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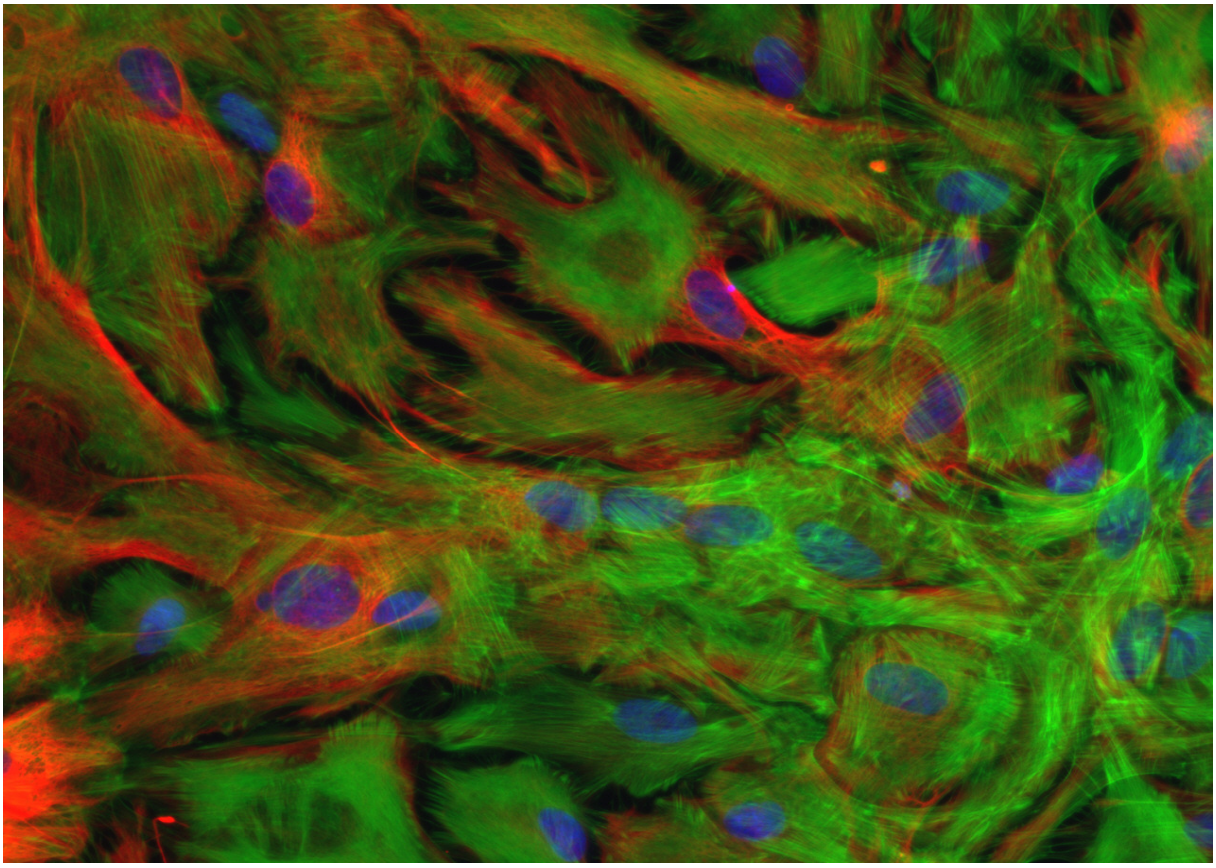

Network coupled astrocytes as a model system to study inflammatory properties

Master's thesis in Biotechnology

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Supervisor: Professor Elisabeth Hansson

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Gothenburg, Sweden 2014

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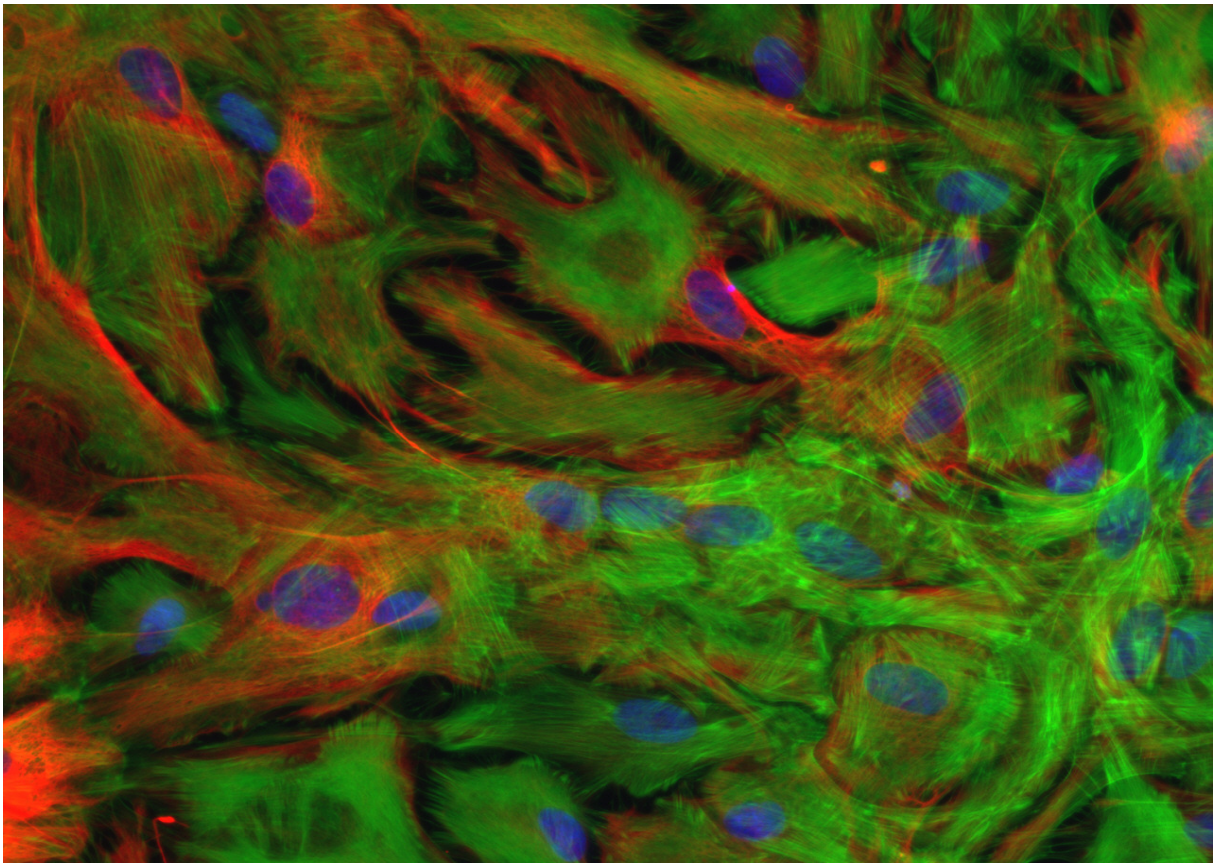

Network coupled astrocytes as a model system to study inflammatory properties

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Abstract

Before antibiotics and vaccines were discovered, microbes were the primary cause of mortality rates. However, with the drastically altered life style that comes with economic development, diseases that are non-infectious, chronic and recurrent have replaced microbial diseases as the biggest challenge to human health. Cancer, cardiovascular- and neurodegenerative diseases are some examples with potentially debilitating outcomes, and the common denominator among these have been recognized to be the pathways of chronic low-grade inflammation. However, inflammation is not well understood and research must thus be conducted to better understand the condition and elucidate potential therapeutic solutions. Because of this, scientists have employed both *in vivo* and *in vitro* systems to study inflammation but because of the lack of knowledge, especially regarding neuroinflammation, *in vitro* assays have produced many contradictory results. A reliable *in vitro* system is an attractive option to *in vivo* systems, as it is relatively inexpensive, quick, less complex and eliminates the ethical concern for animals.

The aim of this project was to develop a model cell system consisting of network coupled astrocytes and analyze changes in biomarkers when exposed to the pro-inflammatory mediators, lipopolysaccharide (LPS) and interleukin-1 beta (IL-1β), for 24 hours, 3, 6 and 9 days. Analyses were done by enzyme-linked immunosorbent assay, immunofluorescence, and Western blot. The astrocyte cell cultures were investigated under different cultivation parameters, of which two different seras were tested; one of which has been shown to promote microglial proliferation.

The cultures with the serum that has been shown to be beneficial to microglial proliferation had drastic effects on some of the investigated biomarkers, with a stronger induction of the toll-like receptor 4, reduced the Na^+/K^+-ATPase instead of an increase and an induced transcription of excitatory amino acid transporter 2. However, both seras reorganized astrocytic actin filaments similarly, caused a polymerization of globular actin, neither released the pro-inflammatory cytokines IL-1β or tumor necrosis factor alpha and in both serum groups the alteration to the biomarkers returned to control levels during longer stimulations than 24 hours. These results suggest that the astrocytes and microglia are not capable of initiating an inflammation by themselves, but microglia do appear to be at least partly responsible for observed alterations to known biomarkers. Furthermore, as previous studies have produced inflammatory reactive astrocytes by LPS or IL-1β, the results imply that additional unknown factors are missing, possibly an unrecognized contaminating cell type.

Contents

Introduction

Prior to the advent of antibiotics and vaccines, infections have been a major cause of mortality rates. However, with the epidemiological transition that comes with economic development, diseases that are noncommunicable, recurrent, lasting and have no microbial primary cause are replacing infections as the biggest challenge to human health (Egger 2012). Type two diabetes, cancer, asthma, cardiovascular and neurodegenerative diseases are examples, and a common denominator shared between them has been recognized to be the pathways of chronic low-grade inflammation (Medzhitov 2008).

Typically, when tissue injury or infection is recognized by tissue-resident macrophages and mast cells, a production of inflammatory mediators (e.g cytokines, chemokines and vasoactive amines) takes place. The immediate effect of this reaction is an acute inflammatory response which results in the delivery of leukocytes (primarily neutrophils) and plasma to the area of insult (Medzhitov 2008). When the neutrophils reach the location they are activated by cytokines that are released by tissue-resident cells (Steinbeck & Roth 1989). This activation results in an expulsion of toxic contents, including reactive oxygen species (ROS) and proteases, which not only attacks the pathogen, but the cells of the host as well (Nathan 2006). If the acute inflammatory response is successful, tissue-resident and recruited macrophages mediate a resolution and repair phase through secretion of anti-inflammatory compounds, which promote the recruitment of monocytes. Monocytes then remove dead cells and together with myofibroblasts initiate tissue remodeling (Serhan & Savill 2005; Hinz et al 2007). However, if the pathogen persists neutrophils are replaced with macrophages, and during infection T cells are involved as well. If these cells fail to clear the inflammatory insult a chronic inflammation arises. Beside pathogens, autoimmune disorders (in which the immune system attacks its own cells) and nondegradable foreign bodies may result in chronic inflammation (Medzhitov 2008).

Inflammation in the central nervous system (CNS) occurs on slightly different terms, due to its sensitive and closed nature. The major cells of the CNS are astrocytes, microglia, neurons and oligodendrocytes, of which the major tissue-resident macrophages are microglia. Microglia produce anti-inflammatory and neurotrophic factors under physiological conditions and pro-inflammatory mediators in response to infection or tissue damage (Streit 2002). Some of the pro-inflammatory mediators are toxic, such as NO and ROS, and have been suggested to act additively or synergistically with neurotoxic factors produced by microglia. These factors are continually produced by activated microglia, which also acts on astrocytes, that in turn amplifies the inflammatory reaction (Saijo et al 2009).

Toll like receptors (TLRs) which recognize structural motifs characteristic for pathogens and are known to be expressed by the innate immune system cells (Janeway & Medzhitov 2002) are gaining increasing recognition for having a role in several inflammatory diseases. Beside their exogenous ligands, they have also been shown to have endogenous agonists, e.g heat shock protein 60, which are released during apoptosis and necrosis (Lehnardt et al 2008). ATP, which is released by dead cells, has also been shown to induce inflammatory responses (Di Virgilio 2007). It is thus possible that an initial inflammatory reaction may harm or kill neurons and result in the release of endogenous proteins, forming a vicious cycle which maintains a low-grade inflammation.

The genesis of neurodegenerative diseases is largely unknown but inflammation has been correlated to numerous. If inflammation is indeed the cause or catalyst of neurodegenerative diseases, an attenuation of the reaction is of clinical significance. Due to this, microglia and astrocytes are of particular interest as they are able to initiate and amplify inflammation respectively, and by doing so, release neurotoxic compounds. Therefore, an *in vitro* cell model system for inflammation is desired to study potential treatments.

In this thesis astrocytes were cultured for two weeks. The aim of the project was to make the cells inflammatory reactive through various methods, study the change of known biomarkers and compare the results to previous research. Limitations of the project were the long cultivation time, the low amount of cell material produced in a given cultivation and the dynamic nature of cells which produces contradicting results among researchers, depending on a myriad of factors such as the serum used. Finally, the current knowledge of neuroinflammation is relatively poor.

Background

Central Nervous System

The cells of the CNS constitute a complex system mainly consisting of four cell types; neurons, astrocytes, microglia and oligodendrocytes. The three latter types make up about 90% of the CNS, with astrocytes constituting around 85% of all the glial cells (Blake 2008). Shortly summarized; astrocytes support neurons with energy substrates, inactivates neurotransmitters and K^+ by uptake, they are communicators between blood and extracellular space as well as produce scars in damaged CNS tissue (Sandberg 2013). Microglia mobilizes and phagocytose dead cells and bacteria when detecting an altered chemistry in the extracellular space, in addition to initiating inflammatory responses in astrocytes by the production and release of pro-inflammatory cytokines. Oligodendrocytes mainly produce insulating myelin around the axons of the neurons.

