NOVA SCHOOL OF SCIENCE & TECHNOLOGY

Department of Chemistry

Inês Portela Menezes Barbosa Vieira

Bachelor of Science in Biochemistry

Carbon monoxide-mediated microglial phagocytosis of live neurons – role of CO in microglial engulfment of neurons

Master of Science in Biochemistry for Health

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Abstract

In the Central Nervous System, microglia are the immunocompetent cells that control inflammation. In response to certain stimuli, these cells can be activated and polarize towards a pro-inflammatory response. Microglia have the ability to phagocyte dead cells and toxic factors. The process of phagocytosis is regulated by several cell surface receptors. One of these receptors is CD200R, which is expressed by microglia and, by binding to the CD200 ligand expressed on neurons, elicit anti-inflammatory activity and modulate phagocytosis.

In a scenario of exacerbated inflammation, microglia can phagocyte viable neurons, which enter in a programmed cell death process by phagocytosis, known as phagoptosis. Carbon monoxide (CO), a gas endogenously produced with anti-inflammatory and anti-apoptotic properties. Previous work by our group indicates that CO may also be important in modulating microglial phagocytosis of dead neurons under inflammatory conditions. Therefore, this work set out to evaluate what the role of CO in the microglial phagocytosis of live neurons is and whether CD200/CD200R signalling is involved.

Engulfment of neurons by microglia was assessed by measuring co-localization of microglial BV-2 cell line and neuronal CAD cell line using flow cytometry. It was found that there was an increase of microglial engulfment of neurons after LPS treatment, indicating the activation of phagoptosis. In contrast when microglia were pre-treated with the CO-releasing molecule ALF-826, engulfment of live neurons was reverted. We evaluated whether CD200R was involved in this reversal by silencing this gene expression. Preliminary data indicates, that after silencing CD200R, LPS did not increase engulfment and there were no differences with CO pre-treatment. This data suggests that a lack of CD200/CD200R signalling may be protective against phagocytosis of live neurons.

Finally, because peroxisomes are important organelles modulating inflammation and phagocytosis, we evaluated whether the anti-inflammatory action of CO involves these organelles. Three different BV-2 microglia cell lines were used, the wildtype (WT), a knockout of the ACOX-1 peroxisomal enzyme gene and a double knockout of the ABCD1 and ABCD2 peroxisome transporter genes. The integrity of the peroxisomes was assessed indirectly by lipid peroxidation using the thiobarbituric acid reaction (TBARs) assay. Preliminary data indicates that in the mutant strains there is a decrease of lipid peroxidation under inflammatory conditions.

Keywords: Inflammation, Microglia, Phagocytosis, Phagoptosis, CD200, Peroxisome

Resumo

No sistema nervoso central, as células imunocompetentes que despoletam a resposta inflamatória são as células da microglia. Estas células em respostas a determinados estímulos podem ser ativadas e polarizadas para uma resposta pró-inflamatória. A microglia têm a capacidade de fagocitar células mortas ou debris celular tóxico. O processo de fagocitose é regulado por sinalização por parte de recetores da superfície celular. Um destes recetores é o CD200R que é expresso pela microglia e em ligação com o ligando CD200, expresso por neurónios, tem atividade anti-inflamatória e modula a fagocitose.

Em cenários de inflamação exagerada, a microglia pode fagocitar neurónios viáveis, que entram num processo de morte celular por fagocitose, a fagoptose. O monóxido de carbono (CO), um gás produzido endogenamente, já foi comprovado como uma molécula anti-inflamatória e anti-apoptótica. Trabalho prévio do grupo indica que o CO também seja importante na modulação da fagocitose microglial de neurónios mortos em condições de inflamação. Deste modo foi-se avaliar qual o papel do CO na fagocitose microglial de neurónios vivos e se o par CD200/CD200R está envolvido.

O engulfment de neurónios pela microglia foi avaliado através de citometria de fluxo que permite medir a co localização de microglia BV-2 e neurónios CAD. Verificou-se que houve um aumento de engulfment após tratamento de LPS, mas que pré-tratamento com a CO-releasing molecule ALF-826 revertia este aumento. Avaliou-se se o CD200R estava envolvido nesta reversão ao fazer um silenciamento deste gene. Dados preliminares indicam que com silenciamento, o LPS não aumentou o engulfment e não se verificaram diferenças com pré-tratamento com CO. Estes dados sugerem que a falta de sinalização CD200/CD200R pode ser protetora contra a fagocitose de neurónios vivos.

Por fim, como os peroxisomas são organelos importantes na regulação da inflamação e da fagocitose, foi-se avaliar se a ação anti-inflamatória do CO envolve estes organelos. Para tal utilizaram-se três linhas celulares de microglia BV-2, a linha wildtype, uma com knockout no gene do enzima peroxisomal ACOX-1 e outra com knockout duplo dos genes dos transportadores peroxisomais ABCD1 e ABCD2. Avaliou-se a integridade dos peroxisomas pela sua peroxidação lipídica através do ensaio da reação do ácido tiobarbitúrico (TBARs). Dados preliminares indicam que nas estirpes mutantes há uma diminuição da peroxidação lipídica em condições inflamatórias.

Palavras-chave: Inflamação, Microglia, Fagocitose, Fagoptose, CD200, Peroxisoma

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Abbreviation	Definition
5-TAMRA	5-(and-6)-carboxytetramethylrhodamine N-succinimidyl ester
ABC	ATP binding cassette
ACOX	Acyl-CoA oxidase
Arg-1	Arginase-1
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
CAD	Cath.-a-differentiated
CD200R	CD200 receptor
CNS	Central Nervous System
CO	Carbon monoxide
COHb	Carboxyhaemoglobin
CORM	CO-releasing molecule
DMSO	Dimethyl sulfoxide
FA	Fatty acid
FAD	Flavin adenine dinucleotide
FBS	Foetal bovine serum
Hb	Haemoglobin
HO	Haem oxygenase
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor 1
IgSF	Immunoglobulin superfamily
IL	Interleukin
iNOS	inducible NO synthetase
LPS	Lipopolysaccharide
MDA	Malonaldehyde
NBD	Nucleotide binding domain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domains
P/S	Penicillin/Streptomycin

PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PNS	Peripheral Nervous System
PS	Phosphatidylserine
ROS	Reactive oxygen species
TBARs	Thiobarbituric acid reactive substance
TLR	Toll-like receptor,
TMD	Transmembrane domain
TNF-α	Tumour necrosis factor α
TREM-2	Triggering receptor expressed on myeloid cells 2
VLC-FA	Very long chain fatty acid
WT	Wildtype
X-ALD	X-linked adrenoleukodystrophy

Introduction

1. Central Nervous System

The Nervous System is responsible for the control and communication of the body. It modulates memory and cognitive functions, while also regulating and maintaining body homeostasis [1]. This system can be divided into to two: the Central Nervous System (CNS) and the Peripheral Nervous System (PNS) [1,2].The CNS is constituted by the brain and the spinal cord. The brain is an organ of the utmost importance made of nervous tissue contained within the skull. The spinal cord is found in the vertebral column and it sends the brain's commands to the peripheral body and communicates sensory information from the organs to the brain [1,2]. The CNS controls voluntary movements (walking and speech), involuntary movements (breathing and reflex actions) and is the centre of emotion and cognition [2]. The PNS consists of the nerves outside the brain and spinal cord, connecting the CNS to the rest of the body (muscles, endocrine glands, organs and sensory receptors) [2]. This system can be divided into two systems – Autonomous Nervous System and the Somatic Nervous System. The functions of the PNS include sensory perception, coordination of endocrine functions and motor control [1].

