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Molecular characterization of glial cells in a neuroinflammatory model of *Cx3cr1* $^{GFP/+}$ mutant mouse line

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Glial cells alter their morphology and molecular profile in response to stimuli from their surrounding environment. This ability makes them the foremost defence against inflammatory conditions in the brain. However, information about the inflammatory response and heterogeneity of glial cells still remains limited. The aim of this study was to characterize the molecular profile of glial cells in a LPS-induced neuroinflammatory model of *Cx3cr1*$^{GFP/+}$ heterozygous mice. Flow cytometry analysis was performed in order to determine variations in glial cell abundancy, MHC II expression and expression of multiple microglia-specific markers (CX3CR1, CD11b, CD172a, CD200R and CD115) in three different brain regions – hippocampus, cerebral cortex and cerebellum. We observed several alterations in the receptors' surface expression in response to LPS with notable heterogeneity between different regions, as well as remarkable variation in the basal surface expression levels. This study aims to broaden current knowledge of the heterogeneity and inflammatory molecular profile of glial cells.

Keywords: neuroinflammation, glial cells, microglia, astrocytes
CERCS: B640 (Neurology, neuropsychology, neurophysiology)

Gliia rakkude molekulaarne iseloomustamine neuroinflammatoorses mudelis *Cx3cr1*$^{GFP/+}$ mutantses hiireliinis

Gliia rakkude võime muuta oma morfoloogiat ning molekulaarset profiili vastusena keskkonnast saadud stiimulitele, võimaldab mikrogliial koos astrotsüütidega tagada ajus esmane immuunreatsioon põletikuliste muutuste vastu. Gliia rakkude immuunvastuse detailne toimemehhanism ning heterogeensus on aga endiselt väheuuritud. Käesoleva töö eesmärk oli kirjeldada gliia rakkude molekulaarset profiili LPS-i poolt esile kutsutud neuroinflammatoorses mudelis heterosügootses *Cx3cr1*$^{GFP/+}$ hiireliinis. Voolutsütomeetrilise analüüsi abil hindasime muutusi gliia rakkude arvukuses, MHC II kompleksi ning mitmete mikrogliia-spetsiifiliste retseptorite (CX3CR1, CD11b, CD172a, CD200R ja CD115) ekspressioonis kolmes ajupiirkonnas – hipokampuses, suurajukoores ja väikeajus. Antud töö tulemusena selgus, et sõltuvalt ajupiirkonnast kutsus neuroinflammatsioon retseptorite pinnaekspressioonis esile mitmeid täheldatavaid muutuseid. Regioonipõhised erinevused esinesid ka mitmete retseptorite basaalekspressioonis.

Märksõnad: neuroinflammatsioon, gliia rakud, mikrogliia, astrotsüüdid
CERCS: B640 (Neuroloogia, neuropsühholoogia, neurofüsioloogia)

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ABBREVIATIONS

APC - antigen presenting cells

BBB - blood-brain barrier

CBL - cerebellum

CD - cluster of differentiation

CNS - central nervous system

CTX – cerebral cortex

CX3CR1 - CX3C chemokine receptor 1

DAMP – damage-associated molecular pattern

EMP - erythro-myeloid progenitor cell

GFAP – glial fibrillary acidic protein

GFP – green fluorescence protein

GLAST - glutamate/aspartate transporter

HPC – hippocampus

IFN-γ – interferon gamma

IL - interleukin

LPS - lipopolysaccharide

M1 – classically activated

M2 – alternatively activated

MFI – mean fluorescence intensity

MHC - major histocompatibility complex

NF-κB – nuclear factor kappa B

NO – nitric oxide

O4 - oligodendrocyte transmembrane protein

PAMP - pathogen associated molecular pattern

PRR – pattern recognition receptor

ROS – reactive oxygen species

SAL – saline

TLR - *toll*-like receptor

TNF-α – tumor necrosis factor-alpha

INTRODUCTION

Neuroinflammation, a cascade of inflammatory responses in the central nervous system (CNS), is characterized by the production of inflammatory mediators by resident neuroglial cells, disruption of the blood-brain barrier and the infiltration of peripheral immune cells into the CNS.

Glial cells, mainly including astroglia (or astrocytes), oligodendrocytes and microglia, take cues from the environment and respond to any abnormalities in the CNS through activation, which induces acute inflammatory conditions in the brain. The activated state is characterized by rapid proliferation and expression of different molecular markers, controlling and shaping the progress of inflammation in the CNS.

In order to effectively inhibit overactive inflammatory conditions, pro- and anti-inflammatory responses within the CNS have to be under tight vigilance. Imbalance of the responses can lead to prolonged inflammation, in some cases causing extensive brain damage and ultimately resulting in a variety of different neurodegenerative diseases. In addition, neuroinflammation has been shown to play a role in the progression of different neuropsychiatric disorders.

The aim of this study was to evaluate the molecular signature of different cell surface receptors and cell abundancy changes in astrocytes and microglia, in response to lipopolysaccharide treatment in three different brain tissues (hippocampus, cerebral cortex, cerebellum) in heterozygous *Cx3cr1* $^{GFP/+}$ mice, as well as evaluate the basal surface expression of these receptors under homeostatic conditions.

This study was performed at the Institute of Biomedicine and Translational Medicine of University of Tartu in the Neuroimmune Psychiatry Group of the Department of Physiology.

1 LITERATURE OVERVIEW

1.1 Glial cells

The first mention of glial cells dated back to the 19th century, when the function of these cells was proposed to be solely a binding matter between neurons. The name, originating from the Greek word "glia" meaning "glue", remained, but evidence of glial cells' vast variety of indispensable functions in the brain has grown tremendously over the decades (Parpura et al., 2012).

It has been well established that there are several distinct subpopulations of glial cells in the CNS - microglia, astrocytes, oligodendrocytes and oligodendrocyte precursor cells (OPCs), radial glia, and ependymal cells (Parpura et al., 2012). Glial cells were thought to be much more abundant than nerve cells, however later studies support a rather 1:1 glial to neuron ratio in the average mammalian brains (Herculano-Houzel *et al.*, 2005). Glial cell bodies are smaller than neurons, however, they carry far-reaching extended processes, which form a systematic structure (Lawson et al., 1990).

Contrary to early belief, glial cells hold far more functions than merely providing support for the nervous system. They modulate the functioning of nerve signalling by regulating the neurotransmitter metabolism, help overcome (in some cases worsen) brain and tissue damage, as well as play a role in the early development of the CNS (Jäkel et al., 2017).

Glial cells show identical morphologies in fruit fly *Drosophila melanogaster* with their mammalian counterparts, which indicates that the main functions of glia are most likely to be highly conserved (Freeman et al., 2006).

1.2 Microglia

Microglia roughly account for 10% of all the cells in the CNS. They are the resident immune cells and the only glial cells of non-neural origin in the brain parenchyma (Alliot et al., 1999; Bertrand et al., 2005).

Microglia cells are distributed over different regions of the CNS in a way where they are able to constantly survey the environment of any abnormalities. Their plasticity enables them to change their morphology in response to cues from the environment and acquire a region-specific phenotype (Lopez-Atalaya et al., 2018). They are widely referred to as the resident

macrophages of the CNS, displaying multiple similarities with peripheral tissue resident macrophages, and use their ramified processes to scan the brain parenchyma of pathogenic conditions as well as tissue damage and faulty synapses (Paolicelli et al., 2011).

1.2.1 The role of microglia in CNS development

Although microglia cells were known to be of non-neural origin, their precise lineage has long been debated over. Multiple studies have shown that microglial cells are formed during primitive hematopoiesis and like other tissue macrophage populations, originate from the yolk sac-derived erythro-myeloid progenitor cells (EMPs; Alliot, Godin and Pessac, 1999; Bertrand *et al.*, 2005; Ginhoux *et al.*, 2010).

Microglia migrate to the CNS through the pia basal lamina right around the formation of the blood brain barrier (BBB). In the CNS they are capable of rapid proliferation and achieve a definitive density shortly after birth (Kurz et al., 1998; Nikodemova et al., 2015).

The development of microglia along with its regulatory role in the brain is still notably understudied and has become of interest as of recently. It has been shown that embryonic microglia show an uneven distribution in the early brain. They are mainly located at specific axonal tracts and appear to be formed into distinctive hotspots (Squarzoni et al., 2014).

Microglial cells have shown to promote the differentiation and phagocytosis of neuronal precursor cells in the developing cortex, ultimately regulating the production and proper differentiation of cortical neurons (Cunningham et al., 2013; Hattori et al., 2020, 2018). It also appears that microglial cells support the wiring of the forebrain, as they modulate the development of dopaminergic axons and regulate the migration of interneurons within the neocortex. This activity is achieved by microglial CX3CR1 and DAP12 receptor activity (Squarzoni et al., 2014). Furthermore, microglia contribute to synaptic pruning by engulfing synapses in the developing brain. Synaptic elimination is controlled by many different factors, including CX3CR1, as it has been shown that *Cx3cr1* knockout mice show deficient synaptic maturation (Paolicelli et al., 2011). CX3CR1 signalling has also been demonstrated to play a role in the survival of cortical neurons during postnatal development (Ueno et al., 2013).

In addition, microglia communicate with vascular cells and modulate vascular network formation by stimulating sprouting and branching of vessels in the brain (Arnold et al., 2013; Rymo et al., 2011).

1.2.2 The role of microglia in the adult brain

Microglia were long studied only in the context of pathological and disease-related conditions in the CNS. However, studies have shown that microglia also contribute to maintaining a healthy CNS homeostasis by surveillance, phagocytic activity and self-renewal in the adult brain (Nimmerjahn *et al.*, 2005; Askew *et al.*, 2017).

The local microglia population in the brain is maintained by proliferating resident microglia cells also referred to as the proliferating microglia (Lawson *et al.,* 1992; Askew *et al.*, 2017). The density of microglia also varies greatly in the adult brain. The highest concentrations of microglia cells are mainly found in the cerebral cortex, hippocampus, basal ganglia, olfactory bulb and substantia nigra. Average densities are in the thalamus and hypothalamus and lowest densities in the cerebellum and brainstem (Lawson et al., 1990).

In physiological conditions, microglia cells are preserved in a quiescent state. The quiescent or otherwise known as resting microglia exhibit a unique morphology - the cell body is covered in stems which extend into fine and vastly ramified processes. While the stems stay mainly fixated, the processes are highly motile and move by constant extending and retracting. The free-moving morphology helps resting microglia survey and clear the brain parenchyma of piled up metabolic and tissue components, as well as effectively interact with neighbouring cells and synapses (Davalos et al., 2005; Nimmerjahn et al., 2005b).

Furthermore, microglia cells use microvesicle shedding as a tool of communication. Shedding vesicles into the microenvironment helps microglia interact with surrounding neurons as well as regulate synaptic activity. These microvesicles contain different inflammatory mediators, such as tumor necrosis factor alpha (TNF- α) and interleukin (IL)-1β, and by inducing sphingolipid metabolism in neurons, are capable of regulating the neurotransmitter release and excitatory transmissions (Antonucci et al., 2012).

All of the above mentioned ways of communication and surveillance help to maintain all physiological processes under tightly controlled vigilance and once pathological conditions strike, microglial cells are the first ones to respond (Davalos et al., 2005; Shrikant et al., 1996). For this purpose, microglial cells express a wide range of different receptors on their surface for “eat me”, “don’t eat me” and “come get me” signals (Griffiths et al., 2009; Grimsley et al., 2003). Microglia respond to disease- or injury-related conditions through receptors that recognize neurotransmitters, as well as many pattern-recognition receptors (PRRs). These receptors specifically detect danger-associated molecular patterns (DAMPs). DAMPs are

expressed in the healthy brain and once injury occurs, are released from the damaged cells. DAMPs include adenosine triphosphate (ATP), IL-1α (Eigenbrod et al., 2008), S100 proteins (Rickmann et al., 1995) etc. PRRs also detect pathogen-associated molecular patterns (PAMPs) including pathogenic compounds like lipopolysaccharide (LPS; Li *et al.*, 2014) and viral nucleic acids (Pocock *et al.*, 2007).

Similar to other peripheral antigen presenting cells (APCs) like macrophages, dendritic cells and B lymphocytes, microglia express MHC II complex proteins on their surface. This is vital, as microglia are the main resident APCs in the brain parenchyma (Aloisi et al., 1998) and use the MHC II complex as a means of communication to CNS-infiltrating T lymphocytes (Pocock *et al.*, 2007).