Cerebral blood flow and the molecular exchange between blood and neural tissue are also tightly regulated. In contrast to most endothelia which allow paracellular molecular traffic, the capillaries in the brain are constituents of a physical barrier called the blood-brain barrier (BBB), which forces molecules to cross the BBB through transcellular means (Hawkins & Davis 2005). The BBB is composed of endothelial cells making up the capillaries, surrounded by pericytes and basal lamina, which together are enveloped by astrocytic end-feet (see figure 1) (Abbott et al 2006).

Blood-brain barrier

The BBB is a selective physical, transport and metabolic barrier which, due to tight junctions between the endothelial cells, transport molecules transcellularly rather than paracellularly in contrast to most endothelia (Hawkins & Davis 2005). To limit the entry of harmful compounds, the barrier employs transport systems for required nutrients, although small gaseous- and small lipophilic molecules are still able to cross the BBB freely (Begley & Brightman 2003). In addition to a physical barrier, intracellular enzymes such as the cytochrome P450 family functions by inactivating toxic compounds (El-Bacha & Minn 1999). Besides protecting the CNS from toxic compounds, the BBB also has regulatory functions. Together with astrocytes, it maintains CNS milieu homeostasis by regulating the ion composition and preventing fluctuations that occur in conjunction with e.g meals, as well as allowing similar signaling molecules peripherally and within CNS without interference (Wolburg & Lippoldt 2002; Hawkins & Davis 2005). The known cells of the BBB are endothelial cells making up the vasculature, pericytes surrounding them with astrocytic end-feet enveloping both (see figure 1) (Abbott et al 2006). Mast cells have also been found in the BBB, where 96 % of CNS resident mast cells are located, and 90 % of these make contact with endothelial cells or its extracellular matrix (Khalil et al 2007). The importance of astrocytes, pericytes and perivascular cells regarding BBB regulation and maintenance is largely unknown. Astrocytes have been implicated in the formation of endothelial tight junctions and polarized endothelial expression of transporters, and both astrocytic end-feet and pericytes have transport activities that may contribute to barrier functions (Abbott et al 2010). Pericytes have also been shown to regulate

the formation of tight junctions as well as they regulate vesicle trafficking and displays an inhibition of the expression of molecules that increase vascular permeability (Daneman et al 2010). Furthermore, they are suggested to modulate vasculature and are able to either inhibit or facilitate immune cell migration (Hamzah et al 2008).

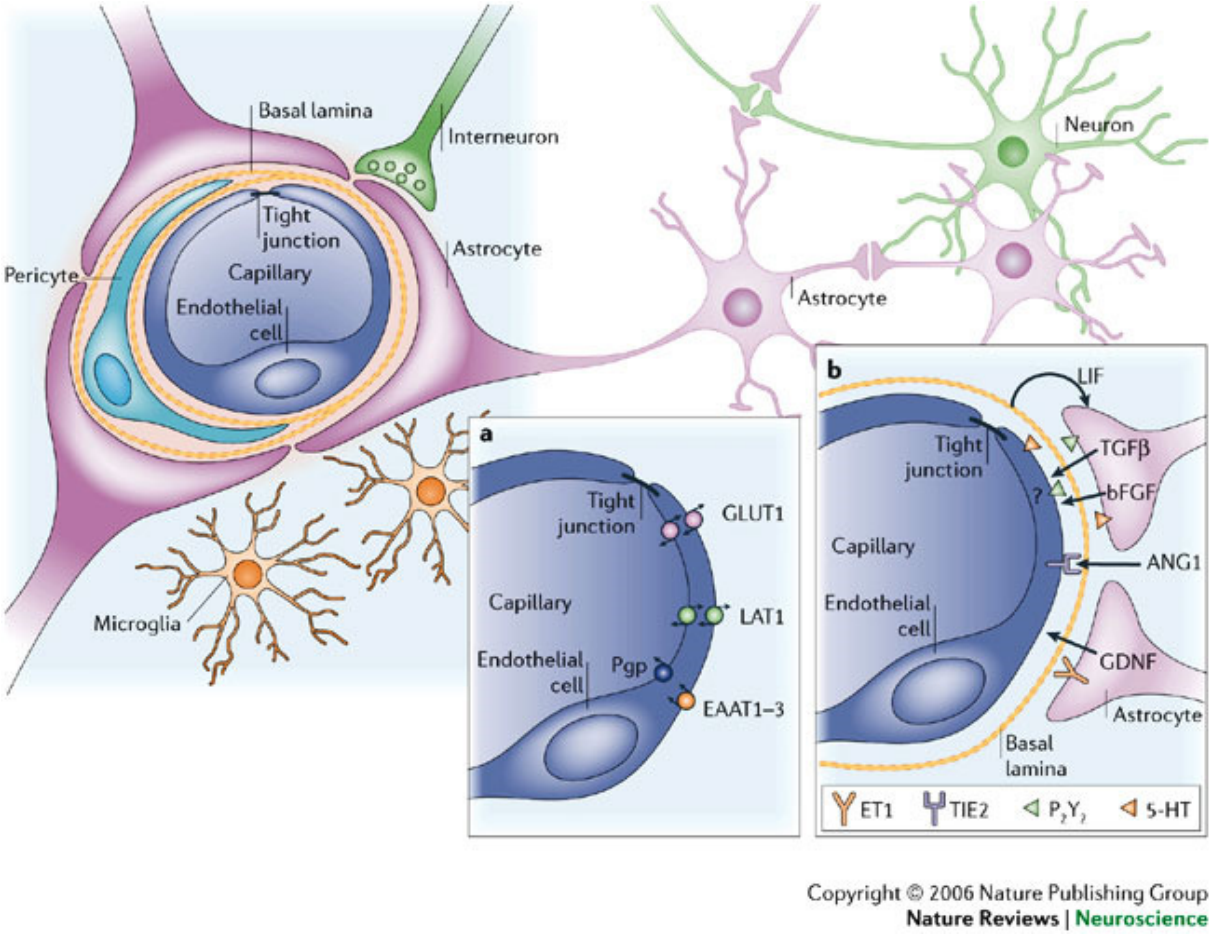

Figure 1. The main cellular constituents of the blood-brain barrier are endothelial cells, pericytes and astrocytic end-feet. a) Endothelial cells have transport proteins for e.g nutrients. b) Astrocytic end-feet responsible for bi-directional signaling in the BBB. Abbott NJ *et al.* (2006) Astrocyte–endothelial interactions at the blood–brain barrier. *Nat. Rev. Neuro.* 7: 41–53 doi:10.1038/nrn1824

Astrocytes

Astrocytes (from Greek *astron* = star and *cyte* = cell), also known as astroglia (*glia* = glue), are named so because of early immunolabeling experiments that stained the glial fibrillary acidic protein (GFAP) (Eng et al 1971; Bignami & Dahl 1974), which resulted in a star-shaped appearance. However, since the finer processes of the astrocytes are GFAP negative, the name does not represent the true morphological form of the cells (Nedergaard et al 2003). Two main types of astrocytes exist; fibrous and protoplasmic. Fibrous astrocytes exist primarily in white matter and have long processes similar to fibers, while protoplasmic astrocytes mainly exist in grey matter with a globular morphology (Ramon & Cajal 1909). Studies have shown that the processes of protoplasmic astrocytes envelop synapses, while the fibrous astrocytes make contact with nodes of Ranvier, and together they make up a heterogeneous cell population. Some common features the subtypes share in common are extensive contact with blood vessels and the formation of gap junctions between distal astrocytic processes which connects neighboring astrocytes (Peters et al 1991). The gap junctions in the processes are evenly distributed and apart from interconnecting astrocytes, they interconnect the processes of individual cells as well. These gap junctions serve to minimize the difference in concentrations concerning molecules of 1000 kDa and less (Kumar & Gilula 1996). In contrast to neurons, astrocytes are electrically non-excitable, and after this finding was made it was concluded that astrocytes have no involvement in signal transmission (Nedergaard et al 2003). However, observations made in the 1990s found that astrocytes react to glutamate by increased cytosolic Ca^{2+} concentrations, followed by an inter-astrocytic propagation of a Ca^{2+} waves (Cornell-Bell et al 1990). This increase in Ca^{2+} concentration has been shown to be part of an astrocyte-astrocyte and astrocyte-neuron intercellular communication (Blomstrand et al 1999). Elevations of Ca^{2+} concentration can be triggered as a result of neurotransmitters released by neurons (Blomstrand et al 1999). The result of released Ca^{2+} includes a release of neurotransmitters into the extracellular space, thereby triggering synaptic action potentials (Shigetomi et al 2008).

Astrocytes make extensive contact with blood vessels. This strategic position allows them to regulate blood flow depending on synaptic activity (Schummers et al 2008), possibly by their production of the auto- and paracrine signaling molecules which includes prostaglandins and nitric oxide (Amruthesh et al 1993; Murphy et al 1993). Another important feature of astrocytes lies with their regulation of synaptic mileu, as they control Ca^{2+}, Na^{+}, K^{+}, Cl^{-}, pH, and neurotransmitter homeostasis (Longuemare et al 1999; Ko et al 1999; Danbolt 2001), which is a critical task to ensure an optimal system.

Microglia

Microglial cells are macrophages of hematopoietic origin, i.e they stem from the progenitor cells of all blood cells, and make up approximately 10% of total cells in the CNS (Alliot et al 1999; Blake 2008)During early development they migrate from the umbilical vesicle to the developing neural tube (Ginhoux et al 2010) and act as the main immunocompetent cells of the CNS throughout life. They are mostly found in gray matter; mainly the hippocampus, basal ganglia, substantia nigra and olfactory bulb (Lawson et al 1990). During normal physiological conditions, microglia remain in a quiescent state due to neuron and astrocyte derived factors (Cardona et al 2006), but are highly active by continually surveying the environment of the CNS with their extended processes (also known as the "resting state") (Nimmerjahn et al 2005). During insult to the CNS, such as infection or damaged cells, the microglia become "activated" and change their morphology into an amoeba-like shape (Streit & Kreutzberg 1988). These insults are sensed through their expressed pattern recognition receptors, which regulate the activity of transcription factors including "nuclear factor kappa-light-chain-enhancer of activated B cells" (NFκB), followed by a mobilization towards the relevant area (Blake 2008; Akira et al 2006). However, microglia are not the only CNS cells capable of sensing injury and infection, as astrocytes are active participants in the reaction as well, and amplifies the reaction initiated by microglia (Saijo et al 2009).

During inflammation, among other CNS diseases, the microglial pool of the CNS expands, which has been believed to be partly due to bone-marrow derived stem cells that migrate across the BBB (Simard & Rivest 2004; Priller et al 2001). All studies that made this finding employed lethal irradiation and transplant of whole bone-marrows by flushing and intravascular injection. However, since the irradiation technique disrupts the BBB and the transplants artificially produce circulating progenitor cells, the results may be skewed as it would allow a higher frequency of cell recruitment. Ajami et al (2007) performed a study to see whether this cell migration is true in non-irradiated animals, and came to the conclusion that the increased microglial pool is primarily a result of the resident microglia proliferating, and not a recruitment of circulating precursor cells through the BBB. Other studies have also supported the finding that there is minimal cellular recruitment through the BBB under physiological conditions (Massengale et al 2005; Hickey & Kimura 1988). Furthermore, Ajami et al (2007) found that bone-marrow derived progenitor cells were not capable of spontaneously entering the blood stream and only when artificially delivered to the blood stream do they enter the CNS and generate microglia. It was then concluded that these findings suggest self-renewing resident cells of the CNS are the major reason for maintenance and local expansion of microglia.

Cytoskeleton

The cytoskeleton has three types of protein filaments and is composed of actin filaments, intermediate filaments and microtubules. As the name implies, the cytoskeleton plays a structural role, e.g. determining the cell shape and positioning of organelles. However, it is also responsible for entire cell movements as well as internal transport of organelles. When the cell changes its shape, e.g. during cell migration, the cytoskeleton reorganize continually (Cooper & Hausman 2009).

