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Molecular characterization of microglial populations in CX3CR1-GFP mutant mouse line in an LPS induced inflammatory model

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Tartu 2019

Molecular characterization of microglial populations in CX3CR1-GFP mutant mouse line in an LPS induced inflammatory model

Abstract: Microglia are the resident macrophages of the central nervous system (CNS). They constitute the first line of defence within the CNS. Microglia have a crucial role in the development of the central nervous system and also in the mature CNS. Microglia are often attributed a 'resting' state under homeostatic conditions. However, studies have shown that resting microglial cells are actively scanning their environment for detection of pathogens. Microglia have a typical ramified shape under homeostatic conditions. Upon detection of external stimuli, they become activated and undergo morphological changes. Activated microglia can produce trophic and anti-inflammatory factors, which serve the purpose of protecting the CNS. However, when microglia are over activated, they release pro inflammatory neurotoxic factors such as superoxide, nitric oxide (NO) and cytokines that contribute to the pathology of neurodegenerative conditions such as Parkinson's disease (PD). Fractalkine (CX3CL1), a unique chemokine which is the only ligand for its receptor CX3CR1, is expressed by microglia. Several studies have shown that fractalkine acts to protect neurons in vitro in lipopolysaccharide (LPS)-activated microglia by limiting the release of inflammatory factors. The purpose of the thesis was to study the molecular signature of microglial cells of $CX3CR1^{GFP/+}$ mice and how it is altered by lipopolysaccharide (LPS) administration in an LPS induced inflammatory model.

Keywords:

Microglia, inflammation, LPS, activation, CX3CR1

CERCS:

B640, B470

LPS-i poolt indutseeritud põletikureaktsiooni mõju CX3CR1-GFP mutantse hiireliini mikrogliia populatsioonidele.

Lühikokkuvõte:

Mikrogliia rakud on kesknärvisüsteemis (KNS) elavad makrofaagid ja on KNS-i esimene kaitseliin. Mikrogliial on oluline roll nii kesknärvisüsteemi arengus kui ka täiskasvanud KNS-is. Mikrogliiast rakkudest räägitakse sageli kui homeostaatilistes tingimustes "puhkeolekus" olevatest rakkudest. Tegelikult, uuringud on näidanud, et puhkavad mikrogliia rakud skaneerivad pidevalt KNS-i otsides patogeene. Mikrogliia on homeostaatilistes tingimustes tüüpilise harunenud kujuga, kuid väliste stiimulite avastamisel muutuvad rakud aktiveerituks ja läbivad morfoloogilisi muutusi. Aktiveeritud mikrogliia võib toota troofilisi ja palavikuvastaseid faktoreid, mis töötavad KNS-i kaitsmiseks. Seevastu , kui mikrogliia on üleaktiveeritud, eritavad need rakud neurotoksilisi faktoreid nagu superoksiid, lämmastikoksiid (NO) ja tsütokiinid, mis aitavad kaasa neurodegeneratiivsete seisundite patoloogiale, näiteks Prakinson'i tõbi (PD). Mitmed uuringud on näidanud, et neuronite poolt eskpresseritud fraktalkiin (CX3CL1) - kemokiin, mis on ligand mikrogliia poolt ekspresseeritud retseptorile CX3CR1, piirab põletikuvastaste faktorite vabastamist in vitro lipopolüsahhariid (LPS)-aktiveeritud mikrogliia rakkudes. Antud töö eesmärgiks oli uurida kuidas CX3CR1(GFP/+) hiirte mikrogliiarakkude molekulaarne muster on mõjutatud LPS manustamise poolt.

Võtmesõnad:

mikrogliia, põletik, LPS, aktivatsioon, CX3CR1

CERCS:

B470, B640

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LIST OF ABBREVIATIONS

ATP: adenosine triphosphate

BDNF: brain derived neurotrophic factor

CD: cluster of differentiation

c-Kit: tyrosine protein kinase

c-Kit: tyrosine-protein kinase Kit

CNS: central nervous system

CX3CL1: C-X3-C motif ligand 1

CX3CR1: C-X3-C chemokine receptor 1

DAPI: 4', 6-diamino-2 phenylindole

E: embryonic day

GD: gestational day

GFP: green fluorescent protein

gw: gestational week

IGF: insulin-like growth factor

IL: interleukin

Inos: inducible nitric oxide synthase

LPS: lipopolysaccharide

NK: natural killer

NPC: neural precursor cell

PBS: phosphate buffered saline

PCD: programmed cell death

PND: post-natal day

ROS: reactive oxygen species

Scl/Tal1: stem cell leukemia/ T cell acute lymphoblastic leukemia 1

SGZ: subgranular zone

TGF-β: transforming growth factor beta

TNF: tumor necrosis factor

INTRODUCTION

Microglia constitute 5-20% of the total glial cell population and are a major part of the central nervous system (CNS) (Benveniste, 1997; Lawson *et al*., 1990; Lawson *et al*., 1992). In the healthy adult CNS, microglia can be distinguished by a ramified morphology, with a small cellular body and several long thin branched processes that are capable of extending up to 50 µm from the soma (Arnoux *et al*., 2013; Kozlowski and Weimer, 2012; Nimmerjahn *et al*, 2005) and they show lower expression of myeloid monocytic markers such as CD11b, CD45, and MHCII (Guillemin and Brew, 2004). During embryonic development and upon detection of a stimulus, microglia can adopt an amoeboid morphology which can be distinguished by a mostly rounded soma with fewer and shorter processes (Kozlowski and Weimer, 2012; Mosser *et al*., 2017; Rigato *et al*., 2011) (Figure 1).

