP = Glial fibrillary acidic protein, hESC = Human embryonic stem cell, hiPSC = Human induced pluripotent stem cell, IP-10 = Interferon gamma-induced protein 10, Il-1β = Interleukin 1β, IL-6 = Interleukin 6, IL-8 = Interleukin 8, Lcn2 = Lipocalin-2, LPS = Lipopolysaccharide, MHC = Major histocompatibility complex, TNF-α = Tumor necrosis factor α

2.4 Co-cultures of neurons and astrocytes

The mechanisms underlying the development and maintenance of neural networks in the human brain remain still poorly understood. There are still unanswered questions in the basic functions of neurons and the complex pathological mechanisms of different CNS diseases. Different *in vitro* models for neurons and their networks could offer more knowledge of their function. However, neurons are not alone in the human brain, but instead form complex networks with other CNS cells. Especially astrocytes are strongly involved in the bidirectional crosstalk with neurons and emerging evidence has shown their role in the pathophysiology of neuronal disorders (Ricci et al. 2009). In the past decade, the interest towards neuron and glial interactions have increased and different co-culture studies have emerged (Gao et al. 2016).

Classical approach for studying neuron and astrocyte interactions includes suspension of embryonic rat neurons over a feeder layer of rat astrocytes (Kaech & Banker 2006). In addition, rat astrocytes have been effectively co-cultured with hiPSC-derived neurons in multi-electrode array (MEA) systems (Odawara et al. 2014). The aim in those was to investigate the long-term electrophysical activity of neurons and drug responsiveness effects. However, rodent models might not be suitable for mirroring results to humans since human astrocyte and neurons have unique gene expression, morphology and functions (Xu et al. 2018; Oberheim et al. 2009). In a recent study, human astrocytes and neurons derived from hPSCs were assembled in co-culture systems utilizing 3D organoid-like spheres (Krencik et al. 2017). There are limitations in these kinds of conventional randomly mixed co-cultures with neurons and glial cells. The physical and biochemical environments in culture cannot be manipulated and controlled properly. In addition, the investigation of localized interactions between axons and glial cells is difficult. (Park et al. 2012) To answer these limitations, co-culture platforms for modeling CNS diseases and injuries using microfluidic technologies have recently been assembled (Park et al. 2018; Shi et al. 2013; Taylor et al. 2005).

2.4.1 Microfluidic devices

The development of engineered microfluidic devices have created new possibilities for biochemically and physically controlled microenvironment for cell culturing (Taylor et al. 2005). Microfluidic devices are microfabricated devices that can manipulate the fluid flow in the microscale level (Gross et al. 2007). Usually this fluid processing is done via series of channels and chambers in which the fluid flow can be controlled. Traditionally, these microfluidic devices can be molded in silicone-type elastomer, polydimethylsiloxane (PDMS), using soft lithography (Park et al. 2009). There are many benefits in PDMS; easy fabrication and sterilization, biocompatibility, permeability to gases and ability to be permanently bonded to glass (Gross et al. 2007; Taylor et al. 2006). However, the field

of microfluidics is developing, and new methods and materials have also been evaluated. The use of nanoscale structures like nanotubes and fibers in the microfluidic devices have suggested to gain potential in the future (Boquet & Tabeling 2014; Soe et al. 2012). In addition, the utilization of 3D printing in fabrication of polymer microfluidic devices has gained interest (Waheed et al. 2016). 3D printing could offer some three-dimensional fabrication that is not possible with current methods (Au et al. 2016). However, 3D printing cannot currently compete with the conventional lithography methods used in microfluidic device fabrications.

Microfluidic devices have established their place in the field of neuroscience, since the devices offer advantages for both spatial and temporal manipulation of microenvironment (Yi & Lin 2017; Jadhav et al. 2016). This kind of precise and controlled manipulation of the extracellular environment is necessary for the study of axonal injury and regeneration in different neurodegenerative diseases (Jadhav et al. 2016). In addition, compartmentalized microfluidic devices allows the isolation of neurites, which allows controlled study of subcellular parts of neurons (Jadhav et al. 2016). In 2003, Taylor and colleagues described a microfluidic multicompartment device, which can be used in neurological research studying axonal injuries, myelination and drug testing (Figure 6A) (Taylor et al. 2003). The device is composed of two compartments (axonal and soma side) separated by micron-size grooves, which allows the neurites to grow between the two compartments. Somas are restricted from the axons in their own compartments. The original platform or modified versions of this microfluidic device have been used in different applications after it was first presented (Southam et al. 2013; Lee et al. 2012; Kanagasabapathi et al. 2011). In a recent study, 3D *in vitro* tri-culture system modeling AD used neurons, astrocytes and microglial cells (Park et al. 2018). They used microfluidic device containing two separate compartments; astrocytes and neurons in the central compartment and microglial in the angular compartment (Figure 6B). These two compartments were linked with migration channels. Their model platform included the key features of AD; Aβ aggregation, phosphorylated tau accumulation and neuroinflammation. They presented that their model is in the right path towards more precise and controlled human brain disease models. However, *in vivo* studies are necessary to test the physiological utility of these kinds of models.

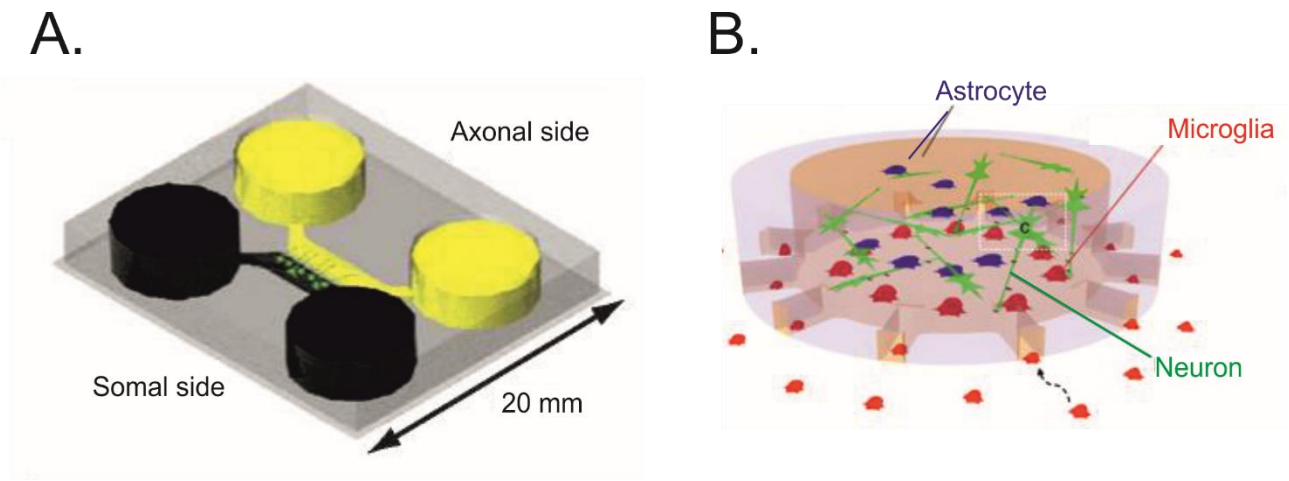


Figure 6. (A) Original model of Taylor and colleagues' multicompartment microfluidic device. Somal (black) and axonal sides (yellow) are separated in two compartments, which are connected by microgrooves. Neurons are plated in the somal side and they are drawn into the somal channel by capillary effect. The axonal growth is guided to the axonal side through the microgrooves. Modified from the figure in Taylor et al. 2005. (B) 3D tri-culture microfluidic platform of Park et colleagues. The platform mimics the in vivo *environment of Alzheimer's disease. Astrocytes and neurons are in mixed cultures in the central compartment and the microglia are in the angular compartment. These two compartments are linked together with migration channels. Modified from the figure in Park et al. 2018.*

3 Aims of the study

The general aim of this thesis project was to induce the reactive phenotype in hiPSC-derived astrocytes and characterize it. In addition, to establish astrocyte and neuron co-cultures utilizing in-house developed microfluidic devices. This general aim can be divided into smaller entities.

First, the aim was to induce the reactive phenotype in hiPSC-derived astrocytes with inflammatory cytokines and characterize the reactivated astrocytes. One major part was to optimize western blot -method for detecting GFAP expression from astrocytes. Characterization also included studying the inflammatory nature of reactive phenotype of astrocytes.

Other aim of this project was to optimize the astrocyte and neuron co-cultures in microfluidic devices and induce the reactive phenotype of astrocytes in those. It was important to optimize the coating conditions for both cells in the devices and to see if they both grow well in those. Overall aim of these co-cultures was to study the potential of these microfluidic devices for modeling different neurological diseases.

4 Materials and methods

4.1 Human pluripotent stem cell culture

The used cell line was hiPSC line 10212.EURCCs. The cell line was derived at Faculty of Medicine and Health Technologies, Tampere University, Finland. Faculty of Medicine and Health Technologies has approval from the Finnish Medicines Agency (FIMEA) to conduct human stem cell research (Dnro 1426/32/300/05) and supportive statements from the regional ethics committee of Pirkanmaa Hospital District for the derivation, culture, and differentiation of hiPSCs (R08070). The hiPSC line was expanded in feeder-free culture as described earlier (Hongisto et al 2017).

4.1.1 Neuronal differentiation

The differentiation protocol from hiPSCs to neurons is modified in-house from Shi et al. (2012). Neurons were differentiated and cultured in neural maintenance medium (NMM) which is described in Table 2. Cells were passaged using Accutase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and plated with medium containing 10 µM ROCK inhibitor (Sigma Aldrich, Saint Louis, Missouri, USA). Plates were coated with 100 µg/ml Poly-L-ornithine (PO, Sigma Aldrich) in 0.1 M Borate buffer and 15 µg/ml human recombinant laminin (LN521, Biolamina, Sundyberg, Sweden) in phosphate buffered saline (PBS) containing magnesium and calcium. First 12 days the cells were cultured using NMM with neural induction supplements 100 nM LDN (Sigma Aldrich) and 10 µM SB431542 (Sigma Aldrich). In the day 13, neural proliferation was started culturing cells in NMM with 20 ng/ml FGF-2 (R&D Systems, Minneapolis, Minnesota, USA). In final maturation phase after day 26, the cells were cultured in NMM supplemented with 20 ng/ml BDNF (R&D Systems), 10 mg/ml glial-derived neurotrophic factor (GDNF, R&D Systems), 500 µM dibutyryl-cyclicAMP (db-cAMP, Sigma Aldrich) and 200µM AA (Sigma Aldrich). At day 32, the cells were ready for final plating and experiments. Cell density on the plastic and glass was 50 000 cells/cm^2 and two different coatings were used; PO with LN521 and Matrigel Matrix (Corning, New York, USA). For glass, PO+LN521 was 30 µg/ml and for plastic, 15 µg/ml. Matrigel was diluted in NMM in 1/100 dilution. During culturing, the cells were kept in an incubator at +37 °C in 5% CO_2 atmosphere and 95% humidity and medium was changed every two or three days.

Table 2. Components of the neural maintenance medium.

Component	Final Conc.	Supplier
D-MEM/F-12 with Glutamax medium	0.5x	Thermo Fisher Scientific 31331-028
Neurobasal	0.5x	Thermo Fisher Scientific 21103-049
N2	0.5%	Thermo Fisher Scientific 17502-048
B27 with RA	1%	Thermo Fisher Scientific 17504-044
GlutaMAX™-I Supplement	0.5 mM	Thermo Fisher Scientific 35050-061
Non-essential Amino Acid Solution (NEA)	0.5%	Thermo Fisher Scientific 11140-050
2-mercaptoethanol	50 µM	Thermo Fisher Scientific 31350-010
Insulin	2.5 µg/ml	Sigma I9278
Pen/Strep antibiotic	0.1%	Lonza DE-602E

4.1.2 Astrocyte differentiation

The used cells were commercial human iPSC-derived astrocyte progenitors (cat. ax0083) derived from healthy donor iPSCs (Axol Bioscience, Cambridge, UK). The cell culture was done according to the manufacturer's protocol, which is based on the differentiation article (Shaltouki et al. 2013). Cell thawing from cryopreserved, pre-differentiated cells was done according to the protocol, and after astrocyte progenitor expansion, the cells could be frozen again for later use in experiments. Astrocyte progenitors were cryopreserved after day 14 of differentiation with 10% DMSO in culture medium. After thawing, the cells were plated on 6-well plate, which was coated day before with Matrigel in 1/100 dilution. Plating density was 100 000 cells/cm^2. Cells were cultured for six days in astrocyte basal medium containing three different astrocyte medium supplements. Together astrocyte basal medium and supplements make Astrocyte Maintenance Medium (AMM). In addition, 0.1% penicillin/streptomycin antibiotic solution (Lonza, Basel, Switzerland) was added to the medium. On day 7, the cells were passaged to Matrigel coated dishes with 80 000 cells/cm^2 density. The cells were cultured one more week and on the day 14, the final plating of the cells was done in the 100 000 cells/cm^2 density. Cells were cultured for additional three days in AMM to achieve fully mature astrocyte phenotype. After day 17, the cells were ready for experiments. AMM was changed every two days. During culturing and maturation, the cells were maintained in an incubator at +37 °C in 5% CO_2 atmosphere and 95% humidity.

4.2 Experimental design

This study is based on two different experimental set-ups (Figure 7A and 7B). First, astrocytes were treated with cytokines for seven days in free cultures and different analysis and sample collection was performed in different time points (Figure 7A). After seven days, cytokines were removed (wash-out) from the cells and the cells were cultured without cytokines for two days until the 9-day (9d) time point (Figure 7A). In the second experiment, astrocytes and neurons were plated in microfluidic devices. Three days after plating astrocytes were treated with cytokines for 72h (Figure 7B). Cells were fixed after 72 hours. During the experiments, cells were observed and imaged with phase-contrast microscope.

