Diplomarbeit

2-chlorohexadecanal and 2-chlorohexadecanoic acid as effectors of microglia polarization

eingereicht von

Bernd Bucnik

zur Erlangung des akademischen Grades

Doktor(in) der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Molekularbiologie und Biochemie

unter der Anleitung von

Ao. Univ.-Prof. Dr.rer.nat Wolfgang Sattler

Sen. Lecturer Dr.rer.nat Eva Bernhart

Graz, 03.02.2016

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 03.02.2016 *Bernd Bucnik eh*

für Rudolf Zechner sen.

Acknowledgements

This diploma thesis was made possible at the Institute of Molecular Biology and Biochemistry, Center of Molecular Medicine at the Medical University of Graz under the direction of Univ.-Prof. Dr.rer.nat. Wolfgang Graier.

First of all, I would like to thank Ao. Univ.-Prof Dr.rer.nat Wolfgang Sattler for the opportunity he gave me to work on my diploma thesis with him and his team. He has been of great help and has supported me in every way to achieve my research goals. He handled every concern or request I had with great care and I am glad to have made the experience of being his diploma student.

In addition, thanks to my second supervisor Dr.rer.nat Eva Bernhart for advising and assisting me.

Special thanks go to Ioanna Plastira, Msc. and Nora Kogelnik, Msc. for introducing me to the world of science and showing me the techniques required for accomplishing this thesis. It was a great pleasure working with both of them. Through their guidance the research and writing process became an unforgettable experience.

Likewise, I would like to thank the whole staff of the Institute of Molecular Biology and Biochemistry, especially Ing. Helga Reicher and Doris Treier for supporting me and answering any arising questions. I had a wonderful time in the laboratory thanks to every single member of the team.

Thanks to Lily Zechner for reading over this thesis and making corrections.

Finally, I want to say thank you to my family and friends. The encouragement and assistance you gave me every single day, not only during the process of writing this diploma thesis, but during my university career and entire life, gave me the strength to achieve my goals. Without you this important part of my life would not have been possible.

I am so grateful for all of your help and support!

Zusammenfassung

Neuroinflammation und die damit einhergehende Neurodestruktion sind häufige Krankheitsbilder in unserer Gesellschaft und stellen ein interessantes Forschungsfeld, sowie Ziel medikamentöser Therapien dar. Eine Schlüsselrolle bei der Pathophysiologie von Erkrankungen wie Morbus Alzheimer, Morbus Parkinson, Multiple Sklerose oder Schlaganfall kommt der Blut-Hirn Schranke zu, welche für den Schutz sowie die Homöostase des zentralen Nervensystems von großer Bedeutung ist. Myeloperoxidase generiert bei oxidativem Stress mit Hilfe von Wasserstoffperoxid eine hypochlorige Säure, welche die Vinyletherbindung von häufig im Gehirn vorkommenden Etherlipiden, so genannten Plasmalogenen, angreift. Dabei kommt es zur Bildung des chlorierten Aldehyds 2-Chlorohexdecanal und der chlorierten 2-Chlorohexadecansäure. Während der Entzündungsreaktion treten die Immunzellen des Gehirnparenchyms, die Mikroglia, in zwei verschiedenen Polarisationsstadien auf. Studien legen nahe, dass die klassisch-aktivierte M1 Mikroglia eher proentzündlich wirkt, während alternativ-aktivierte M2 Mikroglia neuroprotektive Eigenschaften aufweist. Ziel dieser Diplomarbeit war die Untersuchung des Polarisationsverhaltens von BV-2 Mikroglia nach Behandlung mit 2-Chlorohexadecanal und 2-Chlorohexadecansäure.

Die metabolische Aktivität wurde durch den MTT Assay evaluiert und zeigte das zytotoxische Potential von 2-Chlorohexadecansäure. Des Weiteren wurden Zellen nach der Behandlung mit 2-Chlorohexadecanal nach verschiedenen Zeitpunkten mittels Western Blot und Durchflusszytometrie analysiert. Die Ergebnisse dieser Experimente weisen keine eindeutige Tendenz auf. Änderungen der Expressionslevels von reaktiven Sauerstoffspezies, Nitriten und Nitraten konnten Anhand spezieller Nachweisverfahren nicht detektiert werden.

Trotz widersprüchlicher Ergebnisse über klassisch und alternativ aktivierte Mikroglia, steht deren Schlüsselrolle bei der Entstehung neurologischer Veränderungen außer Frage. Die Erforschung neuroimmunologischer Prozesse stellt damit einen wichtigen Schritt zur Entwicklung neuer Therapien für Patientinnen und Patienten mit neuroinflammatorischen und neurodegenerativen Erkrankungen dar.

Abstract

Neuroinflammatory disorders and subsequent neurodegeneration are common in our modern society, thus representing an interesting target of research. The blood brain barrier, which provides protection and secures homeostasis within the central nervous system, plays an important role during neurological disorders like Alzheimer's disease, Parkinson's disease, multiple sclerosis or stroke. Studies showed that during oxidative stress the MPO-H_2O_2-halide system generates hypochlorous acid. This potent oxidant can attack ether-phospholipids, termed plasmalogens, which are important components of cell membranes and very common in the central nervous system. Their vinyl ether linkage is destroyed by hypochlorous acid, resulting in the formation of the α-chloro fatty aldehyde 2-chlorohexadecanal and the α-chloro fatty acid 2-chlorohexadecanoic acid. Under inflammatory conditions, microglia, the brain resident immune cells, are considered to occur in two different polarization states. The classically activated proinflammatory M1 phenotype is believed to be neurodestructive while the alternatively activated anti-inflammatory M2 microglia was shown to have neuroprotective potential. The aim of this thesis was to get preliminary insights into the effects of 2-chlorohexadecanal and 2-chlorohexadecanoic acid on BV-2 microglia polarization.

For this purpose, cell viability was assessed with the MTT assay, which displayed the cytotoxic properties of 2-chlorohexadecanoic acid. 2-chlorohexadecanal treated cells were analyzed by western blot and flow cytometry after different timepoints post-treatment. The results of these experiments were controversial and remained inconclusive. No changes in the levels of reactive oxygen species and NO_2^-/NO_3^- were detected after 2-chlorohexadecanal treatment.

Although definitive statements on classically and alternatively activated pathways cannot be made after these experiments, the important role of microglia is unquestionable. The understanding of the immunological response in CNS pathophysiology is key in order to discover better treatment options for patients with neuroinflammatory and neurodegenerative disorders.

Table of Contents

Abbreviations

2-ClHA	2-chlorohexadecanoic acid
2-ClHDA	2-chlorohexadecanal
α-ClFALD	α-chloro fatty aldehyde
Aβ	amyloid β
AD	Alzheimer's disease
AJ	adherens junction
APC	antigen presenting cells
Arg1	arginase 1
BBB	blood brain barrier
BCSFB	blood-cerebrospinal fluid barrier
bFGF	basic fibroblast growth factor
BLMB	blood-leptomeningeal barrier
BM	basement membrane
BMVEC	brain microvascular endothelial cells
CNS	central nervous system
COX-2	cyclooxygenase-2
DC	dendritic cell
DMSO	dimethyl sulfoxide
EAE	experimental autoimmune encephalomyelitis
eNOS	endothelial nitric oxide synthase
EP	ether-phospholipid
GP	glycerophospholipid
GPCR	G-protein-coupled receptor
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
ICAM	intracellular adhesion molecule
IGF-1	insulin like growth factor 1
iNOS	inducible nitric oxide synthase
IRF-8	interferon regulatory factor 8
JAM	junction adhesion molecule
LPS	lipopolysaccharide
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MAGUK	membrane associated guanylate-kinase

MPO	myeloperoxidase
MS	multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NVU	neurovascular unit
PAF	platelet activating factor
PBS	phosphate buffered saline
PC	pericyte
PD	Parkinson’s disease
PG	prostaglandin
PMSF	phenylmethylsulfonyl fluoride
PSGL-1	P-selectin glycoprotein ligand
RIPA buffer	Radioimmunoprecipitation assay buffer
RNS	reactive nitrogen species
ROS	reactive oxygen species
Rpm	revolutions per minute
SSeCKS	src-suppressed C-kinase substrate
TEER	transendothelial electrical resistance
TJ	tight junction
TLR	toll-like receptors
TREM2	triggering receptor expressed on myeloid cells-2
VCAM-1	vascular cell adhesion molecule-1

List of figures

1 Introduction

Increasing experimental evidence suggests that transient or persistent damage of blood brain barrier (BBB) integrity is associated with or can even induce neurodegenerative diseases. Peripheral inflammation induces activation of leukocytes that can activate their defense systems against brain microvascular endothelial cells (BMVEC) that form the morphological basis of the blood brain barrier. Alternatively, BBB dysfunction can lead to the activation of the innate immune cells of the central nervous system (CNS), the microglia.

1.1 Blood brain barrier

In humans three different types of barriers ensure homeostasis of the CNS. The blood-cerebrospinal fluid barrier (BCSFB) is located within the ventricles of the brain and regulates the interface between blood and cerebrospinal fluid through epithelial cells of the choroid plexus (1). The blood-leptomeningeal barrier (BLMB) is formed by endothelial cells of the leptomeningeal vessel in the space between arachnoid and pia mater (subarachnoid space). The largest and most important part of these barriers is the BBB, consisting of endothelial cells of parenchymal microvessels (2). The CNS is considered ‘immunologically privileged’ not only due to its protection by special barriers, but also because of its unique innate and adaptive immunities. These characteristics will be described in the following section.

1.1.1 Morphology of the blood brain barrier

The BBB is responsible for various important physiological processes, which require a perfectly tuned environment within the CNS. These processes involve CNS homeostasis, protection and supply of nutrients, as well as inflammatory response in the human brain (3). These important tasks are achieved by a surface area of approximately 20 m^2 and a length of the microvascular network of approximately 650 km (4). The cell components of the BBB include BMVEC, astrocytes, oligodendrocytes, pericytes (PC) and microglia, which altogether form the so called ‘neurovascular unit’ (NVU).

1.1.1.1 Brain microvascular endothelial cells

Endothelial cells of microvessels in the CNS, which differ from peripheral microvascular endothelial cells, form the anatomic basis of the BBB (3). They interact with other components of the NVU, regulate metabolism and prevent the invasion of toxic agents and pathogens. For this cause, BMVEC are connected by narrow junctions, which make them up to 100 times tighter than peripheral endothelial cells (5). Interendothelial junctions include tight junctions (TJ) and adherens junctions (AJ), as well as gap junctions.