Knowledge of the pathogenic functions of microglia can be helpful in the development of inhibition and therapeutic targets for disease modulation (Prinz *et al.*, 2011). In the CNS, CX3CR1, also known as fractalkine receptor, is expressed by microglia (Cardona *et al.*, 2006). In vivo models of autoimmune diseases have shown that dysregulation of microglial responses occurs in the absence of CX3CR1 (Garcia *et al*., 2013). CX3CR1 deficiency has been related to neuronal death following lipopolysaccharide (LPS) challenge (Cardona *et al*., 2006).

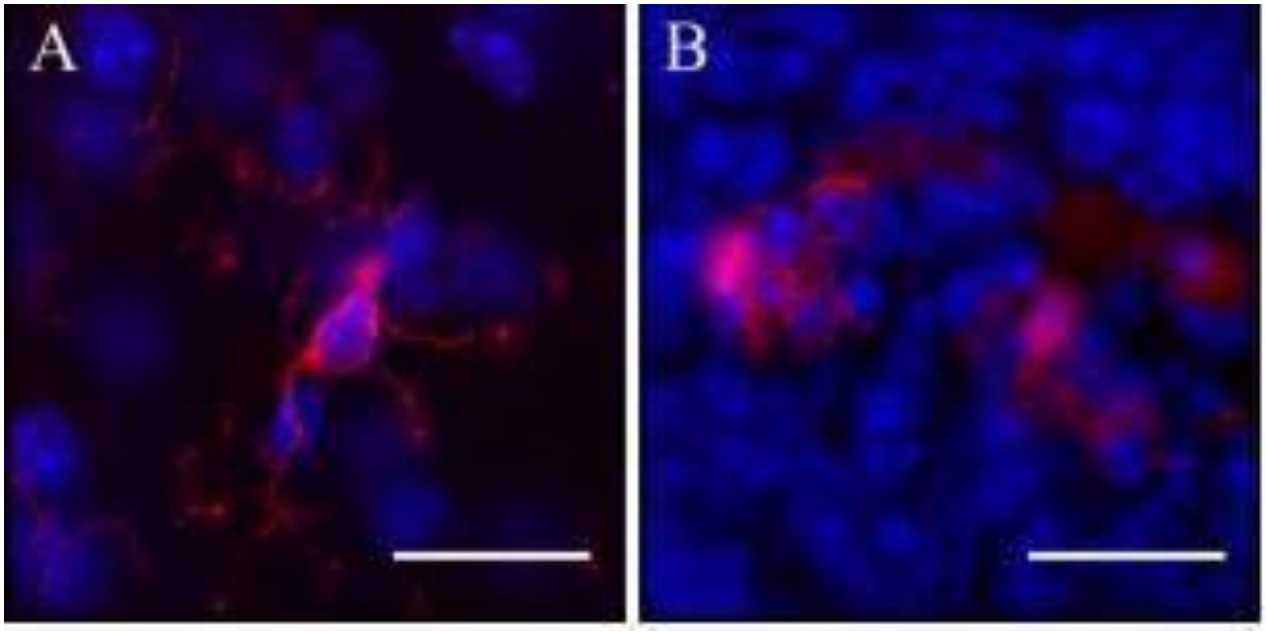

Figure 1: Examples of a ramified and an amoeboid microglial cell morphology. A) A ramified microglial cell with a small cell soma and long thin processes. B) An amoeboid microglial cell, the cell volume is increased, and processes are retracted. Microglial cells ware stained for Iba-1 (red), nuclei were visualized with DAPI (blue). Scale bars = 20 µm. (Adapted from Nina Swinnen, 2013)

1 LITERATURE REVIEW

1.1 Origin of microglia

The mesodermal origin of microglia has been recently confirmed: microglia was absent in mice that lacked the transcription factor Pu.1 (Beers *et al* 2006; Mckercher *et al*., 1996), a crucial regulator of hematopoietic development (Iwasaki *et al*., 2005).

In the mouse embryo, development of erythromyeloid progenitors in the yolk sac starts as from embryonic day 8.5 (E8.5) (Mcgrath *et al*., 2015; Perdiguero and Geissman 2016). This process is dependent on the transcription factors Pu.1 and stem cell leukemia/ T cell acute lymphoblastic leukemia 1 (Scl/Tal-1) (Mcgrath *et al*., 2015; Perdiguero and Geissman 2016).These cells express tyrosine -protein phosphatase cluster of differentiation (CD45) and tyrosine protein kinase c-Kit, and are capable of colonizing the fetal liver and differentiation into erythrocytes and several myeloid cells including tissue resident macrophages (Smolders *et al*., 2019). Maturation of a portion of the erythromyeloid progenitors into CX3CR1+ cells occurs in the yolk sac (Smolders *et al*., 2019). In the mouse embryo, migration of these progenitors to the brain occurs between E9.5 and E14.5, around the time the blood brain barrier is formed (Smolders *et al*., 2019). Invasion of these progenitors in the spinal cord parenchyma occurs by E11.5 (Rigato *et al*., 2011). In the embryonic CNS, CX3CR1, CD45 and adhesion G-protein coupled receptor F4/80 are expressed by microglia (Ginhoux *et al*., 2010; Kierdorf *et al*., 2013). In humans, invasion of microglia into the forebrain begins around 4.5-5.5 gestational weeks (gw) (Menassa and Gomez-nicola, 2018; Rezaie *et al*., 2005; Rezaie and Male, 1999, Verney *et al*., 2010).

After microglial precursors are born in the yolk sac, they travel to the CNS via the developing blood vessel network (Ginhoux *et al*., 2010).

1.2 Role of microglia

1.2.1 Role of microglia in CNS development

Patterning the developing CNS

Programmed cell death (PCD), a process involving apoptosis of cells to achieve the cellular architecture unique to the mature system, is crucial to the development and spatial patterning of all organ systems (Vaux and Kosmeyer, 1999). The absence or malfunctioning of this process gives rise to various developmental abnormalities and diseases (Meier *et al*., 2000).