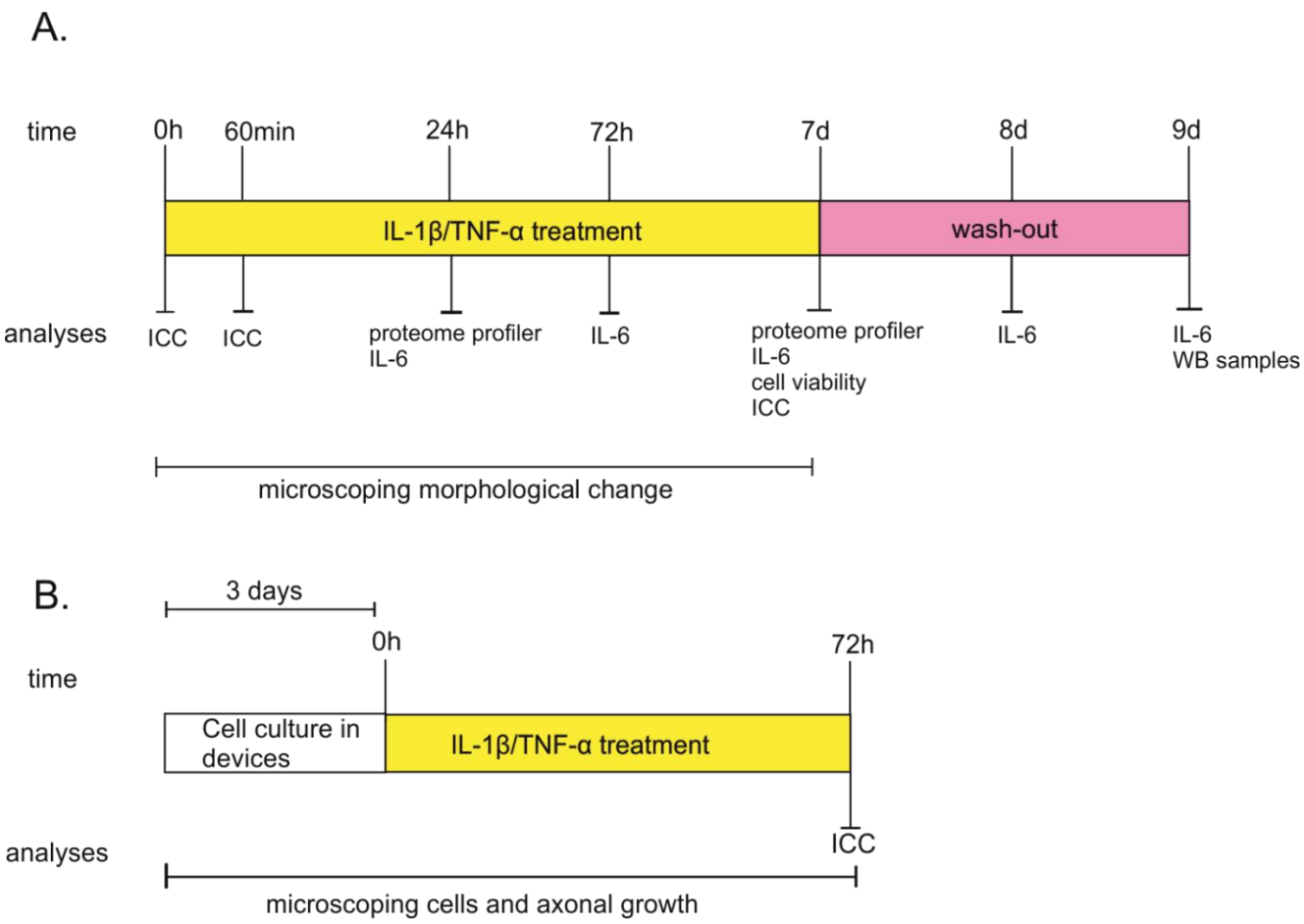


Figure 7. Experimental set-up presented in two timelines. (A) Experiment with astrocytes in free cultures treated with IL-1β and TNF-α for 7 days. Different time points and performed analyses are pointed. (B) Experiment with astrocyte and neuron co-cultures in microfluidic devices. Both cells were cultured for three days in co-cultures before astrocytes were treated with IL-1β and TNF-α for 24h or 72h. Abbreviations: IL-6, interleukin 6; ICC, immunocytochemistry; WB, Western Blot.

4.3 Inducing inflammatory phenotype in astrocytes

Inflammatory environment was created by exposing astrocytes to human recombinant TNF-α (10 ng/ml, PeproTech, New Jersey, USA) and human recombinant IL-1β (10 ng/ml, PeproTech) for seven days. Medium was changed each day for the treated and control cells. The same cytokine treatment was repeated twice with two different astrocyte differentiations. The control and the cytokine treated cells were imaged every day with Zeiss Axio Vert.A1 phase contrast microscope (Carl Zeiss AG, Germany, Oberkochen) to detect the possible morphological change.

4.4 Analysis of protein secretion using ELISA and cytokine array

Medium samples were collected from control and cytokine-treated astrocytes by removing the medium to a falcon tube and centrifuging it for five minutes in 400×g in room temperature (RT) and collecting the supernatant. Samples were kept in -80 °C before use. First, cytokine secretion from astrocyte medium samples was detected using Proteome Profiler Human XL Cytokine Array Kit (Figure 7A, R&D Systems) which is a membrane-based sandwich immunoassay for detecting 105 different inflammatory factors in one sample. Reagent preparation was done according to manufacturer's protocol. Membranes were first blocked for one hour in 4-well multi-dish in a shaker, thereafter membranes were incubated with diluted medium samples O/N +4 °C on a rocking platform shaker. Membranes were washed 3×10 minutes in separate plastic containers and placed back to 4-well Multi-dish with detection antibody cocktail from the kit. Membranes were incubated for one hour in a rocking platform shaker and then washed 3×10 minutes. Membranes were incubated for 30 minutes in 4-Well Multi-dish with Streptavidin-HRP and washed again 3×10 minutes and then placed on the bottom sheet of the plastic sheet protector. Chemiluminescence reagent was added and incubated for one minute. Membranes were imaged with ChemiDoc XRS+ System (Bio-Rad, California, USA).

To characterize the inflammation in more detail, IL-6 detection was performed from astrocyte medium samples with human IL-6 uncoated ELISA kit (Thermo Fisher Scientific). ELISA is enzyme-linked immunosorbent assay designed for detecting and quantifying different substances and in this case, IL-6 from cell medium. Medium samples for this assay were collected in different time points from cytokine treated and control astrocytes (Figure 7A). First, Corning™ Costar™ 96-well plate was coated O/N +4 °C. Wells were washed with washing buffer (1X PBS, 0.05% Tween) with Wellwash™ Microplate Washer (Thermo Fischer Scientific) three times before incubating blocking solution for one hour in RT. In the meantime, samples were diluted in NMM in 1/40 dilution as a result from earlier sample dilution optimizations. Wells were washed, and standard (2-200 pg/mL range) was prepared and added to the wells according to the protocol. Standard was recombinant

human IL-6 and used for generating standard curve and calibrating samples. In addition, diluted samples were added to the well plate with replicates and plate was incubated O/N +4 °C. Plate was washed three times as described earlier and detection antibody was added on the wells and incubated for one hour in RT. Washings were repeated and Streptavidin-HRP was added on the wells and incubated for 30 minutes in RT. Wells were washed six times and substrate solution was incubated for 15 minutes until 1M H_2SO_4 solution was added in each well to stop the reaction. Plate was imaged with Wallac Victor 1420 (PerkinElmer, Waltham, Massachusetts USA) at 450 nm wavelength and IL-6 concentrations were detected according to the standards.

4.5 Cell viability

The viability of the astrocytes after the 7-day cytokine treatment was analyzed from treated cells compared to controls with ApoTox-Glo Triplex Assay (Promega, Madison, Wisconsin, USA), CyQuant assay (Thermo Fisher Scientific) and microscoping the cells (Figure 7A). ApoTox-Glo Triplex assay combines three different assays to measure viability, cytotoxicity and apoptosis. In ApoTox-Glo Triplex Assay, the cells were plated in 96-well plate and cultured and treated with cytokines as presented in chapter 4.2. For positive controls, cells were also treated with 1.25 µM staurosporine (Sigma Aldrich) and 25 µM ionimycin (Sigma Aldrich) for six hours before using the kit. Staurosporine is known to induce apoptosis in cells and ionimycin is toxic to cells and causes necrosis. Besides of positive controls, no-cell condition with only cell medium served as a negative control and as a comparison to cytokine treated cells untreated cells were also used in this assay. All four conditions were run as triplicates. The experiment was performed as in the protocol. Two different substrates (GF-AFC Substrate and bis-AAF-R110) for viability/cytotoxicity were combined with Assay buffer to make Viability/Cytotoxicity reagent and for apoptosis detection the Caspase-Glo® 3/7 Substrate was combined with Caspase-Glo® 3/7 Buffer to make Caspase-Glo® 3/7 reagent. To all wells, 20 µl Viability/Cytotoxicity reagent was added and incubated 30 min in 37 °C after which fluorescence was measured. For the apoptosis detection, 100 µl Caspase-Glo® 3/7 reagent was added and incubated 30 min in 37 °C and luminescence was measured. Fluorescence and luminescence values were measured with Wallac Victor 1420. For viability/cytotoxicity measurement, excitation at 400 nm and emission at 505 nm were used and for cytotoxicity, the values were 485 nm (excitation) and 520 nm (emission).

CyQuant Cell Proliferation Assay Kit (Thermo Fischer Scientific) was also used to analyze cell viability. It is a fluorescence-based method for quantifying amount of DNA and therefore it reflects on the cell number in cell culture. The detection is based on CyQUANT® GR, a green dye, which exhibits strong fluorescence enhancement when bound to nucleic acids. In this assay, cell amount in three wells with control astrocytes and three wells with cytokine treated astrocytes was compared. Assay started with washing the cells in 96-well plate with PBS and adding 60 µl 0.1% Triton-X to lyse the cells and after that the plate was stored -70 °C. On the next day, the cell lysates were collected in Eppendorf tubes and spinned quickly before starting the measurement. Needed volume of working solution including CyQUANT® GR dye, Cell lysis buffer and H_2O was calculated and 180 µl of working solution was added on 20 µl cell lysates in 96-well plate and protected from light. Fluorescence was measured with Wallac Viktor 1420 with 480 nm excitation and 520 nm emission.

4.6 Western blot

Sample preparation. Astrocyte samples for western blot (WB) were collected from 6-well plates after nine days in culture (Figure 7A). Cells were washed with PBS and lysed to 2x Laemmli Sample Buffer (Table 3) and the cell lysis was collected in Eppendorf tubes, which were heated five minutes in 95 ˚C before stored in -80 ˚C.

Protein quantification. To load equal amounts of protein in the wells, the protein concentrations of the samples were measured just before carrying out the WB analysis. Protein quantification was done using Pierce 660 nm Protein Assay (Thermo Fischer Scientific), which is a colorimetric method for total protein quantitation. Samples were collected in Laemmli sample buffer containing bromophenol blue, which normally would interfere fluorescence-based protein assays and bias the result. For making this assay compatible with Laemmli sample buffer, Ionic Detergent Compatibility Reagent (IDCR) (Thermo Fischer Scientific) was added to Pierce 660 nm protein assay reagent to decrease the bromophenol blue interfering with proteins. Bovine serum albumin (BSA) diluted in PBS was used for reference protein standard curve. For the colorimetric detection, 10 µl of each standard dilution and sample dilution was pipetted to 96-well plate and 150 µl Pierce 660 nm protein assay reagent with IDCR was added to the wells and incubated a minute on a shaker and then five minutes off shaker covered from light. Colorimetric detection was performed with Wallac Victor 1420 at 660 nm wavelength. The protein concentrations of the samples were determined using the standard protein curve in comparison.

Western Blot. Lysed cell samples were heated for five minutes in 95 °C before loading into the gel; 10% Mini-PROTEAN® TGX™ Precast Gel (BioRad). Reagents used in different steps in WB are listed in Table 3. After loading samples to the gel, electrophoresis was done first 10-15 minutes with 80 V and 400 mA and then 45 minutes with 120 V and 400 mA. For protein blotting Trans-Blot® Turbo™ Mini PVDF Transfer Packs (Bio-Rad) were used with Trans-Blot® Turbo™ Transfer System (Bio-Rad) with 7-minute transfer program for mixed MW (5-150 kD) in 25 V and 1.3 A. Blocking of the membrane was done for one hour with 5% milk blocking solution. After blocking, the membrane was washed with decreasing TBS-Tween buffers for five minutes each. Blots were incubated with primary antibodies (Table 4) either one hour in RT or O/N in +4 °C in a shaker depending on the antibody. After incubation, the blots were washed with decreasing TBS-Tween buffers and incubated for one hour in RT with HRP-linked secondary antibodies (Table 5) and washings were repeated. Blots were imaged with ChemiDoc XRS+ System (Bio-Rad) using chemiluminescense-based ECL™ Prime Western Blotting System (Sigma Aldrich) reagent. Quantification of the band intensities was done with ImageLab band analysis tool (version 5.2, Bio-Rad Laboratories).

Table 3. Reagents used in western blot.

Step	**Reagent**	**Content**
Gel run	Gel	10% Mini-PROTEAN® TGX™ Precast Gels (BioRad #4561033)
	1x Running buffer	25 mM Tris
		192 mM Glycine
		0.1% SDS
	2x Laemmli Sample Buffer	2x Laemmli Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, Bio-Rad #1610737)
		5% β-mercaptoethanol
	Molecular weight marker	PageRuler Plus Prestained protein ladder (Thermo Scientific #26619)
Transfer	Commercial transfer pack including filter paper, buffer, PVDF membrane	Trans-Blot® Turbo™ Mini PVDF Transfer Packs (BioRad #1704156)
Blocking	5% milk blocking buffer	Milk powder
		1X TBS + 0.05% Tween20
Washing	TBS-Tween buffers	0.5% Tween TBS
		0.1% Tween TBS
		0.05% Tween TBS
Antibody dilution	Antibody dilution solution	5% milk blocking solution
		Antibody
Imaging	Chemiluminescence	ECL reagent (Sigma Aldrich #GERPN2232)

Table 4. Primary antibodies used in the western blot.

Primary antibody	Function	Dilution	Origin	Supplier
anti-Vimentin IgG	radial glia marker	1:1000	mouse	Dako M0725
anti-GFAP	astrocyte marker	1:30 000	chicken	Abcam ab4674
anti-β-actin	cell cytoskeletal marker	1:2000	mouse	Santa Cruz sc-47778

Table 5. Secondary antibodies used in the western blot.

Secondary antibody	Dilution	Origin	Supplier
anti-mouse IgG HRP	1:2000	goat	Santa Cruz sc-2005
anti-chicken IgY HRP	1:30 000	goat	Invitrogen A16054

4.7 Immunocytochemistry

Immunocytochemistry (ICC) staining was performed for both the control and cytokine-treated astrocytes at 0h or 7-day time point in free cultures, and in microfluidic devices 72h time point (Figure 7). Exceptionally, staining for NF-κB was done after 60-minute cytokine treatment in free cultures. The cells were washed two times with PBS and then fixed with 4% paraformaldehyde (PFA) for 15 minutes in RT and then washed two times with PBS for five minutes. Fixed cells were stored at +4 °C in PBS until staining. Fixed cells were incubated in blocking solution containing 10% normal donkey serum (NDS), 0.1% Triton-X and 1% BSA in PBS for 45 minutes, after which the cells were washed with primary antibody solution (1% NDS, 0.1% Triton-X and 1% BSA). The cells were incubated in diluted primary antibody solutions (Table 6) O/N in +4 °C on a shaker. In the following day, the cells were washed two times with 1% BSA in PBS solution before incubating one hour with secondary antibody solutions (Table 7) in RT covered from light. After 1-hour incubation, the cells were washed two times for five minutes with PBS and in the second washing nuclear stain DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, Thermo Fischer) was added in 1:5000 dilution. The cells were washed once for five minutes with phosphate buffer (27.6 g/l NaH_2PO_4 and 28.6 g/l Na_2HPO_4 in H_2O) and mounted with ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, USA). The stained cells were imaged with Olympus IX51 microscope with Olympus DP30BW camera (Olympus Corporation, Hamburg, Germany) and LSM780 Laser Scanning Confocal Microscope with Quasar spectral GaAsP detector (all from Carl Zeiss, Jena, Germany).