Actin filaments are ~7 nm in diameter and consist of single actin monomers, which has polymerized into long chains by the aid of various proteins, providing e.g. nucleation sites or stabilization of the chain (Cooper & Hausman 2009). Actin monomers are called globular actin (G-actin) and the polymerized form is called filamentous actin (F-actin). Polymerization is possible due to head-to-tail interactions between each monomer, which results in a long actin fiber of monomers oriented in the same direction with the ends of the fiber being called pointed end and barbed end. The barbed end of F-actin grows five to ten times faster than the pointed end. However, proteins that regulate the stability and disassembly of either end may give rise to an effect called "treadmilling", where association of G-actin to the barbed end occurs at the same rate dissociation from the pointed end occurs (Cooper & Hausman 2009). Association and dissociation of G-actin is an important feature to allow for cell movement and formation of cell processes. There are also proteins which assist in cross-linking actin filaments into bundles or networks, and associating filaments with cell structures. One such cell structure is the plasma membrane, where the actin filaments are very abundant and form a network which determines cell shape, allows the cells to migrate and the cell surface to move, engulf other particles, divide as well as providing mechanical support. Together with motor proteins such as myosin, actin allows for transport of organelles and vesicles containing, for example, membrane proteins (Cooper & Hausman 2009)

Inflammation

Inflammation is part of the innate immune system, i.e the nonspecific immune system. Inflammation may initiate as a reaction to pathogens, antigen recognition and tissue damage, for the purpose of eliminating infectious agents and repairing damaged tissue. When these insults are detected, a release of cytokines and other pro-inflammatory mediators occur which initiates an inflammatory cascade (Nathan 2002). This reaction activates mast cells which respond by releasing e.g histamine, cytokines and serine proteases, promoting an infiltration and activation of leukocytes to the area as well as an activation of pro-matrix metalloproteases (MMP) (Medzhitov 2008). MMPs then activate cytokines and cleave constituents of the extracellular matrix (Mohan et al 2002). As inflammation resolves, macrophages release a serine protease inhibitor which also has anti-inflammatory effects (Jin et al 1997). However, inflammation may have deleterious consequences if it is induced or regulated improperly (Barton 2008). Sustained inflammation has been correlated to Alzheimer's disease, Parkinson's disease, multiple sclerosis among other neurodegenerative diseases (Glass et al 2010).

The major leukocyte of the CNS is microglia, which produce anti-inflammatory and neurotrophic factors during its "resting" state (Streit 2002). When pathogens or tissue damage

is recognized, microglia promotes inflammatory responses which typically clear once the insult has been resolved. Microglia has for long been the focus of neuroinflammation, where they have been attributed to initiation of inflammation (Saijo et al 2011). Microglia are considered critical elements of the neurodegenerative aspect related to inflammation, partly due to their nature of releasing ROS when activated, which has been observed to be a common characteristic among many neurodegenerative diseases (Block & Hong 2005). However, mast cells are gaining increased attention (Skaper et al 2014). The mammalian brain contains perivascular mast cells that may disrupt the BBB through the action of e.g the vasodilator, histamine, and production and activation of MMP (Pang et al 1996; Lindsberg et al 2010). Mast cells have also been shown to be the first responders to neuroinflammation, and they are also the only cells that contain pre-formed tumor necrosis factor alpha (TNF-α) (Jin et al 2009; Gordon & Galli 1991). Mast cells are not the only cells in the CNS that have been shown to produce pro-MMP, as microglia and astrocytes have also been observed to produce the proteases (del Zoppo et al 2012). The activation of pro-MMPs has been shown to be mediated by e.g serine proteases secreted by mast cells during inflammation (Lindsberg et al 2010), and active MMPs contribute to inflammation by activating pro-inflammatory cytokines such as pro-TNF-α (Mohan et al 2002). Conversely, both active MMPs and serine proteases have also been shown to degrade pro-inflammatory cytokines (Ito et al 1996; Zhao et al 2005). In addition, mast cells have been implicated in the differentiation of fibroblasts into myofibroblasts mediated by the serine protease, tryptase, and as pericytes have been suggested to differentiate into myofibroblasts during inflammation, mast cells could be responsible for that conversion (Mangia et al 2011; Hinz et al 2007). During inflammation myofibroblasts are mainly involved in the remodeling the extracellular matrix through secreted MMPs as well as their substrate, collagen, and as such they could be a key factor in the progression of inflammation (Hinz et al 2007; Rabkin et al 2001). Due to the destructive nature of inflammation, cells also have several mechanisms to inhibit undue pro-inflammatory signaling (Kovalenko et al 2003; Zhou et al 2012).

Pro-inflammatory mediators

Toll/interleukin-1 receptor ligands

The TLR family is a family of receptors that are expressed by the innate immune system cells. These receptors recognize structural motifs which are characteristic for pathogens, namely bacteria, virus and fungi (Janeway & Medzhitov 2002); these structural motifs are also known as pathogen-associated molecular patterns (PAMPs). The activation of TLR results in an induced transcription of pro-inflammatory cytokines. One well studied PAMP is the gram negative endotoxin LPS (Oneill & Todd 1961) that activates TLR4 and induces systemic inflammation, which occurs from excessive signaling (Beutler & Rietschel 2003). The interleukin-1 receptor (IL-1R) is highly related in the cytoplasmic domain (Gay & Keith 1991), and is activated by e.g the pro-inflammatory cytokine, IL-1β (Akira & Takeda 2004). When either TLR4 or IL-1R receptor is activated, a cascade of cellular signals occurs through the myeloid differentiation primary response gene 88 (MyD88) pathway and MyD88-independent pathways, which results in the activation of NFκB. NFκB then proceeds with the transcription

of pro-inflammatory cytokines such as TNF-α and IL-1β (Azzolina et al 2003; Baeuerle & Henkel 1994; O'Neill & Bowie 2007).

Even though TLR exists as a system that contributes to the defense of the host, excessive signaling has also been correlated to inflammatory diseases such as cardiovascular diseases and atherosclerotic lesions (Frantz et al 1999; Xu et al 2001; Edfeldt et al 2002). TLR4 has previously been shown to be up-regulated during LPS induced inflammation (Forshammar et al 2011), which may be due to mobilization of Human antigen R (HuR) from the nucleus into the cytoplasm, which stabilizes the TLR4 mRNA. LPS also promotes binding of HuR to the 3'-UTR of TLR4 mRNA as well as enhancing gene expression due to this interaction, in smooth muscle cells (Lin et al 2006). Analysis of cultured human adult astrocytes have shown that exposure to inflammatory cytokines did not increase the expression of TLR4 (Bsibsi et al 2006; Carpentier et al 2005). In addition, Lehnardt et al (2002) did not find any TLR4 mRNA in murine astrocytes, whereas TLR4 was detected in microglia.

The pro-inflammatory cytokine IL-1β is synthesized in its latent form (pro-IL-1β). The enzyme responsible for the maturation of IL-1β is caspase 1, also known as interleukin-1 converting enzyme (ICE) (Thornberry et al 1992). However, Fantuzzi et al (1997) observed that the latent cytokine was processed in ICE-deficient mice as well, and Schönbeck et al (1998) saw that MMPs are able to process pro-IL-1β into its active form, as well as degrade the matured cytokine.

Tumor necrosis factor alpha

TNF-α is a pro-inflammatory cytokine that causes a signal cascade leading to the activation of NFκB. However, studies in endothelial cells have shown that it does not go through the MyD88 pathway (Li et al 2003). TNF-α is synthesized as an inactive form (pro-TNF-α) and is anchored to the cell membrane prior to activation (Decker et al 1987; Moss et al 1997). Activation of pro-TNF-α has been shown to occur by the metalloprotease TNF-α converting enzyme (TACE) but also other, more general MMPs (Moss et al 1997; Mohita et al 2002).

Excitatory amino acid transporters

Excitatory amino acid transporters (EAATs), also known as glutamate transporters, are transmembrane proteins which exist in both neurons and glial cells. These transporters are responsible for the majority of glutamate removal from the extracellular fluid, which implicates their importance in maintaining a low and non-toxic concentration of glutamate (Danbolt 2001). Since glutamate functions as a neurotransmitter, the EAAT family is able to modulate neurotransmission by e.g modifying the extent and pattern of activation. These transporters are also involved in providing glutamate for synthesis of e.g GABA, glutathione and provide glutamate for energy production (Mathews & Diamond 2003; Rimaniol et al 2001; Peng et al 2007). Glutamate itself is considered the major excitatory neurotransmitter in the mammalian CNS and is believed to be involved in most aspects revolving normal brain function, e.g memory, learning and cognition (Fonnum 1984). Glutamate is also involved in the development of the CNS including cell migration and differentiation (Komuro & Rakic 1993; Moran & Patel 1989). The importance of extracellular glutamate removal is stressed by the fact that should it diminish, synapse firing will increase in intensity, eventually resulting in excitotoxicity (Cheung et al 1998).

Glutamate transporters can be divided into two groups, sodium-dependent- (EAAT) and sodium-independent (VGLUTs and xCT) glutamate transport systems (Danbolt 2001). This thesis will focus on two of the sodium-dependent variants.

GLT-1 and GLAST

GLT-1 (EAAT2) is the dominating glutamate transporter in all regions of the mammalian CNS with the exception for regions where GLAST (EAAT1) is the major transporter, and both GLAST and GLT-1 exist primarily in astroglial cells (Danbolt 2001). The expression of GLAST and GLT-1 varies with maturity. The canonical isoform of GLAST has a molecular weight of 59 kDa and following glycosylation it increases to 64 and 70 (Schulte & Stoffel 1995), while canonical and glycosylated GLT-1 is 62 and 75 kDa respectively kDa (Kalandadze 2004). However, the glycosylation has been shown to not affect the transport activity of GLAST and GLT-1, and glycosylated GLT-1 was transported to the plasma membrane with a similar efficiency to that of the non-glycosylated isoform (Conradt et al 1995; Raunser et al 2005). GLAST mRNA is primarily expressed during cell migration of the CNS, whereafter the expression diminishes and, during the postnatal development, GLT-1 mRNA expression shows a significant increase (Sutherland et al 1996). Swanson et al (1997) showed that monocultured astrocytes only expressed the GLAST subtype whereas astrocytes co-cultured with neurons were more differentiated and expressed both subtypes.

The loss of various EAAT transporters have been observed in the CNS under infectious and inflammatory disorders (Werner et al 2001; Wang et al 2004; Vercellino et al 2007). Following exposure to the pro-inflammatory cytokine TNF-α, the function and expression of GLT-1 and GLAST has been shown to be suppressed (Wang et al 2003; Korn et al 2005), and Prow & Irani (2008) showed that IL-1β resulted in a loss of GLT-1 *in vivo*. NFκB has been shown to function as an activator as well as a repressor of GLT-1 transcription, depending on the mediating molecule; TNF-α represses while EGF induces the transcription (Sitcheran et al 2005).

Conversely, O'Shea et al (2006) found a delayed, increased astroglial GLT-1 cell-surface expression which was increased to 172% compared to the control after 72 hours of stimulation with LPS. A clustering of GLAST, but no significant alteration in the expression of the protein, was found to appear as well and the glutamate uptake rate was increased to 129%, suggesting higher EAAT activity, which is in accordance with the findings of Namekata et al (2008). Namekata et al (2008) also found that the GLAST expression levels were unaffected by the treatment of IL-1.

Na^+/K^+-ATPase

Three Na^+ are required for the transport of one glutamate by EAAT, and as such, the transport is dependent on the intra- and extracellular concentration of Na^+ (Longuemare et al 1999; Szatkowski et al 1990). The intracellular concentration of Na^+ is maintained by the Na^+/K^+-ATPase, which imports K^+ and exports Na^+ out of the cell. As the name of the transporter implies, the function is dependent on ATP; and should ATP deplete, Na^+/K^+-ATPase will not be able to expulse the Na^+, which would result in failing glutamate transport and ultimately, excitotoxicity (Allan et al 2001; Erecinska & Silver 1994; Loungemare et al 1999). Studies made on the Na^+/K^+-ATPase have shown that both LPS and IL-1β decrease the content of the pump in renal and hepatocytic cells (Schmidt et al 2007; Green et al 1996). However, Forshammar et al (2011) observed an increase of the Na^+/K^+-ATPase during the first eight hours from LPS stimulation, followed by an attenuation. Namekata et al (2008) studied IL-1 in conjunction with GLAST and Na^+/K^+-ATPase in Müller cells (a type of astroglia in the retina), showing that IL-1 possibly increased Na^+/K^+-ATPase membrane localization. IL-1 was shown to activate the p38 mitogen-activated protein kinase (MAPK)/caspase 11 pathway, which results in a destabilization of the actin cytoskeleton, and in turn, allows for Na^+/K^+-ATPase membrane redistribution. Furthermore, studies have observed that LPS activates the Sp1 transcription factor, which has a binding site in the promoter region of Na^+-K^+-ATPase α_1-subunit, among other common and cell type-specific transcription factors (Suzukiyagawa et al 1992; Ma et al 2001).

Aim

The aim of this project was to develop a model cell system for inflammation and find suitable biomarkers to detect an inflammatory reaction. The well-established endotoxin, LPS, and pro-inflammatory cytokine, IL-1β, were utilized to achieve an inflammatory reaction. To ascertain whether an inflammatory reaction had occurred, analysis with the different methods; enzyme-linked immunosorbent assay (ELISA), immunofluorescence and Western blot was undertaken to investigate changes of biomarkers.

Astrocyte cell cultures were investigated with different cultivation parameters, including different sera that will henceforth be dubbed astroglia serum and microglia serum. The groups were as follows;

- Cell cultures grown solely in astroglia serum.

- Cell cultures with astroglia serum, added microglia day zero and 30 min shaking on days of serum exchange.
- Cell cultures with astroglia serum the first three days, microglia serum from day four and forth, when astrocytes had established, as well as 30 min shaking on days of serum exchange.
- Cell cultures with astroglia serum the first three days and microglia serum from day four and forth, when astrocytes had established.
- Cell cultures with microglia serum from day zero and shaking for 30 min on days of serum exchange.
- Cell cultures grown solely in microglia serum.

Methods

Primary astrocyte cultures and stimulation

Rat primary cortical astrocytes (Thermo Fischer Scientific, Waltham, MA, USA) were cultured with a density of 1 x 10^4 cells per cm^2 on glass cover slips (Nr 1, diameter 20 mm, BergmanLabora, Stockholm, Sweden) placed in a 12-well plate. The cells were incubated at 37°C, 90% humidity and 5% CO_2 and the medium was replaced twice a week. The experiments were conducted after cultivating the cells for 17 days and stimulating them with LPS (10 ng/ml) or IL-1β (10 ng/ml) respectively, for 24 hours, 3 days, 6 days and 9 days prior to harvest.