The nervous system, having diverse and complex cell populations, is dependant on PCD and is therefore a model system for studying this process (Oppenheim 1991; Yeo and Gautier 2004; Rogulja-Ortmann *et al*., 2007). Approximately 50% of neurons born during development must undergo PCD and the corpses must be cleared (Yeo and Gautier, 2004). Studies done in primates and rats have shown that microglia contact, engulf and phagocytose neural precursor cells (NPCs), including live and proliferating progenitors from gestational day 17.0 (GD17.0) to post-natal day 6 (PND6) (Cunningham *et al*., 2013). Another study revealed that microglia having phagocytic features were associated with a decrease in the number of NPCs at GD17.5 (Tronnes *et al*., 2015). It was shown in sections of cerebellum from PND3 mice that during cerebellar development, reactive oxygen species (ROS) are produced by microglia, thus promoting engulfment-mediated Purkinje neuron death (Marin Teva *et al*., 2004).

Furthermore, microglia are also involved in events associated with neuronal differentiation during development: studies have shown that neurogenesis of embryonic cortical cells is improved by microglia (Aarum *et al*., 2003). Differentiation of nasal forebrain progenitors into cholinergic neurons is promoted by exogenous addition of microglia or conditioned medium from microglial cell culture (Jonakait *et al*., 1996, 2000).

Microglia also play an active role in promoting neuronal death by the secretion of soluble factors or contact mediated signals (Marin Teva *et al*., 2004).

During early development, neurons and synaptic connections are formed in excess (Purves and Lichtman 1980., Lichtman and Colman,2000; Kano and Hashimoto 2009). These are later removed during synapse elimination which is important for synaptic connectivity (Purves and Lichtman 1980., Lichtman and Colman, 2000; Kano and Hashimoto 2009).

An impairment in synapse development was observed in mice deficient in CX3CR1, a chemokine receptor specific to microglia and which binds to the chemokine fractalkine (CX3CL1) expressed by neurons (Harrison *et al*., 1991; Jung *et al*., 2000; Ransohoff *et al*., 2009).

1.2.2 Role of microglia in mature CNS

Microglial phagocytosis of apoptotic newborn neurons

Microglia phagocytose apoptotic debris in the adult brain (Neumann *et al*., 2009).

Newborn granule cells, arising from stem and progenitor cells that persist in the subgranular zone (SGZ) in the adult hippocampus, maturate and incorporate in the hippocampal circuitry over a period of a month (Kempermann *et al*., 2004).

These newborn cells are involved in some forms of learning and memory, mood regulation, and fear conditioning (Kempermann 2008; Ming and Song, 2011) but most of them are pruned during early development and are subject to apoptosis in the first few days of cell life (Marie-Eve Tremblay *et al*., 2011). These are phagocytosed by ramified, unchallenged microglia via terminal or en passant branches forming 'ball-and-chain' structures (Sierra *et al*., 2010), contrary to the phagocytosis by amoeboid microglia observed during neurodegeneration (Kettenmann, 2007).

Microglial reorganization of neuronal circuits

In the in vivo adult somatosensory and visual cortex, there is direct contact between microglial processes and synaptic terminals during four to five minutes at a frequency of once per hour (Wake *et al*., 2009). There is a decrease in the frequency of these contacts with decreasing neuronal activity and there is an increase in the duration of contact in pathological circumstances, thus the contact between microglial processes and synaptic terminals depends on neuronal activity (Smolders, 2017). It has been revealed through research in zebrafish larvae that regulation of neuronal activity is done by microglial contact (Li *et al*., 2012). It was found that the mechanism behind microglial scanning activity and contact formation is dependent on extracellular ATP, which is released through neuronal and astrocytic Pannexin-1-hemichannels, that binds to purinergic P2 receptors on microglia (Davalos *et al*., 2005; Fontainhas *et al*., 2011).

It can be inferred from the dynamic contact between microglia and synapses that they perform local tasks (Smolders, 2017). Indeed, there is remodelling of these structures, depending on activity and age, by inducing the formation of dendritic spines and by elimination of synaptic elements, a process called synaptic pruning (Wake *et al*., 2009; Tremblay *et al*., 2012). Research between P8 and P11 have revealed that there is involvement of microglial brain derived neurotrophic factor (BDNF) in the formation of new spines (Parkhurst *et al*., 2013; Bessis *et al*., 2007). Synapse elimination is dependent on transforming growth factor β (TGF-β) induced expression of complement (C)1q followed by C3 tagging of the synapses to be pruned (Stevens *et al*., 2007; Schafer *et al*., 2012; Bialas and Stevens., 2013).

In vitro research has revealed that there is secretion of exosomes by neurons, based on their activity and, synaptic pruning by microglia is stimulated by these exosomes (Bahrini *et al*., 2015).

Functional synaptic plasticity, which comprises strengthening or weakening of the synapse based on its activity is also mediated by microglia (Smolders, 2017). Regulation of long term potentiation (which is an increase in synaptic strength involved in learning and memory) is done by microglia, through CX3CL1/CX3CR1 signaling (Rogers *et al*., 2011).

In the adult neurogenic zones, namely the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of lateral ventricle (LV), proliferation and differentiation of neuronal precursors is regulated by microglia (Tay *et al*., 2017; Buttgereit *et al*., 2016; Sato, 2015; Xavier *et al*., 2015; Sierra *et al*., 2010). There is an involvement of tumour necrosis factor alpha (TNF-α) signaling via TNF receptors 1 and, IGF-1, IL-1β and CX3CL1 signaling in the regulation of neurogenesis (Sato, 2015). The number of neurons born in the hippocampus is also controlled by microglia (Smolders, 2017). Phosphatidylserine, expressed by apoptotic cells, may be recognized by microglia through their phosphatidylserine receptors (Tay *et al*., 2017; Buttgereit *et al*., 2016; Sato, 2015; Ribeiro Xavier *et al*., 2015; Sierra *et al*., 2010).