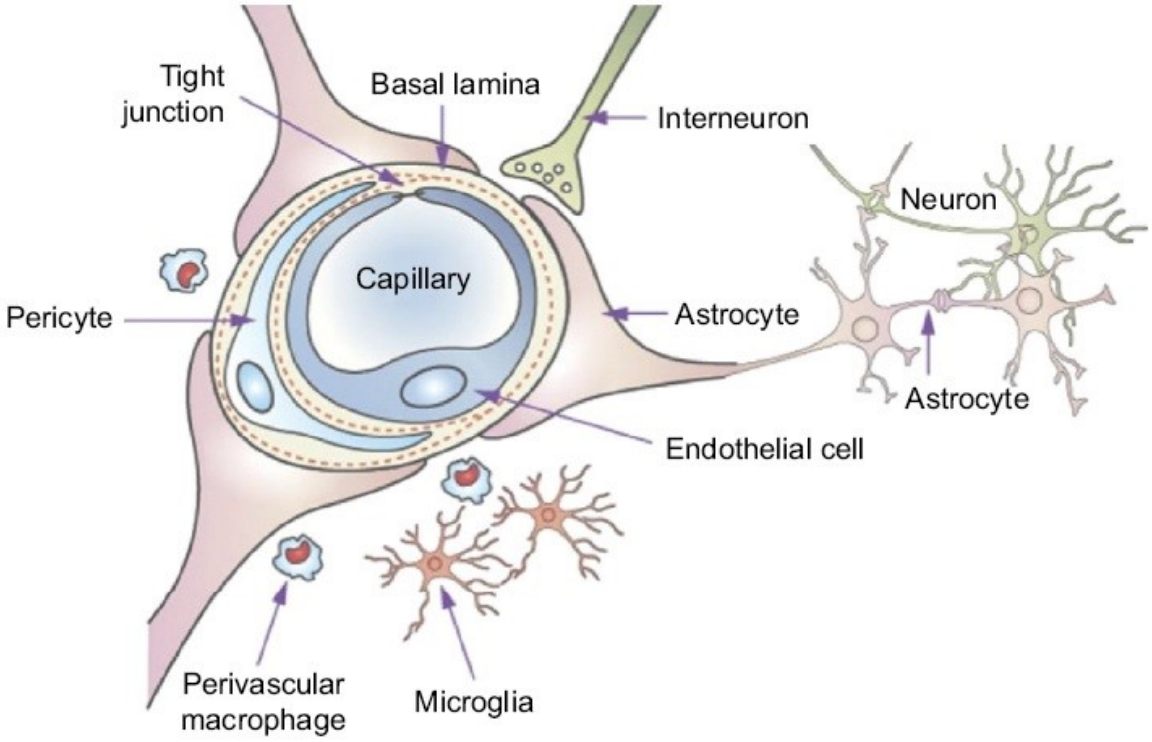

Fig. 1: Morphology of the blood brain barrier
The main components forming the neurovascular unit: BMVEC, basement membrane, astrocytes, pericytes and immune cells. (From Advanced Drug Delivery Review, 64(7), Chen Y, Liu L. Modern Methods for delivery of drugs across the blood-brain barrier. 640-665. Copyright 2012)

At the BBB, TJ regulate lateral diffusion between basolateral and apical plasma membrane domains and paracellular permeability, thus making them the most important structure for barrier maintenance (3). They are formed by claudins, a family of 24 TJ proteins known so far, and occludin, a transmembranous protein of approximately 65 kDa. Claudins, proteins of 20-27 kDa, form the primary seal of TJ, therefore being responsible for permeability restriction (6). Occludin, which is believed to provide additional support for claudins (7), ensures high transendothelial electrical resistance (TEER) and decreased paracellular permeability of BMVEC (8). Other important molecules of TJ are junction adhesion molecules (JAM), proteins from the IgG superfamily, which are involved in organizing junctional structure, cell-cell adhesion, developmental processes and transendothelial migration of leukocytes (9, 10). Also cytoplasmatic components, such as membrane associated guanylate-kinase (MAGUK) proteins, serve as signal transduction and recognition elements (8).

AJ are responsible for a variety of tasks in the BBB. Although paracellular permeability is mainly restricted by TJ, AJ mediate cell-cell adhesion, cell polarity, vascular growth and remodeling. AJ are formed by vascular endothelial-cadherin, which regulates extracellular interactions between neighboring cells and binds to cytoplasmatic proteins like catenins to stabilize the complex (11).

1.1.1.2 Basement membrane

The specific roles of the basement membrane (BM) are still object of study. First of all, the extracellular matrix of the basal lamina is anchoring cells of the endothelium and the CNS and holding them in place. Moreover, it provides a platform for intercellular interactions and allows adaption to environmental changes. The BM includes structural proteins (elastin and collagen), specialized proteins (fibronectin and laminin), proteoglycans, cell adhesion molecules and signaling proteins expressed by vascular cells, neurons and supporting glial cells. The integrity of this complex structure, also regarding its composition, is very important for maintenance of the BBB. Therefore, disruptions of the BM under pathological conditions can lead to increased endothelial permeability (3).

1.1.1.3 Neurons

The brain is in need of high energy supply through a sufficient blood flow in the central nervous microvessels. It was believed that neurons regulate blood flow through vasoactive neurotransmitters such as serotonin, GABA, acetylcholine and noradrenalin, being the main component of the so called ‘neurovascular coupling’. However, studies have shown that astrocytes and PC play a prominent role in modulating microcirculation (12). Attwell et al. (13) showed that PC regulate constriction and dilation at the capillary level, while astrocytes are mediators between contractile elements (pericytes and smooth muscle cells) and neurons. Eventually, the data suggest that both neurons and astrocytes release vasoactive substances during synaptic activity, including neuromodulators and neurotransmitters such as catecholamines, GABA, acetylcholine and glutamate. These neurotransmitters are potent vasoactive mediators, which facilitate neurovascular coupling, thereby determining nutrient and oxygen supply of the CNS.

1.1.1.4 Astrocytes

Astrocytes play a critical role in BBB function, since they provide a cellular link between BMVEC, which they almost completely ensheath with their endfeet and neurons, thereby mediating many different processes in the BBB. Their star-shaped morphology allows them to get into physical contact with a great number of neighboring cells, also including other glia cells of the NVU, through thousands of connections via their endfeet processes. Astrocytes are involved in the supply of substrates and energy in neurotransmission, recycling of neurotransmitters and ions, regulation of brain homeostasis and control of neuronal function (14). The role of astrocytes in brain vascularization remains unclear, since studies showed intact barrier function after extensive astrocyte loss (15). However, one study showed disruption of barrier integrity due to focal loss of astrocytes (16).

Astrocytes contain aquaporin 4 as well as dystrophin and dystroglycan, which links the cytoskeleton to the BM binding agrin (17). They secrete a large number of substances like chemokines, growth factors, and peptides modulating TJ, enzyme and transporter systems. Many of them, such as basic fibroblast growth factor (bFGF) and TGF-β1, were shown to play an important role in barrier permeability (14).

1.1.1.5 Pericytes (PC)

Like vascular smooth muscle cells, PC belong to the group of mural cells of the BBB. In contrast to the peripheral vasculature with a pericyte:endothelial cell ratio of 1:100, the PC:EC ratio in brain microvessels is estimated between 1:3 and 1:1 (18). PC are embedded in the BM, connected with the endothelium by so called 'peg-and-socket junctions', incompletely covering the walls of the microvasculature. Due to a lack of specific markers produced by PC, their role in the BBB is not clear. Studies have shown that contractile proteins of PC can change the flow of blood in the vessels of the CNS (19). Besides regulation of blood flow, PC play an important role in angiogenesis, immune cell infiltration, mechanical stabilization and deposition of extracellular matrix (17). Since they express occludin, an essential protein for TJ, PC are believed to be an important stabilizer of BBB integrity (20).
Eventually, BMVEC, astrocytes and PC influence each other. Although little is known, it has become clear that these tricellular interactions, for example via increased TEER or protein expression, have great effects on BBB permeability and CNS homeostasis (21-23).

1.1.1.6 Immune cells

Important cells of BBB integrity include leukocytes, which regulate inflammatory and immune response. Microglia, which are derived from a primitive yolk sac and represent the resident immune cells within the CNS parenchyma, are discussed in section 1.4, as their role in neuroinflammation was object of this thesis.
Macrophages, leukocytes and neutrophils are derived from blood-borne progenitor cells and are located in the CNS vasculature. If activated, they can interact with the endothelium and enter the brain parenchyma by passing the BBB. Their main function is injury and infection response via phagocytosis, antigen presentation, release of proinflammatory cytokines or release of reactive oxygen and/or nitrogen species (ROS, RNS) that are able to kill pathogens, but can also induce damage to bystander cells. As these cells are an important part of immune modulation, they are critical for BBB integrity and in the development of neuroinflammation and neurodegeneration.

1.1.2 The blood brain barrier in health and disease

BBB, BCSFB and BLMB ensure a homeostatic environment, which is required for proper electrical activity of neural networks in the CNS. In this section, the physiological and pathophysiological role of the BBB will be described. Besides the vascular endothelium and BM, which prevent the invasion of toxins and pathogens, the brain parenchyma is protected by immune cells, especially T-cells. In order to migrate across the BBB, a well-organized mechanism that rigorously controls leukocyte transmigration is required. Under non-inflammatory conditions the number of peripheral immune cells is well regulated and therefore lower compared to that of other organs (24). However, during pathophysiological processes, e.g. multiple sclerosis (MS) or stroke, immune cells are able to cross these barriers causing neuroinflammation.

Leukocyte diapedesis across the BBB is achieved by sequential steps interacting with the endothelium of post-capillary venules. Initially, circulating leukocytes tether to the endothelial cells mediated by adhesion molecules of the selectin family (E-, L- or P-selectin) or α4-integrins. Endothelial counter receptors include P-selectin glycoprotein ligand (PSGL-1), vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). After the initial contact, leukocytes reduce their velocity and roll along the vessel. Chemokines presented by endothelial cells bind to G-protein coupled receptors (GPCR) on immune cells increasing their affinity and avidity. Adhesion is accomplished via ligands of the IgG superfamily including intracellular adhesion molecules (ICAM-1, ICAM-2), VCAM-1 and MAdCAM-1. The last step is transmigration through the endothelium into the perivascular space. This is achieved by two different pathways, either paracellular through endothelial junctions or transcellular through pore like structures (25). Behind the BBB, leukocytes recognize antigens on antigen presenting cells (APC) triggering inflammation through cytokines and recruitment of additional immune cells (26).

Although neuroinflammation is a protective reaction of the organism, it became clear that uncontrolled invasion of leukocytes leads to persistent inflammation and eventually neurodegeneration. BBB integrity can be compromised through physical injury, inflammatory or degenerative processes causing hypoxia and invasion of cells driving the immune response, resulting in a vicious circle.

Important contributing factors are ROS, RNS, TNF-α, IL-1β, IFN-γ as well as vasoactive mediators like bradykinin, histamine, nitric oxide (NO) and matrix metalloproteinases. Cells involved in the inflammatory response are mainly BMVEC, activated microglia, astrocytes and macrophages (27). Other substances influencing BBB integrity are oxidative degradation products from unsaturated lipids, e.g. reactive aldehydes (with the prototypic member 4-hydroxy-2-nonenal) following oxidative stress. In addition, the expression of prostaglandins (PG), leukotrienes and platelet activating factor (PAF) can compromise BBB function (28).