The following variants were performed;

- Cell cultures grown solely in astroglia serum.

- Cell cultures with astroglia serum, added microglia day zero and 30 min shaking on days of serum exchange.
- Cell cultures with astroglia serum the first three days, microglia serum from day four and forth, when astrocytes had established, as well as 30 min shaking on days of serum exchange.
- Cell cultures with astroglia serum the first three days and microglia serum from day four and forth, when astrocytes had established.
- Cell cultures with microglia serum from day zero and shaking for 30 min on days of serum exchange.
- Cell cultures grown solely in microglia serum.

Immunofluorescence

The media covering the stimulated cells was removed and followed by fixation for 10 min using 4 % paraformaldehyde (Histolab, Gothenburg, Sweden). After fixation the cells were washed with phosphate-buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.45) (Invitrogen) (PBS) containing 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) (PBS-BSA). After the washing, PBS-BSA containing 0.05% saponine (Sigma-Aldrich, St. Louis, MO, USA) (PBS-BSA-sap) was used to permeabilize the cells for the duration of 20 min. The cells were then incubated for an hour with a rabbit polyclonal antibody cocktail targeting GFAP (Dako, Glostrup, Denmark), diluted 1:100 in PBS-BSA-sap. For assays where OX42 was targeted as well, a mouse monoclonal antibody was utilized (Serotec, Oxford, UK) with identical dilution. The cells were subsequently washed with PBS-BSA-sap three times and then incubated for an hour with the secondary antibodies, donkey anti-rabbit IgG $F(ab')_2$ fragment conjugated with Dylight 594 and donkey anti-mouse IgG $F(ab')_2$ fragment conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Europe Ltd, Suffolk, UK), diluted 1:150 in PBS-BSA-sap. The cells were afterwards washed with PBS-BSA-sap three times and PBS-BSA a single time. For imaging of actin, 10 µl of the probe Alexa 488 (Invitrogen) was incubated with 400 µl PBS-BSA per cover slip, with the fluorescent DNA dye Hoechst 33258 (Life technologies) in a dilution of 1:1000 when used, for 20 min in room temperature. The cells were finally washed with PBS three times and then mounted on microscope slides with fluorescent mounting medium (Dako). Nikon Eclipse 80*i* microscope was used for imaging and the pictures were taking with Hamamatsu C5810 color intensified 3CCD camera.

Protein determination assay

The DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) was performed in accordance with the supplied manual (Lowry et al 1951). However, the standard ranged from 0-4 mg/ml BSA. Samples and standards were mixed with the reagents, the microplate shaken and then incubated for 15 min in room temperature. The absorbance was read at 750 nm using a Versa-max microplate reader and subsequently analyzed by SoftMax Pro 4.8 (Molecular Devices, Sunnydale, CA, USA).

Sandwich ELISA

Rat IL-1β (R&D Systems, Minneapolis, MN, USA) and TNF-α (BD Bioscience, San Jose, CA, USA) were both performed as advised by respective manufacturers. Each sample was done in duplicate and the standard curve was done in triplicate. The absorbance was read with Versa-max microplate reader and the data analyzed by Softmax Pro 4.8 (Molecular Devices, Sunnydale, CA, USA.). The amount of cytokine release was correlated with the Lowry assay described above.

SDS-PAGE and Western blot

The media covering the stimulated cells was collected and the cells were immediately lysed in radio-immunoprecipitation assay (RIPA) (Sigma-Aldrich, St. Louis, MO, USA) mixed with a protease inhibitor cocktail (Sigma-Aldrich). A cell scraper was employed and the lysate was collected. Total protein content was measured by the Lowry et al (1951) assay described above, and the samples containing a total of 13 µg protein were each mixed with 10 µl 4x LDS sample buffer (Invitrogen), 4 µl 10x reducing agent (Invitrogen) and diluted to 40 µl with distilled water. They were then heated at 70 ^{o}C for 10 minutes before being loaded into the gel lanes. β -actin was stained to control equal loading. SDS-page was performed utilizing the Novex pre-cast gel system (Invitrogen) and recommended method, with 4-12% Bis-Tris gels (Invitrogen) at 200 V for 50 min. Following the electrophoresis, the proteins were transferred to a nitrocellulose membrane (Invitrogen) at 30 V for an hour using NuPAGE transfer buffer (Invitrogen) complemented with methanol (Sigma-Aldrich) and NuPAGE antioxidant (Invitrogen). After the transfer the membranes were rinsed 2 times with distilled water and the transfer confirmed by Ponceau S solution (Sigma-Aldrich). Ponceau S solution was then washed off and a blocking solution consisting of 0.5 % fat-free skim milk (Semper AB, Sundbyberg, Sweden) in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.5 % Tween 20, pH 7.5) was added to the membranes to block overnight. Ensuing blocking, the membranes were probed separately with primary antibodies against CX43, Na^{+}/K^{+}-ATPase (α-subunit), TLR4, GLT-1, with β –actin being probed on all membranes except CX43. The antibodies and dilutions were as follows; 1:150 rabbit polyclonal antibody (Invitrogen), 1:250 mouse monoclonal antibody (Sigma-Aldrich), 1:500 rabbit polyclonal antibody (Santa Cruz), 1:1000 rabbit polyclonal antibody (Covalab), 1:1000 rabbit polyclonal antibody (Covalab) respectively. The dilutions were done in 4 ml blocking solution. After incubation of primary antibodies, the membranes were washed 3x3 min and then incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for an hour. The antibodies used were donkey anti-rabbit IgG $F(ab')_2$ fragment and donkey anti-mouse IgG $F(ab')_2$ fragment (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) diluted 1:10000 in blocking solution. The membrane was again washed with TBST 3x3 min. Detection of antibodies was carried out using the enhanced chemiluminescence kit (PerkiElmer Inc., Waltham, MA, USA) and Fuji Film LAS-3000 (Tokyo, Japan) employed for visualization.

Actin assay

Actin quantitation was performed as recommended by the F-actin/G-actin In Vivo Assay Biochem Kit (Cytoskeleton, Inc., Denver, CO, USA) and SDS-page performed as described above, with the exception of sample preparation. The primary antibody against actin, rabbit polyclonal antibody (Cytoskeleton) was diluted 1:500 in blocking solution, and the secondary antibody, donkey anti-rabbit IgG $F(ab')_2$ fragment conjugated to HRP (Jackson ImmunoResearch) was employed and diluted 1:10000 in blocking solution.

Results

Immunofluorescence

Astroglia serum

Astrocytes were stained with FITC for actin and Dylight 594 for GFAP. F-actin organized in stress fibers dominated the untreated culture whereas LPS had the most pronounced ring structures and IL-1β slightly more than the control group (see figure 2).

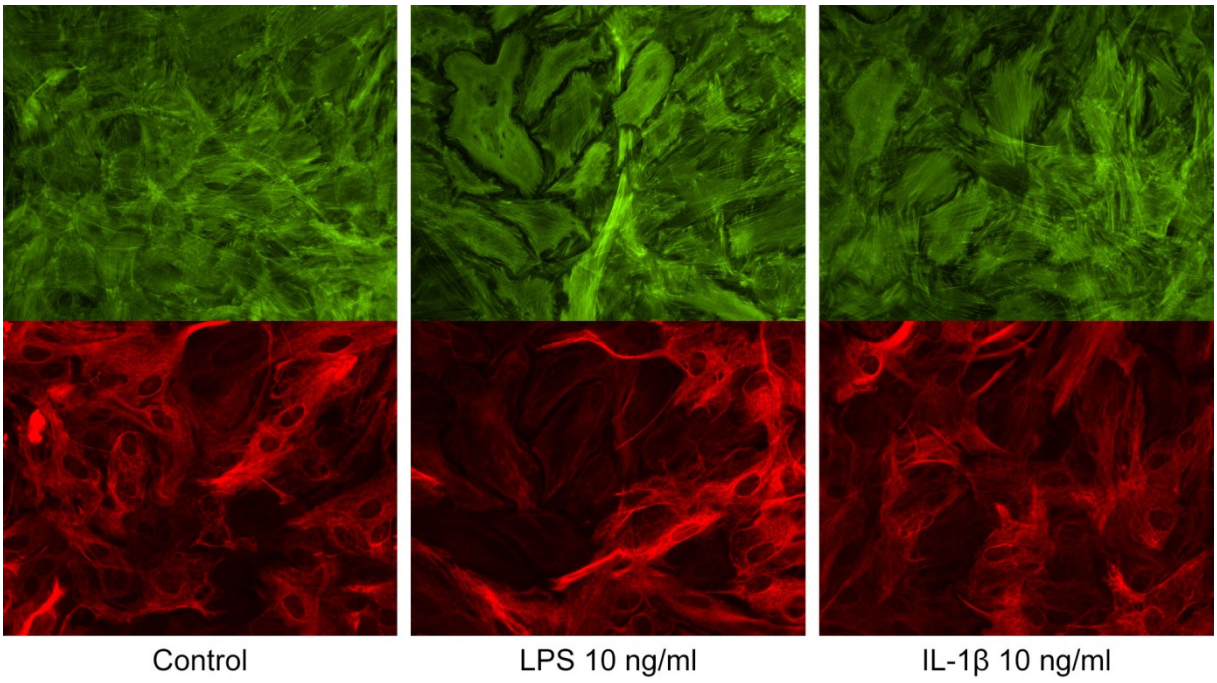

Figure 2. Astroglia serum cultures were stimulated with LPS or IL-1β for 24 h. Actin was stained with FITC (green) and Dylight 594 (red) for GFAP.

Astroglia serum and added microglia with shake on day 4 and forth

Astrocytes were stained with FITC for actin and Dylight 594 for GFAP. Cell debris and diffuse organization of actin was observed in all three groups, with the IL-1β stimulated group exhibiting more ring structural reorganization (see figure 3).

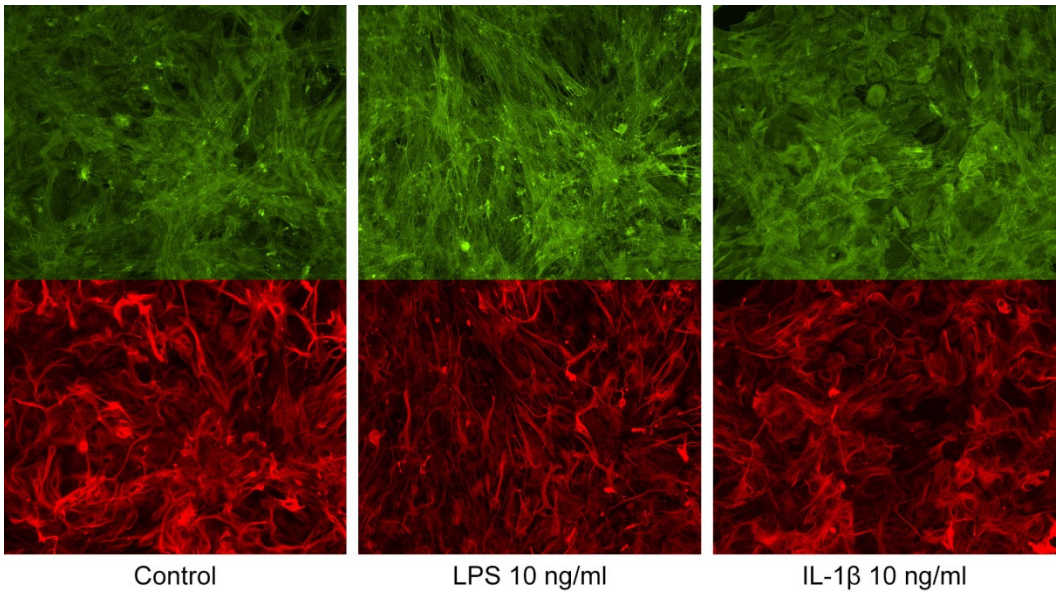

Figure 3. Astroglia serum cultures were stimulated with LPS or IL-1β for 24 h. Actin was stained with FITC (green) and Dylight 594 (red) for GFAP.

Microglia serum from day 4 with shake from day 4 and forth

Astrocytes were stained with FITC for actin and Dylight 594 for GFAP. All three groups displayed cell debris, damage and F-actin in the form of stress fibers (see figure 4).