1.3 Major histocompatibility class II (MHCII)

Microglial activation can be distinguished by their morphology and cell surface markers using immunohistochemistry, and indirectly through assessment of cytokine expression (even if multiple cell types could be the source, such as astroglia; Bedi *et al*., 2013; Beynon & Walker, 2012; Colton & Wilcock, 2010). MHCII is one of those cell surface markers. The primary role of major histocompatibility complex (MHC) class II molecules is to present processed antigens, which are derived primarily from exogenous sources, to CD4T-lymphocytes (Tjadine *et al*., 2004). Major histocompatibility class II (MHCII) is expressed on the surface of antigen presenting cells and plays a role in antigen recognition and the activation of the adaptive immune system (Hopperton *et al*., 2018). Within the brain, MHCII is primarily expressed on microglia, where it is generally considered a marker of activated cells, though it may have weaker expression in resting cells (Lee *et al*., 2002).

1.4 M1/M2 polarization

It has been shown that microglia in the brain are very plastic and they can adopt distinct phenotypes including the classically activated (M1) state and the alternatively activated (M2) state as a response to several stimulations (Ma *et al*., 2016).

The M1-like phenotype is marked by the production of pro-inflammatory mediators including IL-1β, TNF-α, and IL-6 and also, an increased expression of surface markers such as CD16/32, CD86, CD40 and inducible nitric oxide synthase (iNOS), which power the inflammatory process (Kalkman and Feuerbach, 2016).

On the other hand, microglia could adopt the M2 phenotype, which could enhance the phagocytosis function and release several protective and trophic factors stimulating anti-inflammatory and immunosuppressive responses (Park *et al*., 2016).

1.5 Fractalkine (CX3CL1)/CX3CR1 signaling

In the adult brain, interactions of microglial cells with neurons and synapses occurs not only in pathological conditions but also in physiological conditions (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009; Morris *et al*., 2013; Eyo and Dailey, 2013). Several chemokine signaling pathways, including the fractalkine (CX3CL1) pathway (Ransohoff and Perry, 2009). In the CNS, fractalkine is mostly expressed on neurons and its unique receptor, CX3CR1, is expressed by microglia (Wolf *et al*., 2013). Fractalkine is synthesized as a transmembrane protein having 371 amino acid residues, comprising a 76-amino acid glycosylated mucin-like stalk, and a 37 -amino acid intracellular C terminal domain (Wolf *et al*., 2013). Cleavage of this protein can be done by the lysosomal cysteine protease, cathepsin S, and members of the disintegrin and metalloproteinase (ADAM) family, releasing a soluble form of fractalkine that contains the chemokine domain (Sheridan and Murphy, 2013). These two isoforms of fractalkine have the capability of interacting with the microglial receptor CX3CR1, a Gαi-coupled seven transmembrane receptor which modulates several intracellular signaling pathways upon activation (Sheridan and Murphy, 2013).

Microglial activation is modulated by the fractalkine/CX3CR1 pathway (Arnoux and Audinat, 2015). In pathological conditions, several phenotypic changes occur in microglia (Hanisch and Kettenmann, 2007). These include morphological modifications, proliferation, release of mediators, migration to the site of injury, and engulfment of cellular debris and dead cells (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009).

There is a lot of evidence indicating that constitutive expression of membrane-tethered fractalkine has a tendency to inhibit microglial activation (Biber *et al*., 2007). In several animal models of neurological disorders, including Parkinson's disease, amyotrophic lateral sclerosis, stroke and Alzheimer's disease, deficiency of fractalkine or of CX3CR1 causes an increase in the production of proinflammatory molecules (Arnoux and Audinat, 2015).

However, fractalkine/CX3CR1 can also produce neurotrophic effects and inactivating this signaling prevents progression of the disease (Arnoux and Audinat, 2015). Thus, neuroprotective and neurotoxic roles of the fractalkine/CX3CR1 pathway depend on the stimuli activating microglia and also on pathological contexts (Arnoux and Audinat, 2015).

Importance of CX3CL1/CX3CR1 pathway

Fractalkine/CX3CR1 pathway regulates dynamics of basal motility and thus, interactions between microglia and synapses (Arnoux and Audinat, 2015). It has been revealed through confocal imaging of retinal explants that microglial processes move slower in CX3CR1 deficient mice (Eyo *et al*., 2014).

During adult neurogenesis in the subgranular zone, majority of cells are subject to apoptosis and very few of the newborn neurons survive and are integrated in the pre-existing neuronal circuits (Arnoux and Audinat, 2015). Genetic disruption of CX3CR1 causes a decrease in cellular proliferation in the subgranular zone of the dentate gyrus, implying that fractalkine/CX3CR1 aids adult neurogenesis (Bachstetter *et al*., 2011; Rogers *et al*., 2011).

Fractalkine/CX3CR1 pathway also has a crucial role in synaptic pruning by microglia (Arnoux and Audinat, 2015). Comparative analyses, using STED and electron microscope to show the presence of synaptic material engulfed by microglial processes in the hippocampus during the first postnatal weeks, between wild type and CX3CR1 deficient mice showed that CX3CR1 deficiency is related to a greater number of dendritic spines (Arnoux and Audinat, 2015). There were also impairments in the functions of the hippocampal excitory synaptic network in CX3CR1 deficient mice during postnatal development (Aroux and Audinat, 2015).

2 AIMS OF THE THESIS

The main objective of the thesis is to characterize how inflammation caused by systemic administration of LPS alters microglial phenotype in the mouse brain. For this purpose we used CX3CR1-GFP mouse line, in which green fluorescent protein GFP is under the promoter of *CX3CR1* gene (active in microglia).