1.1.3 Myeloperoxidase and BBB function

Under inflammatory conditions the brain is under attack of reactive chemical species that compromise BBB function (29). Neuroinflammatory and neurodegenerative syndromes such as Parkinson's disease (PD) and Alzheimer's disease (AD) are chronic disorders which manifest later in life. Their pathophysiology is associated with oxidative stress that contributes to protein and/or lipid modification and subsequent disruption of cellular homeostasis (30). It was demonstrated that myeloperoxidase (MPO) (which is undetectable in a healthy brain) is abundantly expressed in PD and AD, but not in normal human brain (31, 32). MPO was also localized in plaques of MS patients (in white and grey matter) (33). In experimental autoimmune encephalomyelitis (EAE), a mouse MS model, a paramagnetic sensor that is activated specifically by MPO (34) was utilized to reveal MPO activity in the brain (35). In preclinical models MPO was identified as a potential therapeutic target in stroke (36) and MPO could also be responsible for barrier dysfunction, as observed in bacterial meningitis (37, 38).

Under physiological conditions MPO is considered a front-line defender against phagocytosed microorganisms (39). The MPO-H_2O_2-halide system generates the potent oxidant hypochlorous acid (HOCl; among other pseudo-halides depending on the availability of co-substrates) that is primarily responsible for the microbicidal action of neutrophils. However, there is now compelling evidence that under chronic inflammatory conditions MPO-derived HOCl attacks amino acids, proteins, nucleic acids, carbohydrate components as well as lipids (40). Among the lipid targets that are subject to HOCl modification are ether-phospholipids (EP), so called plasmalogens that are highly abundant in the CNS and essential for normal brain function.

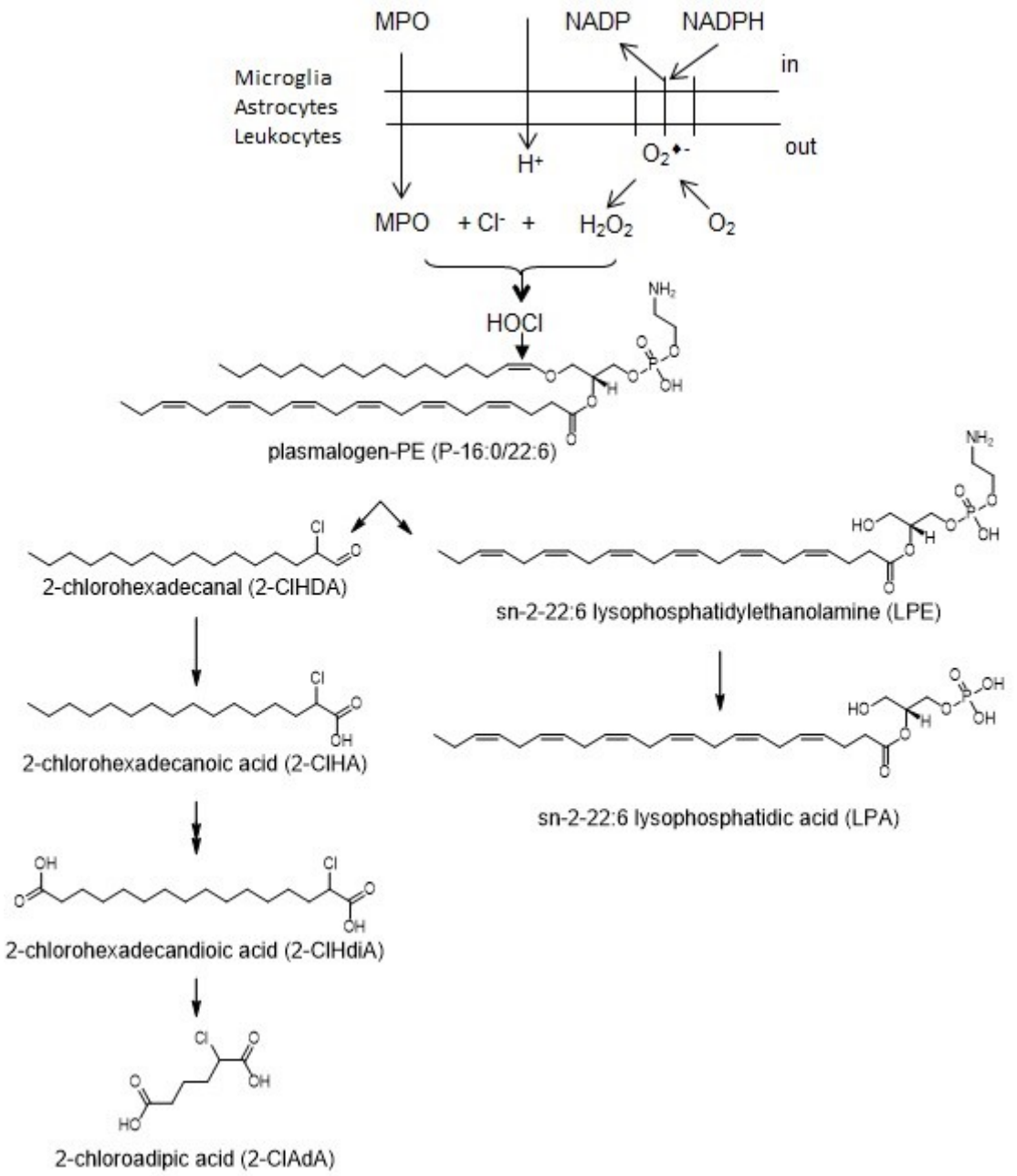

Fig. 2: The MPO-H_2O_2-halide system generates 2-ClHDA and 2-ClHA
MPO derived HOCl attacks the vinyl ether bond of plasmalogens in BBB cells forming a lysophospholipid and the α-ClFALD 2-ClHDA, which is further oxidized to the α-chloro fatty acid 2-ClHA. (Modified from W. Sattler)

1.2 Plasmalogens

Plasmalogens, which belong to the group of glycerophospholipids (GP), are important plasma membrane lipids responsible for proper membrane structure and function. Thus, under pathophysiological conditions their malfunction is associated with neuroinflammation and neurodegeneration.

Plasmalogens are characterized by their polar head group at the sn-3 position of the glycerol backbone, mainly being ethanolamine. A fatty alcohol is connected via a vinyl ether linkage at the sn-1 position. The fatty alcohols are mainly C16 and C18 species linked via a 1-0-alkenyl ether bond (plasmanyl GP) or 1-0-(1Z-alkenyl) bond. The latter compounds are called plasmalogens. At the sn-2 position, EP carry a polyunsaturated fatty acid, e.g. docosahexaenoic acid or arachidonic acid. They are synthesized on the luminal side of the peroxisomal membrane by matrix enzymes glyceronephosphate-O-acyltranferase and alkylglycerone phosphate synthase. Plasmalogens make up for 15-20% of total phospholipids in cell membranes, being especially abundant in the brain, the heart, neutrophils and eosinophils. Besides their role in cell membranes, they are important components of intracellular organelles such as ER, mitochondria and nuclei.

In neurodegeneration, plasmalogen deficiency following loss of peroxisome function and degradation by ROS promotes further neurodestruction. Increased lipid oxidation, for example during chronic inflammation, type II diabetes or metabolic syndrome, is related to low plasmalogen levels (41). Furthermore, plasmalogens enriched in leukocytes are attacked by HOCl due to their sensitivity of the vinyl ether bond. The resulting α-chloro fatty aldehydes (α-ClFALD) emerge as important modulators of the inflammatory response (see below).

1.3 Chlorinative stress and chlorinated species

The human brain is prone to oxidative stress due to its high oxygen metabolic rate and high concentrations of polyunsaturated fatty acids. This is an important fact for understanding the pathophysiology of neuroinflammatory diseases. Phagocytes, such as microglia or macrophages, produce oxidants resulting in the production of HOCl, which causes tissue destruction, cell damage and apoptosis (42). The MPO-H_2O_2-halide pathway uses hydrogen peroxide (H_2O_2) (generated via NADPH-oxidase and superoxide dismutase) as a substrate to oxidize the halides and pseudohalides to their corresponding hypohalous acids (43). In case of Cl^-, the end product of this cascade is HOCl, which attacks plasmalogens to form α-ClFALDs and a remnant lysophospholipid. The prototypic α-ClFALD is 2-chlorohexadecanal (2-ClHDA; for review see (44)) which is oxidized to 2-chlorohexadecanoic acid (2-ClHA), an α-chloro fatty acid.

2-ClHDA accumulates in activated neutrophils (45) and is elevated in atherosclerotic plaque material and upon myocardial infarction (46, 47). 2-ClHDA induces neutrophil chemotaxis (45), endothelial dysfunction (48), inhibits endothelial nitric oxide synthase (eNOS) activity (49) and activates cyclooxygenase-2 (COX-2) via NF-κB-mediated pathways (50). An earlier study demonstrated that a single peripheral lipopolysaccharide (LPS) injection in mice resulted in significantly elevated cerebral MPO protein levels. This treatment induced the formation of 2-ClHDA, which led to a significant decrease of brain plasmalogen content (51).

This presumably leads to a translation of peripheral inflammation to deeper regions of the brain, corroborated by the fact that peripheral inflammation in mice induces significantly elevated levels of MPO and 2-ClHDA in treated animals. This MPO-derived chlorinated fatty aldehyde can trigger apoptosis and ER stress and might produce detrimental effects in the CNS.

These events would trigger the activation of microglial cells and promote localized damage to oligodendrocytes and the myelin sheath, ultimately compromising myelination and the integrity of neural circuits.

1.4 Microglia

The CNS is a unique environment concerning anatomical specialties such as the choroid plexus and meninges as well as immunological specialties, such as lack of lymphatic vessels or reduced ability to present antigens (52). Within these surroundings specialized innate cells, including microglia, macrophages and dendritic cells (DC), are necessary. The most common innate immune cells in the brain parenchyma are microglia. These so called 'tissue-resident macrophages of the CNS' account for 10-15% of all glial cells (53). Their development, function and role in pathophysiological processes will be described here.

1.4.1 Microglia development

Early studies propose that microglia is of hematopoietic origin and derive from circulating monocytes entering the CNS parenchyma and converting to microglia (53). In the 1990s experiments could locate microglia precursors during embryogenesis in the yolk sac and the brain rudiment on embryonic day 8 (54). However, the origin did not become evident until 2010 when Ginhoux et al. (55) detected primitive macrophages originating from the embryonic yolk sac. Depending on heart activity and therefore blood flow, these progenitor cells reach and colonize the brain rudiment becoming microglia. At a later stage, this hypothesis was supported by other experiments (56, 57).

Microglia development and homeostasis are modulated by various molecules like transcription factors, growth factors, chemokines and microRNAs, maintaining a balance between cell proliferation and apoptosis. Among those factors, Runx1, a transcription factor, regulates several genes in embryonic and adult hematopoiesis. At embryonic day 6.5 Runx1 is expressed and yolk cell derived precursor cells migrate to the CNS and become microglia. At postnatal day 10 microglia transform from amoeboid to ramified stage coinciding with downregulation of Runx1. A different transcription factor, PU.1, plays a key role in maturation of yolk-sac-derived progenitors in embryonic development, but was also shown to promote maturation of myeloid cells during inflammation and stress (58). Other factors in microglial differentiation and homeostasis are Interferon regulatory factor 8 (IRF-8) modulating transcription and controlling the state of activation and CSF1R regulating cell survival, development and chemotaxis (for review see (53)).