Table 6. Primary antibodies and dilutions used in the immunocytochemical staining.

Primary antibody	Function	Dilution	Origin	Supplier
anti-β-tubulin	neuronal marker	1:1000, 1:500	rabbit	GenScript A01627
anti-Ki67	proliferation marker	1:800	rabbit	Merck-Millipore AB9260
anti-Vimentin	radial glia marker	1:500	mouse	Dako M0725
anti-GFAP	astrocyte marker	1:4000	chicken	Abcam ab4674
anti-Cl-cas3	apoptosis marker	1:400	rabbit	Cell Signaling Technology #9664
anti-NF-κB	transcription factor	1:400	rabbit	Cell Signaling Technology D14E12
anti-NF-H	neuronal axons	1:500	chicken	GenScript A00136
anti-S100β	astrocyte marker	1:500	mouse	Abcam ab11178
anti-Glutamine Synthetase	astrocyte marker	1:200	rabbit	Abcam ab73593
anti-Aldh1L1	astrocyte marker	1:100	rabbit	Abcam ab87117
anti-GLT-1	astrocyte marker	1:100	mouse	Abcam ab77039
anti-GLAST	astrocyte marker	1:100	rabbit	Abcam ab416

Table 7. Secondary antibodies and dilutions used in the immunocytochemical staining.

Secondary antibody	Dilution	Origin	Supplier
Alexa Fluor® 488 IgG	1:200	donkey-anti-rabbit	Thermo Fischer Scientific A21206
Alexa Fluor® 488 IgG	1:400	donkey-anti-chicken	Thermo Fischer Scientific A11039
Alexa Fluor® 568 IgG	1:200	donkey-anti-mouse	Thermo Fischer Scientific A10037
Alexa Fluor® 568 IgG	1:400	goat-anti-chicken	Thermo Fischer Scientific A11041
Alexa Fluor® 647 IgG	1:200	goat-anti-chicken	Thermo Fischer Scientific A21449

4.8 Microfluidic devices and PDMS reservoirs

Co-cultures with neurons and astrocytes were performed with in-house engineered microfluidic devices (Depending patent: WO 2015/0921419), which were developed in Micro and Nanosystems Research Group, Tampere University. Used microfluidic device was composed from three parts: 1) medium reservoir 2) microfluidic device and 3) 30 mm cover glass. Design of the device is illustrated in the Figure 8. The used cells in these devices were commercial hiPSC-derived astrocytes (Axol Bioscience, UK) and in-house derived cortical neurons (cell line 10212.EURCCs). Co-culture experiment consisted of 25 devices of which 20 contained both neurons and astrocytes and the rest five devices were controls with only neurons plated.

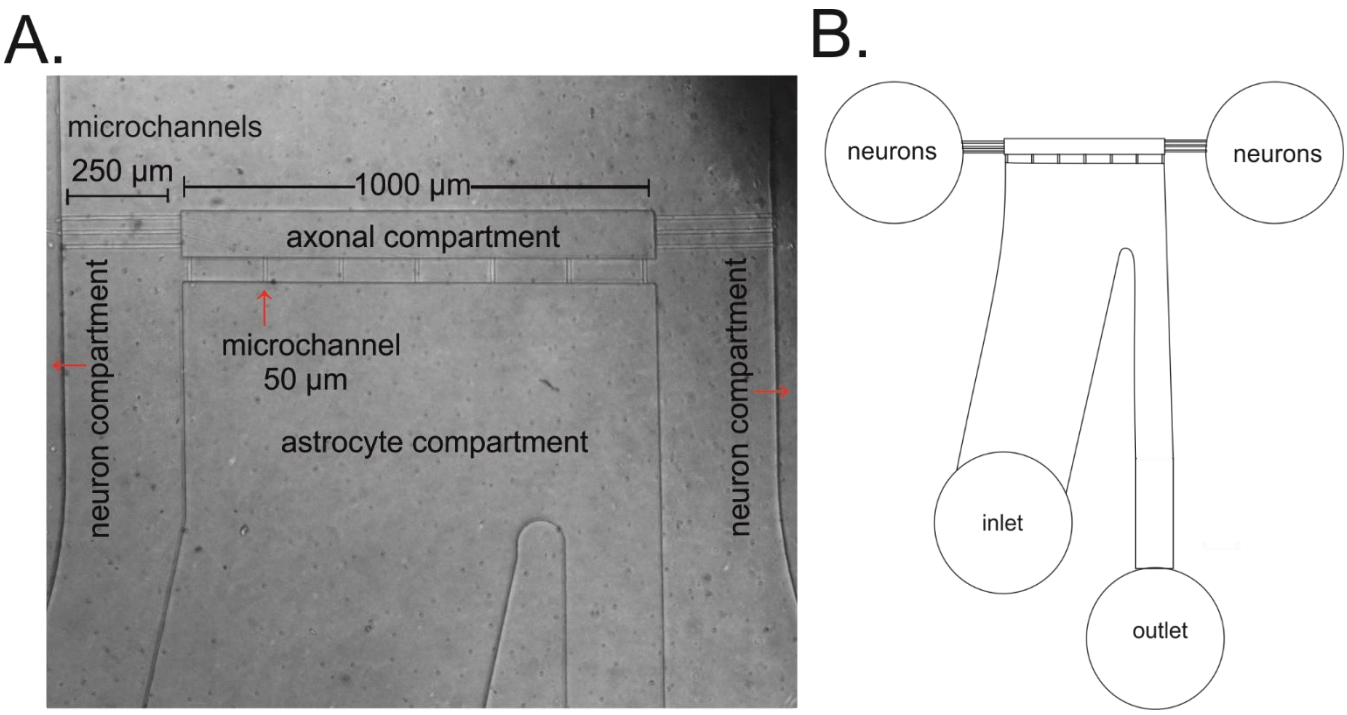


Figure 8. Illustration of in-house developed microfluidic device. (A) Partial image of used in-house microfluidic device for culturing neurons and astrocytes together. Exact dimensions in device; process compartment: width 100 µm, height 110 µm, length 1000 µm, axon channels from neuron compartments: width 10 µm, height 3.5 µm, length 250 µm, microfluidic channels: width 10 µm, height 3.5 µm, length 50 µm. (B) Schematic illustration of the device with all the cell compartments showing, including inlet and outlet. Astrocytes were plated to the inlet compartment and the fluid flow transported the cells across the astrocyte compartment to the outlet compartment.

4.8.1 Optimization of coatings for devices

To test the optimal coating to be used for microfluidic devices, neurons (10212.EURCCs) were cultured on Matrigel and PO+LN521 coated glass and plastic well plates. Viability of the cells were visualized with Live/Dead fluorescent cell viability assay (Thermo Scientific). The cells were cultured in NMM. Live/Dead assay is based on the discrimination of dead cells from the live. Cells are simultaneously stained with green-fluorescent calcein-AM to indicate intracellular esterase activity and red-fluorescent ethidium homodimer-1 (EthD-1) to indicate loss of plasma membrane integrity i.e. cell death. Cells were treated with 0.5 µM ethidium homodimer-1 (EthD-1), which interacts with dead cells, and 0.1 µM Calcein-AM to stain the live cells. Cells were incubated for 30 minutes in RT and protected from light and imaged with Olympus IX51 microscope with Olympus DP30BW camera (Olympus Corporation).

4.8.2 Preparation of devices

Preparation of the devices started with reversible oxygen plasma treatment, which was done using oxygen plasma cleaner (PICO, Diener electronic, Plasma Surface Technology). The oxygen plasma treatment was performed with previously optimized parameters (4 min 30 W, 30 mbar). All the cover glasses were HCl-treated and sterilized with 70% ethanol before use and PDMS medium reservoirs were autoclaved. The following preparations including oxygen plasma treatment and assembling the device depended on the type of coating (PO + LN521 or Matrigel) used in the glasses. In the case of PO+LN52, preparation started with coating the cover glasses with PO, then bonding the PDMS medium reservoir with the device using the oxygen plasma treatment. Finally, the device with the medium reservoir were placed on the top of the PO coated glasses. After assembling the device, 20 µg/ml LN521 was added in the microtunnels by pipetting solution on the neuron compartments and inlet compartment. From the inlet compartment, the coating solution flowed to the outlet compartment. With Matrigel coating, all three elements (PDMS medium reservoir, device and cover glass) were put together and bonded with oxygen plasma treatment. Matrigel was added to all compartments. Matrigel was kept on ice during the handling and the tips used when pipetting Matrigel were stored in freezer to avoid Matrigel from forming a gel. After oxygen plasma treatments and coatings, devices were stored in +4 °C.

4.8.3 Cell culture in devices

Neurons and astrocytes were plated simultaneously to the devices. Neurons were plated 20 000 per neuron compartment on both sides of the device. Astrocytes were plated 45 000 in the inlet compartment from where the fluid pressure pushed the cells to move across the astrocyte area to the outlet reservoir. Used medium was NMM supplemented with 20 ng/ml BDNF, 10 mg/ml GDNF, 500 µM db-cAMP and 200 µM AA. NMM was added both neuron and astrocyte compartments. Devices with the cells were kept in incubators at +37 °C in 5% CO_2 atmosphere and 95% humidity. Figure 9 shows the devices stored in petri dishes. Cells were imaged with Zeiss Axio Vert.A1 phase contrast microscope (Carl Zeiss AG, Germany) every day and axonal growth in axon compartment was followed. Cells were cultured in devices for three days before starting the experiment (Figure 7B). Astrocytes were treated with human recombinant TNF-α (10 ng/ml) and human recombinant IL-1β (10 ng/ml) as before. Cytokines were added in the inlet and outlet reservoirs of the device. Cytokines should not be able to drift into axonal compartment, but little amounts of them might still get in touch with axons via the microchannels.

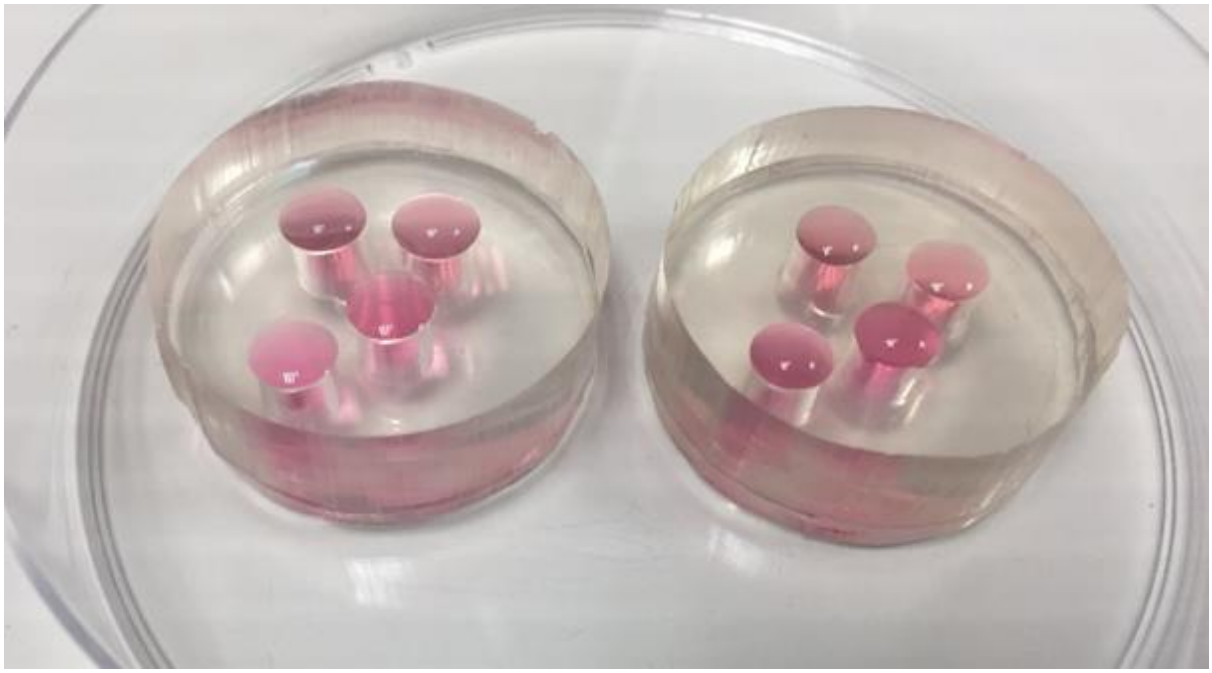


Figure 9. Two microfluidic devices and PDMS medium reservoir on top of them filled with neural maintenance medium.

4.8.4 Immunocytochemical staining in devices

The devices were fixed after 72h treatment with IL-1β and TNF-α (Figure 7B). First, medium was aspirated from all the four wells and the medium reservoir was detached from the device. Then, 10 µl 4% PFA was added to the four holes in the device before carefully detaching the device from the cover glass without breaking the glass and ripping the cells. Cover glass was then placed to 6-well plate and incubated with 4% PFA for 15 minutes. After incubation, the glasses were washed three times with PBS and stored in +4 °C in PBS. Devices were stained for GFAP, β-tubulin and vimentin and staining was done as described earlier in the chapter 4.7. After the final wash, cover glasses were placed on the top of the microscopy slides and mounted with droplet of ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, USA). Samples were stored in +4 °C and imaged with Olympus IX51 microscope with Olympus DP30BW camera and LSM780 Laser Scanning Confocal Microscope with Quasar spectral GaAsP detector.

4.9 Statistical analysis

Normality of the data was tested, and the obtained data was analyzed with independent samples T-test and a p-value of less than 0.05 was considered significant. The SPSS statistical software package (versions 23.0, IBM SPSS Inc., USA) was used to perform the statistics.

5 Results

5.1 Astrocyte characterization

Before starting the cytokine experiments, hiPSCs-derived astrocyte progenitors were cultured for 17 days to reach the mature state. The resting mature astrocytes were stained with different astrocyte specific markers to show that the hiPSC-derived astrocyte progenitors were differentiated to mature astrocytes (Figure 10). The used markers were Aldh1L1, GFAP, EAAT1, EAAT2, GS and S100β. Immunocytochemical staining showed that all the markers were expressed in astrocytes.