Detailed aims:

1. Effect of immersion fixation time on endogenous fluorescence of GFP. Since the morphology of fluorescent microglia is possible to analyse in histological sections, this first goal was to optimize the tissue fixation time.
2. Flow cytometric (FC) MHCII staining of CX3CR1-GFP mouse glial cells from LPS treated and untreated mouse brains (cortex).
3. Analyse the ratio of relatively bright and dim GFP microglial populations in LPS treated and untreated mouse brains (cortex) as well as the MHCII existence in these populations.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

Mice

2-5 months old male CX3CR1$^{GFP/+}$ transgenic mice on a B6JRcc/B6N background were used for the thesis. CX3CR1-GFP mice have chemokine (C-X3 motif) receptor 1 second exon replaced by GFP and thus, they express green fluorescent protein (GFP) in monocytes, dendritic cells, natural killer (NK) cells and microglia, under control of the endogenous Cx3cr1 locus. In this thesis heterozygous mice were used, because they have functional fractalkine receptor, and cells that express this receptor appear green in both heterozygous and knockout mice. Animals were bred and housed in the Laboratory Animal Centre at University of Tartu. Mice were kept under standard conditions with unlimited access to food and water on a 12/12-hour light/dark cycle (lights on from 07:00 to 19:00 hours).

LPS treatment

Lipopolysaccharide (LPS; derived from *E.coli* serotype 01111:B4; Sigma-Aldrich, St.Louis, MO, USA) was dissolved in 0.9% NaCl solution (saline solution). Injections were administered intraperitoneally at a dose of 500 μg/kg. The vehicle consisted of 0.9% saline in an equivalent volume.

There were 13 male mice used for the experiment. They were divided randomly into two groups.

One group, consisting of 7 mice, were injected intraperitoneally (i.p) with 500 μg/kg body weight of LPS.

The second group, consisting of 6 mice, received injection with an equal volume of 0.9% NaCl solution as control.

All animal experiments were done by a certified specialist.

Body weights of the mice were recorded prior to LPS and saline injection and 24 hours after injection.

Transcardial perfusion

After 24 hours LPS challenge, mice were deeply anesthesized with dexmedetomidine (1 mg/kg) and ketamine (100 mg/kg) solution intraperitoneally and transcardially perfused with Phosphate Buffered Saline (1x PBS).

Before perfusion, responses were assessed by tail/toe pinches and proceeded with perfusion only after the mouse was unresponsive to noxious stimuli and the reflexes were absent. Mice were secured onto Styrofoam board lying on the back face upward.

Next, an incision was made through the skin with surgical scissors along the thoracic midline and ribcage was opened. Needle was inserted into the left ventricle and a cut was made into the right atrium. All mice were perfused with about 50 ml PBS or until fluid exiting from right atrium was clear. After perfusion, mice were decapitated and somatosensory cortex (SSC) was dissected.

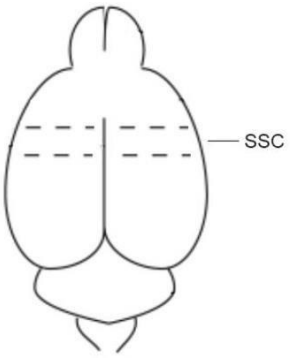

Figure 2: Illustration of defining somatosensory cortex in mice

Immersion fixation of brain tissue

After transcardial perfusion, brains were dissected, and immersion fixed for 2 hours or 24 hours in 4 % PFA/PBS. After immersion fixation, tissues were cryoprotected in 30% sucrose/PBS solution, frozen and kept in -80 ºC until further processing. 40 µm thick sections were cut with cryomicrotome (Leica CM1850-Cryostat) and collected to PBS. Afterwards, sections were mounted on microscope slides with 0.5% gelatin/PBS solution, air dried and mounted in Fluoromount mounting media. Images were acquired using DP71 CCD camera (Olympus, Japan), mounted on a BX51 microscope (Olympus,Japan).

Flow cytometric staining

Tissue preparation

The dissected SSC was gently chopped with a scalpel and minced with a 2-ml syringe plunge in 1 ml ice-cold FC buffer (PBS+ 1% fetal bovine serum) through a 70 µm filter (BD Biosciences) in a small dish on ice. The filtered homogenates were transferred into 1.5 ml microcentrifuge tubes. The tissue homogenates were blocked with 10% rat serum for 30 minutes with gentle rotation at 4 ºC.

Flow marker staining and acquisition

Brain cells were stained with anti-mouse Glast-PE (cat no. 130-118-344, Miltenyi), MHCII-PERCP/Cy5.5 (cat no. 107626, Biolegend) and incubated for 1 hour at 4 ºC under gentle rotation with light protection. After staining, samples were fixed with 4% PFA for 30 minutes. Afterwards cells were washed with 1 ml FC buffer and centrifuged at 2000 rpm for 6 minutes. The supernatant was discarded, and the cell pellets were resuspended in 0.5 ml ice-cold FC buffer and filtered through 35 μm strainer caps into flow cytometry tubes.

The tubes were stored on ice with light protection until acquisition. Samples were acquired with BD LSRFortessa cell analyser under CD45-gating. Astrocytes were defined as Glast positive cells, microglia dim cells as GFP+/CD45(dim) positive cells and bright microglial population as GFP++/CD45+.

The acquisition time and flow rate for each sample was recorded.

Statistical analysis

Flow cytometry data was analysed by Kaluza software (Beckman Coulter). All graphs were made using Graphpad Prism 6. Body weight data was analysed with student's T-test and flow cytomety data was analysed by two-way ANOVA (microglia population x treatment).

3.2. RESULTS

3.2.1 Effect of immersion fixation time on endogenous fluorescence of GFP

Sections from cerebellum, cortex and hippocampus were viewed under the microscope after 2 or 24 hours of immersion fixation (Fig.3). It could be seen that the expression was homogenous in all three sections since the intensity of GFP is fairly similar throughout the sections. A sharper image of microglial cells was observed after 24 hours of fixation.