1.4.2 Microglia physiology

In the developing and adult CNS, microglia both influence neuronal survival as well as neuronal death and synaptogenesis, maintaining health and vital homeostasis. By secreting insulin-like growth factor-1 (IGF-1) many cell lineages of the CNS like oligodendrocytes are protected from apoptosis (59). Other trophic factors, such as nerve growth factor, brain-derived growth factor, bFGF and platelet-derived growth factor secreted by microglia, facilitate neuronal health and survival throughout life.
The role of microglia as inducers of programmed cell death during development is equally important. During CNS development, neurons with defective differentiation or migration need to be eliminated. Microglia can initiate apoptosis by releasing ROS and are able to phagocytose the resulting neuronal debris. One interesting aspect about microglia response is their ability to induce neuronal death without causing an inflammatory response. This is achieved via triggering receptor expressed on myeloid cells-2 (TREM2) signaling inducing cytoskeleton reorganization and phagocytosis. In addition, TREM2 represses transcription of proinflammatory cytokines like TNF-α and IL-1β, but also inducible nitric oxide synthase (iNOS) (60). Besides their role in neuronal survival and death, microglia participate in synaptogenesis and synaptic homeostasis. During CNS development, DAP12, a transmembrane immune receptor protein expressed by myeloid and lymphatic cells, leads to enhanced synaptic function and plasticity. Experiments in mouse brains revealed that DAP12 expression is restricted to microglia suggesting a crucial role in synaptic homeostasis (61). By remodeling synaptic architecture as well as regulating dendritic spine numbers, synapse densities and glutamatergic receptors, microglia have a direct impact on synaptic activity (62, 63). In addition to these findings, microglia seems to be modulated by the complement system. It appears that synaptic complement activation is linked to neuronal apoptosis and phagocytosis, promoting synaptic connections in the developing brain (64). Altogether, these data indicate that various effects of microglia are responsible for maintaining health in the CNS.

1.4.3 M1 and M2 polarization

For a long time it was believed that two states of microglia existed to maintain homeostasis and therefore a healthy CNS. There was resting and activated microglia, which were supposed to regulate environmental changes, such as neuroinflammation and neurodegeneration. Over time, studies showed that activated cells mainly occur in two different types of polarization (65). Classical activation, so called M1 microglia, is characterized by producing ROS and cytokines, e.g. TNF-α, IFN-γ or IL-6, therefore driving a proinflammatory response (66). The alternatively activated M2 microglia have been shown to downregulate inflammation via protective proteins, such as ornithine, YM1, and polyamine synthesis as well as upregulating phagocytosis and restoring homeostasis (67). Although the M1/2 paradigm, which was originally developed for T cells and macrophages, does not fully apply to microglia, it is a valuable nomenclature to differentiate between neurotoxic and neuro-regenerative states of these cells.

The phenotypes of the different polarization states are described in this section, because they are important for understanding the role of microglia in neuroinflammation. Key tasks of M1 cells are eliminating intracellular pathogens and antigen presentation. Main receptors expressed are MHC II, CD86 and Fcγ, leading to an increased antigen presenting activity and communication with other immune cells. Additionally, the M1 phenotype is characterized by production of proinflammatory cytokines such as IL-12 (68), reactive oxygen and nitrogen species and NO (69), which is synthesized by iNOS (70).

M2 microglia is not uniform, but rather forms a diverse group of cells sharing the same profile of mediator or receptor expression downregulating inflammation and maintaining homeostasis (71). One of the main M2 specific markers is arginase 1 (Arg1), which can downregulate NO production, thereby representing a counterpart of iNOS. Other markers used for M2 classification are CD206, a mannose receptor, which is not expressed in parenchymal microglia (72), YM1, a heparin-binding lectin, FIZZ1, a promoter of extracellular matrix deposition and TREM2. Studies mostly carried out in the periphery identified three different subtypes of M2 microglia based on their induction by cytokines. M2a, mainly triggered by IL-4 and IL-13, modulate upregulation of Arg1 and phagocytosis, having a great anti-inflammatory impact (73). M2b, induced by immune complexes and toll-like receptors (TLR), seem to be close to M1-like microglia and are the least understood subtype. Experiments suggest that M2b macrophages, although included in the group of alternatively activated phagocytes, express some marker proteins that are also found in M1 cells. (74). The third activation state, M2c microglia, also described as ‘deactivated’ macrophages, are induced by IL-10, TGF-β and glucocorticoids (73). They appear to play an important role in tissue remodeling and matrix deposition after the inflammatory response has been downregulated (75).

Although there are many remaining questions about microglia polarization, it can be assumed that classical and alternative activation has a great impact in brain pathophysiology. Therefore, they are object of study and interesting targets of pharmacological therapy in CNS disorders, such as stroke, AD and MS. In acute neuroinflammation, e.g. after spinal cord injury, Kigerl et al. (76) observed an initial expression of M1 and M2 markers, favoring a balance between pro- and anti-inflammatory conditions. However, three days after the injury M2 markers were downregulated as M1 related proteins remained on higher levels, leading to extended damage and lack of repair. Similarly, studies demonstrated increased expression of M1 markers, such as proinflammatory cytokines, after traumatic brain injury and decreased levels of the M2 marker Arg1 (77).

Chronic neuroinflammatory disorders like AD and MS lead to excessive destruction of brain parenchyma resulting in neurodegeneration. Most MS patients show the remitting type of the disease, suffering from inflammation and demyelination with intermittent periods of remission. This form often proceeds to secondary progressive MS (78). While the origin of MS remains unclear, it was shown that Th1 and Th17 cells modulate inflammation leading to expression of IFN-γ and TNF-α inducing proinflammatory M1 cells (79). Prolonged inflammation regulated by proinflammatory cytokines and inhibition of neuroprotective M2 cells results in axonal demyelination and neurodegeneration, while M2 proteins like IL-10 or IL-33 were shown to decrease the toxic effects on oligodendrocytes, which form the myelin sheath of axons (80).

AD is characterized by neurotoxic plaques formed by amyloid β (Aβ) and hyperphosphorylated Tau protein causing massive neuroinflammation and ultimately amnesia. Experiments in rat CNS suggest the ability of M2 microglia to phagocytose Aβ (81). However, TNF-α and IFN-γ, cytokines expressed by M1 cells were shown to inhibit this process favoring inflammation (82).

A lot more in vivo studies on microglia polarization as well as phenotypes and effects have to be carried out until definitive statements can be made about neuroprotection and neurodestruction by M1 and M2 macrophages. Nevertheless, the data suggest an anti-inflammatory role of alternatively activated microglia, enabling a better understanding of the pathophysiology of neuroinflammation as well as therapeutic modalities in CNS disorders.

2 Hypothesis and aims

Unraveling the pathophysiological mechanisms of neuroinflammation is important for the development of new and targeted treatment modalities to improve disease outcome and avoid consequential damages.

Microglia plays an important role in neuroinflammation and is therefore an interesting target of study. While it has been widely accepted that these tissue-resident macrophages occur in a classically activated M1 and an alternatively activated M2 polarization, the complex roles of the activation states have yet to be unraveled. While M2 was shown to be rather neuroprotective, M1 could have a proinflammatory potential. During oxidative stress the potent oxidant HOCl derives from the MPO-H_2O_2-halide system and attacks the vinyl ether bond of plasmalogens to form 2-ClHDA and 2-ClHA. The role of these products deriving from chlorinative stress was object of this thesis.

The aim of this pilot study was to characterize initial effects of 2-ClHDA and 2-ClHA on cell viability and how 2-ClHDA affects polarization states of BV-2 microglia over time by analyzing specific markers. Both chlorinated species were expected to reduce cell metabolism. Moreover, it was presumed that 2-ClHDA would rather induce classically activated M1 polarization favoring a proinflammatory environment.

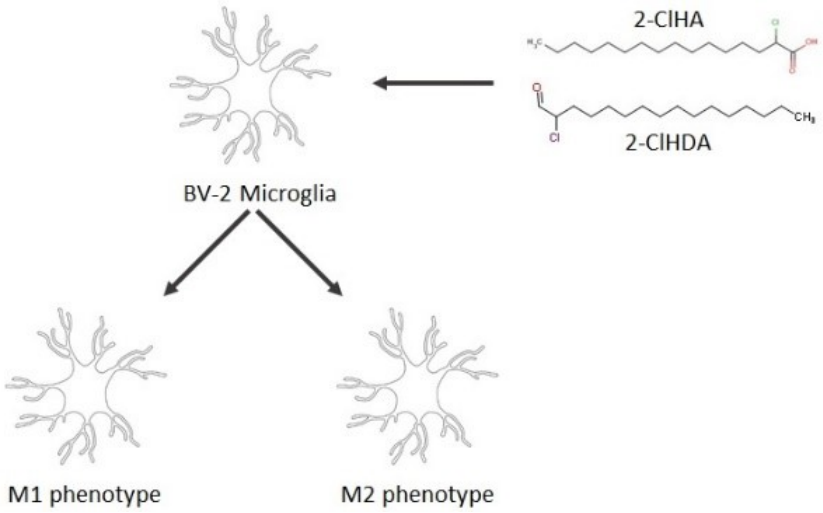

Fig. 3: Effects of 2-ClHA and 2-ClHDA on BV-2 microglia polarization states
Object of this thesis were the effects of 2-ClHA and 2-ClHDA on cell viability and polarization states of microglia.

3 Materials and methods

3.1 Materials

3.1.1 Immortalized BV-2 mouse microglia

BV-2 (mouse, C57BL/6, brain, microglial cells)	Banca Biologica e Cell Factory, Italy

3.1.2 Plastic- and glassware

Cell culture flasks	Greiner Bio One, Germany
Pipette tips	Greiner Bio One, Germany
Micro test tubes	Greiner Bio One, Germany
Vials	Greiner Bio One, Germany
Centrifuge tubes	Greiner Bio One, Germany
Microwell plates	Greiner Bio One, Germany
Cell scrapers	Greiner Bio One, Germany

3.1.3 Laboratory equipment

Pipettes	Gilson, Inc. USA
Multi stepper	Eppendorf, Austria
Centrifuge	Eppendorf, Austria
Cell counter	Thermo Fisher Scientific, USA
Electrophoresis chambers	Bio-Rad, USA
Victor 1420 multilabel plate reader	Perkin Elmer, USA
Guava easyCyte flow cytometer	Merck Millipore, Germany

3.1.4 Other laboratory materials

PVDF membrane	GE Healthcare, UK
Nitrate/nitrite colorimetric assay kit	Cayman Chemicals, USA
Cellular ROS detection assay kit	Abcam, UK

3.2 Media and reagents

3.2.1 Media and reagents for cell culture and treatment

RPMI 1640	Thermo Fisher Scientific, USA
Fetal calf serum	Thermo Fisher Scientific, USA
Trypsin	Thermo Fisher Scientific, USA
Penicillin/streptomycin	GE Healthcare, UK
0,2% DMSO	Sigma-Aldrich, USA
2-ClHDA	Synthesis Sattler lab
2-ClHA	Synthesis Sattler lab
Trypan blue	Thermo Fisher Scientific, USA

3.2.2 Buffers and solvents for MTT assay

MTT stock solution	1.2 mM, dissolved in serum-free medium
Lysis buffer	Isopropanol/1 M HCl, 25:1 (v/v)