In addition to MHC II, microglia express a wide range of other receptors on their surface: CD11b (Mac-1), CD172a (SIRPα), CD115 (CSF1R), CD200R etc. All of these receptors can be used to characterize different microglial activation states and in general help to keep the inflammatory response under control. CD11b is one of the most commonly used microglia specific markers in order to distinguish these cells from infiltrated macrophages, as resting microglia are $CD11b^{hi}$, $CD45^{low}$ and macrophages $CD11b^{hi}$, $CD45^{hi}$ (Vainchtein et al., 2014). CD11b expression also increases when microglia are in an activated state and is therefore considered a specific marker for activated microglia. On the contrary, several of these microglial receptors hinder microglial activation. CD172a, also expressed by peripheral macrophages, controls microglial activation through the interaction with its ligand CD47 (Sato-Hashimoto et al., 2019). CD200R is an inhibitory receptor capable of keeping microglia in a quiescent state. Its ligand CD200 is expressed by neurons, T and B lymphocytes, endothelium and follicular dendritic cells (Shrivastava et al., 2012). Similar to peripheral macrophages, microglia are also dependent on CD115 signalling for their development and survival, as CD115-deficient mice are severely depleted of microglial cells (Chen et al., 2006).

1.2.3 Microglial polarization

Once pathological conditions occur, the morphology of microglia cells rapidly changes accompanied by increased proliferation (Shankaran et al., 2007). The quiescent ramified cells transform into amoeboid cells, which start to upregulate and downregulate a wide range of surface molecules (Cho et al., 2006; Davalos et al., 2005).

Microglia are capable of forming various distinctive phenotypes in response to different stimuli. The previously wide spread stereotypical M1/M2 classification model is now considered rather simplified, as it has been noted that the polarization might be much more complicated, especially *in vivo* - recent research has shown that there is a wide expand of different phenotypes of microglial polarization (Tan et al., 2020; Wells et al., 2003; Williams et al., 2002).

The M1/M2 polarization model divides microglial cells into two distinct phenotypes - classically activated (M1) and alternatively activated (M2) (Figure 1). Classically activated microglia are considered pro-inflammatory while alternatively activated microglia are described as anti-inflammatory. Both of these phenotypes are defined by the production of different inflammatory mediators, as well as different cell surface and intracellular markers (Xie et al., 2003).

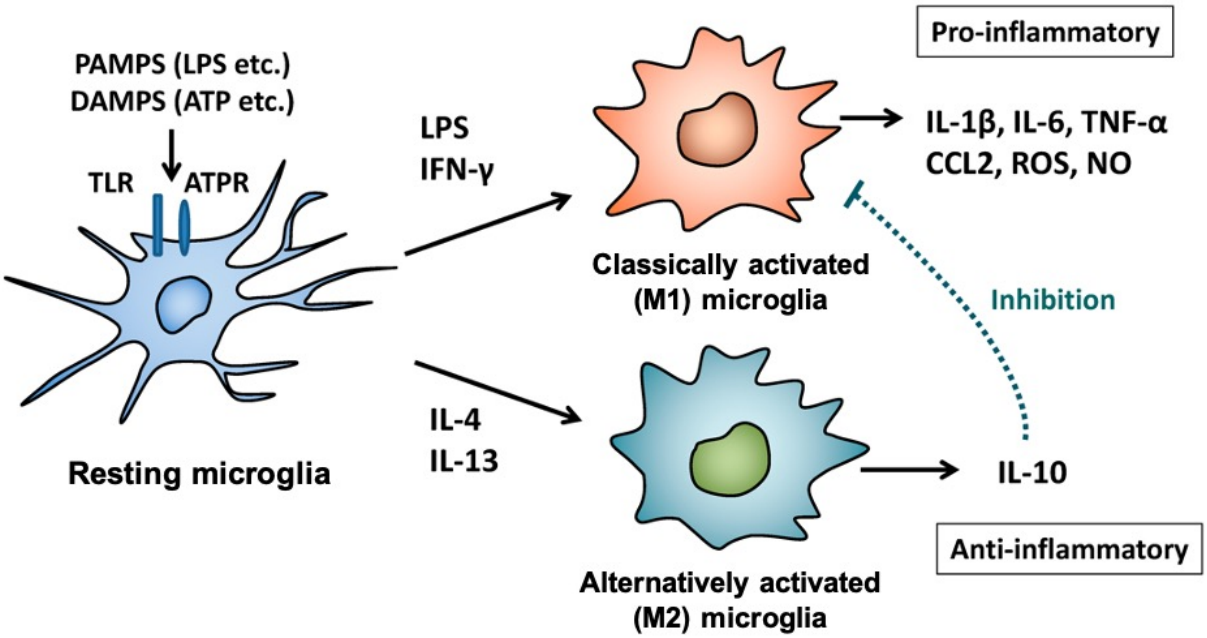

Figure 1. M1/M2 polarization of microglia. The factors inducing microglial polarization and pro- and anti-inflammatory markers produced by M1 and M2 polarized microglia, respectively. PAMPS - pathogen-associated molecular patterns; DAMPS - danger-associated molecular patterns; LPS – lipopolysaccharide; IFN-γ – interferon gamma; IL – interleukin; TNF-α – tumor necrosis factor alpha; CCL2 – chemokine (C-C motif) ligand 2; ROS – reactive oxygen species; NO – nitric oxide (Modified by Nakagawa and Chiba, 2014).

The polarization into M1/M2 phenotype is also largely determined by the microenvironment. It is mainly activated through *toll*-like receptors (TLRs) on microglial cell surface, which are capable of detecting different PAMPs and DAMPs in the environment (Jack et al., 2005). Once polarized, microglia are capable of switching between the two phenotypes in response to the changes in the microenvironment (Figure 1; Gao et al., 2018).

M1 microglia create the foremost defence against pathological conditions in the brain parenchyma. The M1 phenotype is mostly induced by TNF-α and interferon gamma (IFN-γ), released by T helper type 1 (Th1) cells or astrocytes. It is also promoted by pathogen produced compounds like LPS, which is detected by TLR-2 and -4. In addition, TLRs are capable of detecting viral nucleic acids or tissue damage debris. Once polarized, M1 microglia start to produce proinflammatory cytokines and chemokines, as well as express different receptors and MHC class II proteins on their surface to provoke inflammatory conditions (Mantovani et al., 2004). M1 microglia produce IL-1β, IL-6, TNF-α and CCL2. They also express NADPH oxidase in order to produce reactive oxygen species (ROS). The production of nitric oxide (NO) enhances the accumulation of toxic glutamate, which leads to increased neurotoxicity (Figure 1; Boje et al., 1992; Colonna et al., 2017).

All of these factors help M1 microglia effectively phagocytize and eliminate foreign components or cell debris from damaged tissue. The polarization further attracts monocytes and T cells to pass the disrupted BBB. M1 microglia promote T cell differentiation into Th1 cells and recruitment to the CNS by launching a comprehensive immune response (Prajeeth et al., 2018).

Afterwards, the inflammatory response starts shifting in order to protect the tissues from excessive damage. The shift to anti-inflammatory conditions is provoked by M2 microglia. The polarization is stimulated by IL-4 or IL-13 (Figure 1), generally produced by Th2 cells (Ghosh et al., 2016). M2 polarized microglia produce anti-inflammatory cytokines like IL-10, which stimulate the reparation of damaged tissues and calm down the inflammatory response (Figure 1).

Whenever microglial polarization function is damaged, the brain parenchyma can face prolonged inflammatory and pathophysiological conditions. It has been referred to as reactive microgliosis and can lead to a number of degenerative diseases such as Alzheimer's,

Parkinson's, Huntington's, amyotrophic lateral sclerosis and multiple sclerosis (Levesque et al., 2010; Liddelow et al., 2017).

1.3 Astrocytes

Astrocytes, also known as astroglia, are the most abundant cell type in the mammalian brain. Similar to neurons and oligodendrocytes, astrocytes are of ectodermal lineage and originate from radial glial cells. Therefore, they are not considered among immune cells. Nevertheless, they play an important role in battling pathological conditions and tissue damage alongside microglia in the brain (Agulhon et al., 2008; Skoff, 1990).

Astrocytes have a particularly versatile ability to respond to different signals and stimuli and thus play a considerably vital role in keeping the brain's homeostasis under tight control (Gee et al., 2005). Similar to microglial cells, astrocytes display a ramified profile. Their cell bodies are covered with stems branched into fine processes, which scavenge the surrounding environment and form a non-overlapping surveillant system, spanning across the whole CNS. However, astrocytes remain rather static in this process, compared to microglia, which constantly extend their processes to explore the environment (Butt et al., 1994).

Two distinct astrocyte morphologies, protoplasmic and fibrous, were first described by W. Lloyd Andriezen as early as in 1893 (Andriezen, 1893). Protoplasmic astrocytes can be found mainly in the grey matter, their processes wrapping synapses and neuronal cell bodies as well as surrounding vasculature. Fibrous astrocytes on the other hand strictly inhabit the white matter, assembling with neuronal axons and oligodendrocytes, and are in tight connection with nodes of Ranvier and blood vessels (Miller et al., 1984). In addition to the two main subtypes, a series of other astrocyte-like cells have been characterized: Bergmann glia (specific for cerebellum), Müller glia (specific for retina; Reichenbach, 1989), radial glial cells (Reichenbach, 1989; Campbell and Götz, 2002; Grosche *et al.*, 2002) and ependymal cells (specific for ventricles; Spassky *et al.*, 2005)

Astrocytes are connected to one another by gap junctions, which mediate the transferring of small molecules (< ~ 1,2 Da). Gap junctions therefore moderate many of the important signalling and homeostatic functions like spatial buffering of K^+ ions and long-distance transport of signalling molecules, nutrients and other metabolites (Lin et al., 1998; F. Wang et al., 2012).

In addition to their ramified morphology, astrcoytes carry a variety of receptors detecting neurotransmitters, growth factors and cytokines, as well as ion channels and different transporter proteins. Neurotransmitters trigger an increase in astrocytes' intercellular Ca^{2+} level, which carries through adjacent astroglial cells in a wave-like manner. The trespassing increase in Ca^{2+} level triggers the release of neuroactive molecules from secretory lysosomes such as glutamate, glutamine, ATP, D-serine and TNF-α, which in turn regulate the synaptic activity and excitability of neurons (Coco et al., 2003; Cornell-Bell et al., 1990; D. Li et al., 2008; X. Wang et al., 2006).

Astrocytes also control the synaptic activity of neurons by modulating the levels of extracellular ions like K^+, the mechanism of which is still poorly understood. However, it has been shown that neurons cultured in the absence of all glial cells, seem to show little to null synaptic activity. Once cultured with astrocytes, the synaptic activity increases significantly. The same increase is not noted in cultures with other types of glial cells (Ullian et al., 2001). Astrocytes have also been shown to enhance the pre- and postsynaptic remodelling of neurons, forming a "tripartite" component of synapses and contributing to their maturation (Araque et al., 1999). They release thrombospondin, a matrix-associated protein, which has been shown to promote synaptogenesis. Brains lacking thrombospondin show an extensive decrease in synapse numbers (Christopherson et al., 2005). Moreover, complements (C3) release by astrocytes in response to NFκB activation, leads to changes in the synaptic and dendritic morphology of neurons. High levels of complement activation have also been shown to be a common hallmark for neuronal impairment in Alzheimer's disease (Lian et al., 2015).

These data indicate that astrocytes carry a particularly vital role in the maintenance of synaptic activity of neurons (Christopherson et al., 2005; Pfrieger et al., 1997; Ullian et al., 2001).

The terminal branches of astrocytic processes, termed endfeet, are tightly ensheathing endothelial cells and pericytes, located on the outer surface of the brain's vasculature. Astrocytes' endfeet account for the permeability of the blood-brain barrier (BBB) by controlling the disruption and closing of the barrier. The production and release of matrix metalloproteinase (MMP), nitric oxide synthases (NOS; Z. Jiang *et al.*, 2014), vascular endothelial growth factor (VEGF; Argaw *et al.*, 2012; S. Jiang *et al.*, 2014), glutamate and endothelins cause endothelial cell apoptosis and promote the down-regulation of tight junction-related proteins like occludin, F-actin and claudin, which results in the disruption of the BBB (Figure 2; András et al., 2007). This gives way to peripheral monocytes and activated lymphocytes for infiltration and transmigration into the CNS. It has also been suggested that

astrocytes further contribute to this by releasing CCL2, which acts as an chemoattractant to activated T-cells, monocytes, natural killer cells and basophils (Weiss et al., 1998).