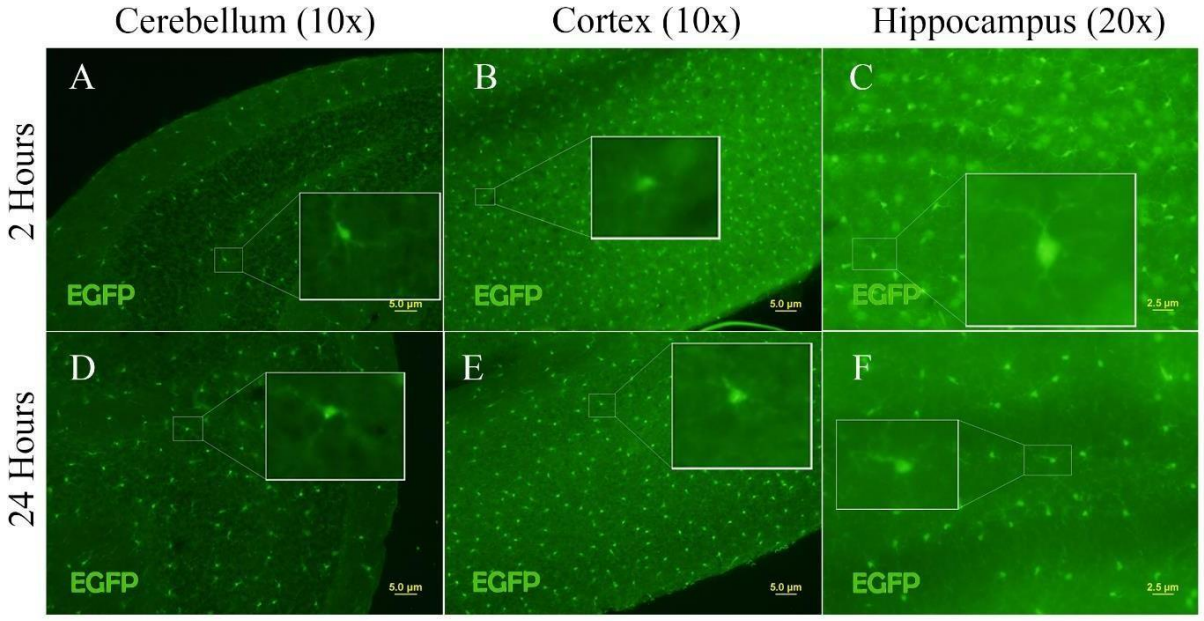

Figure 3: Sections of cerebellum, cortex and hippocampus are shown after being fixated with 4% PFA for 2 or 24 hours. (A) cerebellum (10x magnification) after 2 hours of 4% PFA fixation. (B) cortex (10x magnification) after 2 hours of 4% PFA fixation. (C) cortex (20x magnification) after 2 hours of 4% PFA fixation. (D) cerebellum (10x magnification) after 24 hours of 4% PFA fixation. (E) cortex (10x magnification) after 24 hours of 4% PFA fixation and (F) hippocampus (20x magnification) after 24 hours of 4% PFA fixation.

3.2.2 Change in body weight after LPS and saline injection

The body weights of the mice used in the experiment were measured before and after LPS and saline (control) administration. There was a decrease in the body weight of LPS as well as saline treated mice. Statistical significance analyses were made using t-test on Graphpad.

There was an average decrease of approximately 14% in the body weight of LPS treated mice and an average decrease of approximately 2% in the body weights of the saline treated mice.

Using t-test, the difference in body weight change of LPS treated mice compared to those of saline treated mice was statistically significant ($p > 0.0001$). Figure 4 is a graph showing the percentage decrease in the body weights of LPS and saline treated mice.

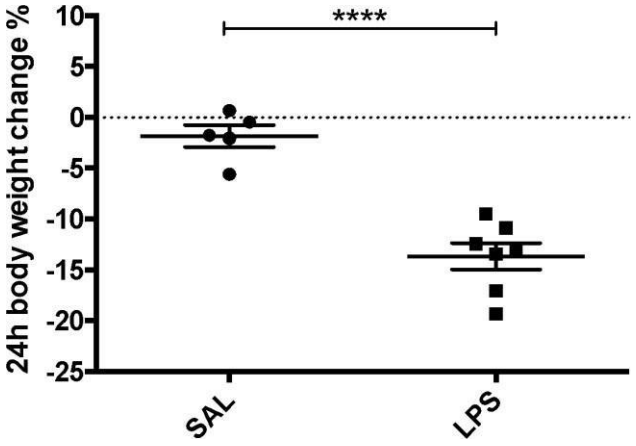

Figure 4: Percentage decrease in the body weights of LPS and saline treated mice. Values are plotted as mean ± SEM and differences (*) are indicated with respect to saline (control). The difference between the average decrease in body weights of LPS and saline treated mice is statistically significant ($p < 0.0001$). There was an average decrease of approximately 14 % in the body weights of the LPS treated mice and a difference of approximately 2% in the body weights of saline (SAL) treated mice

3.2.3 Flow cytometry results

Flow cytometry data was analysed using Kaluza software. Two separate microglial populations could be identified, one population expressing higher levels of GFP and CD45 (labelled as bright microglial population) and one population expressing lower levels of both GFP and CD45 (labelled as dim microglial population). Bright microglial populations from LPS and saline treated mice were similar in number, and dim microglial populations from LPS and saline treated mice were similar in number, since no statistical significance could be observed. Although after LPS administration bright microglial cell population number was diminished, microglial dim cell population number was slightly elevated. Still these differences were not statistically significant, maybe due to small experimental group number.