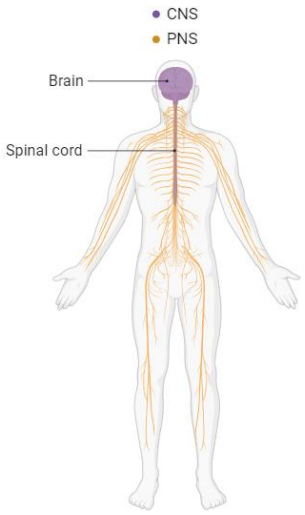

Figure 1 *– Components of the human Nervous System, which include the Central Nervous System (CNS) and its elements (brain and spinal cord) in purple, and the Peripheral Nervous System (PNS) in yellow (adapted from Purves, D. in Neuroscience [2])*

1.1. Neurons

In order to describe the Nervous System, one must first discuss its functional unit - the neuron. Neurons are cells with the capability of generating electrical signals (action potential) through the cell that then is transmitted to other neurons. This transmission of electrical signals is how information is shared within the body [1]. Neurons can present different size and morphology, but most share the following components: cell body or soma, dendrites and the axon (Figure 2) [1].The soma contains the nucleus, ribosomes and important cell organelles like the Golgi complex and the mitochondria [1]. The dendrites are a branched protrusion of the neuron where information from the other neurons is received [1,2]. In the CNS, neurons can have up to 400 000 dendrites [1]. These components increase the surface area of a neuron thus facilitating the communication of signals. The axon is a long process that projects from the neuron soma. In most neurons,

the propagated signals originate in the part of the axon that arises from the cell body which is called the axon hillock [1,2]. Then the signal originated propagates along the neuronal axon until reaching the axon terminal, which in turn will release neurotransmitters from the axon [1]. The axon is insulated by myelin, which enhances the speed of signal transmission (Figure 2).

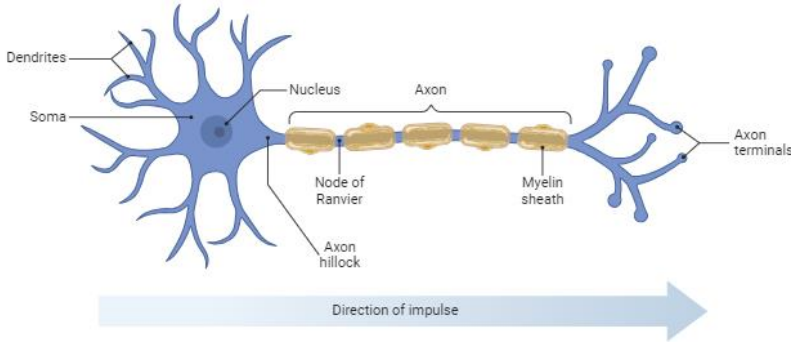

Figure 2 *- Representation of a neuron and its components (adapted from Widmaier, E et al; 2003)*

Neurons can be divided into three functional categories – afferent, efferent and interneurons [1] (Figure 3). Afferent neurons carry out signals from receptors of tissues or organs in the body to the brain and spinal cord in the CNS. These neurons present a specific morphology that consists of a single process (normally an axon) which then divides into a peripheral process and a central process [1]. The peripheral process begins where other afferent neurons terminal branches are. The central process enters the CNS from the periphery and forms junctions with other neurons. On the other hand, efferent neurons carry out information out of the CNS to effector cells like glands or muscles. A typical effector neuron morphology consists of the neuron soma and dendrites within the CNS and the axons in the periphery [1]. Interneurons connect the neurons present in the CNS, thus being restricted to it. They correspond to 99% of the total body's neurons, being associated with many functions and can have varied shapes and properties [1]. The quantity of neurons interposed between specific afferent and efferent neurons depends on the complexity of the process controlled by these neurons.

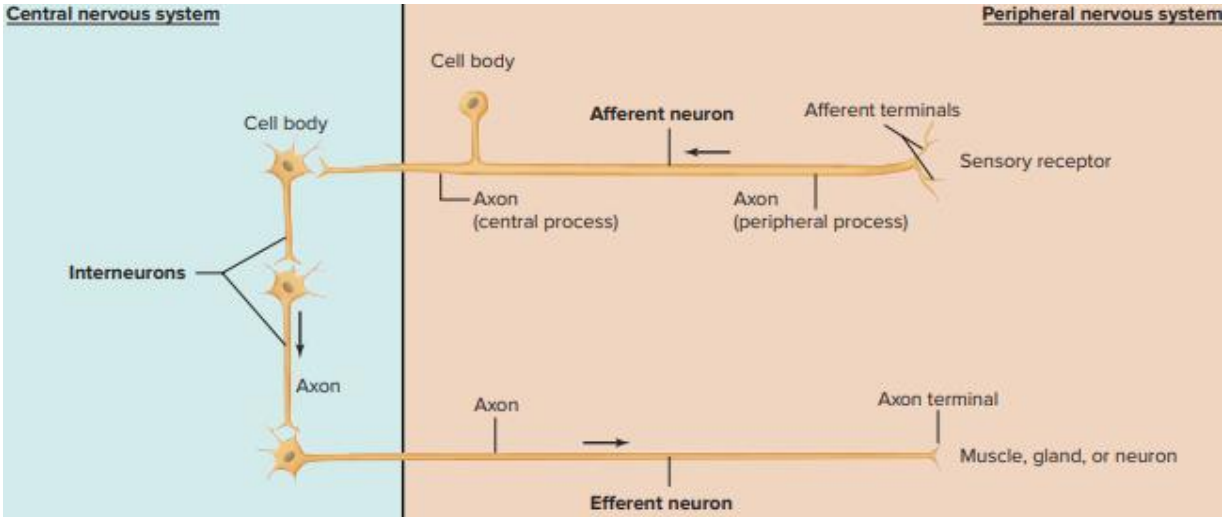

Figure 3 *–The three different types of neurons (afferent, efferent and interneurons). The arrows represent the direction of brain activity transmission (taken from Widmaier, E. et al; 2003)*

1.2. Glial cells

Despite being the functional unit of the CNS, neurons do not represent half of it [1,3,4]. The majority of the cells in the CNS are glial cells. Its name – glia – derives from glue, denoting their important role in the maintenance of the nervous system [4–6]. Glial cells include the macroglia (astrocytes and oligodendrocytes) and the microglia [4]. They are essential in the support and maintenance of neuronal cell function since they provide structural, functional and metabolic support [1,3–7].