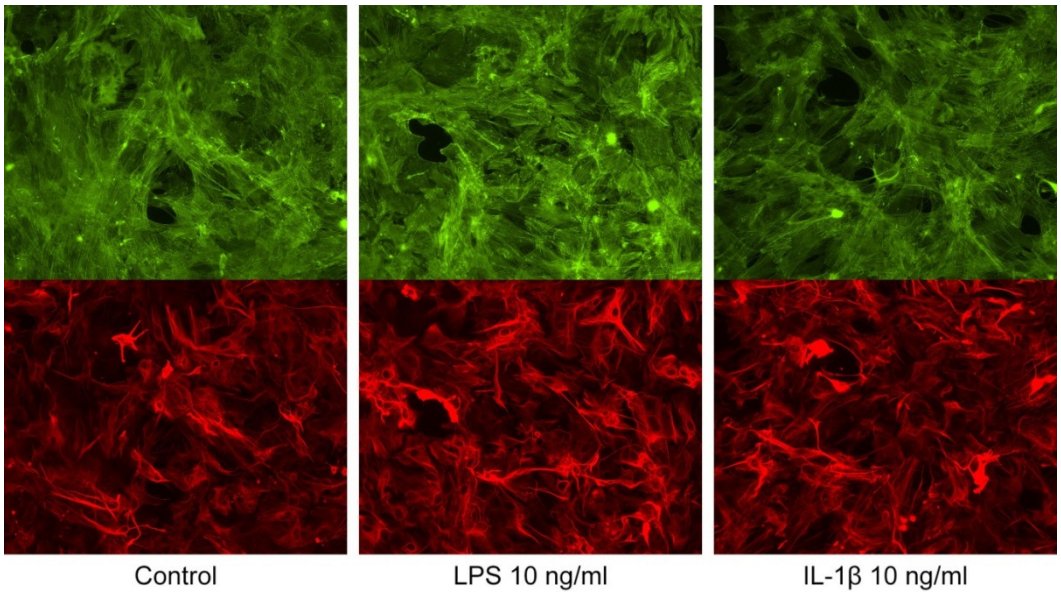

Figure 4. Astroglia serum cultures were stimulated with LPS or IL-1β for 24 h. Actin was stained with FITC (green) and Dylight 594 (red) for GFAP.

Microglia serum from day 4

Astrocytes were stained with FITC for actin and Dylight 594 for GFAP. Both the LPS and IL-1β stimulated group exhibited a slight increase in ring structure reorganization (see figure 5).

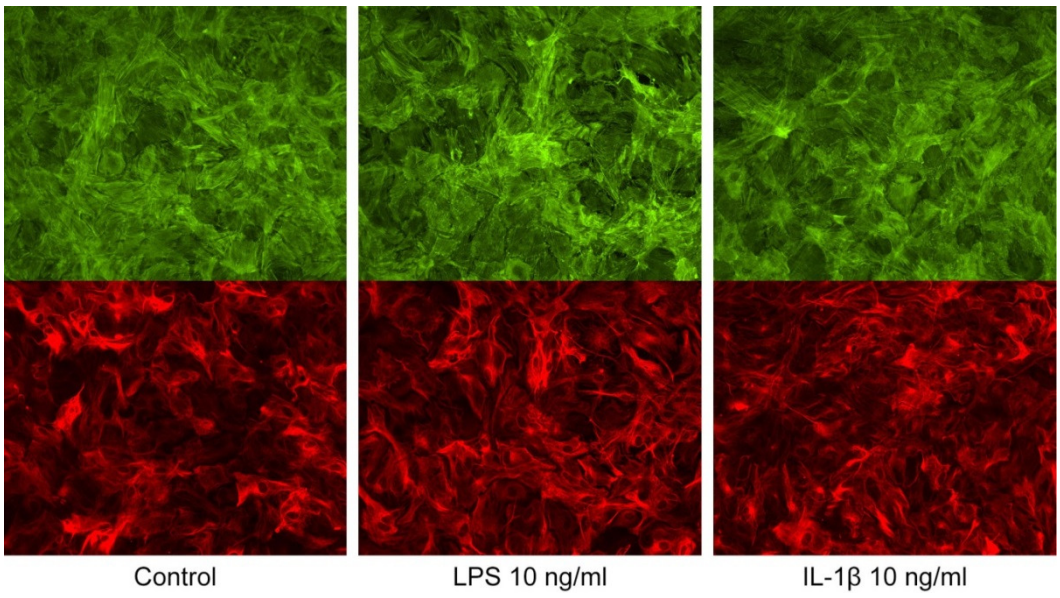

Figure 5. Astroglia serum cultures were stimulated with LPS or IL-1β for 24 h. Actin was stained with FITC (green) and Dylight 594 (red) for GFAP.

Microglia serum from day 0 with shake on day 4 and forth

Astrocytes were stained with FITC for actin and Dylight 594 for GFAP. All three groups displayed cell debris and cell damage. F-actin was observed in the form of stress fibers in all three groups (see figure 6).

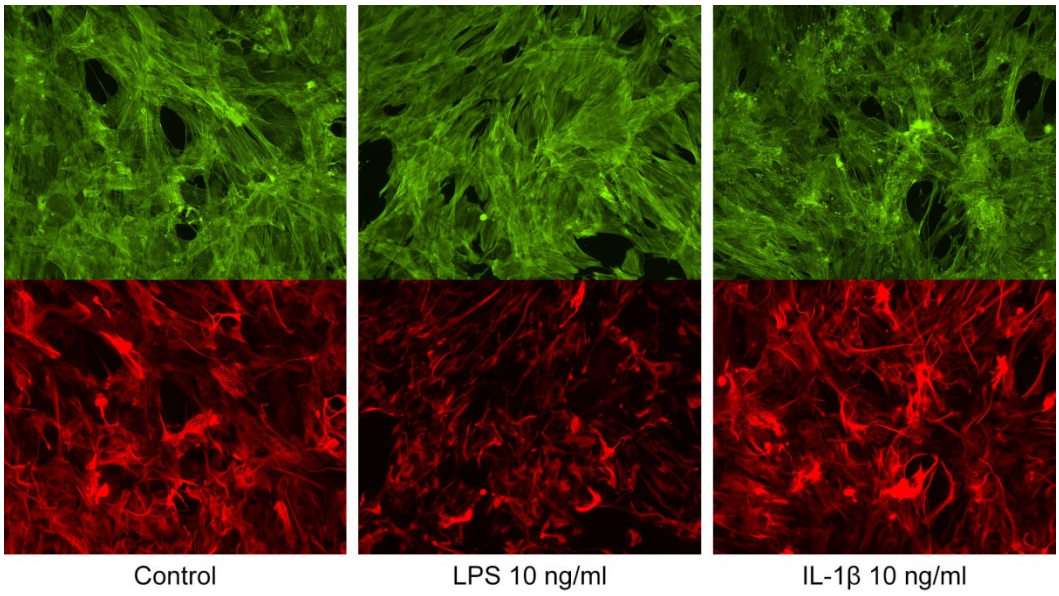

Figure 6. Astroglia serum cultures were stimulated with LPS or IL-1β for 24 h. Actin was stained with FITC (green) and Dylight 594 (red) for GFAP.

Microglia serum from day 0

Astrocytes were stained with FITC for actin and Dylight 594 for GFAP. F-actin organized in stress fibers dominated the untreated culture whereas LPS had the most pronounced ring structures and IL-1β slightly more than the control group (see figure 7).

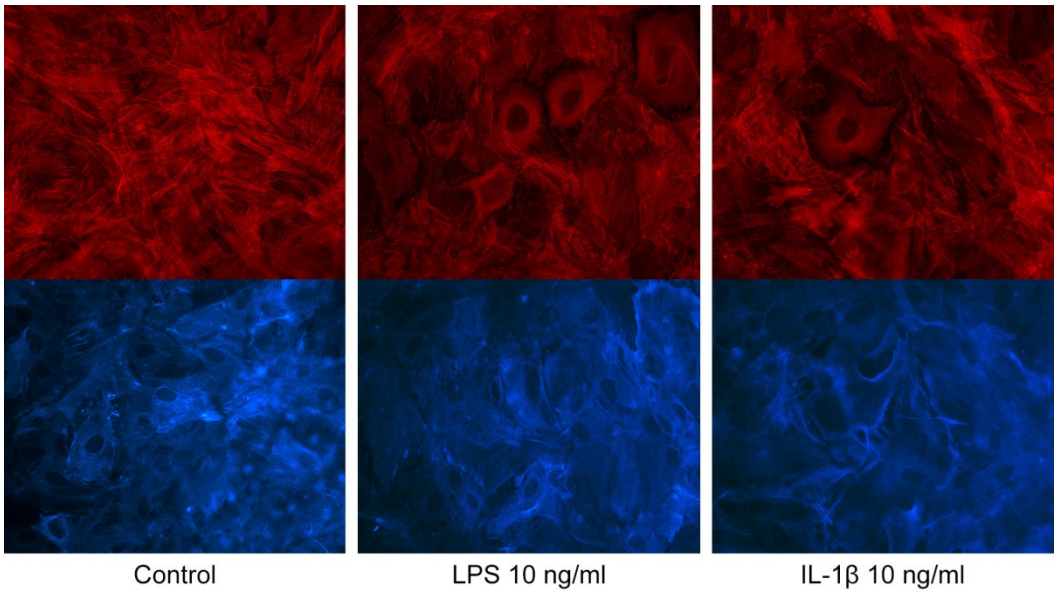

Figure 7. Astroglia serum cultures were stimulated with LPS or IL-1β for 24 h. Actin was stained with Dylight 594 (red) and GFAP was visualized with Alexa Fluor 350 (blue).

Phase contrast microscopy

The microglia serum cultivations displayed more microglia than in the astrocyte serum group (see figure 8).

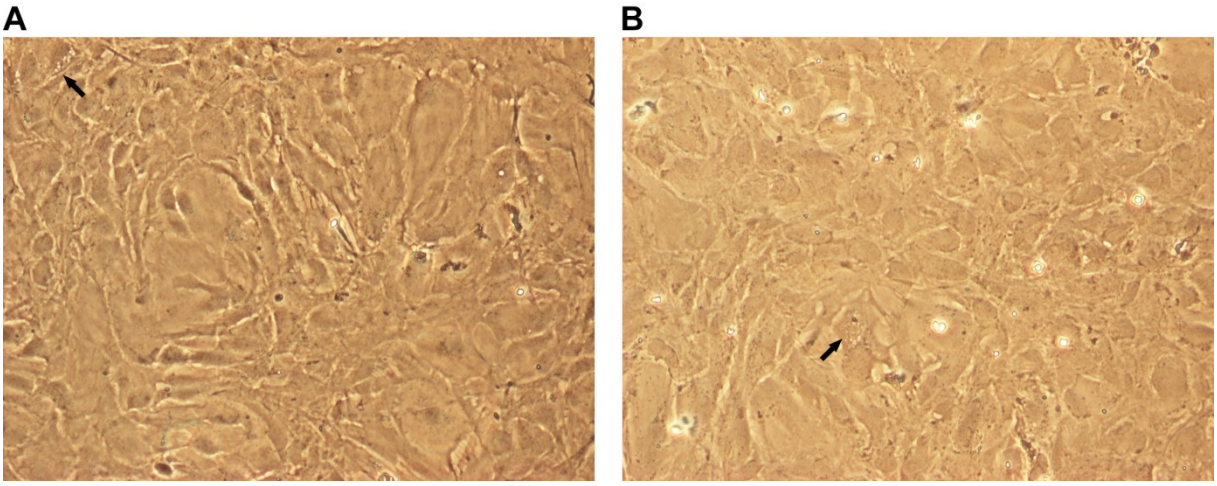

Figure 8. Phase contrast microscope picture of the A) astroglia serum group and B) microglia serum group. Arrows point towards infiltrated microglia and free microglia is represented by the white dots.

Actin assay

The F- over G-actin ratio increased when the cells were stimulated with LPS in the microglia serum group, whereas the replicates in the astroglia serum group were too few to statistically analyze. However, F-actin is increased in both serum groups when the cells have been treated with IL-1β (see figure 9).

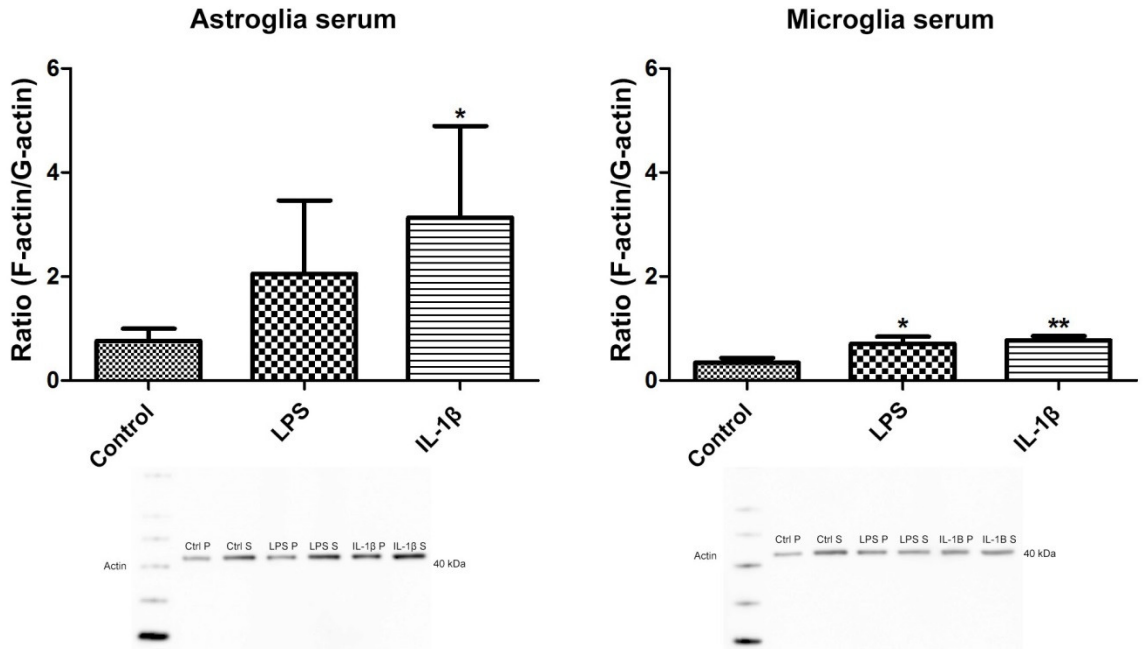

Figure 9. Astrocytes stimulated with LPS or IL-1β for 24 h and the ratio F-actin (P) over G-actin (S) was studied by Western blot. The protein levels are shown as the supernatants relative density compared to the pellet. Error bars show the SEM value. n=2 for LPS and n=3 for IL-1β, for the astroglia serum group, and n=3 for the microglia serum group. Statistical analysis was performed by one tailed student's t test. *p≤0.05 and ***p≤0.001

TLR4

The stimulation with LPS or IL-1β for 24 hours resulted in an increased content of TLR4 in both the astroglia serum group and microglia serum group. The protein content returned to normal levels during the longer stimulations (see figure 10).