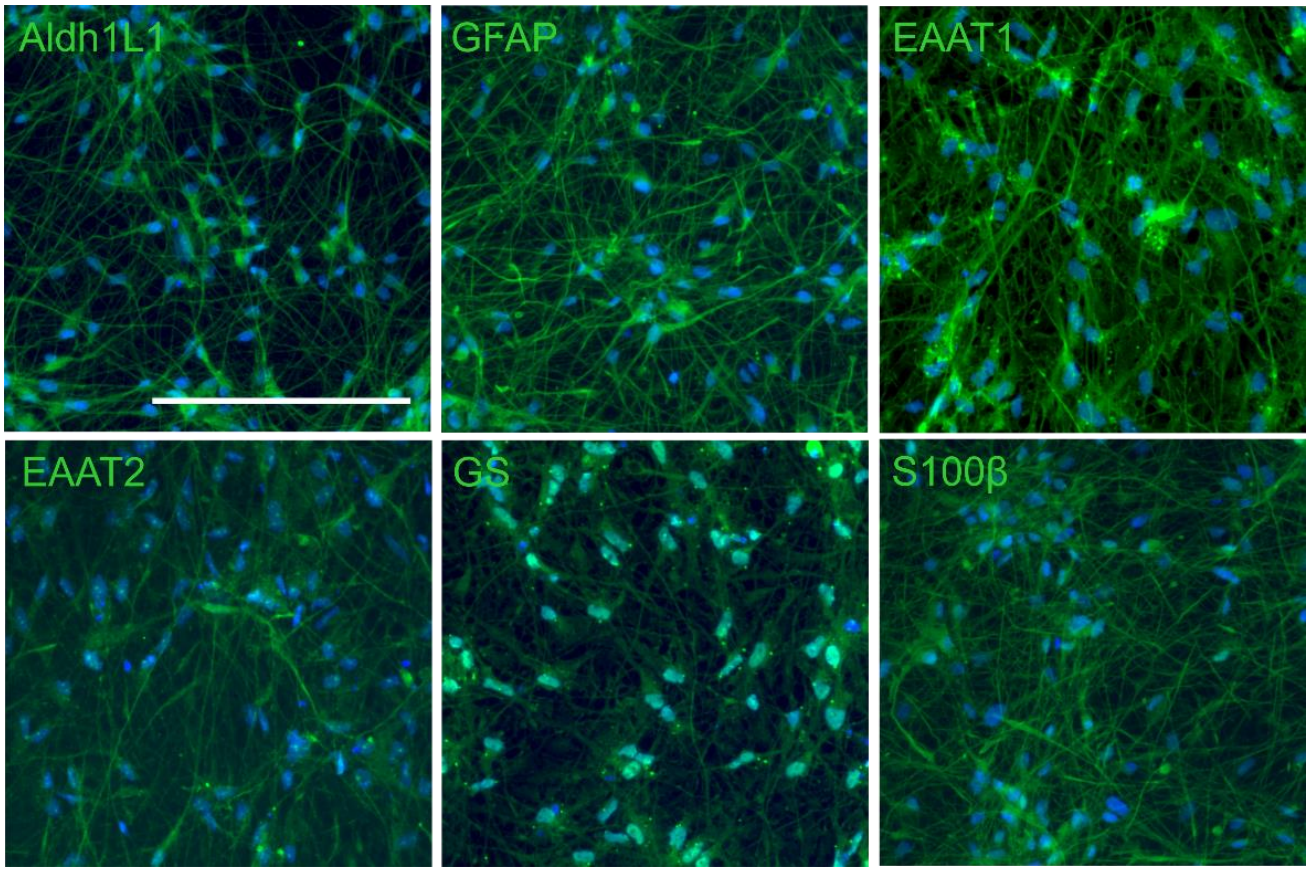


Figure 10. Characterization of hiPSCs-derived astrocytes. hiPSCs-derived astrocytes were stained with astrocyte specific protein markers Aldh1L1, GFAP, EAAT1, EAAT2, GS and S100β (green). Scale bar 100 µm.

5.2 Induction and characterization of reactive phenotype of astrocytes

After astrocyte differentiation, the aim was to induce the reactive phenotype and characterize it. Previous *in vitro* studies have shown that reactive astrocytes can be induced with cytokines IL-1β and TNF-α (Choi et al. 2014; Roybon et al. 2013). Therefore, astrocytes were treated with 10 ng/ml IL-1β and TNF-α for seven days as described earlier (Choi et al. 2014; Roybon et al. 2013). During the 7-day treatment with cytokines, astrocytes were imaged with phase-contrast microscope to see morphological change, which is noted as one of the hallmarks of astrocyte reactivation in literature (Anderson et al. 2014; Wilhelmsson et al. 2006). The 7-day treatment showed some gradual morphological change and it can be seen in the Figure 11. Arrows in the 7d image point the morphological change in astrocyte cell bodies between control and cytokine treated (Figure 11). The cell bodies were more hypertrophied in the cytokine treated cells compared to controls.

Next, the changes in protein expression after cytokine treatment were studied by staining the GFAP and vimentin both in control and cytokine treated astrocytes. According to earlier studies, increased GFAP protein expression has been one hallmark of astrocyte reactivation (Sofroniew 2009; Wilhelmsson et al. 2006). In our study with hiPSCs, GFAP and vimentin upregulation in protein level could not be seen in the ICC staining (Figure 12). However, some alteration in cell cytoskeleton can be seen after 7-days IL-1β and TNF-α treatment compared to control.

GFAP and vimentin expression was also analyzed in protein level with WB analysis. WB samples, both control and IL-1β and TNF-α, were collected in the day nine, after the astrocytes have been without cytokines for two days (Figure 7A). First, WB method was optimized for the experiments. After optimizing the method, antibody concentrations for GFAP and vimentin detection from astrocytes were optimized and β-actin was used as loading control. Representative WB result shows significant ($p=0.01$, Figure 13B) decrease in GFAP protein levels in cytokine treated astrocytes compared to control (Figure 13A). Analysis was performed for three different astrocyte differentiation rounds and three separate cytokine treatments and the result was constant. Since the results of GFAP was so surprising, WB was also shortly done to study the protein expression of vimentin in control astrocytes compared to cytokine treated ones. WB shows no difference in vimentin expression (Figure 13C) and quantification supports the result (Figure 13D). To summarize, both WB results and ICC staining images showed no upregulation in vimentin. WB showed significant decrease in GFAP protein expression, but not in vimentin.

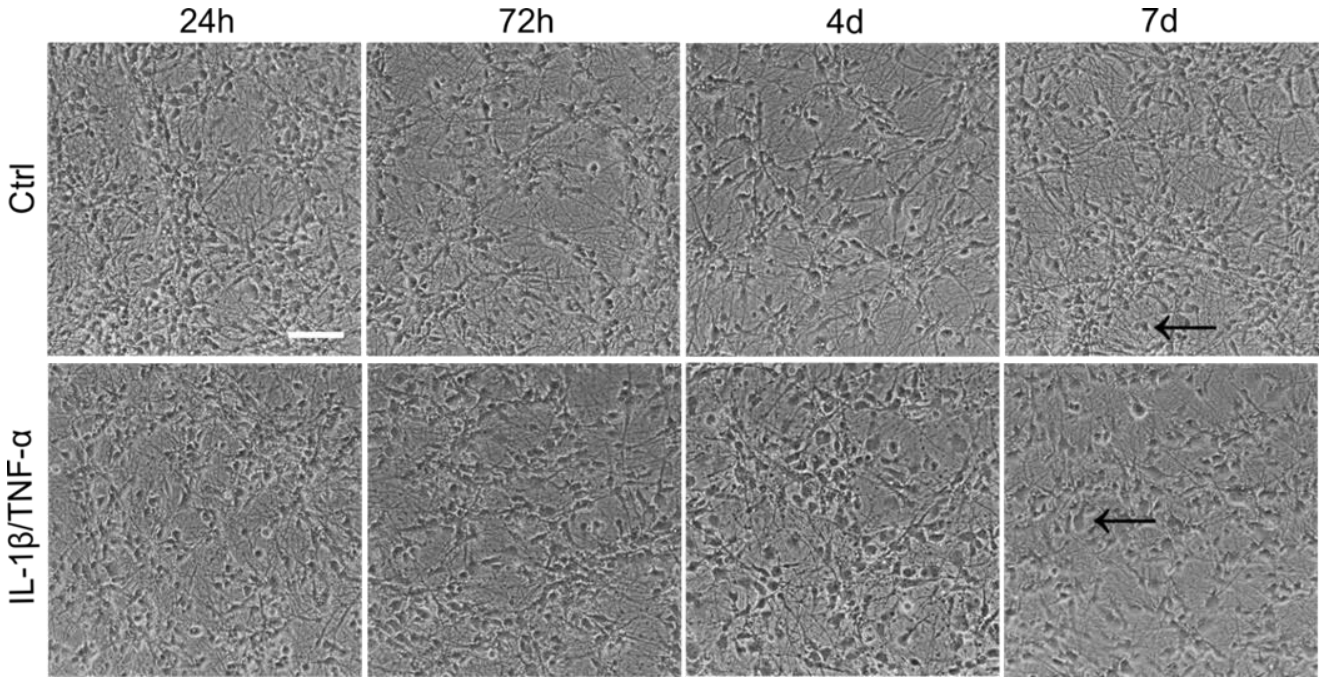


Figure 11. Phase-contrast microscopy images of control and IL-1β/TNF-α treated astrocytes from time points 24h, 72h, 4 and 7 days in culture. Arrows show the cell bodies in the 7d images. Scale bar 100um.

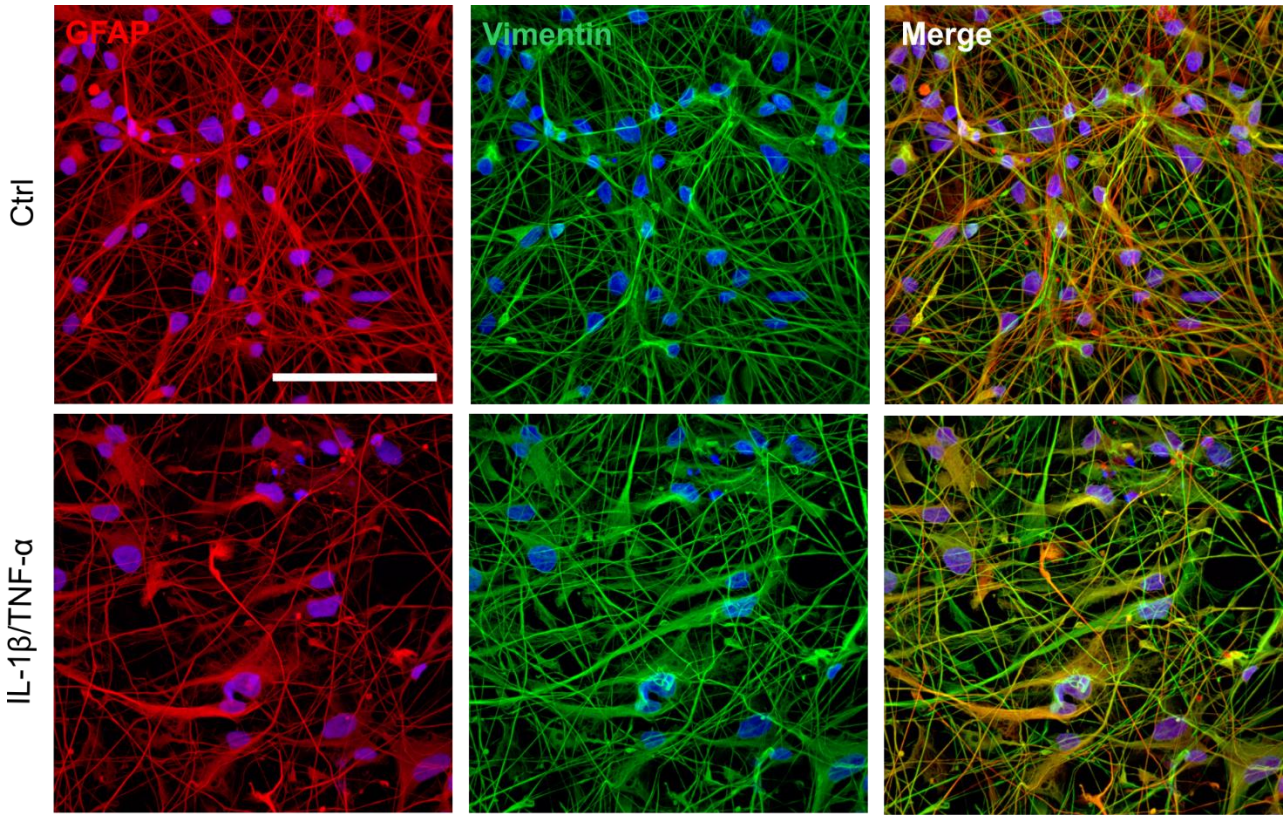


Figure 12. hiPSCs-derived control and IL-1β/TNF-α treated astrocytes stained with GFAP and vimentin after seven days in culture. Scale bar 100μm.

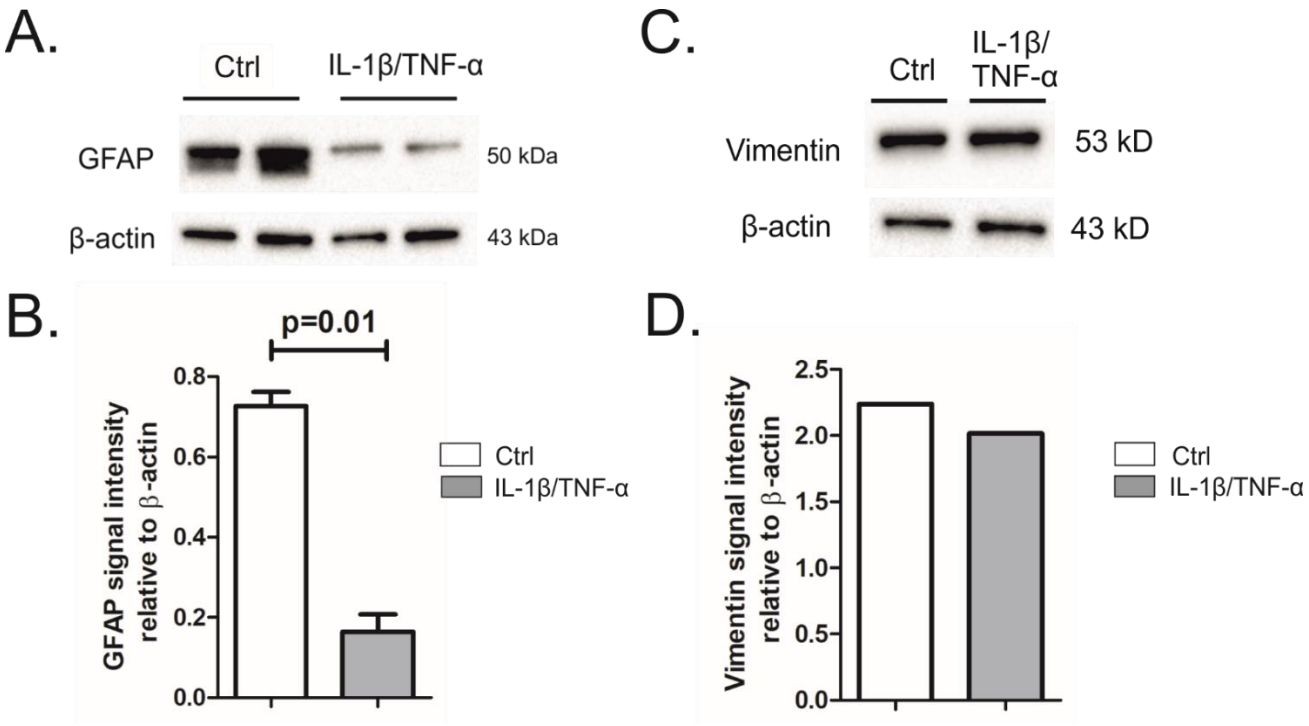


Figure 13. WB results for GFAP and vimentin. (A) Representative WB result for GFAP detection in control and IL-1β/TNF-α treated astrocytes after seven day treatment. β-actin was used as loading control. WB was repeated for three different astrocyte differentiation rounds and cytokine treatments and all showed the same result. (B) Quantification of WB result for GFAP protein expression. GFAP band intentisities are normalized with β-actin. Bars show n=2 and data is representative of three independent differentiations. Statistical test was done using independent-samples T-test. (C) WB result for vimentin detection in control and IL-1β and TNF-α treated astrocytes after 7 day treatment. β-actin was used as loading control and results is representative of two different astrocyte differentiation rounds. (D) Quantification of WB result for vimentin protein expression. Vimentin band intentisities are normalized with β-actin. Bars show n=1.