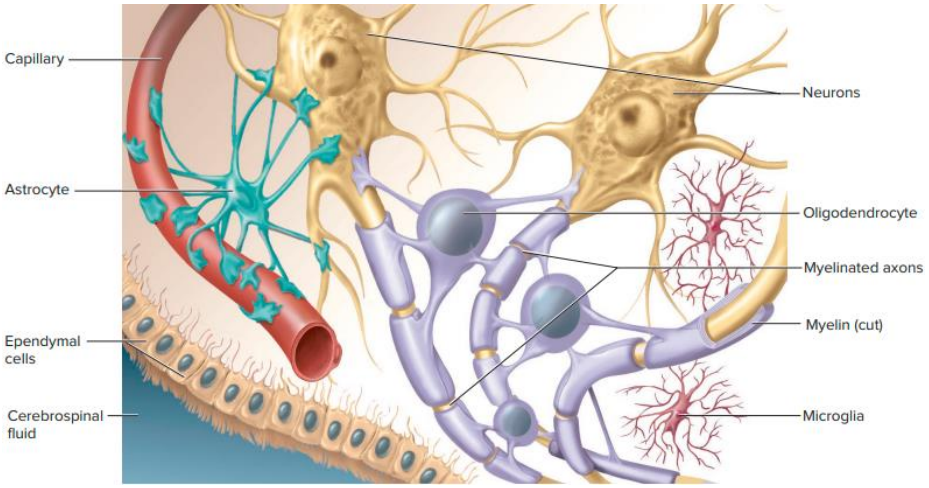

Figure 4 *- Glial cells in the CNS (taken from Widmaier, E. et al; 2003)*

1.2.1. Astrocytes

Astrocytes make up 40 to 60% of cells in the CNS [8]. These cells are characterised by their star shape which gives them their name. The main function associated with astrocytes is the metabolic support of neurons [1,3]. Astrocytes provide to neuronal cells the carbon sources (glucose or lactate) needed to exert their function. Likewise, astrocytes maintain the composition of extracellular fluid of the CNS by removing metabolic products such as potassium ions and neurotransmitters [1]. Astrocytes are also key cells involved in the formation of tight junctions between the endothelial cells near capillaries of the CNS, leading to the formation of the Blood Brain Barrier (BBB) [1]. The BBB is a very important structure in the maintenance of homeostasis in the CNS, by inhibiting the uncontrolled infiltration of blood cells and circulating toxic factors into the brain tissue, and by regulating nutrients entrance and the release of cerebral toxic metabolites.

1.2.2. Oligodendrocytes

The axons of neurons are enveloped by 20 to 200 layers of a modified plasma membrane called myelin (Figure 2). Myelin allows for the conservation of energy and a better conduction of electrical signals [1]. The cell population responsible for myelin production in the CNS are the oligodendrocytes [1,3,7] and in the PNS the Schwann cells [1,3]. For oligodendrocytes in order to exert their myelinating function, they must go through a complex cell differentiation process, evolving from oligodendrocyte precursor cells (OPCs) [7]. The differentiation process is provoked by various internal and external factors, including interactions with factors derived from axons [6]. Besides their myelinating function oligodendrocytes assist in neuron metabolism [3]. In the PNS, axon myelination is done by Schwann cells. Schwann cells are the PNS equivalent of the glia, also having immune and metabolic functions [1,7].

1.2.3. Ependymal cells

Ependymal cells are epithelial cells with multiple cilia [9] that line the spinal cord and cerebral ventricles to regulate the production and flow of cerebrospinal fluid [1,9] (Figure 4). These cells show great heterogeneity, varying in location, surface markers, morphology and function [9].

2. Microglia

After briefly describing astrocytes, oligodendrocytes and ependymal cells, the main focus of this thesis, microglia, will be discussed. Microglia are the phagocytic and immunocompetent cells of the CNS, playing a vital role in maintaining the health and function of the nervous system [4,10,11]. These cells make up 5 to 10% of the CNS [4,12] and are evenly distributed throughout it [6,13]. Microglia were first described by Cajal in the late 1880s, defining them as a 'third element' of parenchymal origin since these cells were neither astrocytes nor neurons, the known neural cells at the time. The concept of the microglia as an individual cell type would be defined later in 1919 by Pio Hortega [14] who using a novel brain staining technique based on silver nitrate was able to identify two cell types in this 'third element' – 'interfascicular glia' (now known as oligodendrocytes) and 'phagocytic cells of mesodermal origin' (the microglia). Pio Hortega defined the microglia as a small cell population of mesodermal origin that migrate to the brain with an amoeboid morphology, acquiring a ramified phenotype in the CNS. In response to stimuli, microglia can change shape and lose their ramifications. The researcher observed that the microglia could migrate within the CNS and phagocytise other cells. All these assessments can still hold up to modern research findings [13].

2.1. Microglial origin

Unlike the other cells of the CNS (neurons, astrocytes or oligodendrocyte) which have an ectodermal origin [6,15], the microglia are thought to have mesodermal origin, meaning that they derive from progenitors from the periphery that migrated to the CNS (Figure 5) [6,13,15]. Microglia originate from primitive myeloid progenitors, that themselves are originated from embryonic yolk sac [6]. It is thought that these myeloid progenitor cells enter the brain tissue through batches before the closing of the BBB [6]. The work of McKercher [16] and his associates in 1996 helped reinforce the notion of microglia as cells with myeloid origin. They noticed that in mice lacking a transcription factor expressed exclusively in hematopoietic cells [16] presented no microglial cells in their CNS [15,16]. In adulthood, microglia seem capable of self-renewal, independently of blood myeloid cells, after colonization of the brain by the myeloid progenitors [17].

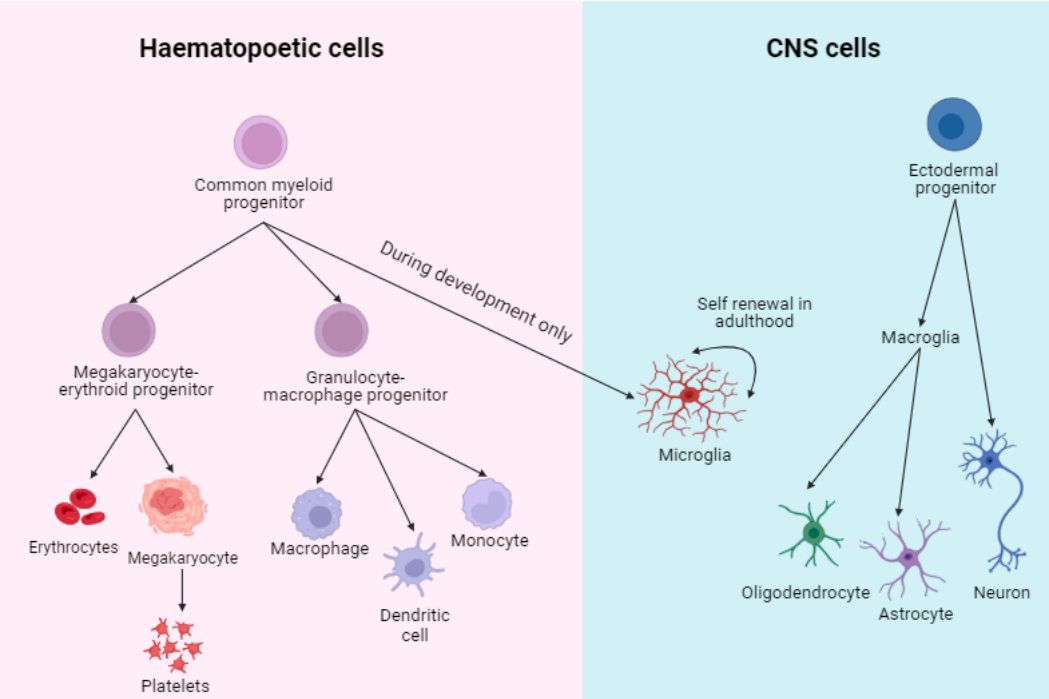

Figure 5 *– Myeloid origin of the microglia, arrows indicate lineage similarity (adapted from Ransohoff, R et al; 2010)*

Three types of microglia phenotypes can be defined. A representation of these different forms of the microglia is on Figure 6. Amoeboid microglia are embryonic, immunocompetent and phagocytic. As they develop and reach maturity, amoeboid microglia can assume a 'resting'/ramified phenotype or an 'activated' phenotype [6,13]. In the so called 'resting' phenotype phagocytosis and immune activity are down-regulated and microglia patrol the microenvironment for any toxic element. Whereas in response to CNS injuries, microglia are 'activated' changing phenotype with the retraction of the microglial processes to the cell body. More importantly, with this phenotype, microglia regain their phagocytic and immune activity. The terms 'resting' or 'activated' can be considered restrictive since the 'resting microglia' are not stagnant as the name suggests, but in constant surveillance of the CNS by extending their processes in search of dead cell debris and possible pathogens [4,6,11,13] . On the other hand, 'activated' microglia does not exist in a single state, microglial activation comes in a spectrum of different morphologies and activity.