The bright microglial populations of both LPS and saline treated mice were higher in number among the total microglial population. Both dim and bright microglial populations of LPS and saline injected mice expressed MHCII. However, microglial cells from LPS injected mice had higher expression level of MHCII than microglial cells from saline injected mice.

The bright microglial populations of LPS and saline injected mice expressed higher levels of MHCII than the dim microglial populations of LPS and saline treated mice.

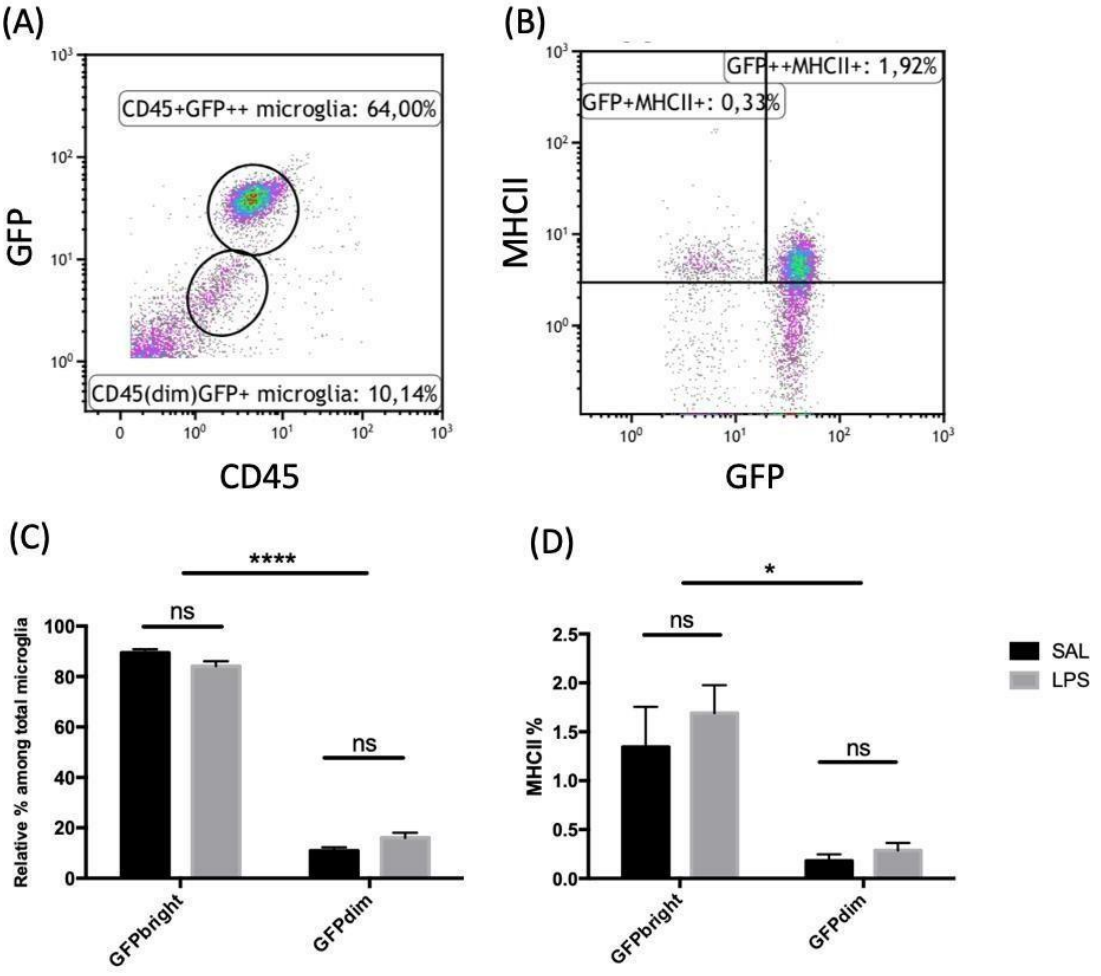

Figure 5: (A) dot plot showing GFP expression along y-axis and CD45 marker's level along x-axis. Two microglial populations are shown: bright microglial population (CD45+GFP++) and dim microglial population (CD45(dim)GFP+). (B) scatter plot showing two microglial populations. GFP+MHCII++ is the bright GFP population and GFP+MHCII is the dim GFP population. The bright GFP population has greater expression level of MHCII but there is no significant difference between the expression level of MHCII by the two populations. (C) the percentage of bright and dim microglial populations of both LPS and saline (SAL) treated mice among the total microglia. (D) the percentage of MHCII expressed by the bright and dim GFP populations of both LPS and saline treated mice. Statistical tests were done by a two-way ANOVA test and significant differences (*) are indicated with respect to the control treatment (saline).