5.3 Cytokine treatment decreases cell viability but no effect on proliferation

During the 7-day cytokine treatment period the viability of astrocytes was evaluated daily with phase contrast microscope. In addition, cell viability and proliferation were studied with different methods. Both control and treated cells were stained with cleaved-caspase 3 (Cl-Cas3), a marker for apoptosis, and with cell proliferation marker Ki67. Both control and cytokine treated cells showed some Cl-Cas3 expression, but no difference between the control and treated cells could be seen (Figure 14). Proliferation seemed to be very minor in both conditions according to Ki67 expression (Figure 14).

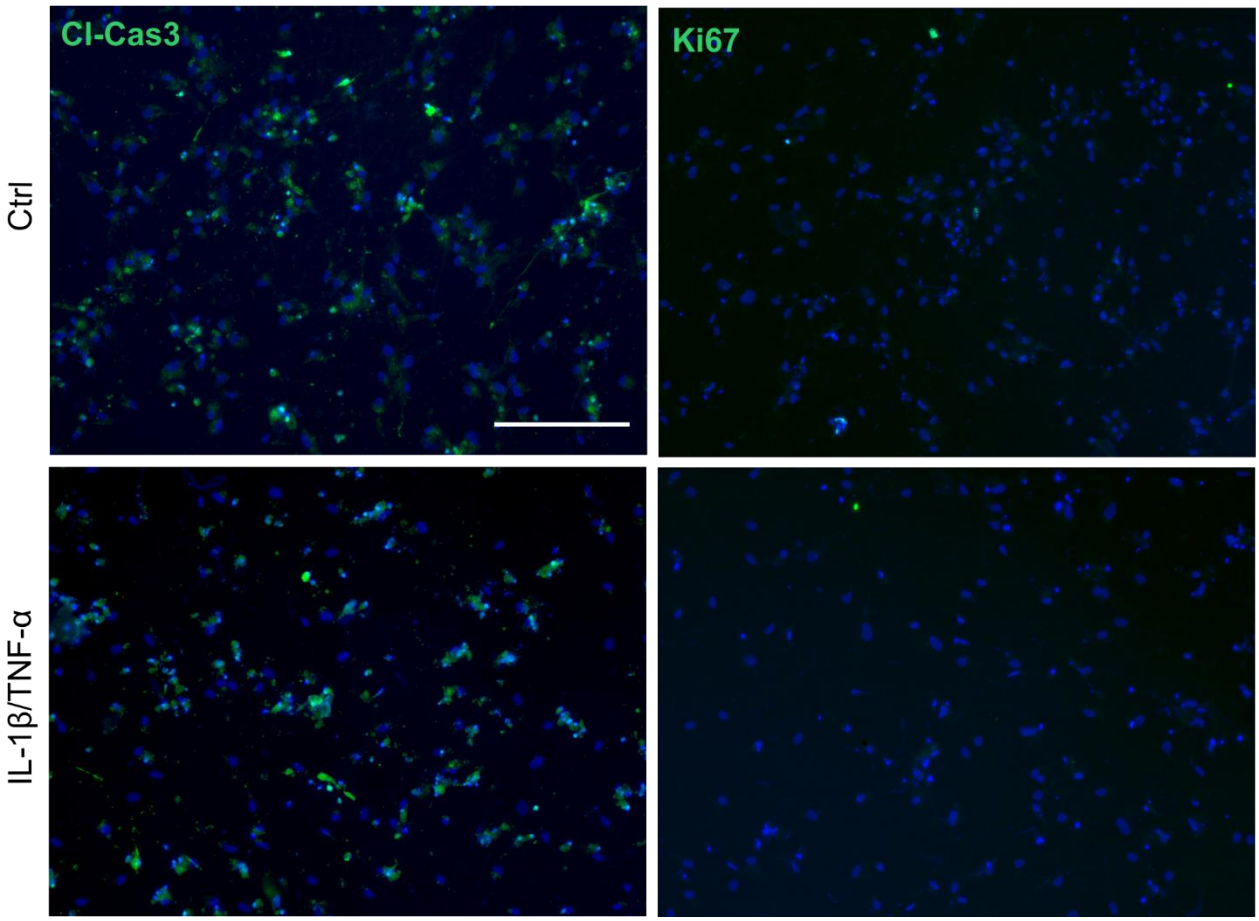


Figure 14. ICC staining for apoptosis marker cleaved caspase 3 (Cl-Cas3) and cell proliferation marker Ki67 both control and IL-1β and TNF-α treated astrocytes after seven days in culture. Scale bar 200 µm.

Cell viability, cytotoxicity and apoptosis was further studied with ApoTox-Glo Triplex Assay kit (Figure 15). Results show that 7-day cytokine treatment decreased astrocyte viability significantly and induced significant cytotoxicity compared to control cells (p-values<0.001). There was no significant difference in apoptosis between treated astrocytes compared to controls (p=0.228). In addition, no significant change in DNA amount (p=0.617) was observed between controls and treated cells after 7-day treatment according to the CyQuant analysis (Figure 16). In conclusion, cytokine treatments decreased cell viability and increased cytotoxicity. Proliferation was minor in both control and cytokine treated cells. No differences in apoptotic activity could be detected between control and treated astrocytes.

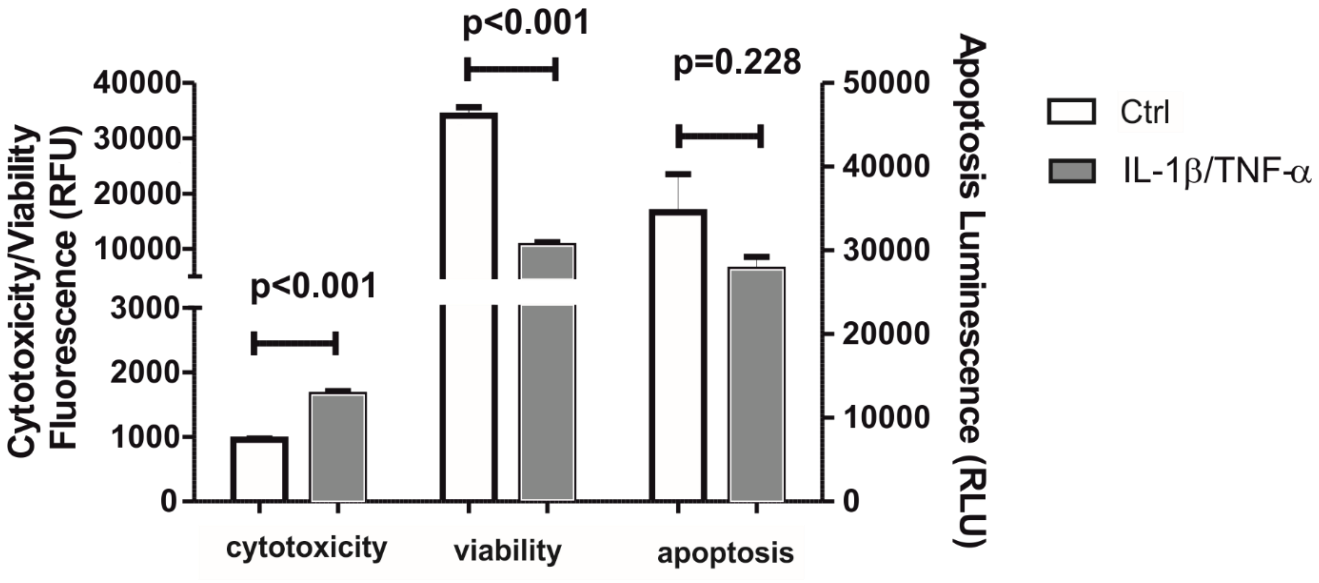


Figure 15. Viability assay measuring cytotoxicity, viability and apoptosis in control and IL-1β/TNF-α treated astrocytes after seven days in culture. Data is from one differentiation and each bar represents three replicates. Statistical tests were done using independent-samples T-test and p-values less than 0.05 were considered significant.

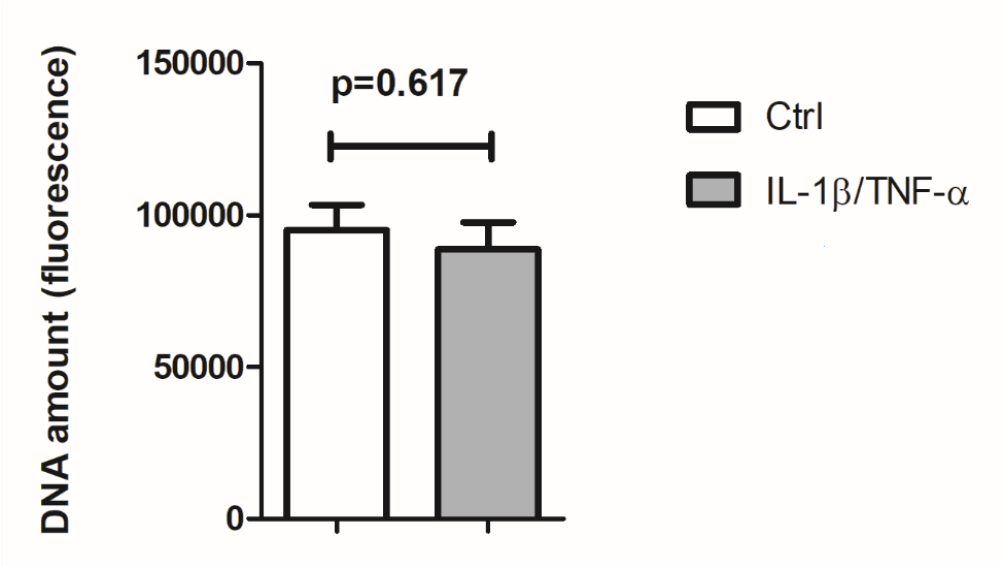


Figure 16. DNA amount comparison between control and IL-1β /TNF-α treated astrocytes after seven days. Data is representative result from two differentiation rounds and each bar represent three replicates. Statistical tests were done using independent-samples T-test and p-values less than 0.05 were considered significant.

5.4 Characterization of inflammatory nature of reactive astrocyte

In the CNS, one of the key regulators of inflammation is activation of transcription factor NF-κB, which has been also related to astrocyte reactivation (Liddelow & Barres 2017; Ben Haim et al. 2015). To study NF-κB activation, astrocytes were treated with IL-1β and TNF-α for 60 minutes and thereafter stained with NF-κB antibody. In the control astrocytes, the NF-κB was ubiquitously expressed in the cytoplasm and was only a slightly seen in the nucleus (Figure 17). However, after 60-minute treatment with IL-1β and TNF-α the transcription factor was localized in the cell nucleus which indicates activation of NF-κB pathway (Figure 17).

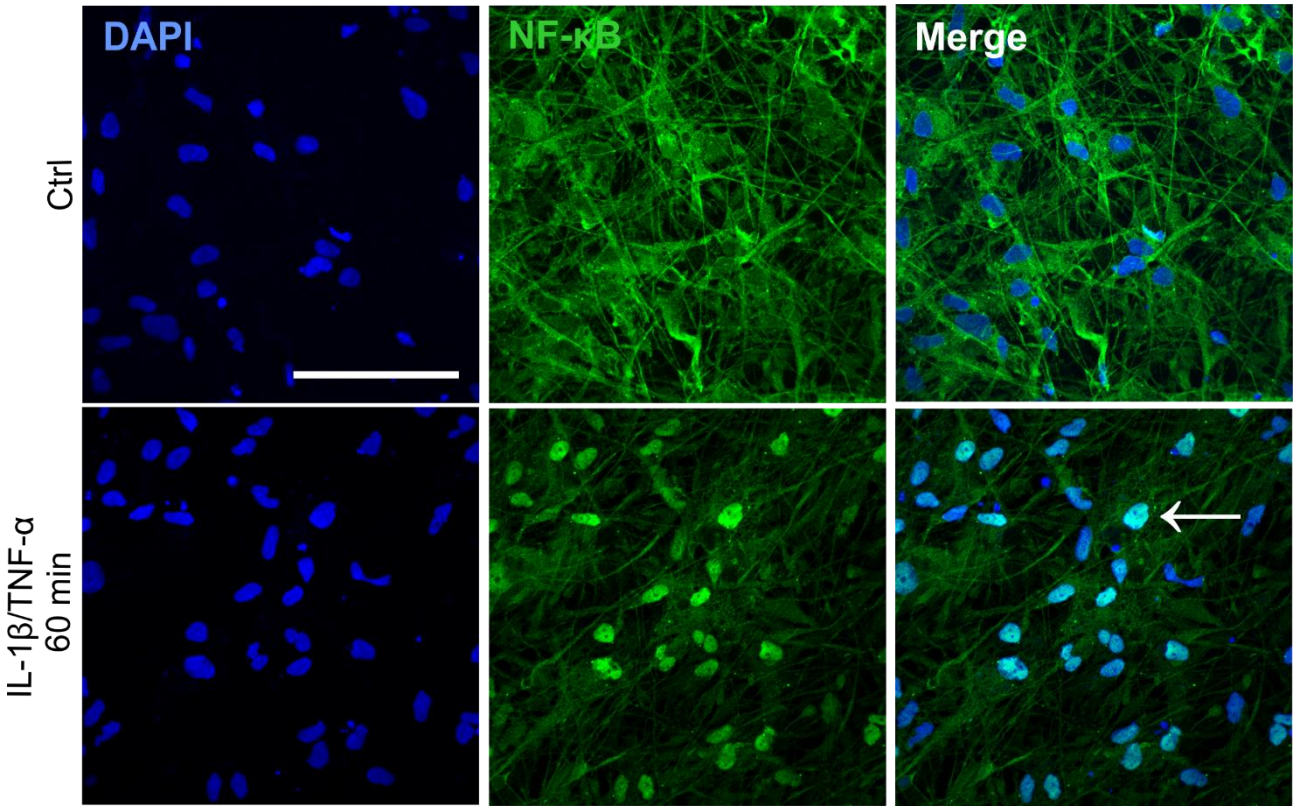
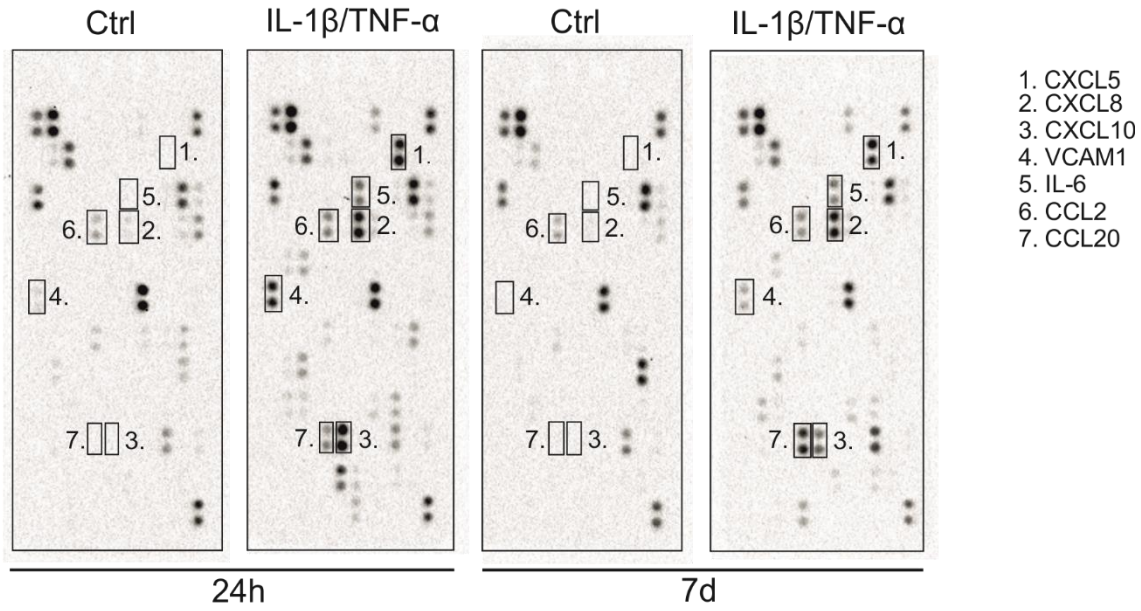


Figure 17. Astrocytes stained with NF-κB after 60 minutes IL-1β/TNF-α treatment and control cells in comparison. Arrow show the cell nucleus where NK- κB translocates. Scale bar 100 µm.