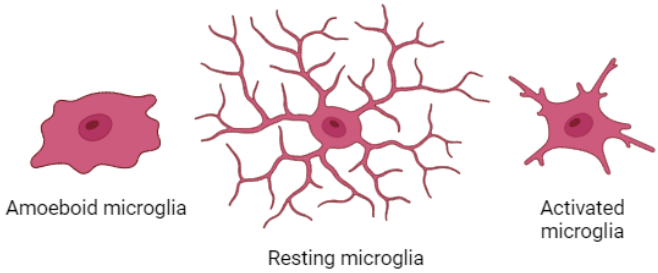

Figure 6 *- Classifications of microglia (adapted from Verkhratsky, A. & Butt, A; 2013)*

2.2. Microglial activation

Microglial activation is a central element of the inflammatory process in the CNS [18,19]. An inflammatory response can be important as it promotes tissue healing and eliminates cellular waste [20]. Chronic or excessive inflammatory reactions, on the other hand, are harmful because they hinder regeneration and may provoke unnecessary cell death [20]. Neuroinflammation is a hallmark of a number of neurodegenerative disorders and is present in brain injury and stroke [18,21]. Considering this, a tight regulation of the inflammatory process is essential to CNS equilibrium and health. Microglia monitor the brain environment for possible system threats and to recognize such threats microglia present immune receptors like toll-like receptors (TLRs), nucleotide-binding oligomerization domains (NODs), NOD-like receptors or ATP receptors [18]. Stimulation by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPS) *via* TLRs or ATP receptors, respectively, can then lead to microglia activation [22].

In phagocytes, activation used to be classified as M1 and M2. This classification of cellular response was made based on T helper lymphocytes *in vitro* activation. Researchers found that depending on the stimuli, different metabolic changes in cytokine or surface marker expression were activated [23]. However, categorising microglia as M1 and M2 can be considered as restrictive [18,23] as the designations 'resting' and 'activated' previously mentioned, since these cells often co-express markers associated with both M1 and M2 activation [23], being the activated state a spectrum.

M1 activation is considered the classical activation and has pro-inflammatory and neurotoxic features [4,18,24]. Exposure to pro-inflammatory cytokines, which include interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α), and/or cellular or bacterial debris, such as myelin debris or lipopolysaccharides respectively, can induce M1 activation [24]. This activation is characterized by (i) the secretion of pro-inflammatory cytokines, such as TNF-α and interleukin-1β (IL-1β), (ii) high expression of inducible NO synthetase (iNOS) for NO production, and (iii) the increased levels of reactive oxygen species (ROS) [18,24]. Cytokines are small proteins produced and secreted by cells that have an important function in cell signalling [25]. Cytokines can act on the cells that release them, neighbouring cells or distant cells [25]. It is to note that the pro-inflammatory cytokines secreted by M1 microglia can polarize further microglia and enhance the inflammatory response [18].

On the other hand, M2 activation is associated with an alternative activation that shows anti-inflammatory and neuroprotective features. M2 is induced by stimuli such as IL-4 or IL-13 [24]. Microglia with M2 activation typically presents the surface markers CD206, CD163 and TREM-2 (triggering receptor expressed on myeloid cells 2). M2 microglia have a secretion pattern distinct from M1 [4], in this instance there is a suppression of secretion of pro-inflammatory cytokines – IL-1β and TNF-α – and an increase of pro-inflammatory ones[18,21,24] – IL-4, IL-10 and transforming growth factor-β (TGF-β). A staple of M2 activation is the enzyme arginase-1 (Arg-1), which competes with the inflammatory mediator iNOS for their common substrate L-Arginine and thus inhibits NO production [4]. Furthermore, M2 microglia can increase the production of neurotrophic factor insulin-like growth factor 1 (IGF-1) to aid in inflammation resolution and neuron survival [20]. M2 microglia counteracts M1 pro-inflammatory responses, contributing to immunosuppression and neuron protection [18]. A key feature of M2

microglia is enhanced phagocytosis, which can allow the clearance of otherwise toxic cell debris resultant from an inflammatory response. The process of phagocytosis will be discussed in the section bellow.

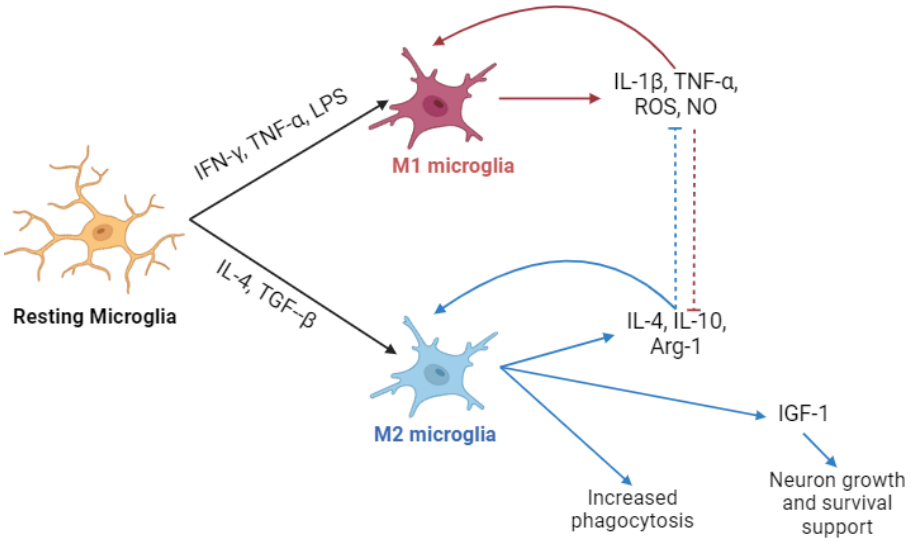

***Figure 7** – M1 and M2 microglia (Adapted from Tang et al; 2016 and Nakagawa and Chiba; 2014)*

3. Phagocytosis

Phagocytosis is a cellular process through which particles bigger than 0.5 µm in diameter, such as bacteria, foreign substances, and apoptotic cells, are ingested and eliminated [26,27]. Phagocytosis is found in many different types of cells and is hence an important process for tissue homeostasis. Nonetheless, only specialised cells known as phagocytes are capable of high-efficiency phagocytosis [26]. Alongside inflammation, this process constitutes the innate immune system's initial line of defence against multicellular organisms [28] and plays an important role in the start of adaptive immune responses [4].

The first step of phagocytosis is the detection of the particle to be internalized. Phagocytes can detect a variety of particles that might be ingested [26,28]. This identification is mediated by a variety of receptors, which detect the particle as a target (eat-me signals [27,28]) and subsequently initiate signalling pathways that promote phagocytosis. The second step of this process is the formation of the phagosome, which can also be termed engulfment [26,28]. Phagocytic receptors activate signalling pathways that cause the actin cytoskeleton and lipids in the membrane to remodel, resulting in the membrane extending to cover the particle [26,29]. The membrane then closes forming an early phagosome, in which the target particle is internalised [26]. The final step of phagocytosis is phagosome maturation. In this maturation, the early phagosome first undergoes a series of fusion and fission processes with endocytic vesicles to form a late phagosome and then the late phagosome merges with lysosomes to form a phagolysosome [26].