3.2.3 Buffers and solvents for SDS-page and western blot

RIPA buffer	50 mM Tris HCl 1% 1% nonoxinol 40 150 mM NaCl 1 mM Na3VO2 1 mM NaF 1 mM EDTA
Aprotinin	Carl Roth, Germany
Pepstatin	Carl Roth, Germany
Leupeptin	Carl Roth, Germany
PMSF	Carl Roth, Germany
BCA protein assay kit	Thermo Fisher Scientific, USA
Sample buffer pH 6.8	0.1 M Tris-HCl 4% SDS 15% glycerol dH2O
β-mercaptoethanol	Sigma-Aldrich, USA
Acrylamid	30g acrylamide 0.8g bisacrylamide dH2O
Separating gel buffer pH 8.8	18.2g Tris dH2O

Protein molecular weight marker	Thermo Fisher Scientific, USA
10x SDS running buffer pH 7.4	30.3g Tris 150.1g glycine 10g SDS dH2O
10x Blotting buffer	12.1g Tris 30g glycine dH2O
Ponceau S	Sigma-Aldrich, USA
Blocking solution pH 7.4	5g powdered milk dH2O
10x Washing buffer pH 7.4	5g Tween 90g NaCl 12.1g Tris dH2O
ECL western blotting detection kit	GE Healthcare, UK
Stripping buffer	2g SDS 100mM NaOH 0.5g DTT

3.2.4 Reagents for Flow Cytometry

Protein block	Thermo Fisher Scientific, USA
Antibody diluent	Dako, Denmark

3.2.5 Antibodies

Arginase-1 antibody	Cell Signaling Technology, USA
iNOS antibody (mouse specific)	Cell Signaling Technology, USA
COX-2 (C-10): sc-1745	Santa Cruz Biotechnology, USA
Monoclonal anti-β-tubulin	Sigma-Aldrich, USA
Monoclonal anti-β-actin	Sigma-Aldrich, USA
PE rat IgG2a, κ isotype control	BioLegend, USA
PE anti-mouse CD40	BioLegend, USA
PE anti-mouse CD206	BioLegend, USA
Goat anti-rabbit IgG-HRP	Thermo Fisher Scientific, USA

3.3 Methods

3.3.1 Thawing cells from liquid nitrogen

The BV-2 cells were stored in liquid nitrogen and therefore needed to be thawed gently. After removing them from the storage Dewar they were quickly put in a 37ºC water bath. After being mixed with 4 ml of growth medium, they were centrifuged at 900 rpm (revolutions per minute) for four minutes. The supernatant was discarded (to remove the cryoprotectant dimethyl sulfoxide (DMSO)) and the cells were plated with growth medium (RPMI 1640) and stored in the incubator at 37ºC in a 5% CO_2 atmosphere.

3.3.2 Splitting cells

Growth medium (RPMI 1640), phosphate buffered saline (PBS) and trypsin were warmed up in a water bath (37°C) for 20 minutes. After the growth medium was removed from the flask, 13 ml of PBS were used to wash the cells. The PBS was removed, 2 ml of trypsin were added and the flask was placed on the heating plate for one minute to detach the cells from the bottom. The suspension was mixed with 6 ml of warm growth medium and centrifuged at 900 rpm for four minutes. After the supernatant was discarded, the pellet was mixed with 20 ml of growth medium. For counting, 20 µl of trypan blue and 20 µl of cell suspension were mixed and counted automatically in a cell counter. According to the number of cells 1 ml of cell suspension was pipetted into a microwell plate.

3.3.3 Cell treatment

Before and during cell culture experiments BV-2 cells were incubated in serum-free RPMI 1640 medium supplemented with penicillin/streptomycin and glutamine at 37°C (5% CO_2). 2-ClHDA and 2-ClHA were prepared as stock solutions in DMSO and applied to BV-2 microglia at the indicated concentrations and for the indicated time periods in serum-free culture medium. The final concentration of DMSO in the medium was 0.2% (v/v).

3.3.4 MTT assay

The MTT assay was used to monitor metabolic activity and cell proliferation in BV-2 cells treated with 2-ClHA and 2-ClHDA. The yellow tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to purple formazan crystals, which are only developed in living and early apoptotic cells. This color change can be measured by using a plate reader.

BV-2 cells were plated in 96-well plates (20,000 cells per well) and cultivated for 24 hours before they were treated with 2-ClHA or 2-ClHDA in serum-free medium at the indicated concentrations for the indicated time periods. MTT was added to the cells (100 µl per well) and incubated for three hours at 37°C under standard conditions. The cells were washed with PBS and cell lysis was performed with 100 µl lysis solution on a rotary shaker (1,200 rpm, 15 min). Absorbance was measured at 570 nm on a multilabel reader and corrected for background absorption (650 nm).

3.3.5 SDS page and immunoblotting

For western blot analysis, cells were lysed in 50 µl RIPA buffer containing a protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF). After keeping the micro test tubes on ice for ten minutes, the samples were centrifuged at 13,000 rpm for ten minutes and the supernatant was collected. Protein concentration was determined using the Thermo Fisher Scientific BCA protein assay kit according to the manufacturer's instructions. The samples were suspended in sample buffer plus 1% β-mercaptoethanol (1:2) and frozen at -20°C. The proteins were separated by SDS-PAGE (10 or 15%; 130 V) and transferred to PVDF membranes (150 mA, 1.5 h). Immunochemical detection of Arg1, iNOS and COX-2 was performed with sequence-specific IgGs as primary antibodies (diluted 1:1000). Immunoreactive bands were visualized with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (dilution 1:5000) using the ECL-detection system. Luminescence was detected using a ChemiDocMP system (BioRad), followed by densitometric analysis with the ImageLab software (BioRad).

3.3.6 Flow cytometry

Flow cytometry was used in order to assess the expression of CD40 and CD206 in microglia cells. BV-2 microglia cells were seeded in duplicate onto six-well plates at a density of $2.5x10^5$ cells per well. After overnight serum starvation, cells were incubated in the presence of 2-ClHDA for 16, 24 and 48 hours. The cells were then collected, blocked using the Ultra V Blocker (Thermo Fisher Scientific) and incubated with PE anti-CD40 or PE anti-CD206 antibody (1:100). Finally, they were fixed and measured using a Guava easyCyte 8 Millipore flow cytometer.

3.3.7 Cellular ROS detection assay

The ROS assay was performed according to the manufacturer's instructions. ROS formation was examined using fluorescent DCF. Rather than being a radical species selective probe, DCFDA (after hydrolysis to dichlorofluorescein and internalization) is converted to fluorescent dichlorodihydrofluorescein by several reactive radical species and allows assessment of general oxidative stress (83). BV-2 cells were plated and cultivated in 12-well plates. The cells were serum starved overnight and then incubated in the absence or presence of 5 µM 2-ClHDA at 37°C for the indicated time periods. One hour prior to completion of the treatment, DCFDA was diluted at 2x of the final concentration desired in the same media used for treatment and warmed to 37°C. 45 minutes prior to completion of the treatment, the cells were supplemented with 2x DCFDA dilution and the plates were returned to the incubator. Finally, the plates were transferred to the microplate reader without washing and fluorescence intensity was measured at 540 nm on a Victor 1420 multilabel counter.

3.3.8 Nitrite/nitrate colorimetric assay

Under inflammatory conditions, NO is produced by macrophages and microglia, among other cells, such as hepatocytes and fibroblasts. The end products are nitrite (NO_2^-) and nitrate (NO_3^-). To get an indication about iNOS activation in 2-ClHDA-treated BV-2 cells, the detection kit was used according to the manufacturer's suggestions. The nitrite/nitrate level in both the culture media and the cells was quantitated by using this colorimetric assay.

First a standard curve was established by using 1 ml of 200 µM stock solution (0.9 ml assay buffer and 0.1 ml nitrate standard). For the assay 8 µl of the samples were pipetted in a 96-well plate and diluted with 72 µl of assay buffer (1:10). Two wells were filled 80 µl of the standard solution. 10 µl of enzyme cofactor mixture and 10 µl of nitrate reductase mixture were added to each of the wells. After covering and incubating the 96-well plate, 50 µl of Griess reagent R1 and 50 µl of Griess reagent R2 were pipetted into the wells. As blank 200 µl of water was used. After ten minutes of development at room temperature, the absorbance was measured at 540 nm using a Victor 1420 multilabel counter.

4 Results

This diploma thesis was thought to be a pilot study in order to get first indications on whether MPO-generated plasmalogen-derived oxidation products can affect microglia polarization states.

4.1 2-ClHDA and 2-ClHA reduce viability of BV-2 microglia

In order to get first indications about potential cytotoxic properties of 2-ClHDA and 2-ClHA, the metabolic activity of BV-2 cells was assessed using the MTT test. The results of these experiments are displayed in Figure 4. These data show that 2-ClHDA severely decreased MTT reduction starting at 10 µM after 3.5 hours and half maximal viability was observed at 25 µM 2-ClHDA. After 24 hours BV-2 cell viability was significantly reduced, following treatment with 5 µM 2-ClHDA, while higher concentrations resulted in cell necrosis and completely detached cells (data not shown).

In contrast to 2-ClHDA, 2-ClHA displayed a higher cytotoxic potential after 3.5 and 24 hours of incubation. While 1 µM of the acid was without effect on cell viability after 3.5 and 24 hours, higher concentrations of 2-ClHA drastically reduced or even obliterated metabolic viability in BV-2 microglia.

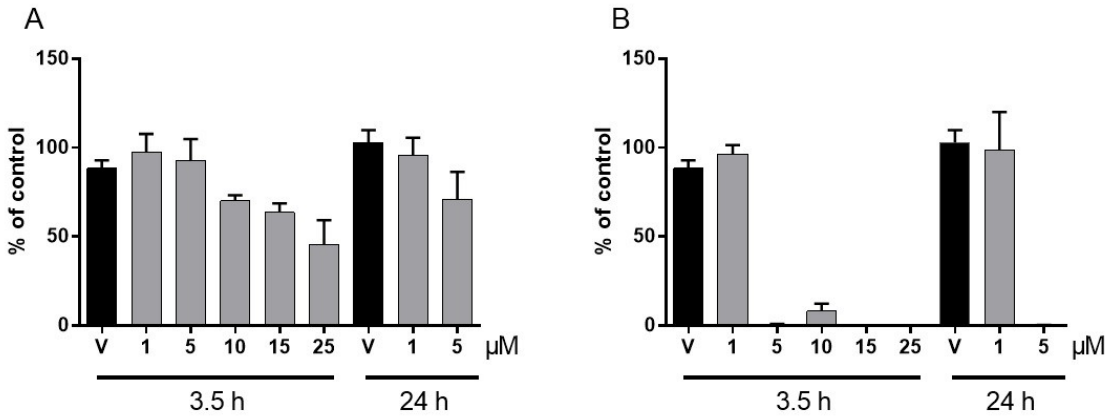

Fig. 4: Effects of 2-ClHDA and 2-ClHA on viability of BV-2 microglia
Cell viability was assessed with the MTT assay. MTT reduction is expressed as % of vehicle (0.2% DMSO, final concentration, 'v'). Serum starved cells were treated with (**A**) 2-ClHDA or (**B**) 2-ClHA at the concentrations and for the times indicated. Data are displayed as mean ± SD of at least triplicate determinations.