The possible inflammatory role of reactive astrocytes was further studied with Proteome Profiler, which is a membrane-based sandwich immunoassay. Here, studied medium samples were from IL-1β and TNF-α treated and control cells in 24h and 7d time points. Both time points showed altered expression in inflammatory factors between the control astrocytes and treated ones (Figure 18A). The most notably altered secretion profile was detected with CXCL5, CXCL8, CXCL10, VCAM-1, IL-6, CCL2 and CCL20 proteins. In the control conditions, these proteins were either detected in low levels or could not be detected at all.

Next, inflammatory role of astrocyte reactivation was studied to detect IL-6. IL-6 is a pro-inflammatory cytokine known to be induced in astrocytes after stimulation of IL-1β and TNF-α (Van Wagoner et al. 1999). In addition, overexpression of IL-6 is connected to astrocyte reactivation and neuroinflammation (Choi et al. 2014). Here, IL-6 levels were determined with ELISA kit from astrocyte medium samples from time points 24h, 72h, 7d, 8d and 9d. IL-6 levels rise quickly after 24-hour stimulation with IL-1β and TNF-α comparing to control astrocytes, where the secretion of IL-6 could not be detected at all in any time point (Figure 18B). Statistical analysis was not performed between the control and treated because control was zero in all time points. Vast secretion continued in the treated astrocytes in the 72h and 7d samples. When the cytokines were removed after the day 7, astrocytes continued secreting IL-6. In the 8d sample, the secretion dropped but rose again slightly in the 9d. As a conclusion, inflammatory characterization of cytokine treated astrocytes showed rapid activation of NF-κB signaling pathway and altered inflammatory secretion profiles.

A.



B.

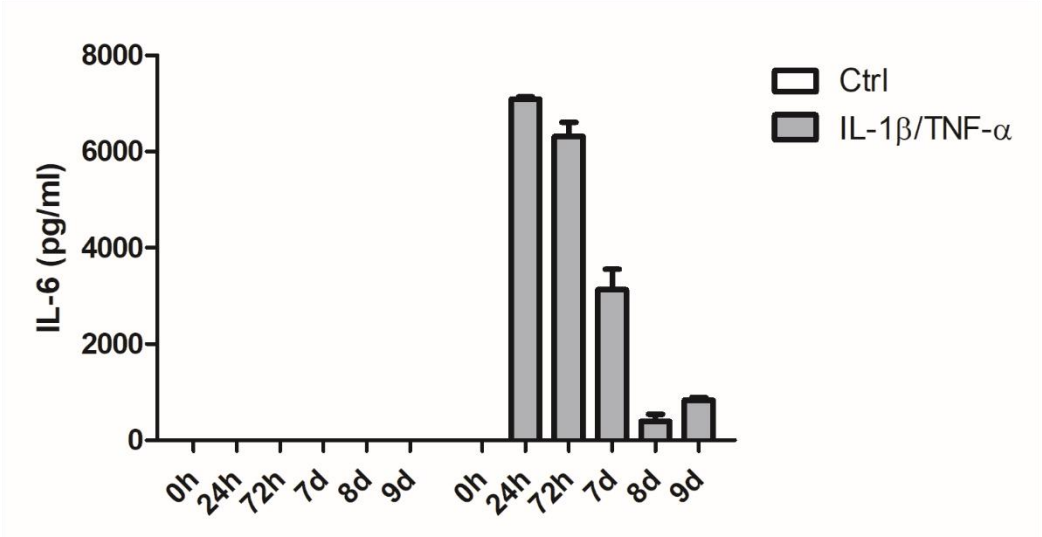


Figure 18. Secretion profile comparison between treated and control astrocytes. (A) Protein expression analysis with Proteome Profiler to detect different inflammatory factors. Analysis done to control and IL-1β/TNF-α treated astrocytes in 24h and 7d time point. Most notable alterations in secreted protein expression between control and treated astrocytes are marked and numbered. (B) Interleukin 6 (IL-6) secretion in control astrocytes and IL-1β/TNF-α treated in different time points measured with ELISA kit. Results shown in pg/ml. Graph is representative result of two different astrocyte differentiation rounds and cytokine treatments and each bar represent two replicates. Statistical analysis was not performed between the control and treated because control was zero in all time points.

5.5 Neuron and astrocyte co-cultures in microfluidic devices with inflammatory environment

The purpose of this co-culture was to study whether it is possible to culture both neurons and astrocytes in our in-house developed microfluidic devices. In addition, astrocytes were treated with cytokines and the effect of this treatment to astrocytes and axonal growth in the devices were observed. The aim was to study whether this microfluidic device would provide a platform for astrocyte reactivation in co-cultures with neurons. Before setting up the microfluidic co-cultures of neurons and astrocytes, different coating combinations were tested in free cultures. Normally in the open well cultures, astrocytes are cultured in Matrigel-coated and neurons in PO+LN521 -coated surfaces. In the microfluidic devices, cells are growing on glass coverslips. To find out the best coating for this co-culture set-up in devices, culturing of neurons in different conditions were tested. hiPSC derived neurons (cell line 10212.EURCCs) were plated on glass and plastic with two different coatings: PO+LN521 and Matrigel. Cells were cultured for eight days and it could be seen that the cells grew differently in different conditions. Live/Dead assay was performed to the cells to assess viability (Figure 19). Cells plated in glass with PO+LN521 coating grew more tightly and in the Matrigel coating the cells aggregated more. The most optimal culturing conditions for neurons seemed to be glass and coated with PO+LN52. Thus, that was the combination that mostly was used in the microfluidic devices. As a comparison, part of the devices was coated with Matrigel since it is the guided coating material in the manufacturer's protocol for astrocytes.

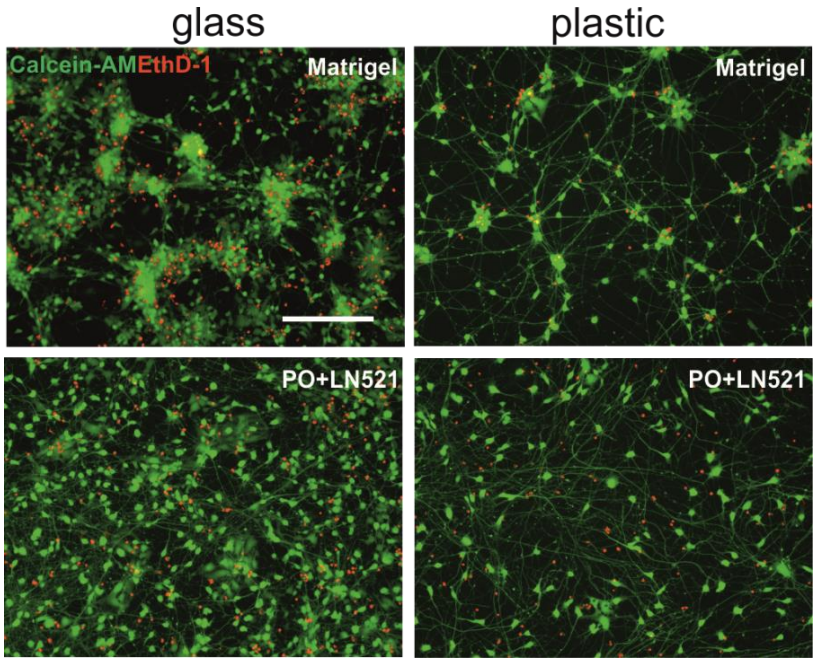


Figure 19. Live/Dead assay for neurons in different coating conditions to optimize cell culturing in the microfluidic devices. Scale bar 200 µm.

Devices were coated with either PO+LN521 or Matrigel to see if it influences the axonal growth or astrocyte attachment. After the cells were plated in the devices, it took about three days that the axons from the neuron compartment reached the axonal compartment. Phase-contrast images of the axonal compartment showed that the axons grew differently in the different coatings after 72h period (Figure 20). In the PO+LN521 coated devices the axons grew more on the edges of the axonal compartment and in the Matrigel coated ones the axons grew also in the middle. Even though neurons and axons grew well in both PO+LN521 and Matrigel coatings, only the PO+LN521 coated devices were used in ICC staining. This is because in the Matrigel coated devices the cells were mostly torn away from the cover slips when the devices were disassembled and fixed for staining. Only the devices with PO+LN521 coating the cells and neuronal axons retained quite well so that the ICC staining could be done for β-tubulin and GFAP (Figure 21). This shows that the co-cultures of neurons and astrocytes in the microfluidic devices are successful and the axons are growing well in the axonal compartment. In addition, astrocytes were growing well and had interactions with neuronal axons via the microchannels.

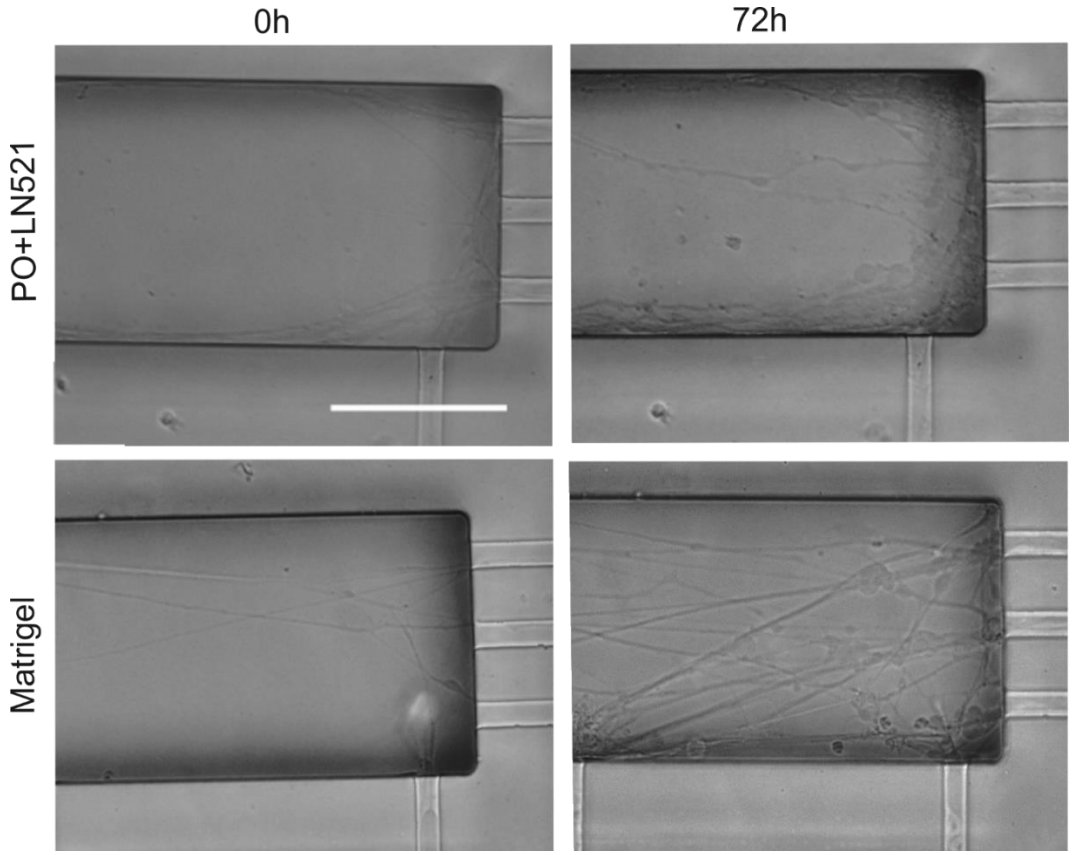


Figure 20. Phase-contrast microscope images of neuronal axons growing in the axonal compartment of PO+LN521 and Matrigel coated devices during 72h period. Cells were cultured in the devices three days before the 0h time point. Scale bar 100 µm.

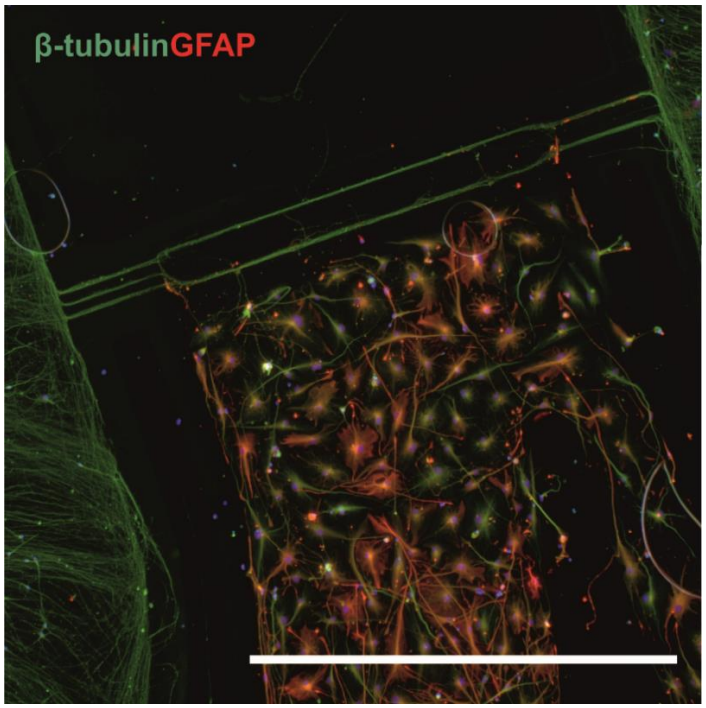


Figure 21. Neurons and astrocytes stained with β-tubulin and GFAP in microfluidic devices coated with PO+LN521. Scale bar 1mm.