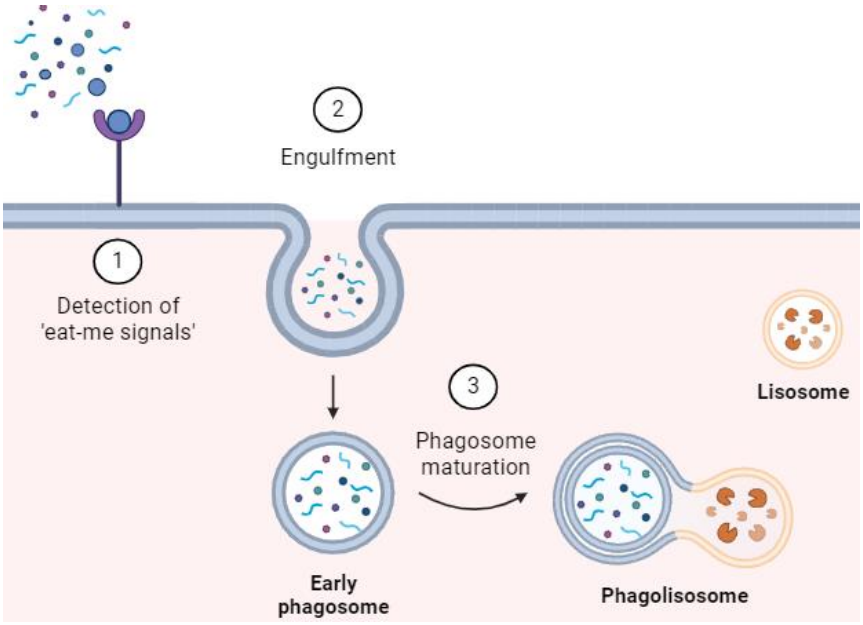

Figure 8 *– Phagocytosis (adapted from Gierlikowski et al; 2020)*

3.1. Microglial phagocytosis

Microglia are the main phagocytes of the brain, containing many of the same surface receptors as other types of phagocytes and perform equivalent physiological tasks [4]. Besides microbes and apoptotic cells, microglia phagocytize content that is specific to the brain which includes axonal and myelin debris in spinal cord injury, amyloid plaques in AD and supernumerary synapses during postnatal development [28]. Phagocytosis of apoptotic cells is indispensable for the normal development of tissues, as during the brain's development, millions of neurons die but leave no trace of toxic debris. This can be attributed to the effective phagocytic action of the microglia [30]. Microglia can undertake the clearance of excitatory inputs in in a process designed synaptic pruning. Synaptic pruning, by reducing excitatory stress, permits injured neurons to recuperate and return to function [11]. Microglia phagocytises immune cells that invade the CNS in cases of a lesion, like neutrophils and leukocytes, [4]. This allows for a control of the CNS immune population.

Nevertheless, there is still a lot that needs to be studied regarding the receptors involved, modes and possible outcomes of microglial phagocytosis and its effect on tissue homeostasis [28].

3.2. Phagoptosis

Phagocytosis is associated with cell death (such as apoptosis or necrosis), being considered a secondary feature of it. Thus, there is the belief that phagocytosis occurs exclusively to consume dead or dying cells. However, research data has shown that phagocytosis can provoke death of live cells in a process known as primary phagocytosis (Figure 9) [31–33]. Brown and associates proposed that cell death can be induced by primary phagocytosis, a process that should be called phagoptosis. In fact, phagoptosis distinguishes from (secondary/normal) phagocytosis, since the first one occurs after cell death, while phagoptosis promotes cell death (Figure 9) [31]. Brown then proposes that phagoptosis is caused by the reversible exposure of phosphatidylserine (PS) or other 'eat-me' signals or by the removal of 'don't-eat-me' signals on the surface of viable cell [31,33]. This change in membrane cell signals are triggered by physiological stimulation or a toxic insult, induced by the phagocyte or elsewhere, to the target cell. The definition of an eat-me signal is a signal that is exposed on or released by a cell to promote phagocytes to phagocytose that cell [27]. Inversely, a don't-eat-me signal is a signal exposed on or released from a cell to prevent phagocytes from phagocytosing [27]. Both these signals are chemotactic factors.

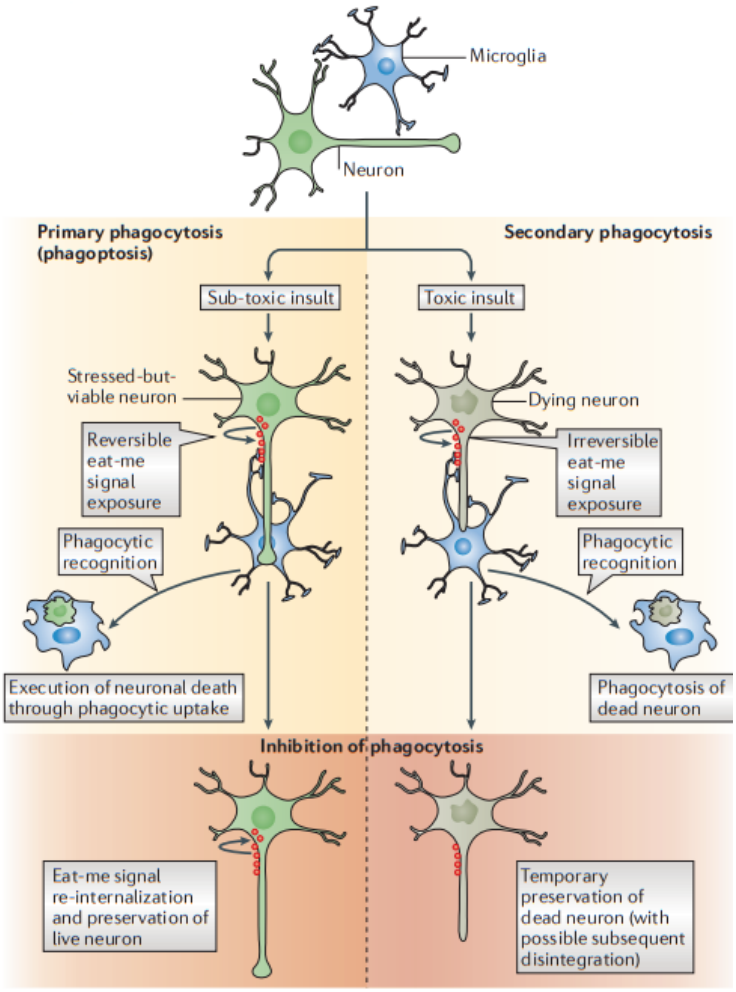

Figure 9 *– Mechanisms of microglial phagoptosis and phagocytosis of neurons (taken from Brown and Neher; 2014)*

3.3. CD200 and CD200 receptor

Ligand-receptor contact is important for the modulation of neuron to microglia communication, as well as microglia activation leading to inflammation or phagocytosis. In this section, the ligand-receptor CD200/CD200R pair which is a key regulator of neuroinflammation and neuron-microglia cross-talk will be discussed.

CD200, previously known as OX-2, is a type I transmembrane glycoprotein that belongs to the immunoglobulin superfamily (IgSF) [34–37]. This protein is composed by two immunoglobulin domains, a transmembrane region, and a short cytoplasmic domain [35]. CD200 is expressed on most immune cells, including T and B cells, and in the CNS it is specifically expressed on neurons. CD200 binds to another transmembrane glycoprotein termed as the CD200 receptor (CD200R) [34–37]. CD200R is expressed on myeloid lineage cells, which include the microglia. The receptor has a similar structure to CD200, but it has more than one isoform [37]. The best studied isoform is CD200R1, which is expressed on myeloid cells and some T cell subsets [37].