4.2 Effects of 2-ClHDA on M1/M2 marker proteins in western blot analysis

While 2-ClHA displayed such strong cytotoxic effects on BV-2 microglia, western blot analysis was performed with 5 µM of 2-ClHDA at different incubation times. The effects of the α-ClFALD are displayed in Figure 5, showing the original blots (Fig. 5A) and their corresponding bar graphs (Fig. 5B).

While the M2 marker Arg1 seems to be downregulated after 8 hours, expression is slowly increased at later timepoints during treatment. 48 hours post-treatment Arg1 is expressed at a 2 ½-fold higher level compared to cells only treated with the vehicle (0,2% DMSO) suggesting an upregulation of M2 microglia after longer incubation times.

iNOS, a marker protein for M1 polarization, displayed increased expression after 8 hours but then declined at 16 and 24 hours to the same levels as the vehicle. Interestingly, iNOS expression again increased 48 hours after treatment up to 250% compared to DMSO. In contrast, the M1 marker COX-2 showed a slight downregulation after 8 hours, but steadily increased afterwards up to almost two-fold levels as the vehicle 48 hours post-treatment.

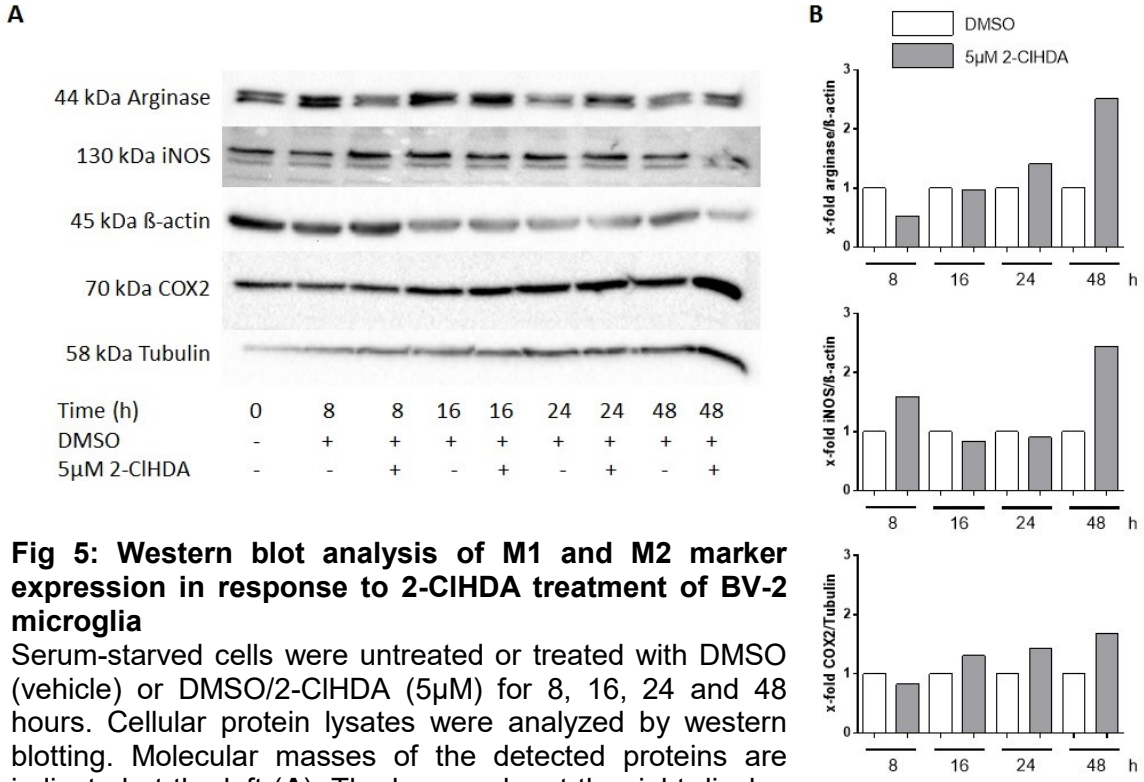

Fig 5: Western blot analysis of M1 and M2 marker expression in response to 2-ClHDA treatment of BV-2 microglia

Serum-starved cells were untreated or treated with DMSO (vehicle) or DMSO/2-ClHDA (5µM) for 8, 16, 24 and 48 hours. Cellular protein lysates were analyzed by western blotting. Molecular masses of the detected proteins are indicated at the left (**A**). The bar graphs at the right display optical density of the protein bands normalized to the corresponding actin or tubulin signal as indicated in the western blots (**B**).

4.3 Flow cytometric analysis of M1/M2 marker expression

Flow cytometry was performed with the M1 polarization marker CD40 and the M2 marker protein CD206. The results of these experiments are displayed in Figure 6, showing the bar graphs and the corresponding scatter plots of both CD40 (Fig. 6A) and CD206 (Fig. 6B) FACS analysis.

16 hours after treatment with 5 µM 2-ClHDA, the M1 marker CD40 (40%) was increased compared to the vehicle 0,2% DMSO (20%). Yet, after 24 hours elevated expression could not be detected. At the last timepoint (48 hours) increased levels compared to DMSO were measured, suggesting a similar trend as iNOS in the western blot experiments. In general, higher levels of CD40 and therefore M1 polarized BV-2 microglia were observed (40% after 16 hours compared to 50% after 48 hours), implying elevated levels of classically activated microglia over time.

CD206 showed a different behaviour compared to its western blot counterpart Arg1. In general, lower levels of M2 polarized microglia and only slightly elevated levels compared to the untreated cells were detected in flow cytometry. Only around 5% of untreated cells, 6% after 16 hours, 10% after 24 hours and 5% after 48 hours of treatment with 5 µM 2-ClHDA expressed CD206. 16 hours post-treatment CD206 marker expression was not elevated compared to the vehicle (0,2% DMSO). After 24 hours DMSO even induced higher levels of M2 polarized microglia (15% of all cells) in comparison to cells treated with 5 µM 2-ClHDA (10% of all cells). 48 hours after treatment, M2 marker expression of BV-2 microglia declined and reached the same level of CD206 expression as untreated cells (5% of all cells). Yet again, DMSO seems to have a greater impact on M2 polarization than 2-ClHDA itself.

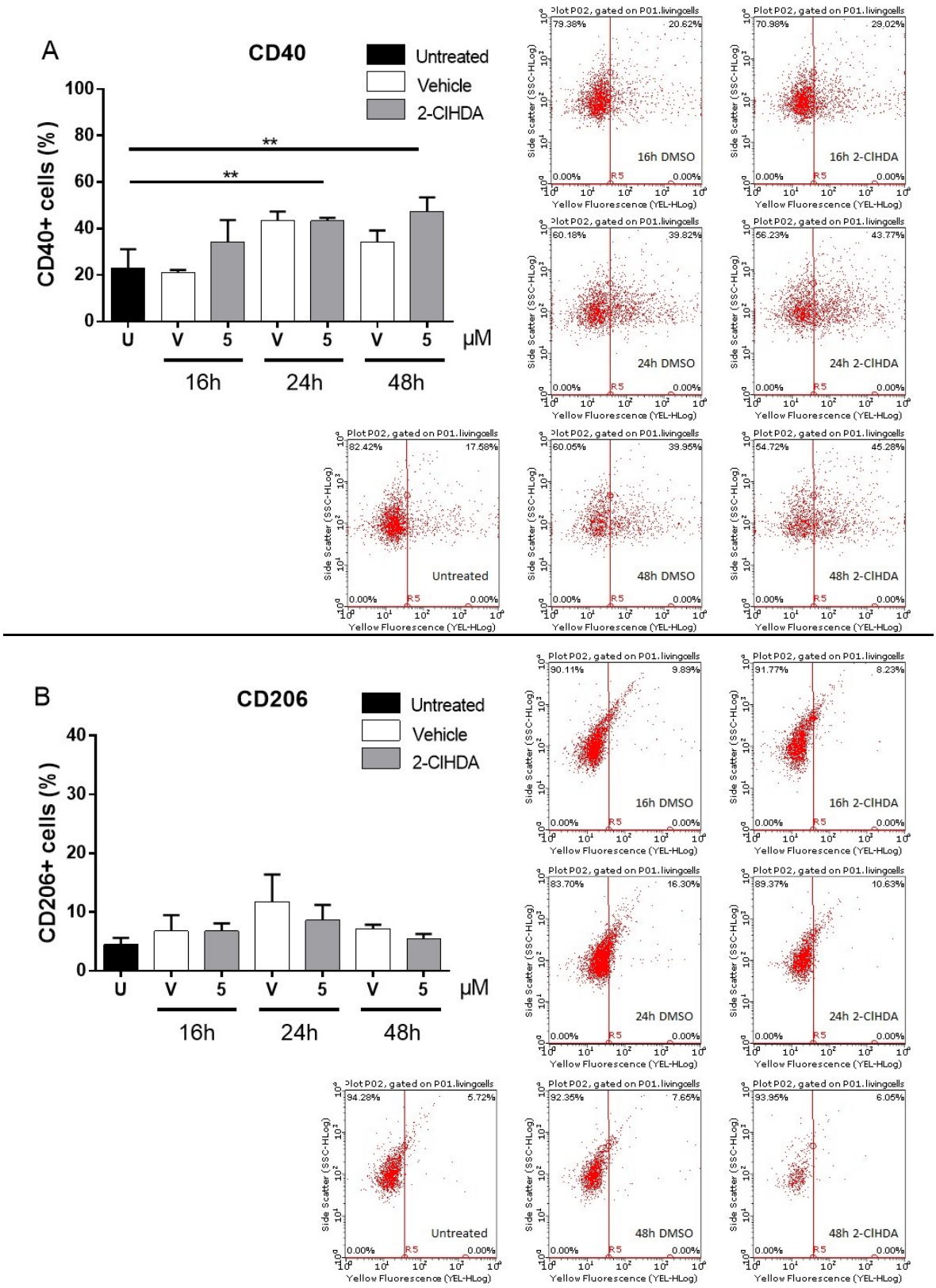

Fig. 6: Flow cytometric analysis of M1 and M2 marker expression in response to 2-ClHDA treatment of BV-2 microglia

Serum-starved cells were treated with 2-ClHDA for the indicated time points. Cells were stained with PE anti-CD40 (**A**) or PE anti-CD206 (**B**) or PE Rat IgG2a as isotype control (1:100) and analyzed using a Guava easycyte flow cytometer. Results (one experiment performed in quadruplicates) are expressed as mean ± SD (*p<0.05, **p<0.01, *** p<0.001; Student's t-test).

4.4 NO_2^-/NO_3^- and ROS levels are unaffected by 2-ClHDA treatment

As readout for iNOS and NADPH-oxidase activity NO_2^-/NO_3^- and ROS concentrations were analyzed. Surprisingly, although immunoreactive iNOS was found to be elevated during western blot analysis (Fig. 5), this was not reflected on the product level since the NO_2^- concentration in the cellular supernatant was unaffected (Fig. 7A).

In addition, DCFDA oxidation was almost unchanged in response to 2-ClHDA treatment (Fig 7B).