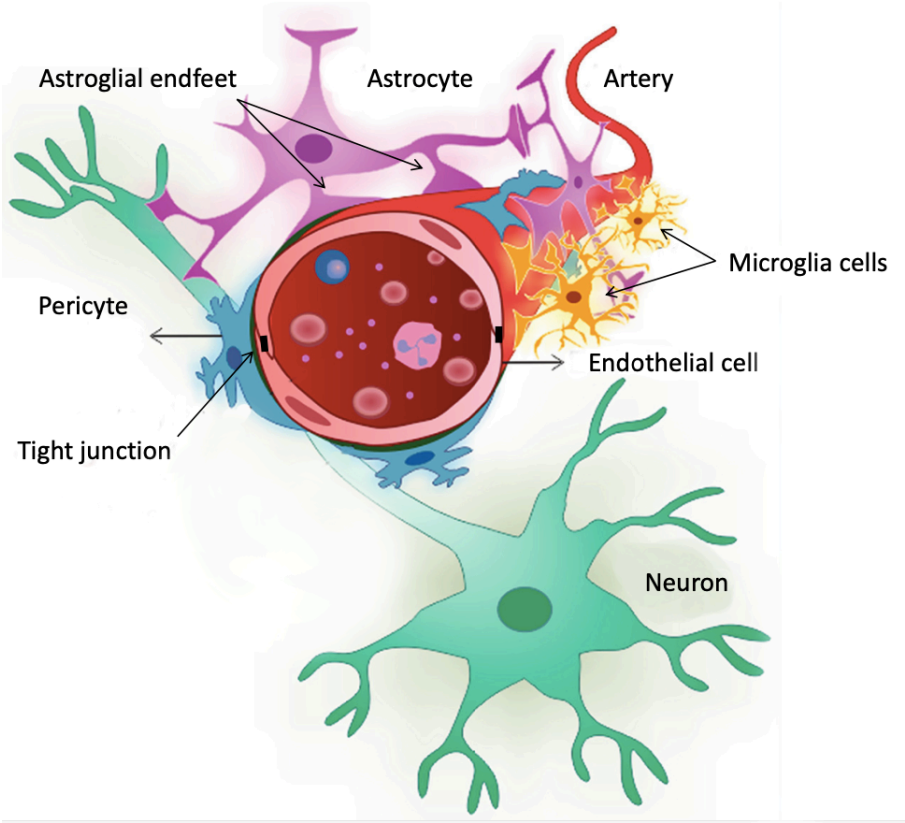

Figure 2. Neurovascular unit and structure of the blood brain barrier (BBB). Monolayer of endothelial cells (pink) connected by tight junctions (black) along with pericytes (blue) and astroglial endfeet (purple) form the BBB. Excitatory neurons (green) are connected to astrocytes in order to signal blood flow alterations and microglia (yellow) protect the brain parenchyma in response to stimuli from other cells (Modified by Barichello *et al.*, 2019).

1.4 Neuroinflammation caused by lipopolysaccharide (LPS)

LPS is an endotoxin present in the outer membrane of Gram-negative bacteria. It induces the production and release of TNF and a variety of other harmful cytokines that can ultimately lead to a septic shock (Poltorak et al., 1998). LPS promotes the activation of glia. The endotoxin is considered a PAMP and is therefore detected by TLRs mainly expressed on microglia, macrophages and astrocytes (Kigerl et al., 2014). Several subtypes of TLRs are present on the microglia surface - TLR2, TLR4 and TLR6 (Fellner et al., 2013; Stewart et al., 2010; Udan et al., 2008).

Because TLRs are present on all of the innate immune cells, it is probable that the endotoxin recognition on peripheral immune cells promotes the systematic secretion of secondary mediators, which then trespass the BBB (Laflamme et al., 1999). In addition, LPS has been shown to increase the permeability of the BBB, however the exact mechanism is still poorly understood (Banks et al., 2015). Alternatively, innate immune response can be initiated within the brain, since TLR4 is present on most of the CNS cells regardless of their lineage, therefore making them capable of secreting pro-inflammatory components (Chakravarty et al., 2005; Lafamme et al., 2001).

1.4.1 The response of microglia in neuroinflammation caused by LPS

As mentioned before, LPS is a ligand to TLR4 (as well as TLR2) present on the surface of microglia cells, which in addition to LPS, detects many other viral components and proteins (Poltorak et al., 1998). The interactions between LPS and TLR4/TLR2 take place through a mediator protein MD-2 (lymphocyte antigen 96; Dziarski et al., 2000) and results in a variety of signalling pathways, which lead to the activation of gene transcription by nuclear factor-κB (NF-κB) and production of distinct pro-inflammatory cytokines and IFNs (Chakravarty et al., 2005).

LPS also promotes a number of indistinct sickness behaviours, like reduction in food intake and anorexia, depression, lethargy etc. (Kim et al., 2013). Several behaviour patterns noted in LPS-induced inflammation models are considered analogous to clinically important symptoms seen in neurodegenerative diseases in humans (J. Zhao et al., 2019).

In response to LPS-induced acute inflammation, TLR4-mediated microglial activation up-regulates the expression of TNF-α, which activates endothelial adhesion molecules and stimulates peripheral leukocyte migration to the injury site (McKimmie et al., 2005; Zhou et al., 2006). The increase in IL-6 transcript levels has also been noted in microglia after LPS treatment (McKimmie et al., 2005).

Administration of LPS also induces the up-regulation of MHC II molecules on activated microglia, which phagocytose and present the foreign antigen bound to MHC II on their surface further to other immune cells like $CD4^+$ T cells (Buttini et al., 1996; Schetters et al., 2018). It has been shown that LPS-induced prolonged inflammation can promote cognitive dysfunction

in the brain (Terrando et al., 2010). Moreover, LPS induces down-regulation of pre- and post-synaptic proteins leading to synaptic alterations, which further contribute to weak cognition and memory formation noted in several neurodegenerative disease models (Y. Zhao et al., 2019).

1.4.2 The response of astrocytes in neuroinflammation caused by LPS

In response to trauma, neurodegeneration, ischemia and infections, astrocytes go through a cascade of proliferation and morphological changes. The process, termed "reactive astrogliosis“, is highly heterogeneous and can be both detrimental and beneficial, depending on the type of injury. Astrogliosis is characterized by the up-regulation of different genes including the glial fibrillary acidic protein (GFAP), which is an early marker for differentiated astrocytes and neurotoxicity (Anderson et al., 2016; Bush et al., 1999; O'Callaghan et al., 2005). The inhibition of NF-κB in GFAP-expressing astrocytes could results in decreased glial scarring. The forming glial scar hinders the regrowth of damaged axons (Brambilla et al., 2005).

Astrocytes have been shown to express TLR2, 3 and 4 in response to LPS treatment, with TLR2 expression being relatively higher in astrocytes than microglial cells. Upon activation by LPS, other TLRs are down-regulated in astrocytes (Ma et al., 2013; McKimmie et al., 2005). TLR-mediated signals induce the production of several inflammatory molecules, like glutamate, NO, TNF-α and ROS. The production of ROS has also been shown to be the most potent inducer of neurotoxicity (Ma et al., 2013).

The astroglial response to neuroinflammation is mainly dependent on IFN-γ, which is the dominant inducer of MHC II class molecules in astrocytes (Fierz et al., 1985). MHC II expression in astroglia is also thought to be controlled by surrounding neurons and astroglial participation in antigen presenting has been noted in several neuroinflammatory diseases (Vardjan et al., 2012).

1.4.3 CX3CR1 receptor

Microglia cells are the only cells present in the CNS that express the fractalkine receptor chemokine (C-X3-C motif) receptor 1 (CX3CR1). CX3CR1 is also expressed on other circulating peripheral immune cells like monocytes, dendritic cells, subtypes of T cells and natural killer cells (Combadiere *et al.*, 1995). Cells expressing CX3CR1 receptor use fractalkine

(CX3CL1) as a mediator for functions like migration, chemotaxis and adhesion (Imai et al., 1997).

CX3CR1 is a G protein-coupled receptor and its polypeptide chain consists of seven α-helical formations spread across the cell membrane. The externally positioned loops of the polypeptide are the binding site for its ligands - CX3CL1 and CCL26 (Mizoue et al., 1999; Nakayama et al., 2010; Raport et al., 1995).

Chemokine (C-X3-C motif) ligand 1 (CX3CL1, also referred to as fractalkine or neurotactin) is a membrane-bound or soluble chemokine synthesised and secreted by neurons in the CNS. Its precise function in the brain is not yet profoundly understood. However, it has been reported that an increase in CX3CL1 expression in neurons takes place once pathological conditions strike (Harrison et al., 1998). The corresponding expression of CX3CL1 in neurons and CX3CR1 in microglia have given rise to a hypothesis that this interaction serves as a unique communication system, where neurons are likely to influence the activation of microglia via CX3CL1 and hence suppress extreme neurotoxicity (A. E. Cardona et al., 2006). It has been shown *in vitro* that the CX3CR1/CX3CL1 interaction endorses the survival of neurons and suppresses microglial cell death (Boehme et al., 2000).

Cx3cr1-deficient mice showed a reduction in microglial numbers and deficit in synaptic pruning during development, resulting in altered numbers of synapses in the adult brain. An impairment of microglial migration into close proximity of developing thalamocortical synapses has been noted in Cx3cr1-deficient mice, leading to alterations in the functional maturation of synapses (Hoshiko et al., 2012). This suggests that neurons communicate early microglial migration and proliferation through the fractalkine signalling (Paolicelli et al., 2011).

2 EXPERIMENTAL PART

2.1 Aim of this study

The main goal of this study was to characterize the molecular changes of glial cells in heterozygous *Cx3cr1*$^{GFP/+}$ mice after inducing an acute systemic inflammatory state including neuroinflammation by LPS treatment.

Detailed aims:

- To evaluate LPS-induced body weight changes;
- To analyse changes in the abundancy of microglial and astroglial cells in different brain regions in control conditions and in inflammatory state;
- To distinguish the LPS-induced changes in MHC II expression on microglia and astroglia in different brain regions;
- To analyse the expression of microglial receptors (CD11b, CD172a, CD115, CD200R, CX3CR) and their possible changes in different brain regions after LPS administration.

2.2 Materials and methods

2.2.1 Mice

In this thesis, 8-10 months old female *Cx3cr1*$^{GFP/+}$ transgenic mice (n=11) on a B6JRcc/B6N strain background were used. In CX3CR1-GFP, the second exon of the endogenous *Cx3cr1* locus has been replaced by a gene encoding green fluorescent protein (GFP), which fluorescently labels monocytes, dendritic cells, natural killer (NK) cells and microglia, under the control of the endogenous *Cx3cr1* locus. More specifically, the mice used in this thesis were heterozygotes, meaning the fractalkine receptor remains partly functional and microglial cells that express this receptor appear green (Feng et al., 2015).

2.2.2 LPS treatment

LPS (derived from *E.coli* serotype 01111:B4; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% NaCl (saline). LPS and saline was administered according to the body weight of mice (300µl/30g). Intraperitoneal (i.p.) injections of LPS were at a dose of 500 µg/kg, control group received vehicles consisting of 0.9% saline in an equivalent volume. Mice were divided randomly into two groups: (1) control group (0.9% saline i.p. administration, n=5) and (2) 24 h LPS treatment group (LPS 500 µg/kg i.p. administration, n=6). After 24 hours, mice were sacrificed by cervical dislocation. Hereafter mice were decapitated, the brains were removed and the hippocampus (HPC), cerebral cortex (hereafter 'cortex'; CTX) and cerebellum (CBL) were dissected.

All animal experiments were done by a certified specialist. Animals were bred and housed in the Laboratory Animal Centre at University of Tartu and were kept under standard conditions with unlimited access to food and water in a 12/12h light/dark cycle (lights on from 07:00 to 19:00 hours). All animal procedures were performed in accordance with the European Communities Directive (2010/63/EU) and permit (No. 141, April 17, 2019) from the Estonian National Board of Animal Experiments.

2.2.3 Body weight determination

Mice were weighed promptly before the LPS and saline injections and the tails were marked with non-toxic marker. After 24h, the mice were weighed once more. Body weight change (BW%) was calculated using the following formula and expressed as change of the initial weight:

$$BW\% = \frac{weight\ post\text{–}administration - weight\ pre\text{–}administration}{weight\ pre\text{–}administration}\ x\ 100\%$$

2.2.4 Tissue preparation

The HPC, CTX and CBL tissues were dissected and collected into centrifuge tubes containing ice-cold cell culture media, Dulbecco's Modified Eagle's medium (DMEM Lonza BioWhittaker) + 10% foetal calf serum. The tissues were minced immediately with a 2 ml syringe plunge in 1 ml ice-cold PBS buffer and filtered through a 70 µm filter (BD Biosciences)

into a small dish. The filtered homogenate was transferred into a 1,5 ml centrifuge tube and then blocked with 10% rat serum for 30 minutes with gentle rotation at +4°C.