Interaction of CD200 and its receptor is regarded as important to immune response modulation and neuron protection [38] as this interaction causes the microglia to remain in a resting state [34,38]. Lyons et al [39] reported that in the brains of aged animals, there was a decrease of CD200 expression, this could explain the increase of microglial activation observed with age [40]. CD200 deficient mice show increased microglial and macrophage activation [34,40] and showed decreased survival after 72 hours of ischaemia [41]. Regarding phagocytosis, there does not seem to be a consensus regarding the action of this interaction. Lyons [40] reported that in microglia prepared from CD200-deficient mice there was an increase of amyloid-β phagocytosis. Whereas Varnum and associates [42] verified that in mice injected with an adeno-associated virus expressing CD200 phagocytosis was increased. Some studies that evaluated the impact of either CD200 or CD200R are summed in Table 1.

Table 1 *– Summary of the reported effects of CD200 or CD200R on microglial phagocytosis*

Effect on phagocytosis	Test model	Disease model	Reference
Increase	Microglia from CD200 $^{-/-}$mice	Parkinson's disease	40
Increase	Microglia from CD200R$^{-/-}$ mice	Ischemic stroke	41
Increase	Injection of WT and APP mice with an adeno-associated virus expressing CD200	Alzheimer's disease	42
Decrease	Microglial cell cultures treated with MPP+ and rotenone (that induced decreased expression of CD200)	Parkinsonian toxicants	43
Increase	CD200 coated PLGA microparticles in contact with macrophages		44

4. Carbon Monoxide

Carbon monoxide (CO) is an odourless, colourless and inert gas [45–48] that consists of a carbon atom triple bonded to an oxygen atom (Figure 10). Most people's first thought when they read CO is to associate it with its toxicity [21]. What confers CO this toxicity? The gas has a binding high affinity with haem proteins [10,45,47], in particular with haemoglobin. In the case of haemoglobin, CO shows 230 times more affinity with this protein than O_2 [46]. Considering that haemoglobin is the protein responsible for oxygen transport to tissues, in a case of extended exposure to CO, haemoglobin will bind to the latter and i) form carboxyhaemoglobin which is regarded as toxic and ii) hinder the transport of oxygen leading to tissue hypoxia, intoxication and possibly death [10,45,47]. In addition, CO inhibits cellular respiration via binding to cytochrome c oxygenase or cytochrome p450, resulting in cell death [47,49]. Nevertheless, the paradigm has started to shift and CO has shown that besides being toxic, it is a metabolite with biological importance. The possibility of CO as a biological molecule would be first introduced in 1949 by Sjostrand [50] when he and his associates discovered the presence of this gas in exhaled air of healthy individuals. This discovery sets CO as an endogenously produced molecule [10,21,45]. In 1968, the source of CO in the human body would be elucidated by Tenhunen [51]. CO is the result of the oxidative degradation of haem catalysed by the haem oxygenase (HO) enzymes.

Figure 10 *- Representation of carbon monoxide molecule*

The reaction catalysed by HO comprises of the first and rate limiting step of haem degradation and consists of the opening Fe – protoporphyrin IX ring into free Fe^{2+}, CO and biliverdin with O_2 and NADPH as electron donors [10,47,52] (Figure 11). HO can be found in all mammalian cells and is an important enzyme to maintenance of redox status and cell equilibrium [49] as it carries out the elimination of potentially toxic free haem group catalyses the production of biliverdin, which is then converted into the antioxidant bilirubin, as well as the production of free iron, which can result in the expression of ferritin promoting further cytoprotection [47,49]. The enzyme has two isoforms: HO-1, the inducible form, and HO-2, the constitutive form [52]. HO-1 in response to stimuli associated with cellular stress (oxidative stress, hypoxia, inflammation, misfolded proteins and UV radiation) has its expression increased so the enzyme can exert its protective action [47–49].

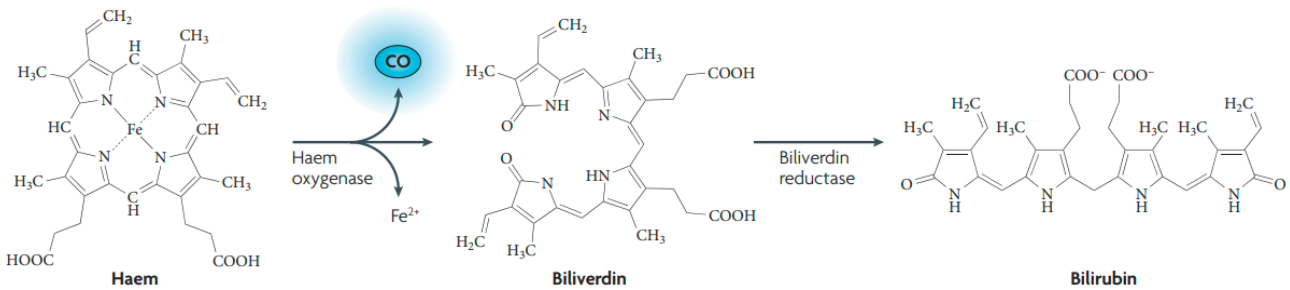

Figure 11 *– Haem degradation pathway (taken from Motterlini, R. & Otterbein, L. E.; 2010)*

As mentioned, HO-1 is an enzyme that show cytoprotective activity and whose activity or expression can be upregulated by stress related stimuli. CO, as a product of HO-1, can exert the enzyme's protective actions [47,48]. While, first regarded as a poison, CO has been found to be an important signalling molecule with functions in inflammation, cell death, cell metabolism and vasodilation [47]. Despite all of CO's reported biological activities, its pathways and targeted molecules in biological systems remain a topic of discussion [10]. It is thought that CO's targets in biological systems include haem-containing proteins [48,49] such as soluble guanylate cyclase, cytochromes, haemoglobin, and myoglobin. CO can only bind to the reduced form of iron (Fe^{2+}), which restricts the number of potential target proteins [10].

In various cell and tissue types, ROS production and signalling mediate CO-induced cytoprotection [47,49]. Two proteins have been identified as directly involved in CO-induced cell redox signalling: cytochrome c oxidase (mitochondrial respiratory complex IV) and NAD(P)H oxidase (plasmatic membrane) [49]. For example, this CO-derived ROS production can activate the anti-inflammatory factor peroxisome proliferator-activated receptor gamma co-activator 1α (PPAR-λ) in macrophages [53], resulting in a decreased release of downstream pro-inflammatory mediators [21].

In the CNS, CO has shown to be neuroprotective as it can reduce neuronal cell death *in vitro* [54] and in *in vivo* models of ischaemia and reperfusion [55,56]. In microglia, it has been observed that CO can inhibits inflammatory response caused by LPS, thrombin, or IFN-γ [57,58]. CO reduces neuroinflammation in microglia by increasing mitochondrial respiration [59].

The action of CO on microglial phagocytosis is not as well studied as CO's action on inflammation. Kaiser et al [60] observed that in the presence of exogenous CO, phagocytosis of erythrocytes was increased in BV-2 microglia cells. Soares et al [21] found that CO treatment can increase the expression of neuronal CD200 and partly reversed the inhibition of expression of microglial CD200R caused by LPS (Figure 12). As discussed in section 3.3, this pair can polarize microglia to a more pro-phagocytic and anti-inflammatory activity.