Next, astrocytes were treated with cytokines and the effect of this treatment to astrocytes and axonal growth in the devices was observed. As mentioned earlier, the cytokine treatment started after the neurons and astrocytes had been three days in the microfluidic devices. Then, the axonal growth during the IL-1β and TNF-α treatment was observed for 72 h before the devices were fixed for ICC. Figure 22 shows the axonal growth in the axonal compartments of control and IL-1β/TNF-α treated devices after 72h. In both cases, the axons grew well in the axonal compartment, and IL-1β/TNF-α treatment did not show to cause any detrimental effects on axonal growth. In addition, ICC staining with β-tubulin and GFAP showed stronger axonal growth in the axonal compartment of IL-1β /TNF-α treated devices compared to control (Figure 23).

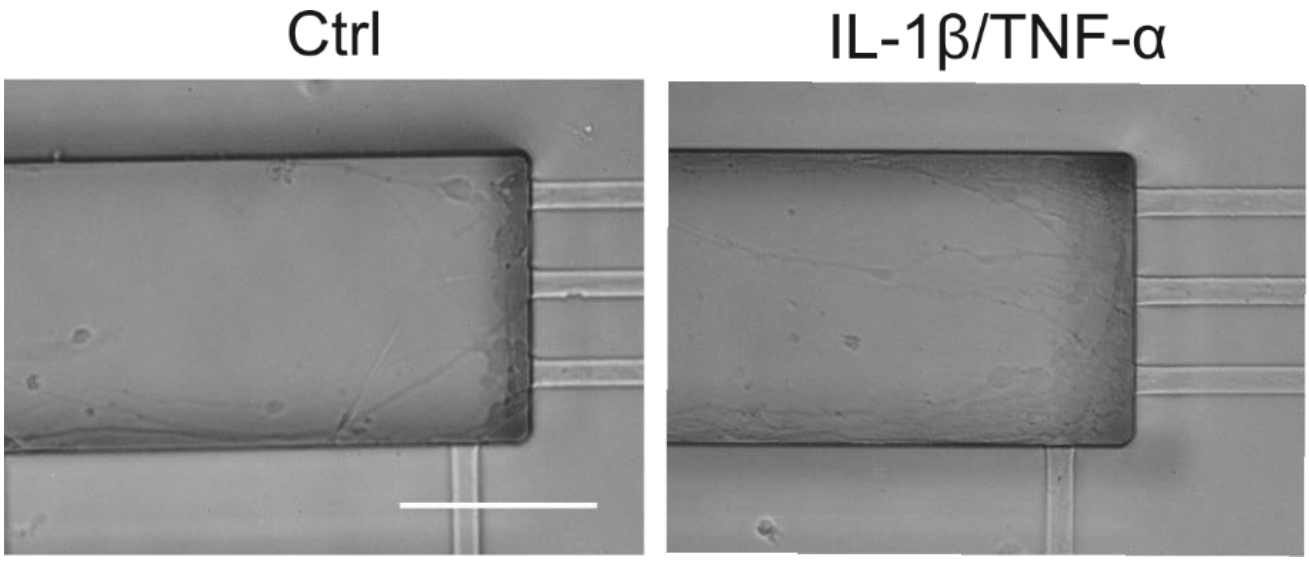


Figure 22. Phase-contrast microscope images of neuronal axons growing in the control and IL-1β/TNF-α treated devices after 72 h. Scale bar 100 µm.

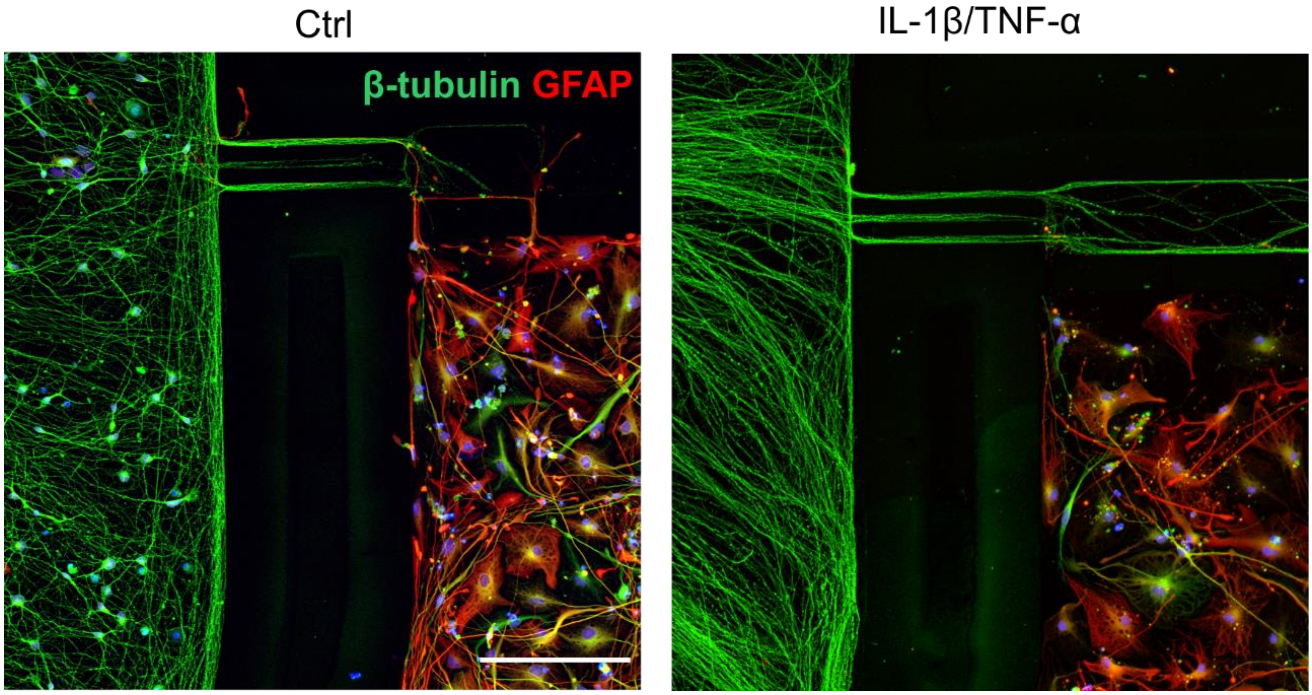


Figure 23. Neurons and astrocytes stained with β-tubulin and GFAP in microfluidic devices after 72h treatment with IL-1β/TNF-α and control. Scale bar 200 µm.

Next, the astrocyte morphology was observed more closely after the cytokine treatment. One of the features describing astrocyte reactivation has been the morphological change to more spread-out form and hypertrophy of the cell body (Ben Haim et al. 2015). GFAP and vimentin were used as markers to see the possible alteration in the cytoskeleton. After 72h treatment with inflammatory cytokines, the morphological change could clearly be seen with ICC staining and the change is pointed with arrows (Figure 24). As a conclusion, co-cultures of neurons and astrocytes in these microfluidic devices can be done. In addition, axons grew both in PO+LN521 and Matrigel coated devices, but the disassembling and ICC staining was only successful in the PO+LN521 coated devices. IL-1β and TNF-α cytokines did not have any detrimental effect on axonal growth. In addition, astrocytes showed alteration in GFAP and vimentin expression after cytokine treatment, which could imply induction of reactive phenotype. These reactivated astrocytes did not have detrimental effects on axons. All in all, reactive astrocytes can be induced in our in-house developed microfluidic devices.

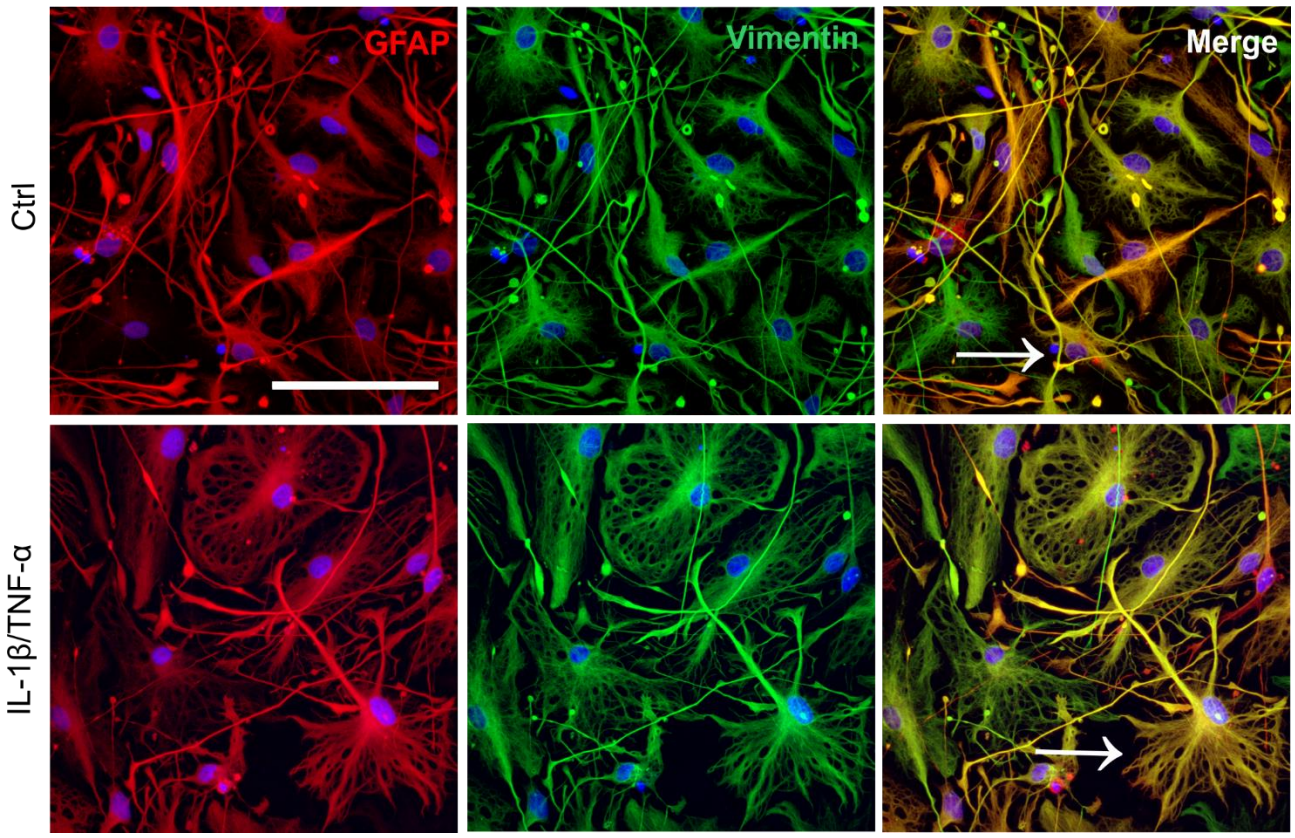


Figure 24. ICC stained hiPSC-derived control and 72h IL-1β/TNF-α treated astrocytes in microfluidic devices. Arrows show the altered morphology in merge staining with GFAP and vimentin. Scale bar 100µm.

6 Discussion

Astrocytes have evoked lot of interest since their role in the CNS have changed from passive supporting cells to key players in many functions, such as sustaining homeostasis in the brain and regulating synapses. The role of astrocytes in CNS injury and disease has also been investigated. Astrocytes respond to inflammatory environment by reactivation, in which they alter their gene expression, morphology and secretion profile (Ben Haim et al. 2015; Sofroniew & Vinters 2010). The effect of reactive astrocytes can be either detrimental or protective to the neurons. Information about reactive astrocytes have mainly been gathered from rodent studies, which most likely are not totally comparable to human because of the differences between species (Liddelow et al. 2017; Zhang et a. 2016; Zamanian et al. 2012). Reactivation studies with hPSCs-derived astrocytes have been done less, but in recent years they have gained more interest (Lundin et al. 2018; Santos et al. 2017). In this study, we used hiPSC-derived astrocytes and exposed them to inflammatory environment using IL-1β and TNF-α to induce the reactivation. We showed that our hiPSC-derived astrocytes express mature astrocyte protein markers and respond to the inflammatory environment. As a respond, astrocytes altered their morphology, gene expression and secretion profiles. In addition, we established co-cultures with hiPSC-derived astrocytes and neurons utilizing in-house developed microfluidic devices. The aim was to see if we could create *in vitro* model system for studying the communication between reactive astrocytes and neurons in controlled microenvironment. Our platform offers a promising disease model since our results suggest that astrocyte reactivation can be induced in these devices.

6.1 Characterization of induced reactive phenotype of astrocytes

Although astrocyte reactivation is studied using rodent astrocytes and human primary astrocytes, studies using hiPSC-derived astrocytes have been done less (Lundin et al. 2018; Perriot et al. 2018; Santos et al. 2017; Roybon et al. 2013). Reactivation in astrocytes have been induced with different inflammatory cytokines, but among the most used ones are IL-1β and TNF-α, either separately used or in combination (Table 1). In addition, many of the reactivation studies include culturing the astrocytes in the presence of FBS, which is shown to induce reactivation in astrocytes already before the cytokine treatments (Zhang et al. 2016). This could bias the results concerning the astrocyte response to cytokine treatments. In our study, hiPSC-derived astrocytes were cultured in serum-free conditions, thus the responses related to reactivation after cytokine treatments did not get affected by serum.

We treated astrocytes with IL-1β and TNF-α for seven days *in vitro* and gradual morphological change could be observed. The cell bodies in the cytokine treated astrocytes were hypertrophied and astrocytes seemed to take more space around them. Alteration in the cell morphology has been regarded as one of the hallmarks for astrocyte reactivation (Ben Haim et al. 2015; Sofroniew & Vinters 2010; Wilhelmsson et al. 2006). Recent reactivation study with hiPSCs-derived astrocytes showed that in their 5-hour treatment with IL-1β, no morphological change could be observed in astrocytes (Santos et al. 2017). However, five hours is relatively short cytokine exposure time and morphological changes need extensive alterations in protein expression which is slower than, for example, mRNA alterations. Thus, in five hours there might not be visible changes in morphology. Many other reactivation studies with hiPSC-derived astrocytes have not clearly shown or studied astrocyte morphology (Lundin et al. 2018; Perriot et al. 2018; Roybon et al. 2013). As a conclusion, our result shows gradual morphology alteration when astrocytes were treated with IL-1β and TNF-α for seven days. This broadens the characterization profile of hiPSC-derived reactive astrocytes, since morphological changes has not often shown before with hiPSCs. Our result supports the longer exposure time to inflammatory cytokines, since the gradual morphological change was clearly shown after four days.