2.2.5 Flow cytometry

Since multiple fluorochromes were used in this experiment, compensation was performed prior to the acquisition. For each staining, mouse glial markers (G3), microglial subpopulation markers (M2) and other microglial markers (Mo), an identical compensation was generated, since the fluorochromes attached to antibodies were the same in all of the three stainings (Table 2). Tubes were labelled for each fluorochrome used in the experiment (PE, PerCP-Cy5.5, PECy7 and APC). UltraComp eBeads compensation beads (Thermo Fischer Scientific) were prepared by adding 1 drop of beads into each of the tubes. Next, 0,5 µl of antibody was added to the corresponding tubes and incubated at +4°C for 30 min in the dark. After incubation 500 µl 1x PBS was added and analysed by a flow cytometer LSR Fortessa (BD BioSciences). Each single stained bead sample was run to perform compensation setup and recorded for compensation controls. LSR Fortessa software collects data from each compensation control tube and automatically calculates accurate compensation values for each fluorochrome combination and makes a compensation matrix (Table 1).

Table 1. Compensation spillover matrix

Spillover (%)					
	PerCP-Cy5-5	**PE**	**Pe-Cy7**	**APC**	**FITC**
PerCP-Cy5-5		33,41	4,35	1,25	3,05
PE	5,27		1,89	0,16	0,33
Pe-Cy7	3,05	1,74		1,31	0,00
APC	7,17	0,06	0,16		0,18
FITC	0,14	1,19	0,20	0,01	

For flow cytometry, Cx3cr1$^{GFP/+}$ mouse tissues were stained by using the following combined antibodies (Biolegend & Miltenyi): mouse glial (microglia, astroglia, oligodendrocyte precursor cells) markers (G3), mouse microglial subpopulation markers (M2) and mouse other microglial markers (Mo) (Table 2). M2 antibodies define microglial cells in an activated pro-inflammatory state, while Mo antibodies tag microglial cells in a quiescent or anti-inflammatory state.

Table 2. Fluorochromes and microglial markers used (Biolegend & Miltenyi)

Fluorochromes	G3	M2	Mo
PE	GLAST (Miltenyi)	MHC II (Biolegend)	CD200R (Biolegend)
PECy7	CD45 (Biolegend)	CD45 (Biolegend)	CD45 (Biolegend)
PerCP/Cy5.5	MHC II (Biolegend)	CD11b (Biolegend)	CD172a (Biolegend)
APC	O4 (Miltenyi)		CD115 (Biolegend)

GLAST – glutamate/aspartate transporter (astrocytes); MHC – major histocompatibility complex (microglia and astrocytes); CD200R – cell surface glycoprotein receptor (microglia); CD45 – lymphocyte common antigen (microglia); CD11b – integrin subunit alpha M (microglia); CD172a - signal-regulatory protein alpha (microglia); O4 – oligodendrocyte transmembrane protein (oligodendrocyte precursor cells); CD115 – colony stimulating factor 1 receptor (microglia).

After blocking, tissue homogenates were incubated with 0,5 µl of each antibody per sample for 1 h at +4°C. Samples were washed using 1X PBS and centrifuged at 2000 rpm (fixed-angle rotor: 15 000 rpm) for 6 minutes. Supernatant was discarded and cell pellets were resuspended in 0,5 ml 1X PBS and filtered through 35 µm strainers. The final volume was 0,5 ml per sample. The tubes were stored on ice protected from the light until collecting the stained cells populations with flow cytometer.

Three lasers in the LSR Fortessa flow cytometer were used in this study: blue (488nm), green (532nm) and red (838nm). The event count varied between stainings. For G3 staining >400 000 cells, and for M2 and Mo stainings each >20 000 cells were collected. For microglial staining (Mo and M2), acquisition was done under live gating of GFP-positive cells. For glial staining (G3), all cells were collected.

2.2.6 Statistical analysis

Results are expressed as mean values ± SEM. Body weight change (BW%) was calculated and expressed as change of the initial weight. Comparison of body weight change between saline and LPS treatment groups was performed using unpaired T-test. Flow cytometry data was first analysed using Kaluza Analysis Software (Beckman Coulter). Data was then transferred and sorted in Excel 2019 and then further analysed in GraphPad Prism 8 (2019) using 2-way ANOVA (tissue x treatment), followed by Tukey's multiple comparisons test. In order to investigate the basal differences between tissues, data from both treatment groups was pooled together (provided that there were no differences between LPS and saline treatments), otherwise simply saline treatment groups were compared. Basal differences between tissues were then further analysed by one-way ANOVA. Statistical significance was set to $p < 0,05$.

3 RESULTS

3.1 Body weight change in response to LPS

To assess LPS induced body weight alterations, mice were weighed before and 24 hours after the administration of LPS and saline. Body weight change (BW%) means for both groups were compared using unpaired t-test. LPS administration induced highly significant weight loss after 24 h compared to the control group ($p < 0,0001$), as LPS injected mice lost 10,21 ± 0,59 % of their initial body weight, whereas saline administration caused merely 0.72 ± 0,66 % body weight loss (Figure 3).

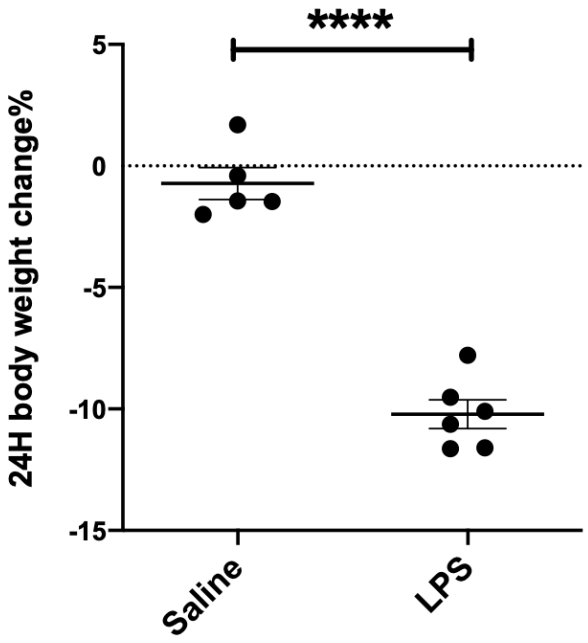

Figure 3. **Effect of lipopolysaccharide (LPS) on body weight.** Body weight was measured before and 24h after intraperitoneal injections of LPS and saline (control group). SAL – saline; LPS – lipopolysaccharide. The data is presented as mean ± SEM (SAL, n=5; LPS, n=6). **** $p < 0,0001$.

3.2 Microglial and astroglial abundance changes in response to LPS

To elucidate the quantity of microglial cells and astrocytes in different brain regions the cells were first analysed based on the cell size and granularity (FSC-A and SSC-A, respectively; Figure 4A). In addition, the gated nucleated cells were further plotted in FSC-A and FSC-H, and single cells were gated to rule out cell debris and doublets (Figure 4B).

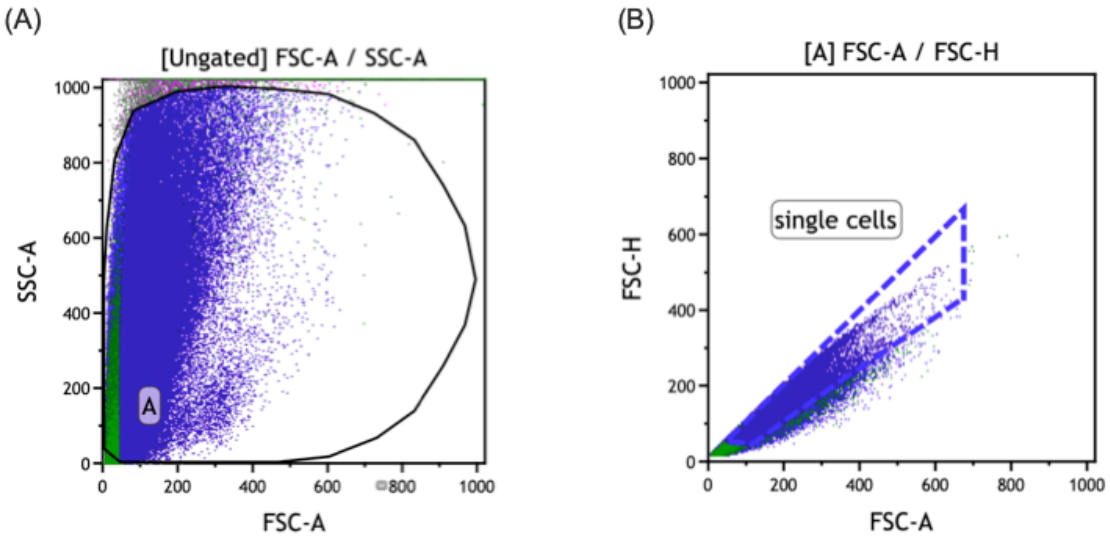

Figure 4. **Determination of nucleated cells and single cells.** (A) Nucleated cells gated based on their size and granularity (FSC-A and SSC-A, respectively). (B) Nucleated cells further plotted in FSC-A vs FSC-H and gated to exclude cell debris and doublets.

Thereafter single cells gate was used as a parental gate in CD45 vs GFP plot, where the microglial cell population was defined as CD45 and GFP positive cells (Figure 5A). Next, astrocytic and nonastrocytic cells were separated based on Glast-PE expression and astrocytes were defined as GLAST+ cells and nonastrocytes as GLAST- cells (Figure 5B).

Our statistical analysis did not reveal any significant differences in microglial and astroglial percentages between LPS and control groups in any of the selected tissues (Figure 5C and E). Thus, we next pooled together the data from LPS and saline groups to give more statistical power to the analysis. Comparison of brain tissues revealed significant regional differences in microglial and astroglial cell abundances. Microglia cell percentage was significantly 2-folds higher in the hippocampus (0,33 ± 0,02 %) compared to cortex (0,14± 0,01 %) and cerebellum (0,11 ± 0,01 %). No significant differences were observed between cerebellum and cortex (Figure 5D). The percentages of astrocytes were highest in cortex (86,06 ± 0,62 %), followed by hippocampus (81,15 ± 0,96 %) and cerebellum (73,59 ± 1,06 %; Figure 5F).