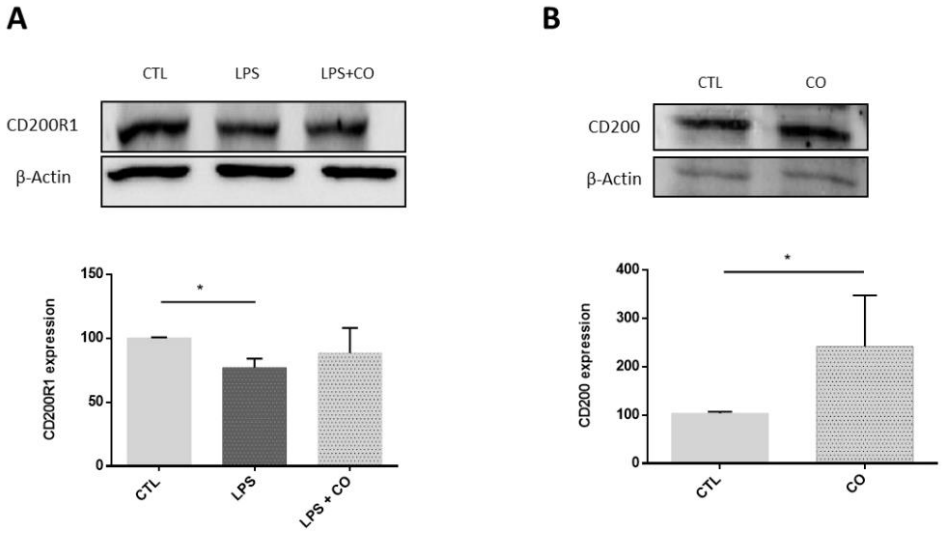

Figure 12 *- CO treatment enhances the CD200-CD200R communication (taken from Soares et al.; 2022)*

4.1. Potential therapeutic use of CO

Considering CO'S cytoprotective effects, the scientific community strived to create CO-based treatments. At first, researchers thought of administering CO in its native gaseous form through inhalation but found it may not the best way. Gaseous CO can interact with haemoglobin once it enters the bloodstream and because of this gas' high binding affinity an increase in the level of toxic carboxyhaemoglobin (COHb) could take place [45,48]. Additionally, because circulating CO cannot distinguish between healthy and diseased tissue, it targets haemoproteins in all tissues, which is the exact opposite of how HO naturally exerts its cytoprotective effect through raising the local concentration of CO at the site of disease through inducible HO-1 expression [45].

Considering the setbacks of direct CO inhalation, another way to administer it was thought – the use of CO-releasing molecules or CORMs [61]. CORMs follow the same principle as a prodrug, meaning that CO is delivered to the organism carried in molecules, which in turn after being activated by a specific stimulus release the gas to the target tissue or cell [45,48]. The interaction of iron in the haemoglobin molecule with CO to generate COHb, a stable iron carbonyl, is a good example of CO's reactivity towards metals [48]. In a similar fashion CO can bind to transition metals forming a transition metal carbonyl complex simpler and with a chemically safer than COHb [10,48,61]. Thus, transition metal carbonyls are often the foundation for CORM design [48]. Since their conception, CORMs have shown their therapeutic potential both *in vitro* and *in vivo* [49]. In the CNS specifically, these chemical compounds have been found to reduce TNF- α and pro-inflammatory cytokine levels *in vitro* [57,62] and reduce brain damage after haemorrhagic stroke [54,63]. Regarding phagocytosis, there is some evidence of CORM's action. Chung et al [64] observed that the injection of a CORM into WT mice, improved macrophage phagocytosis of bacteria and saved HO-1 deficient animals from sepsis-induced death.

In this work, ALF-826 a new molybdenum (Mo) based CORM will be used as a CO source. Mo is a trace metal and therefore can be metabolized by machinery already present in the organism. This CORM has already showed anti-inflammatory properties *in vitro* [21,65].

5. Peroxisomes

Peroxisomes are small organelles (0.1–1 μm) bound by a single membrane [66–71] that mostly contain enzymes. They are found in every eukaryotic cell [66–68], with the exception of erythrocytes [69]. Given their abundance, peroxisomes have important functions in the maintenance of cell viability. Historically, the main function associated with peroxisomes is ROS metabolism. The peroxisomes carry out oxidation reactions leading to the production of hydrogen peroxide (H_2O_2). Because H_2O_2 is toxic, peroxisomes contain great amounts of catalase, which degrades H_2O_2 into water or used H_2O_2 to oxidize organic compounds [70]. Nevertheless, these organelles are also pivotal in lipid metabolism, which includes the β-oxidation of fatty acids (FAs), such as very long chain fatty acids (VLCFA), the α-oxidation of branched-chain FAs [69] and the biosynthesis of ether phospholipids.

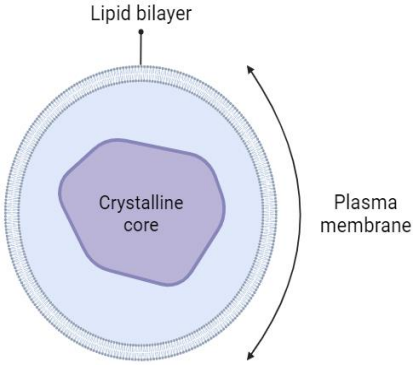

Figure 13 – Peroxisome and its components

5.1. Peroxisomal ABCD transporters

Given the key involvement of peroxisomes in metabolic pathways, it becomes necessary the presence of metabolic transporters to carry various metabolites in or out of the peroxisome [72]. The ATP binding cassette (ABC) proteins are part of the peroxisomal transporters [67,72]. This protein superfamily of membrane transporters is found in almost all organisms and has a conserved structure [66,67,72]. The distributions of ABC transporters within the eukaryotic cell include the plasmatic membrane, peroxisomes, the endoplasmic reticulum and the mitochondria [72]. ABC transporters can translocate various molecules ranging from simple molecules such as FAs, sugars or amino acids, to complex compounds like lipids, polysaccharides and proteins. The translocation is dependent on ATP hydrolysis [67,72]. Functional ABC transporters consist of two structurally analogous hydrophobic halves, each encompassing six transmembrane alpha helices (TMD) and a conserved hydrophilic nucleotide-binding domain (NBD) [66] (Figure 14). By comparing their amino acid similarity and structural arrangement, ABC transporters within the human system can be categorized into seven subfamilies: A to G. In humans, the ABC transporters found in the peroxisomal membrane belong to the D family, being ABCD1, ABCD2 and ABCD3 [69,72]. These ABCD transporters are important in the transport of lipids into to the peroxisome for their shortening by β-oxidation [72].