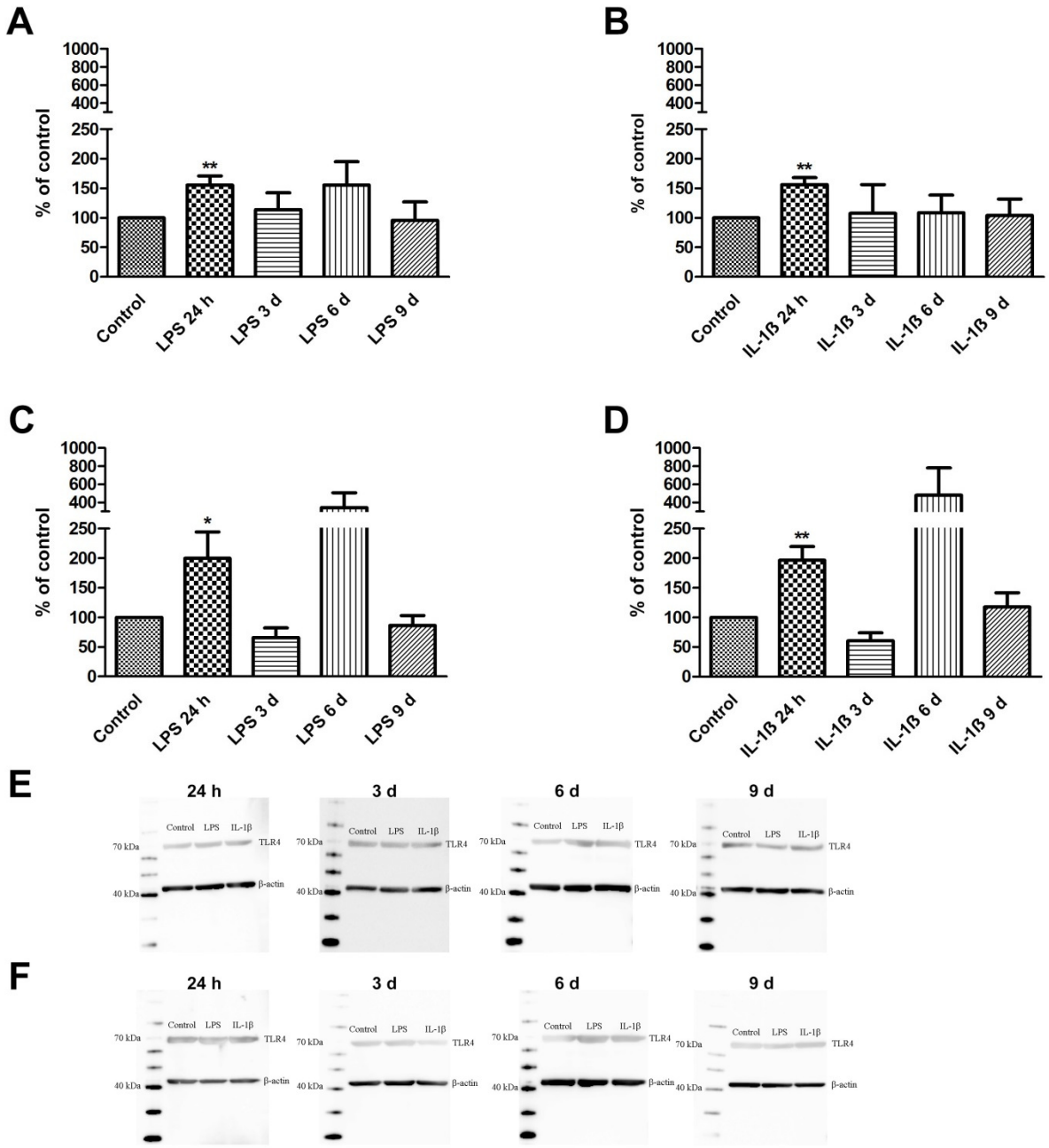

Figure 10. Astroglia serum cultures stimulated with A) LPS or B) IL-1β and microglia serum cultures stimulated with C) LPS and D) IL-1β for 24 h, 3 days, 6 days and 9 days. The expression of TLR4 was studied by Western blot. E) and F) represents membranes from astroglia serum group and microglia serum group, respectively. The protein levels are shown as the relative density compared to control. Error bars show the SEM value. n=7 for the 24 h stimulation and n=3 for the longer stimulations. Statistical analysis was performed by one tailed student's t test. *p≤0.05 and **p≤0.005

Na^+/K^+-ATPase

The astroglia serum group exhibited an increased content of Na^+/K^+-ATPase when stimulated with LPS for 24 hours but no statistically significant increase was observed by the IL-1β stimulation. The microglia serum group showed a decreased content of the protein when stimulated by either LPS or IL-1β. The content of Na^+/K^+-ATPase reverted to normal levels when the cultures were stimulated for 3, 6 and 9 days (see figure 11).

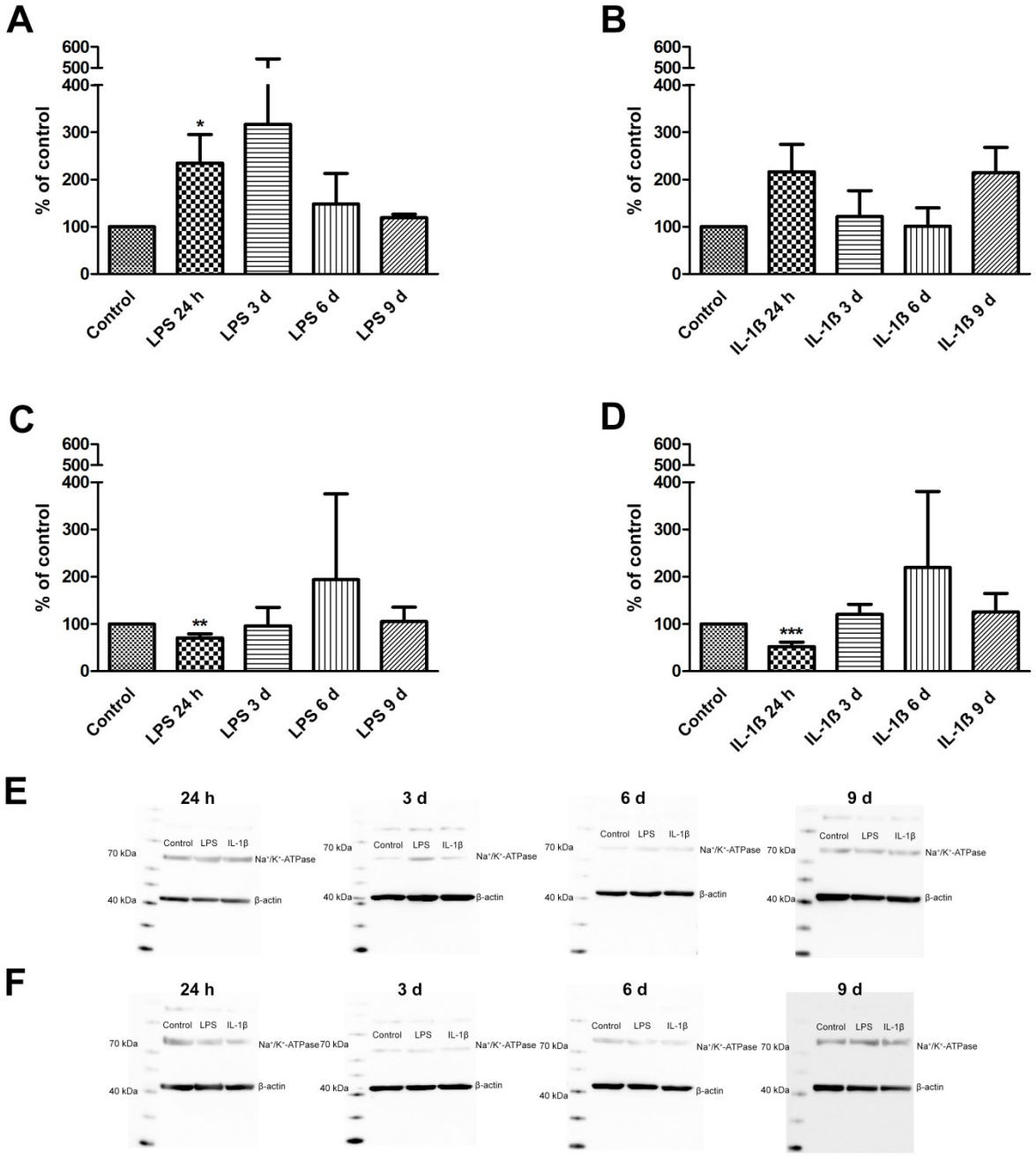

Figure 11. Astroglia serum cultures stimulated with A) LPS or B) IL-1β and microglia serum cultures stimulated with C) LPS and D) IL-1β for 24 h, 3 days, 6 days and 9 days. The expression Na^+/K^+-ATPase was studied by Western blot. E) and F) represents membranes from astroglia serum group and microglia serum group, respectively. The protein levels are shown as the relative density compared to control. Error bars show the SEM value. n=9 for the 24 h stimulation and n=3 for the longer stimulations. Statistical analysis was performed by one tailed student's t test. *p≤0.05, **p≤0.005 and ***p≤0.001

GLT-1

The astroglia serum group exhibited no alteration in the content of GLT-1 when stimulated with LPS or IL-1β with the exception of the 6 day stimulation, which had a decreased content of the 75 and 62 kDa isoforms and an increased content of 38 kDa isoform when stimulated with LPS (see figure 12).

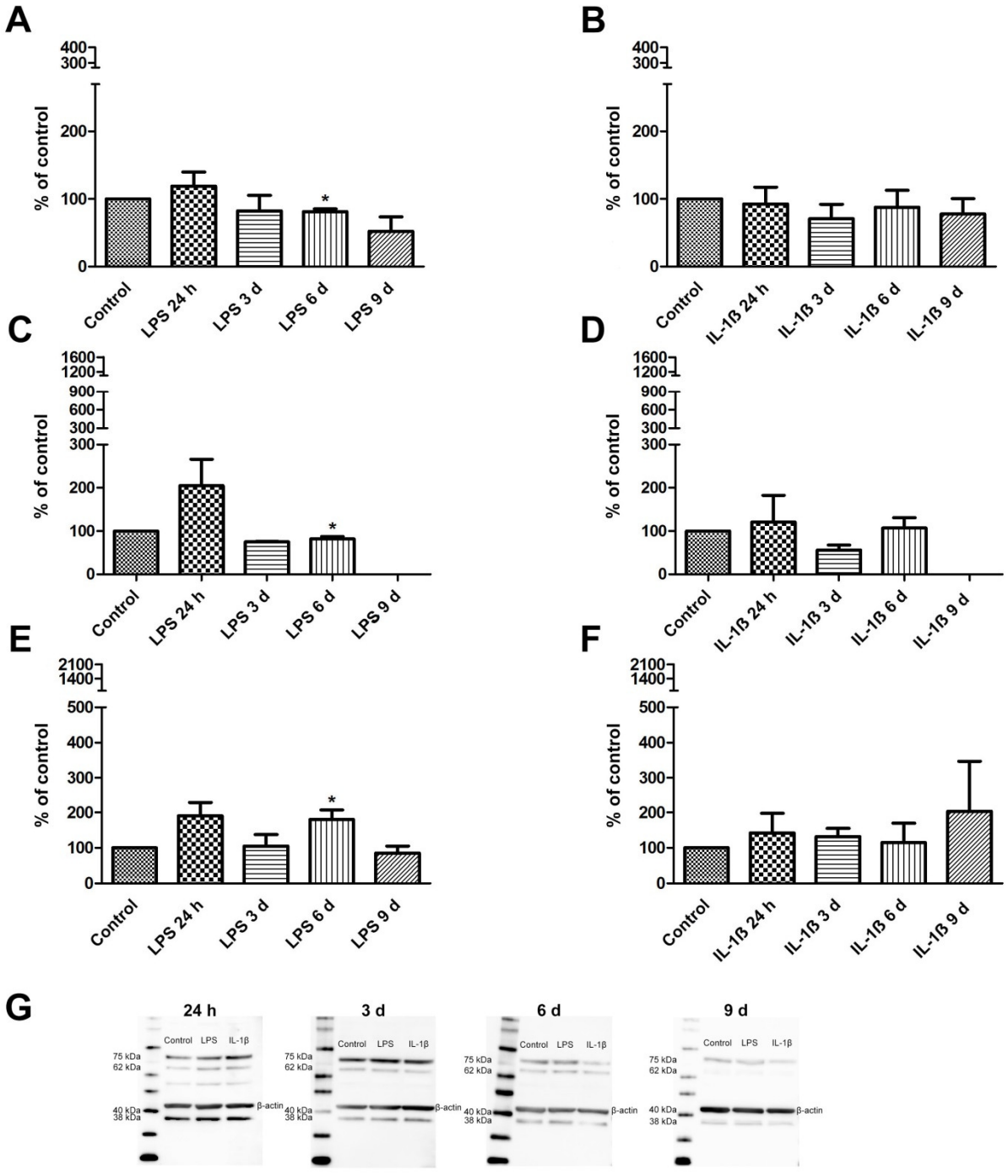

Figure 12. Astroglia serum cultures stimulated with LPS or IL-1β for 24 h, 3 days, 6 days and 9 days. The expression the 75 kDa (A, B), 62 kDa (C, D) and 38 kDa (E, F) GLT-1 was studied by Western blot. G) represents membranes. Protein levels are shown as the relative density compared to control. Error bars show the SEM value. n=9 for the 24 h stimulation and n=3 for the longer stimulations. Statistical analysis was performed by one tailed student's t test. *p≤0.05

The microglia serum group showed an increased content of all the assayed isoforms when stimulated with LPS or IL-1β for a duration of 24 hours. The longer stimulations resulted in a return to normal levels with the exception of an increased 62 kDa isoform content when stimulated with IL-1β for 6 days, and an increased content of the 38 kDa isoform when stimulated with LPS for 9 days (see figure 13).