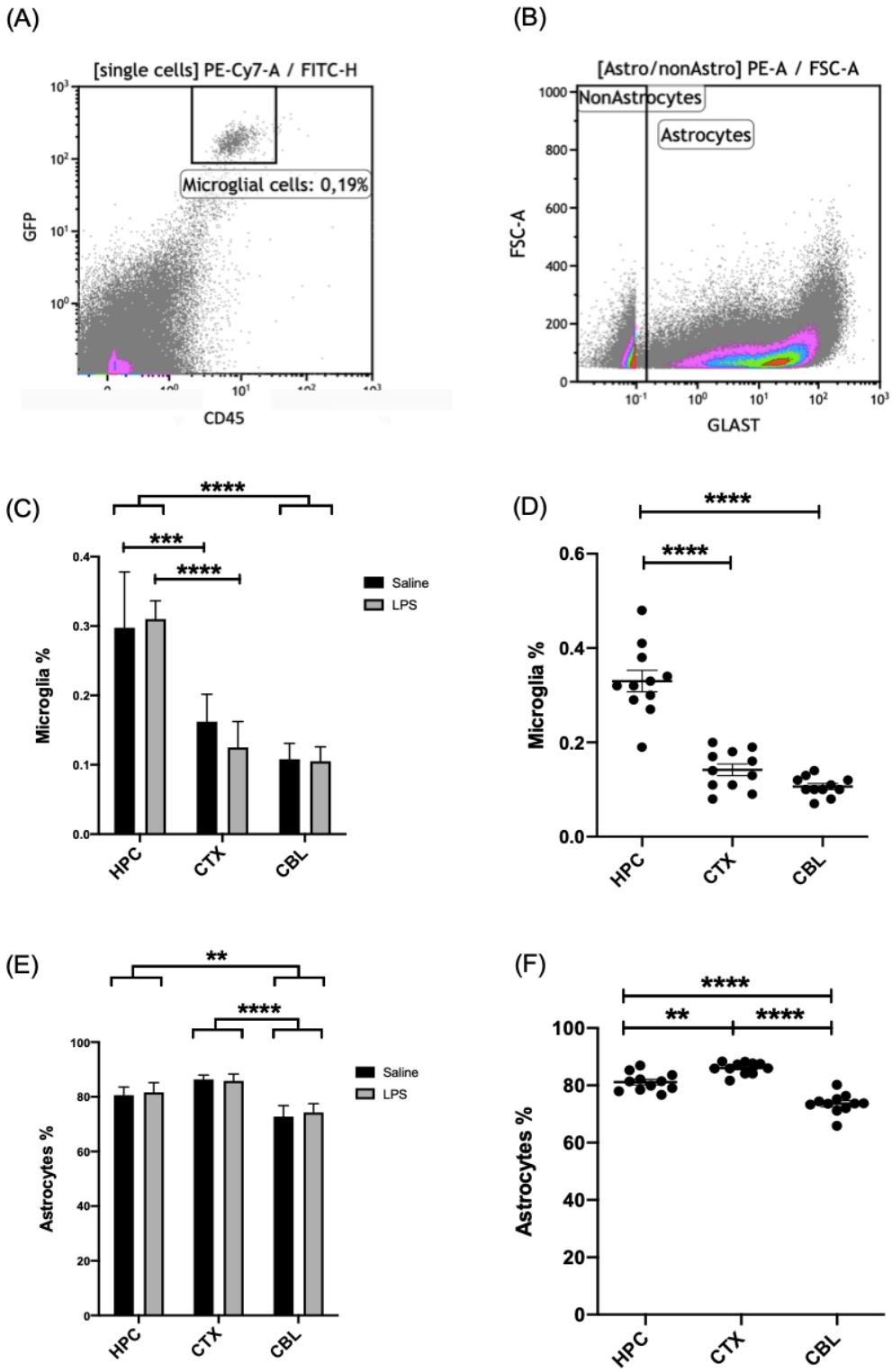

Figure 5. Microglia and astroglia cell abundance changes (%). (A) Determination of the microglial cell population. Microglia were defined as GFP (Y-axis) and CD45 (X-axis) positive cells. (B) Determination of the astrocyte cell population. Astrocytes were defined as GLAST positive cells. Microglial (C) and astroglial (E) abundance changes in response to 24 h LPS treatment. Pooled data of microglia (D) and astrocyte (F) abundance changes in hippocampus (HPC), cerebral cortex (CTX) and cerebellum (CBL) after 24h LPS treatment. SAL – saline; LPS – lipopolysaccharide. Graphed as mean ± SEM; (SAL, n=5; LPS, n=6). ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.

3.3 Marker molecules' expressional changes in response to LPS

3.3.1 MHC II expressional change on microglia and astroglia

In the healthy brain, MHC II expression has been shown to be relatively low in microglia. However, it can be highly up-regulated in inflammatory and neurodegenerative conditions (Wyss-Coray et al., 2002). Therefore, we decided to investigate MHC II surface expression on microglia as well as astroglia in response to LPS-induced acute inflammation.

Firstly, MHC II positive microglia and astrocytes were determined based on their MHC II-PerCP-Cy5.5 expression (Figure 6A and B).

To study tissue specific differences in the basal expression level of MCH II, we compared control group animals (Figure 6D and F). Comparison of tissues revealed substantial differences in MHC II expression both on microglia and astrocytes. MHC II-positive microglia were significantly 2-3-folds less abundant in the hippocampus (both $p<0,0001$) compared to the cortex and cerebellum (Figure 6D). We found no differences between the cortex and the cerebellum (Figure 6D). Similarly, MHC II-positive astrocytes were 2-3-folds less abundant in the hippocampus compared to the cortex ($p<0,01$) and the cerebellum ($p<0,05$) (Figure 6F).

Thereafter, we evaluated the differences in response to LPS in these brain regions. LPS administration induced a slight reduction in the microglial cell surface expression of MHC II, although this alteration was significant only in the cortex (Figure 6C). More specifically, the percentage of MHC II-positive microglia was about 10% lower in the LPS treatment group compared to controls in the cortex ($p=0,046$; Figure 6C). On the contrary, the percentage of MHC II-positive astrocytes was slightly elevated in response to LPS in the cortex, but did not reach the level of statistical significance in any of the selected brain regions (Figure 6E).

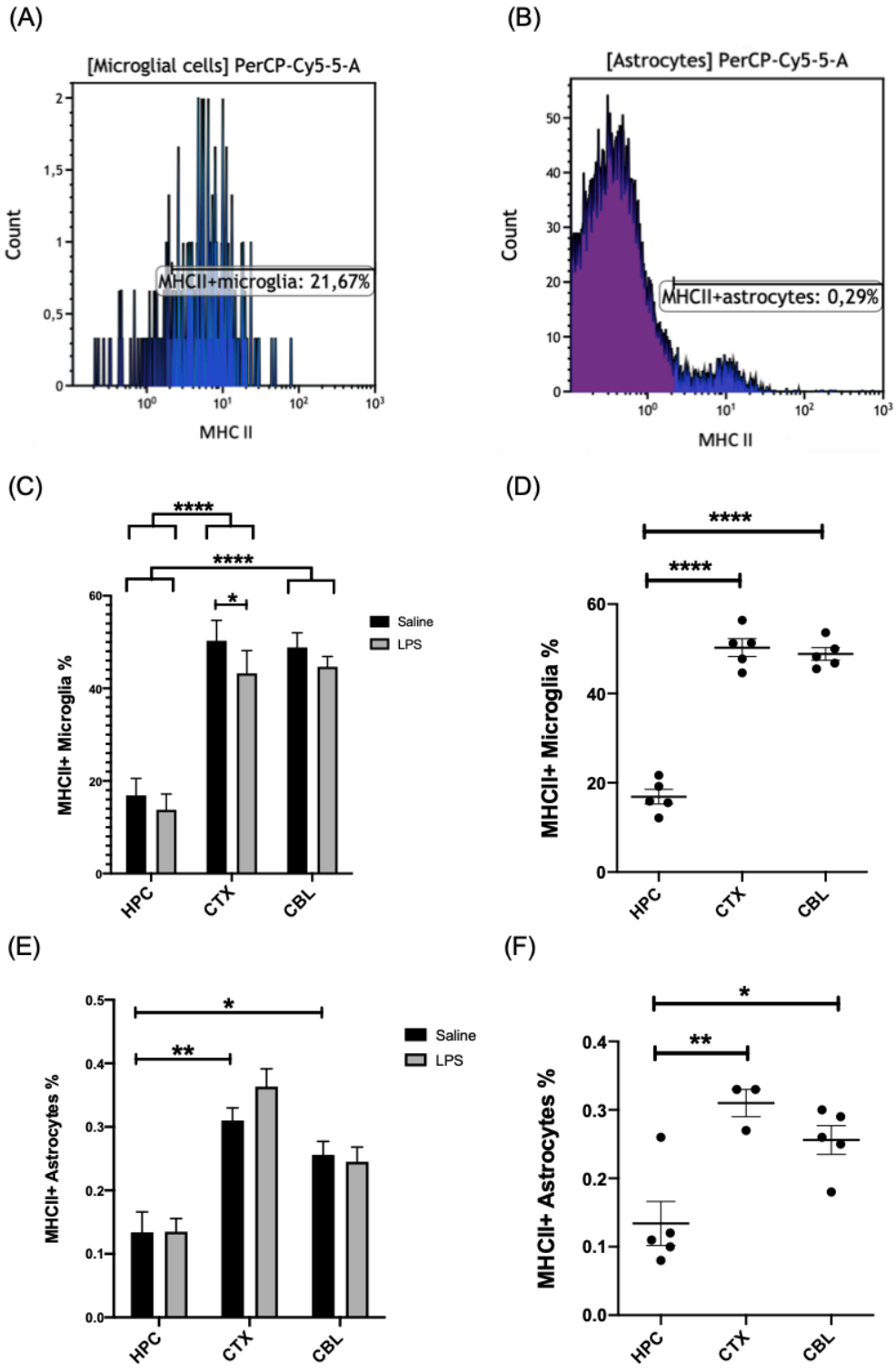

Figure 6. Percentages of MHC II-positive microglia and astroglia (%). Determination of MHC II-positive microglial cells (A) and MHC II-positive astrocytes (B). (C) LPS-induced changes in MHC II-positive microglia percentages. (D) Basal percentages of MHC II expressing microglial cells in HPC, CTX and CBL (comparison of control groups) (E) MHC II-positive astroglia percentages alterations in response to LPS. (F) Basal percentages of MHC II expressing astroglial cells in hippocampus (HPC), cerebral cortex (CTX) and cerebellum (CBL; comparison of control groups). SAL – saline; LPS – lipopolysaccharide. Graphed as mean ± SEM (SAL, n=5; LPS, n=6). * $p < 0,05$; ** $p < 0,01$; **** $p<0,0001$.

3.3.2 CD172a expressional change on microglia

Next, we explored the surface expression of different microglia-specific receptors. CD172a has been previously shown to be a M2 polarization marker and is expressed in the healthy CNS on ramified microglia (Arcuri et al., 2017; Mammana et al., 2018). Our aim was to further investigate whether CD172a surface expression is modified in response to acute LPS-induced inflammation.

CD172a surface expressional level (MFI) was evaluated on a CD172a vs GFP plot (Figure 7A). Firstly, we studied the basal differences in CD172a surface expression. Comparison of control groups revealed significant variations between regions. CD172a expressional level was profoundly lower on hippocampal microglia (1,400 ± 0,31 MFI) compared to cortical (9,46 ± 0,71 MFI; $p<0,0001$) and cerebellar microglia (7,45 ± 0,46 MFI; $p<0,0001$; Figure 7B). CD172a expression also varied between the cortex and cerebellum. The expression of CD172a was significantly higher on cortical microglia compared to cerebellar counterparts ($p<0,05$; Figure 7B).

Further analysis of treatment groups did not show any substantial differences. However, CD172a expression on microglial cells tended to be lower in LPS treatment groups in all tissues compared to control groups (Figure 7B).

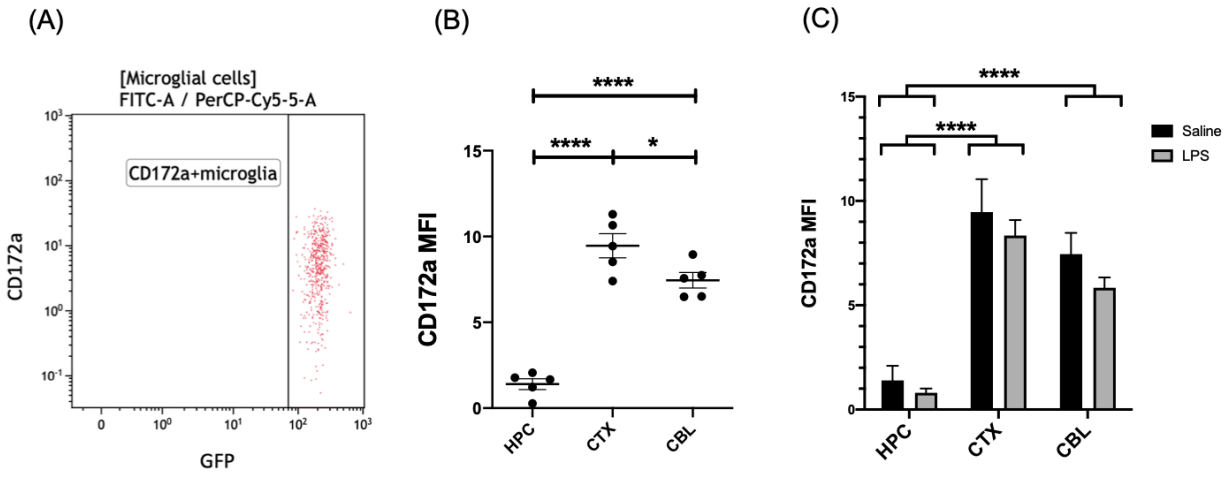

Figure 7. CD172a expression on microglia. (A) Determination of CD172a expressional level (MFI) on microglial cells. (B) Basal tissue differences of CD172a expressional level on microglia (comparison of saline groups). (C) Alterations of CD172a expression in response to LPS in hippocampus (HPC), cerebral cortex (CTX) and cerebellum (CBL). SAL – saline; LPS – lipopolysaccharide. Graphed as mean ± SEM (SAL, n=5; LPS, n=6). * $p < 0,05$; **** $p < 0,0001$.

3.3.3 CD11b expressional change on microglia

CD11b is recognized as a specific marker for activated microglia and is up-regulated during CNS inflammation and several neurodegenerative diseases (Roy et al., 2006). Therefore, we decided to investigate whether LPS-induced acute inflammation induces any differences in the surface expression level of CD11b. We first compared the control groups and found that basal expressional level of CD11b was remarkably different in all of the tissues. We noted nearly 2-folds of lowered expression of CD11b in the hippocampus compared to the cortex and the cerebellum (both p<0,0001). Moreover, there was a significant difference between the cortex and the cerebellum, as the expression level of CD11b was higher in the cerebellum (p=0,0013; Figure 8B).