Cellular cytoskeleton was further studied with staining the intermediate filaments GFAP and vimentin. ICC did not show upregulation in expression of GFAP or vimentin. It is noteworthy, that the used commercial hiPSC-derived astrocytes were strongly GFAP positive already before the reactivation. Even though no upregulation could be seen with ICC, morphological change could be detected in both intermediate filament proteins. Vimentin and GFAP stained parts were thickened and the cell cytoskeleton seemed to be reorganized. Next, GFAP expression was studied with WB and it showed that GFAP protein levels decreased significantly after 7-day cytokine treatment. This WB result was a little bit contradictory compared to ICC staining, since no clear reduction in GFAP expression could be seen with ICC. Since the result for GFAP was a little bit unexpected, WB for vimentin was shortly performed. WB did not show differences in vimentin expression and quantification supported it. Furthermore, upregulation of vimentin has not been highlighted in previous reactivation studies either. The upregulation of GFAP has been used as a marker for astrocyte reactivation (Ben Haim et al. 2015; Pekny & Pekna 2014; Wilhelmsson et al. 2006; Eng et al. 1971), thus decreased GFAP level in our study is contradictory to common view. However, upregulation of GFAP in reactive astrocytes is mainly studied with rodents or seen in human pathological specimens after injury or disease in CNS (Liddelow & Barres 2017; Correale & Fares 2015). Human and rodent astrocytes have many functional and morphological differences, thus the

results from rodent reactive astrocytes cannot be straight compared to human (Zhang et al. 2016). In addition, heterogeneity of astrocytes and their different expression of GFAP is noteworthy. The use of GFAP as a marker for reactivation has been criticized, since some of the subpopulations of astrocytes express GFAP mildly or in undetectable amounts (Sofroniew & Vinters 2010). Related to this, astrocyte reactivation is not all-or-nothing phenomenon but rather a spectrum of different variations of it. Furthermore, not all other reactive astrocyte studies have shown GFAP upregulation. One study with rodents showed that 24h and 72h treatment with IL-1β, TNF-α and LPS induced decrease of GFAP in ICC and mRNA analysis (Ronco et al. 2014). Some reactivation studies with hiPSC-derived astrocytes have not studied GFAP expression at all after cytokine treatments, (Perriot et al. 2018; Lundin et al. 2018; Santos et al. 2017; Tcw et al. 2017) but some of them have shown that the cells express GFAP in variable proportions in non-treated conditions (Perriot et al. 2018). In addition, Roybon and colleagues showed that treatment with TNF-α decreased GFAP expression compared to FBS control in hPSC-derived astrocytes (Roybon et al. 2013). These points support the thought that astrocytes are heterogenous cells and GFAP expression is not standard and thus it might not be a reliable marker for reactivation. Our result about reduction of GFAP expression might be very prominent, since no contradictory results have been achieved with hiPSC-derived astrocytes. However, GFAP expression in hiPSC-derived reactive astrocytes have not been studied much. It is also noteworthy, that the 7-day cytokine treatment period might be too intense for the cells, thus the proteins are degrading including GFAP. As a conclusion, our results suggest that GFAP upregulation is not a standard hallmark for hiPSC-derived reactive astrocytes. In the future, different concentrations in cytokine treatments could be tested to model the spectrum of severity of the insult. All in all, more study related to GFAP expression in hiPSC-derived astrocytes in different treatment conditions is needed.

6.2 Cytokines have detrimental effects on cell viability but no change in proliferation

Cell viability during the 7-day cytokine treatment was studied from control and treated astrocytes. Cytokine treated astrocytes did not show significant difference in apoptosis compared to control astrocytes. However, cell viability was decreased, and cytotoxicity was increased significantly after the 7-day treatment, but the cell amount stayed the same between the control and treated astrocytes. Other reactivation studies using hiPSCs-derived astrocytes have not shown compromised cell viability after cytokine treatments (Lundin et al. 2018; Santos et al. 2017; Roybon et al. 2013). However, one study showed that treatment with IL-1β and TNF-α separately induced Cl-Cas3 activity in carcinoma cell line derived astrocytes after 24h (van Kralingen et al. 2014). This apoptotic activity was correlated to compromised nuclei and finally, considerable cell death. Our cytokine treatment

period was seven days and the cell viability assays were performed then. Based on this, it could be possible that apoptosis occurred in earlier time point and was not detectable in the day seven. In our study, the treated astrocytes did not go through considerable cell death, since the cell amount stayed almost the same level, but they suffered from the treatment. This could mean that the 7-day treatment is too hard for the hiPSC-derived astrocytes as it was discussed earlier in the context of GFAP expression results. Also, the concentration of the cytokines might be too high. Furthermore, there could be variation in the differentiation rounds from hiPSCs since the astrocyte differentiation protocols are not standardized. Thus, there might be considerable heterogeneity among the hiPSC-derived astrocytes and they might respond differently to inflammatory environment. To study this viability matter further, shorter treatment period and earlier cell viability and apoptosis analysis could be combined and tested with different hiPSC-lines and astrocyte differentiations.

Increased proliferation has been one prominent feature in reactive astrocytes, but in our study, both control and treated astrocytes proliferated only mildly. As a support for our finding, it has earlier presented that astrocyte proliferation in not always present in the astrocyte reactivation (Ben Haim et al. 2015; Sofroniew & Vinters 2010). In the case of mild or moderate reactivation, the proliferation of astrocytes is shown to be mild or absent (Sofroniew & Vinters 2010). In addition, rodent studies modeling neurodegenerative diseases AD and ALS showed limited proliferation in cell counts (0-7%) when staining with Ki67 (Sirko et al. 2013; Lepore et al. 2008). However, considerable proliferation has been seen after severe CNS trauma when the purpose is to create a protective scar (Anderson et al. 2016). Thus, proliferation in reactive astrocytes is contradictory matter and proliferation might be induced only certain types of injuries. Our results showing no increased proliferation might imply that our astrocytes express a mild type of reactivation or the cells are compromised by the cytokine treatment and are not capable of proliferation. The latter assumption might be the right one, since cytokine treatment induced significant decrease in cell viability and increase in cytotoxicity. The cytokine treated astrocytes might have suffered and not being able to proliferate that much.

6.3 Cytokines activate NF-κB pathway and induce secretion of inflammatory molecules

In our study, we stained astrocytes with transcription factor NF-κB antibody after 60-minute treatment with IL-1β and TNF-α. In the control astrocytes, the NF-κB was ubiquitously expressed thus not activated. In the treated astrocytes, NF-κB was activated and translocated in the cell nucleus. NF-κB is known to be related in neuroinflammation and it has been thought one of the key signaling pathways in reactive astrocytes (Liddelow & Barres 2017; Colombo & Farina 2016; Shih et al. 2015). This is in line with our result, and NF-κB activation indicated that our hiPSC-derived astrocytes can

respond to inflammatory cytokines by activating inflammatory signaling pathways related to reactive astrocytes.

To study more the effects of inflammatory cytokines to our hiPSC-derived astrocytes, we investigated the secretion profile of both control and treated astrocytes. Proteome Profiler array showed that many inflammatory mediators were altered. The most notably altered proteins were CXCL5, IL-8 (CXCL8), IP-10 (CXCL10), vascular cell adhesion molecule-1 (VCAM-1), IL-6, MCP-1 (CCL2) and CCL20. All of those were not expressed in the control astrocytes or expressed in low levels. In addition, we confirmed strong IL-6 secretion with ELISA after 24h cytokine-treatment whereas IL-6 could not be detected at all from the control astrocytes. In earlier reactivation studies with hiPSC-derived astrocytes, upregulation of IL-8 and IL-6 have been detected (Lundin et al. 2018; Perriot et al. 2018; Santos et al. 2017; Roybon et al. 2013). In these studies, they used the same cytokines than in our study, IL-1β and TNF-α, as stimulants. Thus, our results are in line with other hiPSC-studies. Recently, a wide secretome profile analysis of purified human astrocytes was performed and it showed alterations in many cytokines after IL-1β and TNF-α stimulation (Choi et al. 2013). Similarities to our findings were upregulation of IP-10, IL-8, IL-6 and MCP-1. In addition, astrocytes differentiated from primary embryonic carcinoma cells treated with IL-1β and TNF-α showed upregulated secretion of IP-10 and IL-6 (van Kralingen et al. 2013). Cytokine CXCL5 has not been the most studied and upregulated protein in reactivation studies, but recent study showed strong CXCL5 upregulation in human spinal cord astrocytes treated with IL-1β (Teh et al. 2017). It is suggested that reactive astrocytes can express adhesion molecules, such as VCAM-1, and have a role in the pathophysiology of MS disease (Correale & Farez 2015; Lee & Benveniste 1999). Upregulation of VCAM-1 have been shown *in vitro* study with primary human astrocytes, and human astrocytoma cell lines showed VCAM-1 production after stimulation with TNF-α, IL-1β, or IFN-γ (Rosenman et al. 1995). Previously, CCL20 and MCP-1 were shown to be secreted from rodent astrocytes after stimulation of IL-1β or TNF-α (Li et al. 2014; Wang et al. 2014). This study also suggested that these secreted chemokines would induce migration of immune cells in the CNS. For long, IL-6 secretion has been linked to astrocyte reactivation. According to Colombo & Farina, IL-6 binding to its receptor gp130-IL6R in astrocytes is thought to act as a neuroprotective pathway (Colombo & Farina 2016). IL-6 binding to gp130-IL6 receptor activates STAT3 signaling pathway in astrocytes, which is suggested to have protective effects to neurons and axon regeneration after CNS injury. However, activation of NF-κB signaling pathway is induced by variety of factors, including IL-17. IL-17 have shown to enhance the IL-6 signaling and subsequently IL-6 secretion in astrocytes. (Meares et al. 2012) Thus, IL-6 secretion can be also mediated by NF-κB, which is suggested to be part of the

detrimental nature of reactive astrocytes (Colombo & Farina 2016). NF-κB signaling pathway modulates the expression of many cytokines, chemokines and cell adhesion proteins, including IL-6, IL-8, VCAM-1, IP-10 and MCP-1 (Liu, T. et al. 2017). As a conclusion, altered molecules found in our study have complex effects on the inflammation in CNS. These molecules secreted by reactive astrocytes can have either detrimental or protective effects on neurons. Based on our findings, it is difficult to say if the reactive astrocytes generated in our study represent more the detrimental or protective subtype. Other studies using hiPSC-derived astrocytes have not highlighted or measured at all the secretion of CCL20, MCP-1, CXCL5, IP-10 and VCAM-1 found in our study. Thus, the secretion of those newly found altered molecules could be studied further. For example, using different hiPSC-lines to compare if there are line-specific alterations. Furthermore, it could be evaluated that are these secreted molecules characteristics for hiPSC-derived astrocytes in inflammatory environment.

6.4 Reactivation can be induced in microfluidic devices and co-cultures are successful

Co-cultures with neurons and astrocytes were established utilizing in-house developed compartmentalized microfluidic devices, which had PDMS medium reservoir, device and cover glass combined. The aim was to evaluate the suitability of this device to culture both neurons and astrocytes. More precisely, to study axons growth in the axonal compartment and how the astrocytes interact with them through the connecting microchannels. The experiment started with coating optimization for the both cell types, neurons and astrocytes. The result showed that our neurons grew best on the PO+LN521 coating on glass and that was selected as main coating for the devices. Neurons were growing also quite well on Matrigel coating on glass, and since astrocytes are usually cultured on Matrigel it was used in some of the devices. In the devices, axons grew well in both coatings. Matrigel still might not be good choice for coating for two reasons. In higher concentrations Matrigel may form gel thus there is a risk that it might aggregate into the narrow microchannels when applying the coating material into the device. Also, we experienced problems when the devices were disassembled for the fixing and staining, as most of the axons were torn away from the Matrigel coated glasses. ICC staining of the PO+LN521 devices with β-tubulin and GFAP confirmed that the axons were growing well in the axon compartment. When the neurons and astrocytes had been cultured for three days in the devices, astrocytes were treated with IL-1β and TNF-α. As a result, cytokine treatment did not have detrimental effects to axons or their growth. Almost opposite effects were observed in some of the treated devices where axons seemed to grow even stronger. This could be possible since the effects of reactive astrocytes can be neuroprotective, and they might induce axonal growth as previously was discussed in this thesis. The effect of cytokine treatment to

astrocytes was further analyzed with ICC staining. The cytoskeletal changes in astrocytes were observed by staining the cells with GFAP and vimentin after 72h cytokine treatment. The treated astrocytes expressed both GFAP and vimentin in altered way compared to control. Astrocyte morphology changed in a way that GFAP and vimentin were vastly thickened, and the cell shape was hypertrophied. Previously, astrocyte and neuron co-cultures have been done with rodent cells in a suspension or hPSC-derived cells in co-culture systems utilizing 3D organoid-like spheres (Krencik et al. 2017; Kaech & Banker 2006). However, these randomly mixed co-culture methods have limitations. Rodent studies are not directly comparable to human studies, and many times the physical and biochemical environment cannot be manipulated and controlled properly. Advances in the microfluidics have created possibilities for neurological research. So far, the use of microfluidics for neuron and reactive astrocyte studies is minimal, thus comparison to other findings is limited. However, Park and colleagues introduced 3D triculture microfluidic platform for neurons, astrocytes and microglial cells (Park et al. 2018). In their platform, they tried to mimic the pathophysiology of AD with two different compartments; one with neurons and astrocytes and other for microglial cells. This differs from our set-up, since they had neurons and astrocytes in mixed cultures and we had them separately. In addition, they used activated microglia in astrocyte reactivation instead of separately added cytokines. However, they also showed altered astrocyte morphology and reported increased GFAP expression with activated microglia compared to control devices. At this point, we did not quantified GFAP expression in our study, but that could be something that would be interesting to study next more thoroughly. Their model was specifically modeled for AD whereas the aim in our study at this point was to study if the reactivation of astrocytes is possible in our in-house developed device. All in all, our platform could possibly be optimized and modified for certain CNS disease modelling. We have showed that axons grew well in our device in their own compartment, which offers possibilities for restricted study of axons. According to our study, reactive phenotype could be induced in hiPSC-derived astrocytes with just two cytokines. Since the reactivation of astrocytes have not been well studied with microfluidic devices, our platform could offer a promising tool for more precise study of reactive astrocytes and their crosstalk with neurons. Next, it would be interesting to study the molecules that reactivated astrocytes secrete in this device and how they effect on neurons. Thus, axonal viability and growth should be studied in more detail.

7 Conclusion

According to this thesis study, hiPSC-derived astrocytes can be induced to express reactive phenotype with IL-1β and TNF-α treatment. The treated astrocytes changed their morphology, upregulated pro-inflammatory molecules and altered their intermediate filaments and cytoskeletal structure. However, GFAP upregulation, a common marker for astrocyte reactivity, could not be detected. This indicates that astrocytes are heterogeneous cells and GFAP upregulation might not be a hallmark for hiPSC-derived reactive astrocytes. The inflammatory role of reactive astrocytes was observed and known inflammatory signaling pathway NF-κB was activated. However, astrocyte reactivation should be studied more with hiPSC-derived astrocytes to fully understand it.

Neuron and glial cell co-cultures are essential for understanding the complex mechanisms of human neural networks and CNS diseases. In addition, microfluidics offers a possibility to modify the environment of the cells in controlled way. We showed that neuron and astrocyte co-cultures can be successfully done in our in-house developed compartmentalized microfluidic device. In addition, reactive phenotype can be induced in these devices with inflammatory cytokines. More analysis related to astrocyte and neuron secretion profiles should be done to investigate their crosstalk. To sum up, our in-house developed microfluidic device offers a promising *in vitro* model for studying diseases in a dish.

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