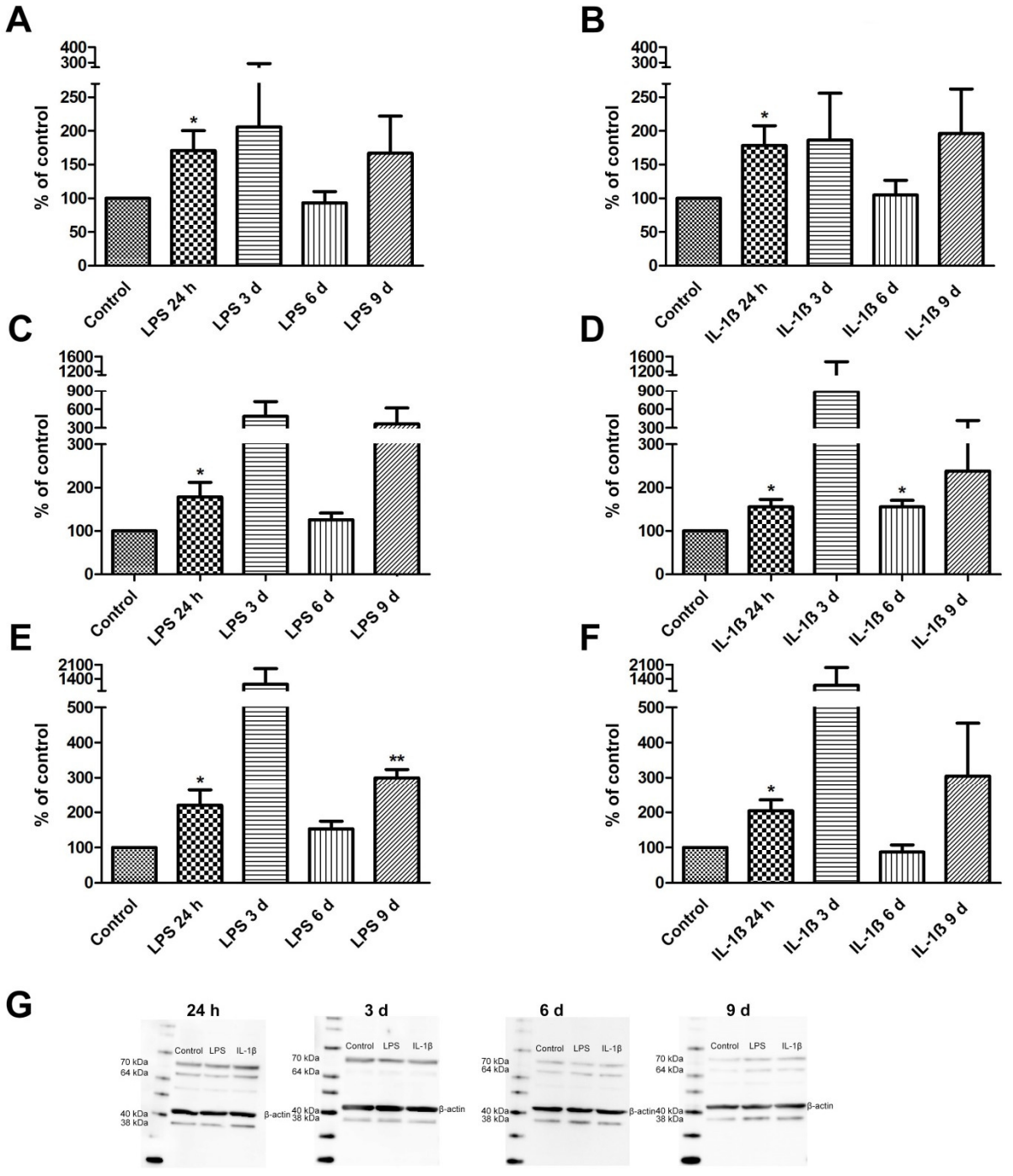

Figure 13. Microglia serum cultures stimulated with LPS or IL-1β for 24 h, 3 days, 6 days and 9 days. The expression the 75 kDa (A, B), 62 kDa (C, D) and 38 kDa (E, F) GLT-1 was studied by Western blot. G) represents membranes. The protein levels are shown as the relative density compared to control. Error bars show the SEM value. n=9 for the 24 h stimulation and n=3 for the longer stimulations. Statistical analysis was performed by one tailed student's t test. *p≤0.05

GLAST

The astroglia serum group exhibited no alteration in the content of GLAST when stimulated with LPS or IL-1β, with the exception of the 3 day stimulation, which had a decreased content of the 38 kDa isoform when stimulated with LPS (see figure 14).

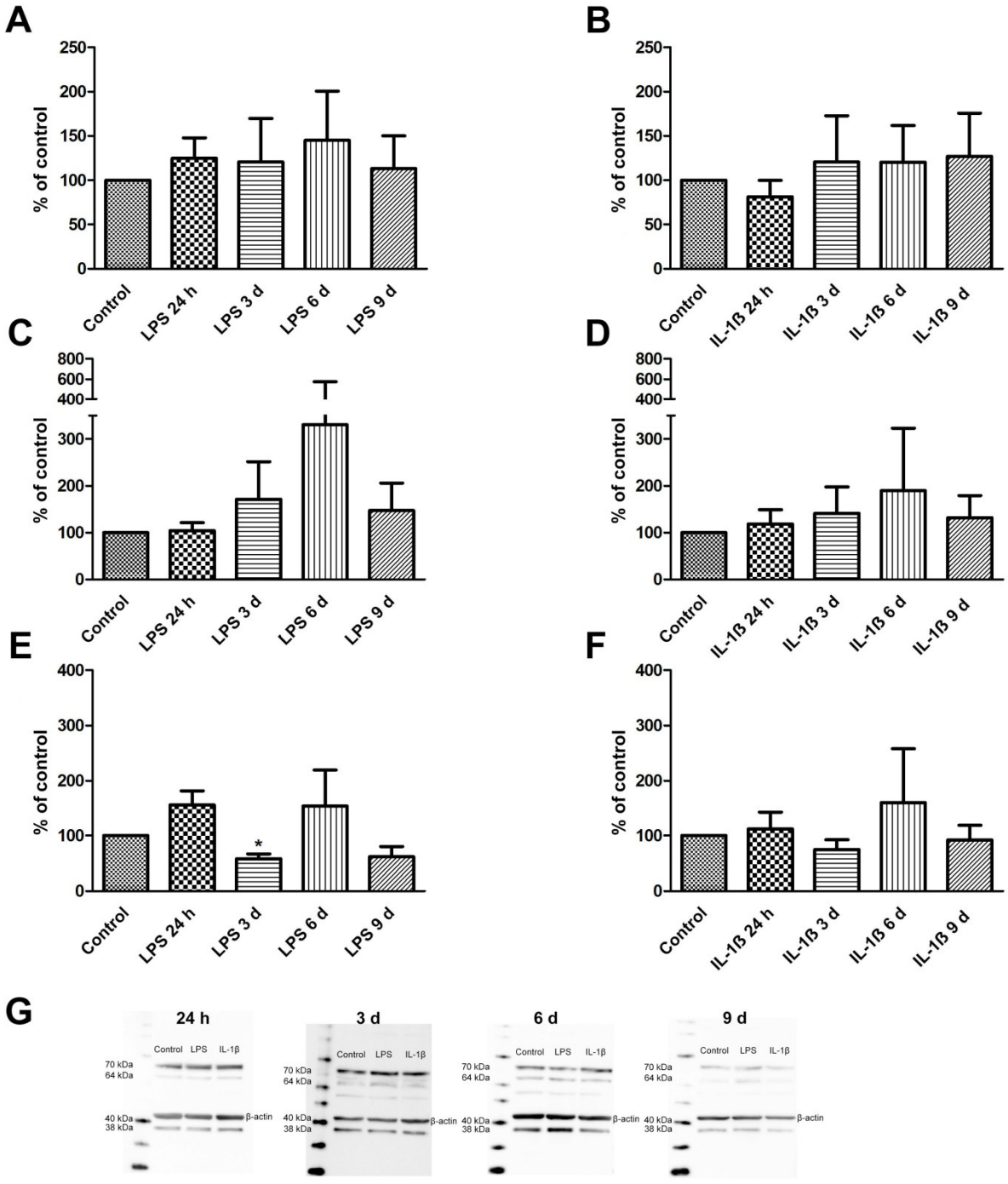

Figure 14. Astroglia serum cultures stimulated with LPS or IL-1β for 24 h, 3 days, 6 days and 9 days. The expression the 70 kDa (A, B), 64 kDa (C, D) and 38 kDa (E, F) GLAST was studied by Western blot. G) represents membranes.The protein levels are shown as the relative density compared to control. Error bars show the SEM value. n=6, 4 and 5 for the 70 kDa, 64 kDa and 38 kDa isoform and n=3 for the longer stimulations. Statistical analysis was performed by one tailed student's t test.

GLAST expression in the microglia serum group remained largely unchanged, with the exception of the glycosylated isoform at day three and six when the cultures were stimulated with IL-1β (see figure 15).

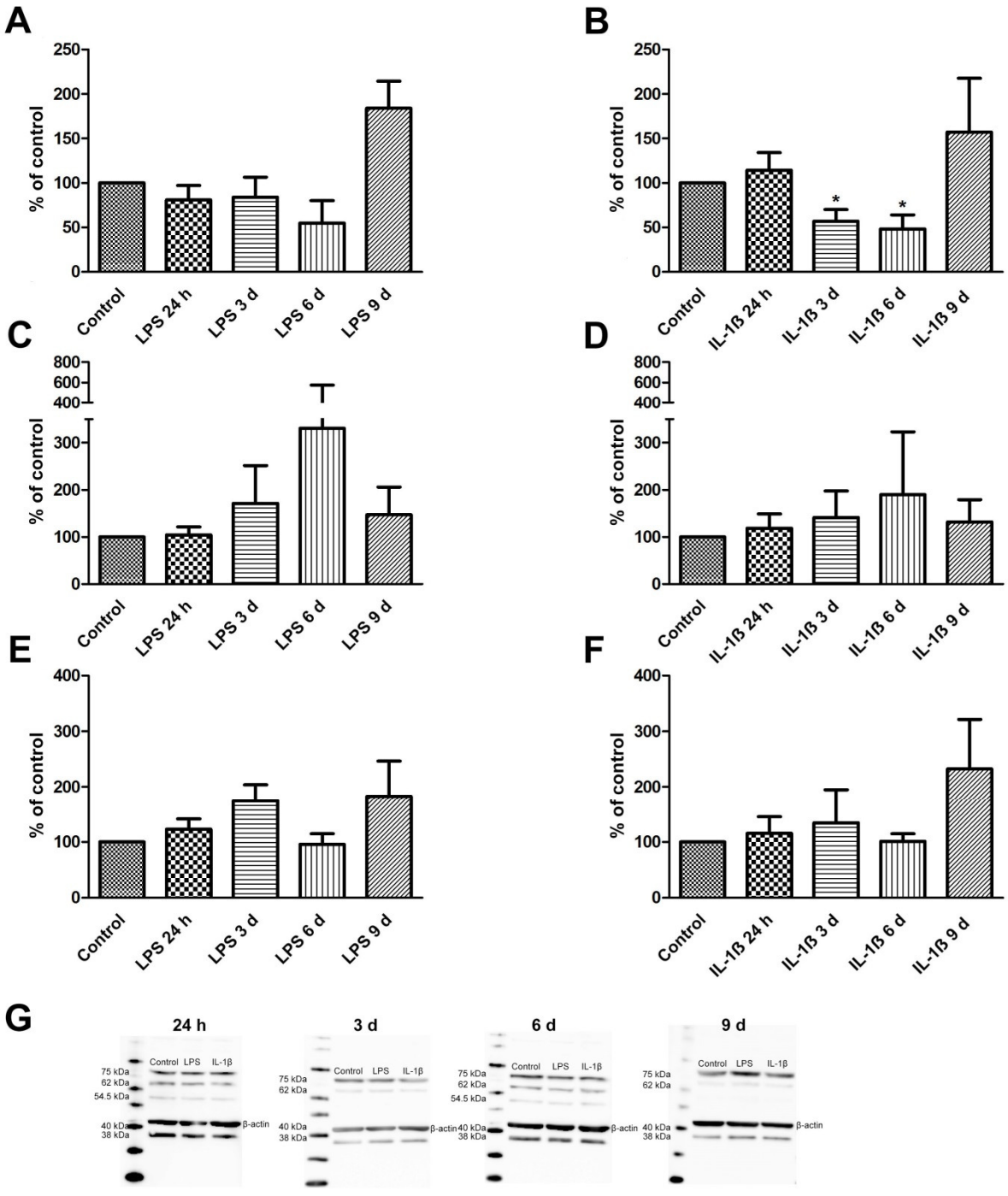

Figure 15. Microglia serum cultures stimulated with LPS or IL-1β for 24 h, 3 days, 6 days and 9 days. The expression the 70 kDa (A, B), 64 kDa (C, D) and 38 kDa (E, F) GLT-1 was studied by Western blot. G) represents membranes. The protein levels are shown as the relative density compared to control. Error bars show the SEM value. n=6, 4 and 5 for the 70 kDa, 64 kDa and 38 kDa isoform and n=3 for the longer stimulations. Statistical analysis was performed by one tailed student's t test.