When comparing the CD11b expression level between LPS and saline treatment groups, we found that it was significantly upregulated in the cortex (p<0,005) and the cerebellum (p=0,0007) of LPS treated mice. The same tendency was also seen in the hippocampus, although not statistically significant (Figure 8C).

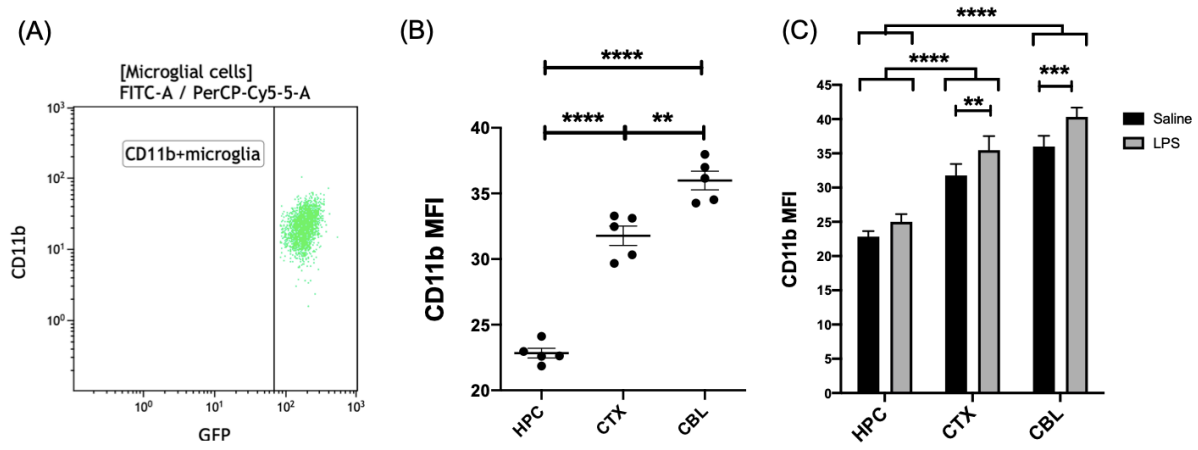

Figure 8. CD11b surface expression level on microglia. (A) Determination of CD11b expressional level (MFI) on microglial cells. **(B)** Basal tissue differences of CD11b expressional level on microglia (comparison of saline groups). (C) Alterations of CD11b expression in response to LPS in hippocampus (HPC), cerebral cortex (CTX) and cerebellum (CBL). SAL – saline; LPS – lipopolysaccharide. Graphed as mean ± SEM; (SAL, n=5; LPS, n=6). ** p < 0,01; *** p < 0,001: **** p < 0,0001.

3.3.4 CD115 expressional change on microglia

Previously, CD115 receptor has been shown to play an important role in microglial development and survival (Elmore et al., 2014). In our study, we decided to evaluate whether LPS-induced inflammation influences the surface expression of CD115 on microglia. While the analysis did not reveal any significant differences in the MFI of CD115 on microglial cells between LPS and control groups (Figure 9C), we found tissue-specific variations in the basal MFI of CD115 between the cerebellum and the cortex. The CD115 positivity was about 15% higher in cortex compared to cerebellum ($p<0,05$) Figure 9B).

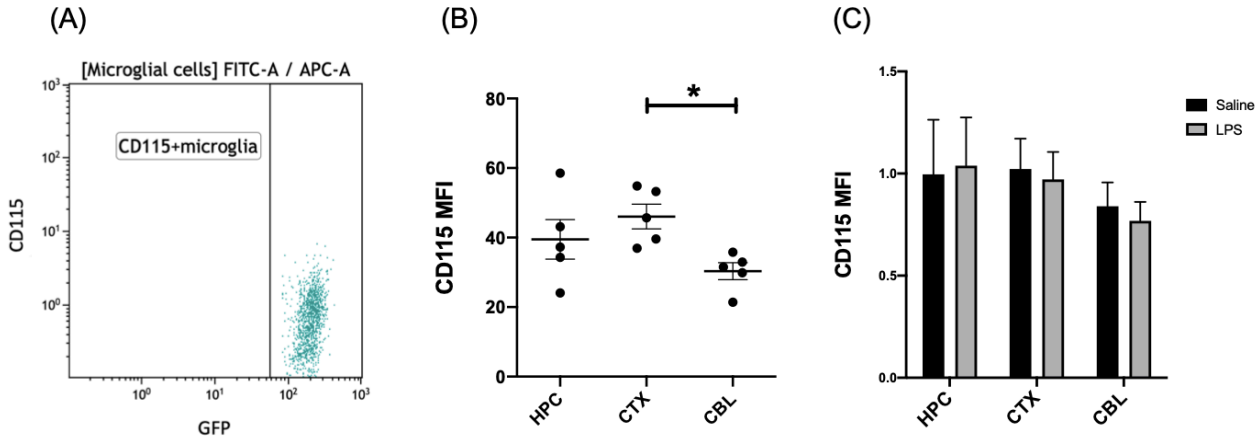

Figure 9. CD115 surface expression on microglia. (A) Determination of the expression level (MFI) of CD115 on microglial cells. (B) Basal tissue differences of CD115 expression level on microglia (comparison of saline groups). (C) Alterations of CD115 expression in response to LPS in hippocampus (HPC), cerebral cortex (CTX) and cerebellum (CBL). SAL – saline; LPS – lipopolysaccharide. Graphed as mean ± SEM; (SAL, n=5; LPS, n=6). * $p < 0,05$.

3.3.5 CD200R expression change on microglia

CD200R is an important inhibitory receptor present on microglia. The receptor has been found to be responsible for the regulation of microglial activation and actively maintains microglia in a quiescent state through its interaction with CD200, which is expressed by neurons (Hoek et al., 2000). Thus, we decided to further analysed CD200R surface expression changes on microglia in response to LPS-induced acute inflammation.

CD200R surface expression level (MFI) on microglia was evaluated on a CD200R vs GFP plot (Figure 10A). We first analysed the basal CD200R surface expression level by comparing only

saline groups and found that CD200R expression was half-reduced in the cerebellum (6,57 ± 0,34 MFI) compared to the cortex (8,85 ± 0,38 MFI; p<0,001) and the hippocampus (1,14 ± 0,20 MFI; p<0,01; Figure 10B).

More interestingly, evaluation of CD200R expression differences between LPS group and control group revealed that CD200R expression on microglia was significantly lowered in LPS treated mice of all three regions (HPC and CBL, p<0,0001; CTX, p<0,001; Figure 10C).

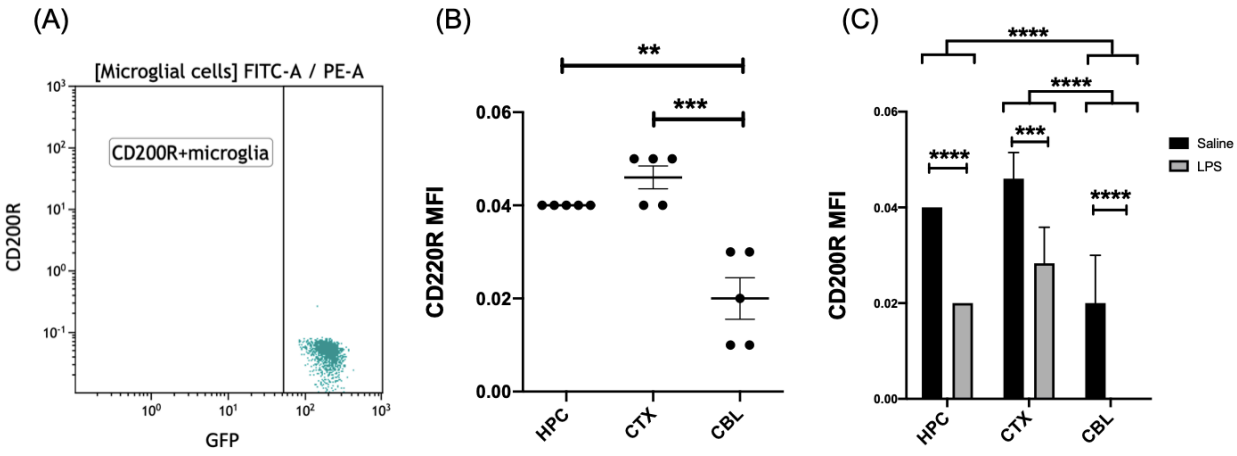

Figure 10. CD200R surface expression on microglia. (A) Determination of CD200R expressional level (MFI) on microglia. (B) Basal tissue differences of CD200R expressional level (comparison of saline groups). (C) Alterations of CD200R expression in response to LPS in hippocampus (HPC), cerebral cortex (CTX) and cerebellum (CBL). SAL – saline; LPS – lipopolysaccharide. Graphed as mean ± SEM; (SAL, n=5; LPS, n=6). ** p < 0,01; *** p < 0,001: **** p < 0,0001.

3.3.6 CX3CR1 response to LPS

It has been suggested previously that CX3CR1-CX3CL1 interaction serves as a communication tool between neurons and microglia and could play a role in the microglial activation process (A. E. Cardona et al., 2006; Hughes et al., 2002). To evaluate the LPS-induced alterations in fractalkine receptor (CX3CR1) expressional level, we quantified the MFI level of GFP on microglial cells (Figure 11A).

Comparison of control groups revealed highly significant basal differences between tissues. The highest CX3CR1 expressional level was observed in the hippocampus (178,5 ± 2,67 MFI), followed by the cortex (147,9 ± 2,8 MFI) and the lowest in the cerebellum (103 ± 1,24 MFI; p<0,0001 between all tissues; Figure 11B).

Furthermore, our data revealed significant alterations in the CX3CR1 expressional level in the hippocampus and cortex in response to LPS. The expressional levels of CX3CR1 in LPS-treated mice were significantly higher compared to their control counterparts in both the hippocampus (SAL, 178,48 ± 2,67 MFI; LPS, 189,24 ± 1,95 MFI; $p<0,05$) and the cortex (SAL, 147,89 ± 2,80 MFI; LPS, 157,42 ± 2,63 MFI; $p<0,05$). We did not observe any statistically significant expression level changes in the cerebellum (Figure 11C).

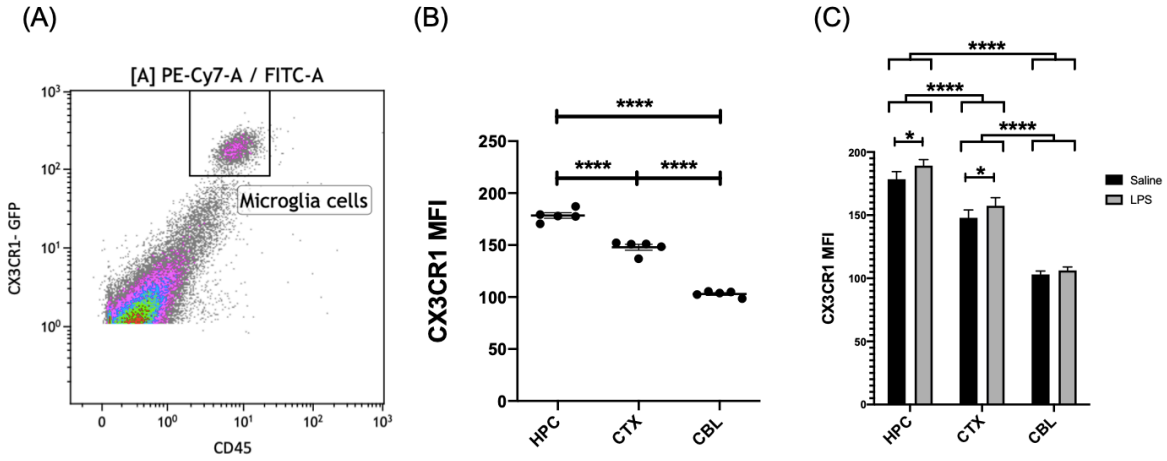

Figure 11. CX3CR1 response to LPS treatment. (A) CX3CR1-GFP expressional level (MFI) on microglia**.** (B) Tissue differences of CX3CR1-GFP basal expressional level on microglia (comparison of saline groups). (C) Alterations of CX3CR1-GFP expression in response to LPS in hippocampus (HPC), cerebral cortex (CTX) and cerebellum (CBL). SAL – saline; LPS – lipopolysaccharide. Graphed as mean ± SEM; (SAL, n=5; LPS, n=6). * $p < 0,05$; **** $p < 0,0001$.