DISCUSSION

Lipopolysaccharide (LPS) is an endotoxin that is a component of the outer membrane of Gram-negative bacteria (Galanos and Freudenberg, 1993). Administration of isolated LPS into experimental animals elicits a broad range of biological activities that are also manifested during Gram-negative septic shock (Galanos and Freudenberg, 1993). Biological activities of LPS are not direct effects of the LPS molecules but endogenous mediators produced after the interaction of endotoxin with LPS sensitive cells induce those biological activities (Galanos and Freudenberg, 1993). Tumor necrosis factor alpha (TNF-α) is a primary mediator of the toxic action of endotoxin (Beutler *et al*., 1985; Leumann *et al*., 1987; Galanos *et al*., 1988; Freudenberg *et al*., 1991). LPS is one of the most powerful stimulators of immune responses known (Lehner *et al*., 2001). Response of immune system to LPS is characterized by a systemic production of proinflammatory cytokines, that recruit and activate immune cells for elimination of invading pathogens (Shahin *et al*., 1987). Toll-like receptor 4 (TLR4) is a pattern-recognition receptor (PRR) which recognizes distinct pathogen-associated molecular patterns (PAMPS) such as LPS and cytokines (Lien *et al*., 2000). Upon ligand binding, TLR4 recruits signaling adaptors and initiates a succession of signaling cascades resulting in the activation of NF-kB and the release of inflammatory cytokines (Cheong *et al*., 2011). NF-kB is a key mediator of pro-inflammatory gene induction and has a role in both innate and adaptive immune cells (Liu *et al*., 2014). Microglial activation can occur via a TLR4-mediated pathway (Zhang *et al*., 2015). It has been shown that TLR4 has deleterious effects in neurodegenerative diseases and stroke models and plays a crucial role in microglial signaling in some disease processes (Kettenmann *et al*., 2011). Microglia is activated by peripheral (systemic) LPS challenge (Hoogland *et al*., 2015). Peripheral LPS challenge in rodent experiments caused a sharp increase in brain tumor necrosis factor alpha (TNF-α) that can persist for months (Laflamme *et al*., 2001; Raghavendra *et al*., 2004; Semmler *et al*., 2008; Sierra *et al*., 2007; Qin *et al*., 2007; Sehgal *et al*., 2011; Wu *et al*., 2012). For the purpose of this thesis, LPS administration was used to induce inflammation in CX3CR1-GFP heterozygous mice, as they have functional CX3CR1 receptor. One of the aims of current thesis was to characterize how microglial phenotype changes in response to this inflammation. Additionally, fixation protocol opimization of brain sections of CX3CR1-GFP mouse line was carried out.

First, immersion fixation of brain tissue was done so as to optimize the immersion fixation time. Brain sections were immersion fixated for 2 or 24 hours and images of cortex, cerebellum and hippocampus were obtained with epifluorescence microscope.

From the experiments, the optimal tissue fixation time is 24 hours because images of the sections after 24 hours of immersion fixation were sharper than those after 2 hours of immersion fixation. Thus, for the following experiments where we plan to characterize microglial morphology, 24 hour 4% PFA fixation protocol will be used.

Flow cytometric (FC) MHCII staining of CX3CR1-GFP mouse glial cells from LPS treated and untreated mouse brains (cortex).

Then, flow cytometric (FC) MHCII staining of CX3CR1-GFP mouse glial cells from LPS treated and untreated mouse brains (cortex) was done. Major histocompatibility class II (MHCII) is expressed on the surface of antigen presenting cells and plays a role in antigen recognition and the activation of the adaptive immune system (Hopperton *et al*., 2018). Within the brain, MHCII is primarily expressed on microglia, where it is generally considered a marker of activated cells, though it may have weaker expression in resting cells (Lee *et al*., 2002).

Body weights of mice were recorded prior to and after 24 hours of LPS and saline (control) administration to assess the effect of LPS challenge on body weights of mice. There was a significant decrease in body weights of LPS treated mice compared to saline treated mice after 24 hours of LPS and saline treatment. Thus, from the experiments, we can say that LPS challenge causes a significant weight loss after 24 hours.

The flow cytometry results enabled the identification of microglial populations using CD45 as marker as well as endogenous GFP expression level of microglial cells in CX3CR1-GFP heterozygous mice. Two separate microglial populations could be found: one expressing higher levels of CD45 and GFP (labelled as bright microglial population) and one expressing lower levels of both CD45 and GFP (labelled as dim microglial population). The microglial population expressing lower levels of CD45 also expressed low levels of GFP. Since green fluorescent protein (GFP) is under the promoter of *CX3CR1* gene, a receptor on microglial cells, expression levels of GFP are related to CX3CR1 expression. Thus, lower level of CD45 expression can be associated with a lower level of CX3CR1 expression.

Microglial cells from LPS as well as saline treated mice expressed MHCII. However, there was a slightly increased expression of MHCII on microglial cells upon LPS administration. Thus, it could be said that LPS did cause the upregulation of MHCII on microglial cells but there wasn't a statistically significant difference between the expression levels of MHCII in microglia of LPS treated animals compared to saline treated controls. This could be due to insufficient animal number in experimental groups and further studies need to be done so as to provide more precise results about alterations in microglial molecular signature.

Furthermore, it seems that LPS altered slightly the amount of cells in different microglial populations. LPS administration decreased the relative percentage of GFP bright microglial cells and increased GFP dim microglial cell numbers compared to saline treated control group. However, when applying two-way ANOVA statistical analysis, this alteration was not statistically significant. There was a significant difference in the number of GFP bright microglial cells of both administration groups (LPS and saline) compared to GFP dim microglial cells. The GFP bright population makes up approximately 80% and GFP dim population about 20% of total microglial cells.

In conclusion, in this thesis we were able to optimize paraformaldehyde fixation protocol and can proceed with characterization of microglial morphology in CX3CR1 mutant mouse line in future experiments. As expected, MHCII levels in microglial cells were elevated in response to LPS challenge, however, to provide more accurate results, more experiments still need to be done. As for changes in microglial subpopulations, we plan to further investigate how these two populations behave under inflammatory conditions.

SUMMARY

Microglia are known as the immune sentinel of the brain. They can be in a resting or activated state. They respond to pathogens and injury by adopting morphological changes and migrating to the site of infection/injury, where they destroy pathogens and remove damaged cells. As part of their response, cytokines, chemokines, prostaglandins, and reactive oxygen species, which help in mediating the immune response, are secreted. Furthermore, they have a major role in the resolution of the inflammatory response, through the production of anti-inflammatory cytokines. Microglia have been widely studied for their roles in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, as well as cardiac diseases, glaucoma, and viral and bacterial infections.

Microglial activation is greatly influenced by their environment, resulting in different activation states, namely the M1 and M2 which are characterised by the production of different mediators and the expression of various markers. Inflammation, an important aspect of several neurodegenerative diseases, is characterised by microglial activation. In this study LPS was used to induce inflammation in mice for the assessment of the microglial response after systemic LPS administration.

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