Discussion

The aim of this thesis was to cause an inflammatory reaction in astroglial cells by the aid of the endotoxin, LPS, or the pro-inflammatory cytokine IL-1β, followed by studying various biomarkers to prove an inflammation. The first cultivation using astroglia serum did not provide any significant cytokine release and did not significantly alter the content of GLT-1 nor Na^+/K^+-ATPase. This suggested a lack of microglial cells which is assumed to initiate inflammatory responses (Saijo et al 2011). A couple of different conditions during cultivation were then explored. A serum previously used in the research group that had been shown to be beneficial for microglial proliferation was tested (Persson et al 2007). Shaking of the cultures has also been shown to proliferate microglia (Giulian & Baker 1986) and was thus undertaken, producing in total six groups. However, due to causation of cell debris in the shaking groups, they were deemed inappropriate. Changing from astroglia serum to microglia serum on day four did not provide any significant advantage either. Therefore only the astroglia serum group and the microglia serum group were continued with, and as can be seen in the phase contrast pictures the microglia serum group had considerably more microglia.

An F/G-actin assay based on Western blot was also employed to detect any changes between the astroglia serum and the microglia serum. The method had shown great discrepancies in the ratio of F-actin over G-actin between the same groups, so the process was evaluated for possible optimizations. The supernatant pipetting step following ultracentrifugation was previously undertaken by carefully pipetting the supernatant with the tube held upright as close to the barely visible pellet as deemed appropriate. This led to some supernatant residues being left with the pellet causing an overestimation of F-actin. As such, tipping the centrifugation tube cautiously to allow for the supernatant to collect by the opening of the tube, while the pellet remained in the bottom, thus produced much more reproducible results. However, the issue of remaining mineral oil in the supernatant is still pertinent, as it may cause an underestimation of G-actin due to dilution. As the mineral oil cannot be poured off separately of the supernatant, the reproducibility issue pertaining dilution thus lies with personal variances in the technique employed by the individual.

Both serum groups had an increased content of F-actin from IL-1β and whereas only the microglia serum displayed the same effect when treated with LPS (the astroglia serum group was disregarded due to insufficient replicates). Both serum groups also displayed reorganization which reduced stress fibers and induced formation of ring structures. This can be explained in part by the fact that IL-1β inhibits the rho-associated protein kinase, a kinase that acts inhibitory on ras-related C3 botulinum toxin substrate 1, which in turn catalyzes actin branching (John et al 2004; Yamaguchi et al 2001; Cooper & Hausman 2009). Since TLR has a highly related cytoplasmic domain to that of IL-1R (Gay & Keith 1991) it may be expected to cause a similar effect. This response could be an effort to reorganize various cell constituents, such as locating the Na^+/K^+-ATPase close to active glutamate transporters (Namekata et al 2008).

Since both LPS and IL-1β activate NFκB, which initiates pro-inflammatory cytokine production such as IL-1β and TNF-α (Azzolina et al 2003; Baeuerle & Henkel 1994; Akira & Takeda 2004), these two stimulations were expected to increase the IL-1β and TNF-α levels. However, the release of aforementioned cytokines was too low to be detected by ELISA in the astroglia serum group and microglia serum group. Furthermore, ELISA assay on IL-1β release displayed a value too low to be detected by ELISA in cultures stimulated with IL-1β, which suggests that the cells have degraded the cytokine. The reason for the lack of cytokine release may be because of the cells not being inflammatory reactive enough. The current scientific consensus attributes an initiation of inflammation to microglia (Saijo et al 2011). However, the results of this thesis suggests that this is not the case, since an increase in microglia did not result in an observable increase in cytokine release, nor did a longer stimulation with LPS or IL-1β which spanned up to nine days. Further reinforcing this notion is the fact that previous experiments within the group, with astrocyte and endothelial co-cultures, yielded cytokine release when stimulated with 10 ng/ml LPS (Delbro et al 2009; Westerlund et al 2013).

The protein expression of TLR4 was increased in both the astroglia serum group and microglia serum group when stimulated with LPS or IL-1β for 24 hours, which is in accordance with Forshammar et al (2011), who stimulated astrocytes co-cultured with endothelial cells for up to 48 hours. The microglia serum group also exhibited a higher expression of TLR4 protein, which may indicate more microglia. However, the protein expression unexpectedly reverted back to control levels in the longer stimulations, spanning from 3 to 9 days. The results might be due to a negative regulation of pro-inflammatory signaling pathways (Kovalenko et al 2003; Zhou et al 2012), but at the time of this thesis there is a lack of studies that have observed TLR4 expression in astrocytes following longer stimulations, so no comparison can be drawn.

A higher concentration of Na^+/K^+-ATPase was observed in the astroglia group when the cultures were stimulated with LPS for 24 hours, followed by attenuation back towards control levels, which is in accordance with Forshammar et al (2011). Conversely, the microglia serum group displayed a decrease of Na^+/K^+-ATPase when stimulated with either LPS or IL-1β for 24 hours, which is in accordance with observations in hepatocytic and renal cells (Schmidt et al 2007; Green et al 1996), followed by an increase to control levels during longer stimulations. However, Namekata et al (2008) did not find an alteration in the total concentration of Na^+/K^+-ATPase in Müller cells stimulated with IL-1 for 24 hours, but increased membrane localization was observed. Explanations for the decrease in Na^+/K^+-ATPase in the microglia serum group might be due to paracrine signaling by microglia that down-regulates the transcription of Na^+/K^+-ATPase in astrocytes, or degradation of the pump by e.g ROS release by activated microglia, which is known to occur (Block & Hong 2005). Therefore, the contradictory results may be dependent on the amount of microglia in the cultures. Furthermore, the Na^+-K^+-ATPase α_1-subunit gene has been shown to have an Sp1 binding site in its promoter region, and LPS has been shown to activate the Sp1 transcription factor in human macrophages (Suzukiyagawa et al 1992; Ma et al 2001). However, Suzukiyagawa (1992) also showed that Na^+-K^+-ATPase α_1-subunit is regulated by both common and several cell type-specific transcription factors. This complex regulation of the pump combined with the lack of studies on astrocytic Na^+/K^+-ATPase makes it hard to draw a conclusion, but the initial increased protein expression in the astroglia

serum group suggests that astrocytes react to inflammatory stimuli by an upregulation of the pump. Future studies could therefore compare the transcription of Na^+/K^+-ATPase between astroglia cultures and astroglia-microglia co-cultures by northern blot. However, the fact that mRNA levels does not always correspond to protein expression should not be ignored (Gebauer & Hentze 2004).

Previous studies have shown that GLT-1 is reduced in cells following stimulation with TNF-α (Wang et al 2004), whereas O'Shea (2006) found an up-regulation of the protein when cells were stimulated with LPS. Furthermore, Prow & Irani (2008) observed a down-regulation of the protein by IL-1β injection *in vivo*. In this thesis, the astroglia serum group exhibited no statistically significant alteration of the protein except for a minor decrease in LPS stimulations with a duration of six days concomitant with an increase of the 38 kDa isoform. On the other hand, the microglia serum group displayed an initial increase in either the LPS or IL-1β stimulations followed by attenuation. The microglia serum group also displayed an increase of the canonical protein by IL-1β at day six, as observed by increased 62 kDa isoform content. Furthermore, an increased 38 kDa isoform was observed by LPS stimulation at day nine. In contrast to the observation by O'Shea et al (2006), who showed that GLT-1 was up-regulated at day three, the up-regulation of GLT-1 in this thesis was present at 24 hours of stimulation, with an attenuation at day three. Interestingly, in both serum groups the 38 kDa isoform was observed at a band intensity on par with the otherwise most intense band, the 75 kDa isoform; and a GLAST related isoform at this size was also detected. At the time of this thesis no studies on the function or cause of the 38 kDa isoform was found, but it has been observed (Russo et al 2013). The occurrence of the isoform may be due to degradation. As such, studies on the amino acid composition of the protein followed by cross referencing with known protease cleavage sites of GLT-1 could therefore be undertaken to determine whether it is a result of self-imposed degradation or not. In addition, the astrocytes used in this project express GLT-1; which means the cells are mature and differentiated since astrocytic monocultures have been shown to only express the GLAST subtype in the absence of neurons (Swanson et al 1997). Reports have shown that LPS and IL-1 have no effect on GLAST expression (O'Shea et al 2006; Namekata et al 2008), however, Korn et al (2005) have shown that GLAST is reduced when astrocytes are exposed to TNF-α. The results in this thesis exhibit a largely unchanged expression of the transporter, although IL-1β did decrease the content of the glycosylated isoform at day 3 and 6 in the microglia serum group. Together with the fact that GLT-1 was not reduced, these results could indicate that TNF-α is absent from the cultures, which supports the findings of the ELISA assay that indicated a lack of TNF-α release.

The lack of cytokine release and the attenuation of the change in biomarkers suggest that the cells are not inflammatory reactive. Contrary to the common belief that microglia initiates inflammation (Saijo et al 2009), the results of this thesis suggest that they do not; or at least not solely. However, activated microglia do seem to drastically contribute to the alteration of astroglial proteins which may be due to the release of paracrine signaling molecules and ROS, as shown by (Saijo et al 2009). A cell that has not received much attention regarding neuroinflammation is the mast cell. The brain contains perivascular mast cells which are able to release serine proteases that activate pro-MMPs (Florenzano & Bentivoglio 2000; Lindsberg et al 2010). These MMPs are in turn are able to activate pro-inflammatory cytokines which are needed to mediate inflammation (Mohan et al 2002). Furthermore, one of the proteases secreted by mast cells, tryptase, has been shown to be a ligand to the protease-activated receptor 2, a receptor that has been shown to act synergistically with TLR4 and induce pro-inflammatory cytokine production (Rallabhandi et al 2008; Zeng et al 2013). As discussed in the review by Nathan (2002), initiation of inflammation may be a set of "stop and go" signals, where multiple signals (binary or higher order) are required to escalate the inflammatory response further. Since mast cells have been shown to be the first responders to neuroinflammation (Jin et al 2009), an astrocyte cell culture lacking mast cells may then be deficient in the first steps towards inflammation, which may have resulted in a stop signal. The initial response could therefore be the result of activated IL-1R or TLR4 and respective signaling pathways, followed by the activation of a negative feedback loop that inhibits further signaling (Kovalenko et al 2003; Zhou et al 2012). As a previous study that isolated microvascular fragments exhibited a contamination of pericytes (Bondjers et al 2006), and since mast cells are closely associated with the BBB they may have contaminated the cultures as well. Therefore, the astroglia and endothelial cell co-culture used in the studies performed by Forshammar et al (2011) and Westerlund et al (2013) could have been contaminated by perivascular mast cells which may have contributed to the inflammatory response of the cells. Furthermore, since pericytes have been suggested to differentiate into myofibroblasts during inflammation, and myofibroblasts secrete a wide range of pro-MMPs (Hinz et al 2007; Rabkin et al 2001), the contaminating pericytes in conjunction with mast cells may also have been responsible for the inflammatory reactive astrocytes. As such, compounds secreted by mast cells or both mast cells together with myofibroblasts may be necessary for an inflammation to occur, which could explain the lack of inflammation observed in the cultures of this thesis.

Conclusion

The purpose of the thesis was to create a model cell system consisting of network coupled astrocytes to study inflammation. Both serum groups displayed actin reorganization as visualized by immunofluorescence, and actin polymerization when stimulated with IL-1β, whereas only the microglia serum group displayed an actin polymerization when stimulated with LPS. Neither cultivation became inflammatory reactive by LPS or IL-1β stimulations, as observed by the lack of cytokine release and return of TLR4, Na^+/K^+-ATPase and GLT-1 to control levels during longer stimulations. The results suggest that astrocytes and microglia are not capable of initiating an inflammation on their own and that another factor is missing. Mast cells could be necessary for an initiated inflammation due to e.g their production of serine proteases, which signals for inflammation and activates pro-MMPs that in turn activates pro-inflammatory cytokines. Furthermore, pericytes differentiated into myofibroblasts may be needed for a continuous production of pro-MMPs to drive inflammation. Mast cells and myofibroblasts may therefore be interesting future venues to explore regarding neuroinflammation.

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