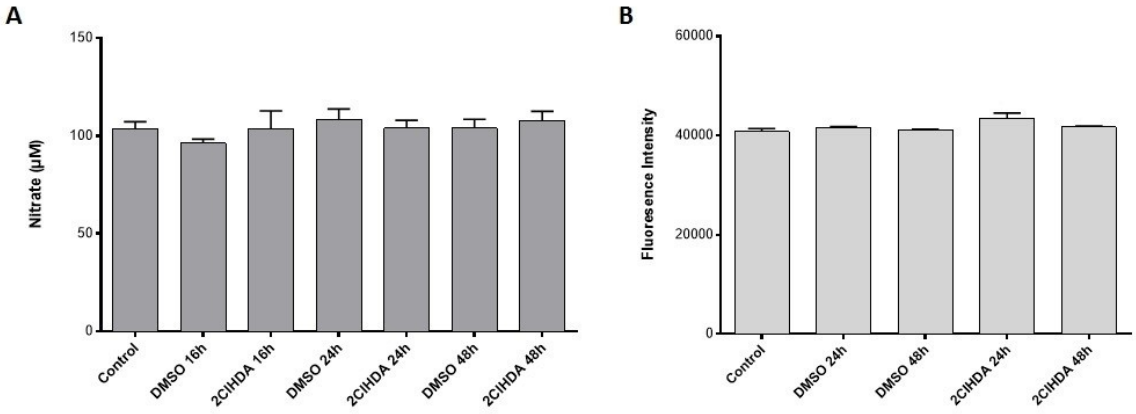

Fig. 7: Effects of 2-ClHDA on NO_2^-/NO_3^- and ROS levels in BV-2 microglia
The production of NO (**A**) in 2-ClHDA-treated BV-2 cells was determined by measuring the content of total nitrite and nitrate in the media. Results (quadruplicate determination) are expressed as mean values ± SD. The cellular redox status (**B**) was determined using fluorescent DCF. Serum-starved BV-2 cells were incubated with DCFDA, treated with 2-ClHDA and fluorescence intensity was evaluated. Results (triplicate determination) are expressed as mean values ± SD.

5 Discussion

The experimental work performed during the present thesis aimed at obtaining initial information on whether there is a connection between plasmalogen-derived oxidation products and microglia polarization states. The major findings can be summarized as follows.

With the MTT assay, the high cytotoxic potential of 2-ClHA after a short period of time was shown, giving first indications on a destructive component of this α-chloro fatty acid in vivo. Compared to the acid the α-ClFALD displayed less harmful properties on BV-2 microglia, although suggesting toxic effects with higher concentrations. With these first results from the cell viability assay, we decided to evaluate the effects of 2-ClHDA after different timepoints using a concentration where the majority of cells is still viable. Western blot and flow cytometry analysis displayed somewhat controversial effects. While a trend toward classically activated M1 microglia was expected, the experimental findings did not confirm the hypothesis, showing an increase in both M1 and M2 markers over time in western blot analysis. However, flow cytometry showed rather different effects of 2-ClHDA on BV-2 microglia. As expected, M1 polarization was induced 48 hours post-treatment and a slight decrease of the M2 marker CD206 was detected. At last, ROS and NO_2^-/NO_3^- levels were unaffected by 2-ClHDA, which was fairly unexpected and leaves room for interpretation and requires additional experimental work.

Since this thesis was thought to be a pilot study on microglia polarization, the results only serve to show a trend of the effects of 2-ClHA and 2-ClHDA on BV-2 cells. One important circumstance is the effect of the vehicle DMSO, which could have a major influence on microglia activation itself. An important limitation to this thesis arises from the fact that specific markers for M1 and M2 microglia do exist, but should be used in a larger set of marker proteins as suggested recently (84).

The effects of MPO and MPO-derived oxidants on BBB function was investigated in earlier studies (85). These data suggest decreased barrier function after 2-ClHDA treatment on BMVEC. Furthermore, it was shown that the MPO-H_2O_2-halide system leads to decreased plasmalogen levels in endothelial cells and generation of α-ClFALDs. In accordance with these findings, a similar reaction to the brain resident immune cells is likely, favoring further neuroinflammation and neurodestruction. This fact makes MPO an interesting target for the medical therapy of neuroinflammatory diseases (86).

Although the concept of M1 and M2 microglia is a useful categorization, it is a debated issue that has yet to be proved. New studies on this object are investigating the benefit of M2-like microglia in vivo. Chakrabarty et al. (87) showed increased M2 polarization accompanied by an exacerbation of amyloid plaques after expression of the anti-inflammatory cytokine IL-4. Yet, it is widely accepted that an inflammatory environment favors M1-like microglia and impedes an M2 switch. Several studies using EAE demonstrated beneficial effects on chronic inflammation through M2 promoting cytokines, such as IL-10 (80) and IL-33 (88). In AD, microglia activation states could have a great impact on disease progression. Although these cells are a source of inflammatory factors, they take part in Aβ phagocytosis and the removal of harmful material (89). On the other hand, Michelucci et al. (90) demonstrated that microglial reactivity and degradation of Aβ was decreased during disease. Another interesting aspect is the alteration of polarization as treatment for neuroinflammatory diseases. Nakajima et al. (91) used mesenchymal stem cell transplantation to promote alternatively activated microglia after spinal cord injury resulting in axonal extension and functional recovery in rats. All these data point toward the complex structure of immunological response in acute and chronic neuroinflammatory diseases and the highly dynamic function of microglia in restoring health.

In conclusion, the influence of 2-ClHDA and 2-ClHA on microglia polarization needs further experimental work and requires more in vitro studies to be able to provide definitive answers. Moreover, in vivo experiments are necessary in order to understand the role of microglia and blood brain barrier dysfunction in pathophysiological processes in humans. If these mechanisms are better understood, it might be possible to pharmacologically target MPO (with e.g. aminobenzoic acid hydrazide), thereby being able to interfere with microglia polarization.

6 References

1. Brown PD, Davies SL, Speake T, Millar ID. Molecular mechanisms of cerebrospinal fluid production. Neuroscience. 2004;129(4):957-70.

2. Engelhardt B, Ransohoff RM. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. Trends Immunol. 2012;33(12):579-89.

3. Cardoso FL, Brites D, Brito MA. Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. Brain Res Rev. 2010;64(2):328-63.

4. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;57(2):178-201.

5. Abbott NJ. Astrocyte-endothelial interactions and blood-brain barrier permeability. J Anat. 2002;200(6):629-38.

6. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nature reviews Molecular cell biology. 2001;2(4):285-93.

7. Persidsky Y, Ramirez SH, Haorah J, Kanmogne GD. Blood-brain barrier: structural components and function under physiologic and pathologic conditions. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology. 2006;1(3):223-36.

8. Huber JD, Egleton RD, Davis TP. Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. Trends in neurosciences. 2001;24(12):719-25.

9. Vorbrodt AW, Dobrogowska DH. Molecular anatomy of intercellular junctions in brain endothelial and epithelial barriers: electron microscopist's view. Brain research Brain research reviews. 2003;42(3):221-42.

10. Stamatovic SM, Keep RF, Andjelkovic AV. Brain endothelial cell-cell junctions: how to "open" the blood brain barrier. Current neuropharmacology. 2008;6(3):179-92.

11. Hawkins BT, Davis TP. The blood-brain barrier/neurovascular unit in health and disease. Pharmacological reviews. 2005;57(2):173-85.

12. Kim JH, Kim JH, Park JA, Lee SW, Kim WJ, Yu YS, et al. Blood-neural barrier: intercellular communication at glio-vascular interface. Journal of biochemistry and molecular biology. 2006;39(4):339-45.

13. Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. Glial and neuronal control of brain blood flow. Nature. 2010;468(7321):232-43.

14. Engelhardt S, Patkar S, Ogunshola OO. Cell-specific blood-brain barrier regulation in health and disease: a focus on hypoxia. British journal of pharmacology. 2014;171(5):1210-30.

15. Krum JM, Kenyon KL, Rosenstein JM. Expression of blood-brain barrier characteristics following neuronal loss and astroglial damage after administration of anti-Thy-1 immunotoxin. Experimental neurology. 1997;146(1):33-45.

16. Willis CL, Nolan CC, Reith SN, Lister T, Prior MJ, Guerin CJ, et al. Focal astrocyte loss is followed by microvascular damage, with subsequent repair of the blood-brain barrier in the apparent absence of direct astrocytic contact. Glia. 2004;45(4):325-37.

17. Daneman R, Prat A. The blood-brain barrier. Cold Spring Harbor perspectives in biology. 2015;7(1):a020412.

18. Shepro D, Morel NM. Pericyte physiology. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1993;7(11):1031-8.

19. Hall CN, Reynell C, Gesslein B, Hamilton NB, Mishra A, Sutherland BA, et al. Capillary pericytes regulate cerebral blood flow in health and disease. Nature. 2014;508(7494):55-60.

20. Hori S, Ohtsuki S, Hosoya K, Nakashima E, Terasaki T. A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. Journal of neurochemistry. 2004;89(2):503-13.

21. Al Ahmad A, Gassmann M, Ogunshola OO. Maintaining blood-brain barrier integrity: pericytes perform better than astrocytes during prolonged oxygen deprivation. Journal of cellular physiology. 2009;218(3):612-22.

22. Nakagawa S, Deli MA, Nakao S, Honda M, Hayashi K, Nakaoke R, et al. Pericytes from brain microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. Cellular and molecular neurobiology. 2007;27(6):687-94.

23. Nakagawa S, Deli MA, Kawaguchi H, Shimizudani T, Shimono T, Kittel A, et al. A new blood-brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes. Neurochemistry international. 2009;54(3-4):253-63.

24. Greenwood J, Heasman SJ, Alvarez JI, Prat A, Lyck R, Engelhardt B. Review: leucocyte-endothelial cell crosstalk at the blood-brain barrier: a prerequisite for successful immune cell entry to the brain. Neuropathology and applied neurobiology. 2011;37(1):24-39.

25. Feng D, Nagy JA, Pyne K, Dvorak HF, Dvorak AM. Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. The Journal of experimental medicine. 1998;187(6):903-15.

26. Greter M, Heppner FL, Lemos MP, Odermatt BM, Goebels N, Laufer T, et al. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nature medicine. 2005;11(3):328-34.

27. Larochelle C, Alvarez JI, Prat A. How do immune cells overcome the blood-brain barrier in multiple sclerosis? FEBS letters. 2011;585(23):3770-80.

28. Stanimirovic D, Satoh K. Inflammatory mediators of cerebral endothelium: a role in ischemic brain inflammation. Brain pathology (Zurich, Switzerland). 2000;10(1):113-26.

29. Halliwell B. Oxidative stress and neurodegeneration: where are we now? Journal of neurochemistry. 2006;97(6):1634-58.

30. Freeman LR, Keller JN. Oxidative stress and cerebral endothelial cells: regulation of the blood-brain-barrier and antioxidant based interventions. Biochimica et biophysica acta. 2012;1822(5):822-9.

31. Maki RA, Tyurin VA, Lyon RC, Hamilton RL, DeKosky ST, Kagan VE, et al. Aberrant expression of myeloperoxidase in astrocytes promotes phospholipid oxidation and memory deficits in a mouse model of Alzheimer disease. J Biol Chem. 2009;284(5):3158-69.