4 DISCUSSION

In this thesis, we chose *Cx3cr1*$^{GFP/+}$ heterozygous female mice to study how the activation of innate immune responses may affect immune cells in the brain. Cx3cr1-GFP heterozygous mice are widely used as a tool for studying microglial cells. Although they are partially deficient in fractalkine signalling, nevertheless, microglial morphology, surveillance and interactions with synaptic elements have been shown to be comparable to wild-type mice (Jung et al., 2000).

Our first goal was to evaluate the body weight changes in response to LPS. We observed that LPS treatment induces significant body weight loss in mice 24 h after LPS treatment. Reductions in food intake (loss of appetite) and weight loss have been considered common hallmark physiological responses (sickness behaviours) that occur in many infectious and inflammatory diseases. Thus, the credibility of our finding is backed up by multiple other studies reporting similar results (Henry et al., 2008; Kim et al., 2013; Lawrence et al., 2012; Strassmann et al., 1993; J. Zhao et al., 2019). This is also in line with previous experiments done in our laboratory, as we have observed LPS induced body weight loss in multiple different mouse lines.

Strassmann *et al.* demonstrated that pretreatment with an anti-IL-6 antibody 20F3 reduces the LPS-induced body weight loss, exhibiting a critical role of IL-6 in the progression of inflammatory reactions that may occur both in the periphery and in the CNS (Strassmann et al., 1993). A study by Lawrence *et al.,* performed with diet-induced obese (DIO) mice, revealed that DIO mice exhibited an altered response to LPS treatment – they displayed significantly greater weight reduction than the control group (Lawrence et al., 2012).

Together with previous research, our result implicated that weight loss in inflammatory conditions may be caused by many different factors, including, but not withstanding, production of cytokines such as IL-6 as well as metabolic conditions such as obesity, which exacerbates inflammation. Persistent immune response to endotoxic microbial components such as LPS, which we recapitulated in this study, reflects chronic inflammatory status and may serve as a precipitating risk factor for chronic inflammatory and metabolic diseases such as obesity.

Our LPS treatment paradigm induced systemic inflammatory responses, including that in the CNS. Inflammatory responses in the CNS is generally termed as neuroinflammation and LPS is a potent agent to cause both acute and chronic neuroinflammations (Layé et al., 1994; Nava Catorce et al., 2016). Details on quantitative responses of different CNS cellular components

to LPS have not been abundantly studied, which is why this thesis focused to address this matter.

We did not find any significant differences in microglial abundancy between the LPS treatment group and control group. Our results seem not to match with previous observations that LPS inflammation induced rapid increase of microglial population size in the CNS (Hoogland et al., 2018; McGuiness et al., 2016; Shankaran et al., 2007). This may be mainly due to the different LPS treatment periods and dosages in different studies. Consequently, considering we only studied acute LPS effect after 24h with a relatively low dose, it is somewhat not surprising that an increase in microglial population was not observed in our study. Hoogland *et al.* noted a significant increase in microglial numbers in the cortex, hippocampus, thalamus and caudate nucleus 48h after a dose of 5 mg/kg LPS treatment. The increase may be a result of microglial proliferation in LPS-treated mice. Shankaran *et al.* reported microglial proliferation after daily i.p. treatments of increasing LPS doses over 5 days. They demonstrated that the increase in microglial abundance was LPS dose-dependent and from 0,5 mg/kg on was significantly higher compared to the control group, the highest being at a dose of 4 mg/kg (Shankaran et al., 2007). Their data imply that excessive microglial proliferation increase could be detected at higher doses of LPS, and variations at lower dosages, such as what we used, are not significantly evident.

McGuiness *et al.* described an elevated LPS-induced proliferation in non-obese diabetic (NOD) mice, where hippocampal microglial treated with BrdU showed an increased proliferation rate in both NOD mice and control group (McGuiness et al., 2016). As we did not further assess the proliferation of microglia in response to LPS in our study, our data cannot confirm whether microglia proliferated and died in response to LPS treatment and further evaluations of proliferation and apoptosis are needed.

Interestingly however, a study by Rogove *et al.* implicates that microglial proliferation is not crucial for the progression of neurotoxicity caused by microglial activation, as resident hippocampal microglia were capable of inducing inflammatory conditions in the hippocampus without the need of proliferation (Rogove et al., 2002).

The basal differences in microglial abundance between tissues noted in our experiment were similar to data collected by other studies suggesting that larger microglial densities can be found in the hippocampus and cortex, lower densities in the cerebellum (Lawson et al., 1990; Yang et al., 2013).

The evaluation of astroglia numbers between the LPS treatment and control group did not show any differences either. Multiple studies have evaluated the effect of LPS in the activation of astroglial cells as well as particular mechanisms for astrogliosis in the brain (Acaz-Fonseca et al., 2019; Levison et al., 2000; Röhl et al., 2007). Acaz-Fonseca *et al.* studied the effect of Notch activity in astrocytes in the context of neuroinflammation. Upon treatment with LPS, immunocytochemistry analysis revealed that astrocytes treated with BrdU did not show any signs of elevated astroglial proliferation (Acaz-Fonseca et al., 2019).

On the contrary, Levison *et al.* reported that IL-6, also produced by activated M1 microglia, was seen to enhance the induction of astroglial proliferation by epidermal growth factor (EGF), suggesting IL-6 and related cytokines could act as competent factors for astroglial cell proliferation. When studying the effects of microglial activation on astrogliosis, Röhl *et al.* reported that activated microglia induced both astroglial proliferation and IL-6 mRNA expression. All things considered, and similar to microglial response, the data we observed in astroglial abundance changes in response to LPS suggest astrocytic proliferation within 24h is less likely. However, an evaluation of proliferative and apoptotic rates in astrocytes may be needed to confirm this.

The comparison of different tissues revealed that astroglial abundance was significantly lower in the cerebellum compared to the cortex and the hippocampus. In general, the heterogeneity of astrocytes between different brain regions is still poorly understood. On one hand, this is due to the fact that glia:neuron ratios vary significantly among brain regions. Similar to our results, the astroglia to neuron ratio has been suggested to be lower in the cerebellum compared with the hippocampus and cortex. (Keller et al., 2018). On the other hand, different astroglial markers can show specificity depending on the tissue. For example, GFAP, a widely used marker for astrocytes, has also been identified in Schwann cells and non-neuronal cells, such as fibroblasts and myoepithelial cells (Bianchini et al., 1992; Hainfellner et al., 2001). This suggests that some astroglial markers, including the GLAST marker used in this study, could potentially show incomplete coverage of the whole astroglial population, as well as mark various cell populations, and therefore it may be difficult to firmly evaluate the whole astroglial population in the brain.

The multifariousness of astrocytes is reflected in their wide variety of different functions in health and disease and it is probable that the 2 subtype classification of fibrous and protoplasmic astrocytes needs re-evaluation (Batiuk et al., 2020). The need for further investigation of the

heterogeneity of astroglial populations is also highlighted by various studies suggesting that astrocytes may take cues from their environment, ultimately shaping their function and morphology in a brain region-specific manner (Chai et al., 2017; D'Ambrosio et al., 1998).

Taken together, our results provide additional support for data collected by previous studies. We essentially still lack conclusive information about the mechanisms under-lying differences of astrocyte subtypes from different brain regions and the heterogeneity of astroglial cells is a topic, which certainly needs further investigation, in order to better understand their function in health and disease.

We also analysed the abundance of oligodendrocyte precursor cells, however the results were inconclusive due to the rareness of O4-positive cells. The statistical analysis bar graphs are included in the supplementary material (Supplementary figure 1).

We next explored the surface expression of MHC II on microglia and observed mild reductions in the abundancy of MHC II-positive microglia in the cortex of LPS treated mice. Other regions only showed a slight tendency of lower MHC II expression in response to LPS, which did not however reach a level of significance. Based on the findings of similar studies, it is more plausible that MHC II expression should in fact increase during CNS inflammation and be a sensitive marker for microglial activation (Buttini et al., 1996; Xu et al., 1995). The discrepancy may be due to that unlike ours, many of these studies used immunohistochemistry to study MHC II expression and did not quantitatively measure the cell abundancy. For instance, immunohistochemistry analyses by Buttini *et al.* and Xu *et al.* reported an increased staining intensity of MHC II in microglia in the rat brain after LPS treatment (Buttini et al., 1996; Xu et al., 1995).

Our observations are however in line with two studies exploring the effect of ageing in the context of neuroinflammation, which likewise, reported no differences in MHC II expression between the control group and LPS group in adult mice (Henry et al., 2008; Matt et al., 2016). Interestingly, both studies found that MHC II expression was significantly higher in aged mice. This serves as an intriguing future prospect in understanding the development of neurodegenerative diseases, as they are often related to persistent and/or chronic microglial hyper-activation. Understanding the complex mechanisms of microglial activation, can make microglia a fundamental targets of therapy (Thameem Dheen et al., 2007).

Comparison further revealed that MHC II expressing hippocampal microglia were significantly less abundant compared to in other tissues. This is expected, as the hippocampus, although

among one of the most densely populated tissues by microglia in the brain (Lawson et al., 1990), has been shown to express less immune defence molecules while having more immune regulatory molecules than other regions such as the cerebellum (Grabert et al., 2016). Microglia were long considered a homologous population, but as of recently, with the emergence of single-cell transcriptomics, new studies have been tackling the unexplored subject of microglial diverseness throughout the whole brain (Tan et al., 2020). Our findings and the unravelled differences in gene expression and responsiveness to environmental cues reported by other studies, provide a new insight into the multifariousness of this cell type (De Biase et al., 2017; Sousa et al., 2018).

We also explored the MHC II expression on astrocytes. There seems to be a small tendency of MHC II up-regulation in response to LPS in the cortex, however it did not reach the level of significance. Our results build on an existing piece of evidence that the induction of MHC II expression on astrocytes after LPS treatment did not occur (Carpentier et al., 2005). This could be due to the short treatment period in our LPS regimen or the bluntness of astrocytic response to LPS compared to microglia. Moreover, several studies have found that MHC II expression in astrocytes is controlled by multiple external factors like IFN-γ and TNF-α released by T-lymphocytes (Constantinescu et al., 2005; Fierz et al., 1985; Panek et al., 1994; Pulver et al., 1987). Furthermore, it has been suggested that MHC II expression in astrocytes is tightly regulated by neurons and is induced only during extreme neuroinflammation and neuronal loss (Neumann et al., 1996; Tontsch et al., 1993).

We demonstrated minor basal differences in MHC II-expressing astrocytes in different tissues, with hippocampal astrocytes having the lowest abundance. The regional differences in MHC II surface expression on astrocytes are still understudied and our results offer considerable insight into the heterogeneity and antigen presenting role of astrocytes. Future studies should also take into account all of the above-mentioned factors influencing the MHC II expression in astrocytes.

We noted an increase in the expression level of CX3CR1 in microglia in the LPS group of the hippocampus and the cortex. In the healthy rodent brain, Hughes *et al.* found CX3CR1 to be highly expressed in microglial cells, however, they did not observe significant upregulation of CX3CR1 expression 24h after LPS treatment (Hughes et al., 2002). Cardona *et al.* demonstrated CX3CR1-CX3CL1 interaction as a neurotoxicity regulator in LPS-stimulated inflammation. *Cx3cr1*-knockout mice also exhibited less microglial migration from the injection sites (A. E. Cardona et al., 2006).

Our data also showed a significantly lower expression of CX3CR1 in the cerebellum compared to other regions, which is in line with the regional transcriptomic data that we recently reported (Tan et al., 2020). Another recent study by Cardona *et al.* found broader demyelination and neuronal loss in the cerebellum of *Cx3cr1*-knockout mice, as well as in mice carrying a CX3CR1 polymorphism I249/M280, which exhibits a weaker interaction with CX3CL1 (S. M. Cardona et al., 2018). As CX3CR1- CX3CL1 interaction is a neurotoxicity regulator, these data in combination with our findings suggest that the cerebellar region could be more vulnerable to reduced CX3CR1-CX3CL1 interaction, with compromised control of microglial activation as well as increased susceptibility to neuronal damage. However, the regional diversity of CX3CR1 expression noted in our study needs further investigation.

We further evaluated the expression of microglial specific markers, CD11b, CD172a, CD115 and CD200R. CD11b expression was significantly higher in the LPS treatment groups of the cortex and cerebellum. Roy *et al.* have demonstrated that bacterial LPS induces a heightened expression of CD11b mRNA in mice. Furthermore, inhibition of NO production prompted a decrease in LPS-mediated CD11b expression (Roy et al., 2006). Heightened expression of CD11b was also noted by Hoogland *et al.* 48h after treatment with LPS (Hoogland et al., 2018). Hart *et al.* reported an increase in CD11b expression after a dose of 5 mg/kg LPS in brain region-dependent manner, and demonstrated that CD11b expression was heightened in aged mice (Hart et al., 2012). Thus, our findings corroborate previous evidence that expression of CD11b reflects a stable and fundamental activation status of microglia.

We also noted a significantly lower basal expression of CD11b in the hippocampus. The heterogeneity of microglial marker expression between different brain regions has been demonstrated before by several studies (de Haas et al., 2008; Grabert et al., 2016; Hart et al., 2012; Tan et al., 2020). De Haas *et al.* studied the expression of 11 markers present on *in vivo* microglia both in health and disease. Similar to our results, their flow cytometry analysis of microglia in healthy young adult mice exhibited analogous differences in the expression patterns of CD11b throughout the hippocampus, cortex and cerebellum (de Haas et al., 2008). Our findings therefore coincide with existing evidence that CD11b expression is heightened by LPS treatment and that the expression levels of CD11b vary between different brain tissues.

We further demonstrated that although CD172a expression was not changed by LPS, it was significantly lower in the hippocampus compared to the cortex and cerebellum as well. Kong *et al* showed a reduction of CD172a expression in macrophages after LPS treatment (Kong et al., 2007). Information about the expression levels of CD172a in microglia and its expressional

heterogeneity in different brain regions is scarce, therefore our findings provide novel knowledge in this regard. It has been suggested that the CD172a-CD47 interaction down-regulates inflammatory mechanisms like phagocytosis, complement activation and cytokine production. However, it is still less known how this specific pathway controls microglial activation in neuroinflammatory diseases, as previous research has been done mainly on CD172a-CD47 interactions in the peripheral immune system (Brown et al., 2001; Oldenborg et al., 2001). It was once shown that CD172a plays an important role in the control of spinal microglial activation in neuropathic pain (Z. Li et al., 2016). Our results demonstrate evident diversity of CD172a expression levels between tissues and warrant further research to better understand this receptor. Investigating the CD172a-CD47 pathway in the brain could give us a better understanding about the regulation of microglial activation, as well as unravel new possibilities in the therapy of neuroinflammatory diseases.

We did not observe any LPS-induced alterations in the surface expression of CD115 on microglia. Previously, CD115 signalling has only been shown to play a role in the survival of microglia and that CD115 knockout mice are entirely depleted of microglial cells (Erblich et al., 2011). Our findings could indicate that CD115 does not play a primary role in LPS-induced activation of microglia. However, we observed a lower expression of CD115 on cerebellar microglia compared to other tissues.

Evaluation of CD200R revealed substantial results – unlike CD11b, LPS induced significant CD200R down-regulation in all of the regions. Furthermore, basal surface expression of CD200R was the lowest in the cerebellum. These data correlate favourably with previous studies demonstrating that CD200R-CD200 interaction serves as an inhibitory mechanism for microglial activation (Cox et al., 2012; Meuth et al., 2008). Interestingly, Cox et al. demonstrated in their study that CD200 fusion protein could act as an immunomodulator by activating CD200R and decreasing microglial activation in the hippocampus of aged rats (Cox et al., 2012). Significantly lower basal CD200R expression along with CX3CR1 and CD115 in the cerebellum, as observed in our study, could indicate that this region is more vulnerable to the damaging effects of microglial activation and the heterogeneity of CD200R receptor expression should be taken into account in future studies. Our results combined with previous studies suggest that CD200R surface expression can be down-regulated in both acute and chronic inflammation and stimulation of CD200R function could act as a tool for calming down microglial overactivity in different brain pathologies.

Further experimental investigations are needed to evaluate the sex-dependent differences in the molecular changes induced by LPS in microglia and astroglia, as it has been established that the brain is sexually differentiated, however most of the studies tackling sex differences in microglia have thus far been done in the context of CNS development (Schwarz et al., 2012; Thion et al., 2018; Weinhard et al., 2018). For this reason, we intend to carry out these experiments also on male mice to further our research.

Conclusions

Considering the findings and data demonstrated in this study the following conclusions can be drawn:

- We confirmed that lipopolysaccharide induces significant body weight loss in mice and our findings are supported by previous studies reporting similar results.
- Our data did not exhibit changes in microglial and astroglial cell abundancy in response to lipopolysaccharide, indicating no proliferation of these cell types. However, our finding of the regional differences in both cell populations suggest that additional research is necessary to unravel mechanism underlying microglial and astroglial heterogeneity in the CNS.
- Our data exhibited various effects of LPS on microglial marker molecules, such as increases of CX3CR1 and CD11b whilst a reduction of CD200R on activated microglia in different brain regions, suggesting they play different roles in microglial activation and progression of neuroinflammation.
- We demonstrated that basal expressions of CX3CR1, CD115, and CD200R are the lowest in the cerebellum, whereas MHCII, CD11b, CD172a are the least expressed in the hippocampus. Our findings suggest region-specific vulnerability to neuroinflammation, the biological significance of which needs further interrogation.

SUMMARY

Acute neuroinflammation is characterized by the rapid response of microglia, the resident immune cells of CNS. Microglia are activated through the interactions of several surface receptors and are capable of modifying their morphology and molecular profile accordingly. Signals released by activated microglia can further activate other glial cells, including astroglia, which in turn contribute to the progression of inflammation and prevent excessive neuronal damage.

In this thesis, we characterized the molecular changes of glial cells in response to LPS treatment in heterozygous Cx3cr1$^{GFP/+}$ female mice. Our aim was to describe the changes in the cell abundancy as well as glial receptors' surface expression induced by acute inflammation in three different brain regions – hippocampus, cerebral cortex and cerebellum. We analysed the surface expression of MHC II, CX3CR1, CD11b, CD172a, CD115, CD200R on microglia and MHC II expression on astrocytes, along with cell abundancy and body weight changes.

Our data suggest that LPS induces significant body weight loss as well as alterations in the molecular characteristics of glial cells. We found that the specific marker for activated microglia CD11b was up-regulated in LPS-treated mice accompanied by the down-regulation of the inhibitory receptor CD200R of microglial activation. We also noted substantial dissimilarities in the basal surface expression of different astroglial and microglial receptors between different brain regions. These observations may be useful for further research on the heterogeneousness of glial cell populations.

This study highlighted the diversification of glial subpopulations in the healthy brain and in response to acute inflammation and therefore has raised many questions in need of further investigation. Additional experiments on other microglia specific markers would help to better characterize the basal heterogeneity and inflammatory response of this cell type. Future studies should certainly examine a bigger cohort and further address sex-dependent differences in glial receptor responses.

Gliia rakkude molekulaarne iseloomustamine neuroinflammatoorses mudelis *Cx3cr1*$^{GFP/+}$ mutantses hiireliinis

Helen Paapstel

RESÜMEE

Aju põletikulisele seisundile reageerivad esmalt kesknärvisüsteemi makrofaagide rolli täitvad mikrogliia rakud. Lisaks mikrogliiale on mitmeid toetavaid immunoloogilisi rolle täheldatud ka makrogliias. Gliia rakke iseloomustab morfoloogiline ja molekulaarne plastilisus, mis võimaldab neil vastavalt keskkonnast saadud signaalidele muuta nii oma kuju, molekulaarset profiili kui ka funktsiooni. Gliia rakkude immuunvastust on varem peetud ühetaoliseks kogu kesknärvisüsteemi ulatuses, kuid üha enam pälvib tähelepanu selle rakupopulatsiooni arvukuse, funktsioonide ja molekulaarse profiili lai varieeruvus aju eri piirkondades.

Käesoleva töö eesmärk oli kirjeldada bakteriaalse endotoksiini, lipopolüsahhariidi (LPS), põhjustatud akuutse neuroinflammatsiooni poolt esile kutsutud muutusi gliia rakkude molekulaarses profiilis heterosügootses Cx3Cr1$^{GFP/+}$ hiireliinis. Eesmärgist lähtuvalt kirjeldasime emaste hiirte kehakaalu muutusi ning viisime läbi voolutsütomeetria analüüsi, hindamaks muutusi gliiarakkude arvukuses, MHC II kompleksi ja erinevate mikrogliia spetsiifiliste retseptorite (CX3CR1, CD11b, CD172a, CD115 ja CD200R) pinnaekspressioonis. Kõiki parameetreid hindasime kolmes erinevas ajuregioonis – hipokampuses, suurajukoores ja väikeajus.

Analüüsi tulemused näitasid, et LPS poolt indutseeritud põletikureaktsioon kutsub esile olulise kehakaalu languse ning mitmete retseptorite pinnaekspressiooni tõusu (CD11b, CX3CR1) või languse (CD200R). Nende retseptorite pinnaekspressiooni muutuste amplituud erines ajupiirkonniti. Saadud tulemused ühtivad ka varem läbi viidud uuringutega, kus gliia rakkudes on täheldatud sarnaseid immuunvastuseid.

Selleks, et hinnata retseptorite baasekspressiooni erinevusi ajupiirkondade vahel, võrdlesime kontrollgruppe. Kontrollgruppide vahel ilmnesid retseptorite pinnaekspressioonis mitmed statistiliselt olulised varieeruvused aju eri piirkondade vahel ning saadud tulemused viitavad gliia rakkude populatsiooni kõrgele heterogeensusele.

Edaspidiste uuringute raames tuleks kindlasti analüüsida veel teiste gliia-spetsiifiliste retseptorite ekspressiooni piirkondlikke erinevusi ning lisaks hinnata ka gliia rakkude immuunvastuse soost sõltuvat varieeruvust. Gliia rakkude molekulaarset varieeruvust

ajuregioonide vahel, nii homöostaasi kui infektsiooni kontekstis, on endiselt vähe uuritud, kuid nende rakkude mitmekesisuse täpsem mõistmine võib luua uuenduslikke meetmeid mitmete neuropsühhiaatriliste ja -degeneratiivsete haiguste ennetuses ja ravis.

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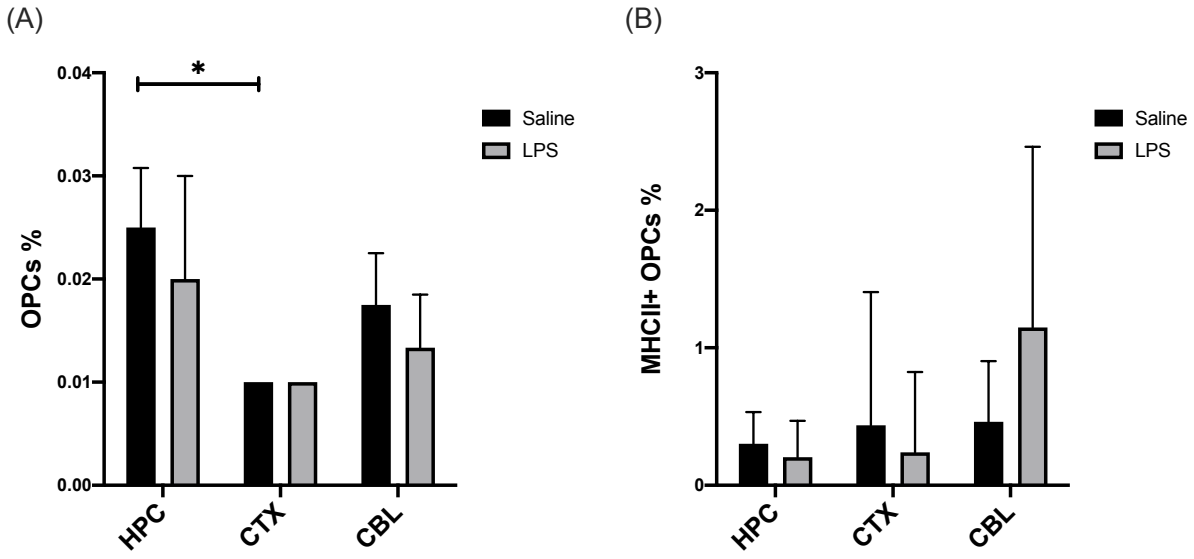
Supplementary material

Supplementary material 1. Oligodendrocyte precursor cells



Supplementary figure 1. Abundance and MHC II expressional level of oligodendrocyte precursor cells (OPCs; defined as O4 positive cells). (A) Abundance changes in OPCs after 24h LPS treatment (B) LPS induced changes in MHC II positive OPCs. Data represented as mean ± SEM (SAL, n=5; LPS, n=6). SAL – saline; LPS – lipopolysaccharide. * $p < 0,05$.

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