32. Choi DK, Pennathur S, Perier C, Tieu K, Teismann P, Wu DC, et al. Ablation of the inflammatory enzyme myeloperoxidase mitigates features of Parkinson's disease in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005;25(28):6594-600.

33. Gray E, Thomas TL, Betmouni S, Scolding N, Love S. Elevated activity and microglial expression of myeloperoxidase in demyelinated cerebral cortex in multiple sclerosis. Brain pathology (Zurich, Switzerland). 2008;18(1):86-95.

34. Chen JW, Breckwoldt MO, Aikawa E, Chiang G, Weissleder R. Myeloperoxidase-targeted imaging of active inflammatory lesions in murine experimental autoimmune encephalomyelitis. Brain : a journal of neurology. 2008;131(Pt 4):1123-33.

35. Forghani R, Wojtkiewicz GR, Zhang Y, Seeburg D, Bautz BR, Pulli B, et al. Demyelinating diseases: myeloperoxidase as an imaging biomarker and therapeutic target. Radiology. 2012;263(2):451-60.

36. Forghani R, Kim HJ, Wojtkiewicz GR, Bure L, Wu Y, Hayase M, et al. Myeloperoxidase propagates damage and is a potential therapeutic target for subacute stroke. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2015;35(3):485-93.

37. Miric D, Katanic R, Kisic B, Zoric L, Miric B, Mitic R, et al. Oxidative stress and myeloperoxidase activity during bacterial meningitis: effects of febrile episodes and the BBB permeability. Clinical biochemistry. 2010;43(3):246-52.

38. Christen S, Schaper M, Lykkesfeldt J, Siegenthaler C, Bifrare YD, Banic S, et al. Oxidative stress in brain during experimental bacterial meningitis: differential effects of alpha-phenyl-tert-butyl nitrone and N-acetylcysteine treatment. Free radical biology & medicine. 2001;31(6):754-62.

39. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. Journal of leukocyte biology. 2013;93(2):185-98.

40. Davies MJ, Hawkins CL, Pattison DI, Rees MD. Mammalian heme peroxidases: from molecular mechanisms to health implications. Antioxidants & redox signaling. 2008;10(7):1199-234.

41. Braverman NE, Moser AB. Functions of plasmalogen lipids in health and disease. Biochimica et biophysica acta. 2012;1822(9):1442-52.

42. Yap YW, Whiteman M, Cheung NS. Chlorinative stress: an under appreciated mediator of neurodegeneration? Cellular signalling. 2007;19(2):219-28.

43. Babior BM. Phagocytes and oxidative stress. The American journal of medicine. 2000;109(1):33-44.

44. Ford DA. Lipid oxidation by hypochlorous acid: chlorinated lipids in atherosclerosis and myocardial ischemia. Clinical lipidology. 2010;5(6):835-52.

45. Thukkani AK, Hsu FF, Crowley JR, Wysolmerski RB, Albert CJ, Ford DA. Reactive chlorinating species produced during neutrophil activation target tissue plasmalogens: production of the chemoattractant, 2-chlorohexadecanal. J Biol Chem. 2002;277(6):3842-9.

46. Thukkani AK, McHowat J, Hsu FF, Brennan ML, Hazen SL, Ford DA. Identification of alpha-chloro fatty aldehydes and unsaturated lysophosphatidylcholine molecular species in human atherosclerotic lesions. Circulation. 2003;108(25):3128-33.

47. Thukkani AK, Martinson BD, Albert CJ, Vogler GA, Ford DA. Neutrophil-mediated accumulation of 2-ClHDA during myocardial infarction: 2-ClHDA-mediated myocardial injury. American journal of physiology Heart and circulatory physiology. 2005;288(6):H2955-64.

48. Ullen A, Fauler G, Bernhart E, Nusshold C, Reicher H, Leis HJ, et al. Phloretin ameliorates 2-chlorohexadecanal-mediated brain microvascular endothelial cell dysfunction in vitro. Free radical biology & medicine. 2012;53(9):1770-81.

49. Marsche G, Heller R, Fauler G, Kovacevic A, Nuszkowski A, Graier W, et al. 2-chlorohexadecanal derived from hypochlorite-modified high-density lipoprotein-associated plasmalogen is a natural inhibitor of endothelial nitric oxide biosynthesis. Arteriosclerosis, thrombosis, and vascular biology. 2004;24(12):2302-6.

50. Messner MC, Albert CJ, Ford DA. 2-Chlorohexadecanal and 2-chlorohexadecanoic acid induce COX-2 expression in human coronary artery endothelial cells. Lipids. 2008;43(7):581-8.

51. Ullen A, Fauler G, Kofeler H, Waltl S, Nusshold C, Bernhart E, et al. Mouse brain plasmalogens are targets for hypochlorous acid-mediated modification in vitro and in vivo. Free radical biology & medicine. 2010;49(11):1655-65.

52. Galea I, Bechmann I, Perry VH. What is immune privilege (not)? Trends Immunol. 2007;28(1):12-8.

53. Nayak D, Roth TL, McGavern DB. Microglia development and function. Annual review of immunology. 2014;32:367-402.

54. Alliot F, Godin I, Pessac B. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Brain research Developmental brain research. 1999;117(2):145-52.

55. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science (New York, NY). 2010;330(6005):841-5.

56. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science (New York, NY). 2012;336(6077):86-90.

57. Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, Perdiguero EG, et al. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nature neuroscience. 2013;16(3):273-80.

58. Mossadegh-Keller N, Sarrazin S, Kandalla PK, Espinosa L, Stanley ER, Nutt SL, et al. M-CSF instructs myeloid lineage fate in single haematopoietic stem cells. Nature. 2013;497(7448):239-43.

59. Ness JK, Wood TL. Insulin-like growth factor I, but not neurotrophin-3, sustains Akt activation and provides long-term protection of immature oligodendrocytes from glutamate-mediated apoptosis. Molecular and cellular neurosciences. 2002;20(3):476-88.

60. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. The Journal of experimental medicine. 2005;201(4):647-57.

61. Roumier A, Bechade C, Poncer JC, Smalla KH, Tomasello E, Vivier E, et al. Impaired synaptic function in the microglial KARAP/DAP12-deficient mouse. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2004;24(50):11421-8.

62. Tremblay ME, Lowery RL, Majewska AK. Microglial interactions with synapses are modulated by visual experience. PLoS biology. 2010;8(11):e1000527.

63. Ji K, Akgul G, Wollmuth LP, Tsirka SE. Microglia actively regulate the number of functional synapses. PloS one. 2013;8(2):e56293.

64. Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, et al. The classical complement cascade mediates CNS synapse elimination. Cell. 2007;131(6):1164-78.

65. Colton CA. Heterogeneity of microglial activation in the innate immune response in the brain. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology. 2009;4(4):399-418.

66. Boche D, Perry VH, Nicoll JA. Review: activation patterns of microglia and their identification in the human brain. Neuropathology and applied neurobiology. 2013;39(1):3-18.

67. Chen Z, Trapp BD. Microglia and neuroprotection. Journal of neurochemistry. 2015.

68. Skeen MJ, Miller MA, Shinnick TM, Ziegler HK. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. Journal of immunology (Baltimore, Md : 1950). 1996;156(3):1196-206.

69. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annual review of immunology. 1997;15:323-50.

70. Bagasra O, Michaels FH, Zheng YM, Bobroski LE, Spitsin SV, Fu ZF, et al. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(26):12041-5.

71. Varin A, Gordon S. Alternative activation of macrophages: immune function and cellular biology. Immunobiology. 2009;214(7):630-41.

72. Galea I, Palin K, Newman TA, Van Rooijen N, Perry VH, Boche D. Mannose receptor expression specifically reveals perivascular macrophages in normal, injured, and diseased mouse brain. Glia. 2005;49(3):375-84.

73. Cherry JD, Olschowka JA, O'Banion MK. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. Journal of neuroinflammation. 2014;11:98.

74. Edwards JP, Zhang X, Frauwirth KA, Mosser DM. Biochemical and functional characterization of three activated macrophage populations. Journal of leukocyte biology. 2006;80(6):1298-307.

75. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004;25(12):677-86.

76. Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29(43):13435-44.

77. Hsieh CL, Kim CC, Ryba BE, Niemi EC, Bando JK, Locksley RM, et al. Traumatic brain injury induces macrophage subsets in the brain. European journal of immunology. 2013;43(8):2010-22.

78. Antel J, Antel S, Caramanos Z, Arnold DL, Kuhlmann T. Primary progressive multiple sclerosis: part of the MS disease spectrum or separate disease entity? Acta neuropathologica. 2012;123(5):627-38.

79. Jadidi-Niaragh F, Mirshafiey A. Th17 cell, the new player of neuroinflammatory process in multiple sclerosis. Scandinavian journal of immunology. 2011;74(1):1-13.

80. Yang J, Jiang Z, Fitzgerald DC, Ma C, Yu S, Li H, et al. Adult neural stem cells expressing IL-10 confer potent immunomodulation and remyelination in experimental autoimmune encephalitis. The Journal of clinical investigation. 2009;119(12):3678-91.

81. Koenigsknecht-Talboo J, Landreth GE. Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005;25(36):8240-9.

82. Yamamoto M, Kiyota T, Walsh SM, Liu J, Kipnis J, Ikezu T. Cytokine-mediated inhibition of fibrillar amyloid-beta peptide degradation by human mononuclear phagocytes. Journal of immunology (Baltimore, Md : 1950). 2008;181(6):3877-86.

83. Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? British journal of pharmacology. 2004;142(2):231-55.

84. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14-20.

85. Ullen A, Singewald E, Konya V, Fauler G, Reicher H, Nusshold C, et al. Myeloperoxidase-derived oxidants induce blood-brain barrier dysfunction in vitro and in vivo. PloS one. 2013;8(5):e64034.

86. Malle E, Furtmuller PG, Sattler W, Obinger C. Myeloperoxidase: a target for new drug development? British journal of pharmacology. 2007;152(6):838-54.

87. Chakrabarty P, Tianbai L, Herring A, Ceballos-Diaz C, Das P, Golde TE. Hippocampal expression of murine IL-4 results in exacerbation of amyloid deposition. Molecular neurodegeneration. 2012;7:36.

88. Jiang HR, Milovanovic M, Allan D, Niedbala W, Besnard AG, Fukada SY, et al. IL-33 attenuates EAE by suppressing IL-17 and IFN-gamma production and inducing alternatively activated macrophages. European journal of immunology. 2012;42(7):1804-14.

89. Lee CY, Landreth GE. The role of microglia in amyloid clearance from the AD brain. Journal of neural transmission (Vienna, Austria : 1996). 2010;117(8):949-60.

90. Michelucci A, Heurtaux T, Grandbarbe L, Morga E, Heuschling P. Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-beta. Journal of neuroimmunology. 2009;210(1-2):3-12.

91. Nakajima H, Uchida K, Guerrero AR, Watanabe S, Sugita D, Takeura N, et al. Transplantation of mesenchymal stem cells promotes an alternative pathway of macrophage activation and functional recovery after spinal cord injury. Journal of neurotrauma. 2012;29(8):1614-25.