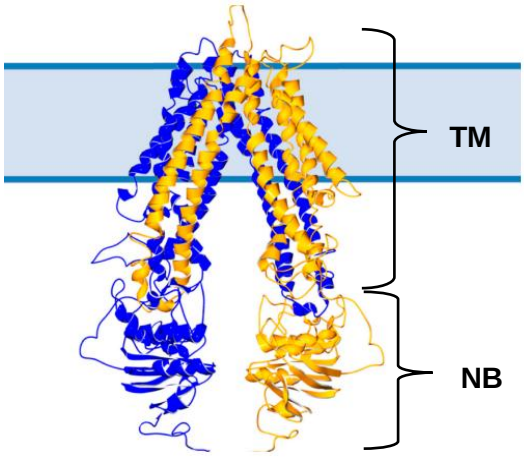

Figure 14 – *Ribbon illustration of the ABCD1 homodimer, showing each of the two subunits coloured blue and yellow, respectively. The hydrophobic transmembrane domain (TMD) and hydrophilic nucleotide binding domain (NBD) are indicated (adapted from Tawbeh, A. et al; 2021)*

5.2. ACOX-1

In mammals, degradation of FA chains occurs in both mitochondria and peroxisome, with each organelle having its specific β-oxidation pathway [71]. The β-oxidation of very long-chain fatty acids (VLC-FAs), which have a number of carbon atoms greater than 22 [72], only occurs in peroxisome [71,72]. Peroxisomal β-oxidation consists of a four step enzymatic loop in which FAs are shortened at each cycle by two carbons with the release of one acetyl-CoA molecule [66,68]. The first and rate limiting step of peroxisomal β-oxidation is catalysed by acyl-CoA oxidase (ACOX) [66,68,71]. The reaction catalysed by ACOX consists of the α,β-dehydrogenation of acyl-CoA esters resulting in the production of a corresponding trans-2-enoyl-CoA esters and $FADH_2$ in the reductive half reaction. FAD's re-oxidation requires O_2 and results in the production of H_2O_2 [73] (Figure 15). In humans, Peroxisomal acyl-coenzyme A oxidase 1 (ACOX-1) catalyses the first step of β-oxidation of straight chain FAs, among these long chain FA (LC-FA) and VLCFAs [68]. While, ACOX-2 is involved in the peroxisomal β-oxidation of bile acids, and both ACOX-2 and ACOX-3 are involved in branched chain FAs [68].

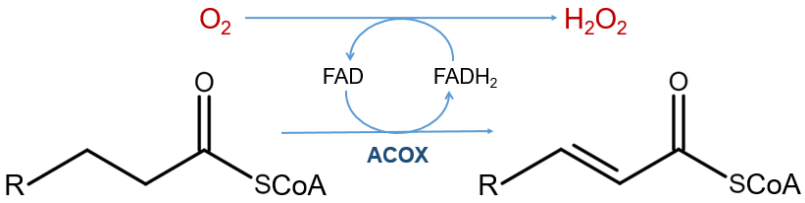

***Figure 15** – Peroxisomal β-oxidation reaction (adapted from Schrader, M. et al.; 2015)*

5.3. Peroxisome disorders

The majority of peroxisomal disorders manifests themselves through neurological impairments [74]. Peroxisomal disorders can either result from defective peroxisome biogenesis or from mutations of individual peroxisomal enzymes or transporter proteins (such as ACOX-1 and ABCD).

The ABCD1 gene encodes the ABCD1 transporter and can be mutated in humans manifesting in a rare disorder that affects the nervous system called X-linked adrenoleukodystrophy (X-ALD) [67,75]. The most recurrent phenotype of X-ALD is an axonopathy of the spinal cord characterized by disordered microglia. While studying a mouse model for this disorder, Gong [75] and colleagues found that the microglia in the spinal cord that lacked ABCD1 were geared to phagocytosis.

Similarly to ABCD, ACOX-1 deficiency is associated with a rare neurological disorder in humans. The disorder, called pseudo neonatal adrenoleukodystrophy, is characterized by seizures, visual impairment, loss of motor abilities, and progressive white and grey matter degeneration [68]. With the purpose of assessing the effect of ACOX-1 deficiency on microglia Raas [68] and colleagues developed an ACOX-1 deficient cell line using CRISPR/Cas9 gene editing. The authors found in the KO cells altered expression levels of genes coding for IL-1β and IL-6 (pro-inflammatory cytokines) and TREM-2, implying a change in microglial polarization and phagocytosis capabilities. Overall, the mutant cells showed a difference in lipid content (accumulation of VLC-FA) and increased phagocytosis.

So, by looking at these examples and other studies, a link between peroxisomal dysfunction and microglial modulation can be made.

6. Objectives

6.1. Engulfment of live neurons and CD200

In a context of inflammation, microglial phagocytosis of neurons can be beneficial since it allows for the elimination of pro-inflammatory dead neurons and neuronal debris [76]. Nevertheless, microglial phagocytosis of neurons may be harmful when it eliminates neurons that otherwise are functioning and healthy [32,76] – a process called phagoptosis. In general, healthy neurons do not signalize microglia to phagocytise them, but in cases of neuronal sub-toxic injury or excessive microglial inflammatory stimuli such can happen, leading to the death of viable neurons. Thus, this work sets out to understand if CO's action influences microglial phagocytosis of live neurons.

It has been previously established by our research group that CO pre-treatment of BV-2 cell cultures reverts the effect of LPS on microglial engulfment of dead neurons. It was observed that under inflammatory conditions there was a decrease in dead neuron engulfment, indicating an unsuccessful clearance, and that CO pre-treatment increased engulfment [77] (Figure 16). The neuronal ligand CD200 and the microglial receptor CD200R pair was found to be mechanistically involved with CO's action on engulfment. In fact, silencing CD200R expression in microglial culture hindered CO's reversion of LPS' effect. Likewise, Soares et al [21] found that CO increases CD200R and CD200 expression in microglia and neurons (Figure 12).

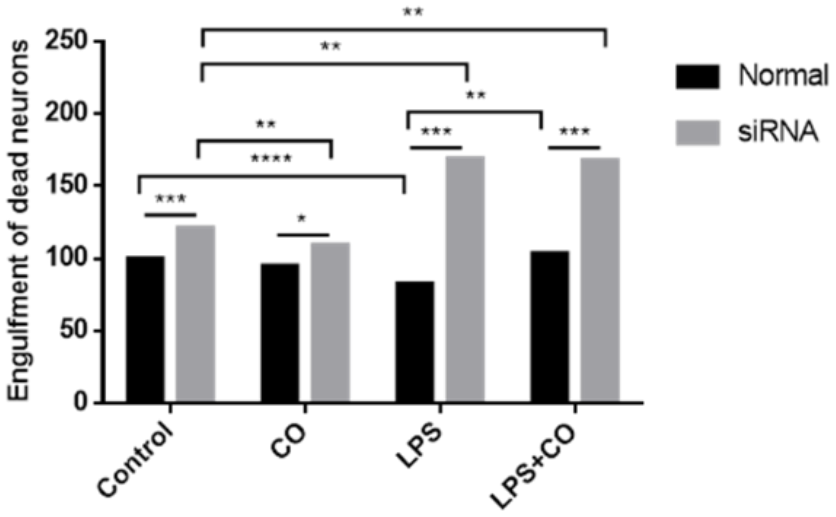

Figure 16 *– Previous data from the research group showing that CO treatment improves the microglial clearance of dead neurons under inflammatory conditions, via the CD200-CD200R neuron-microglia communication pathway. Results are given as a percentage compared to control conditions (100%). Error bars represent mean ± SEM, *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001 by unpaired two-tailored t-test. (Taken from Catarina Simões' thesis; 2022)*

Considering the possible pathological effect of phagoptosis and the previous findings, this work set out to:

i) Assess the effect of CO on the engulfment of live neurons and;
ii) Evaluate if the CD200/CD200R pair is also mechanistically involved.

BV-2 cell line was pre-treated with ALF-826, a molybdenum based CORM. The inflammatory stimuli used was LPS. To assess the involvement of C2000/CD200R, CD200R1 gene was knocked down on microglia using transfected siRNA. Flow cytometry was used to measure engulfment by assessing co-localization of live neurons and microglia.

6.2. Peroxisome and Inflammation

CO has shown to be anti-inflammatory, as well as a possible regulator of microglial phagocytosis [21,49]. Peroxisomes are also regulators of neuroinflammation [78] as peroxisomal deficiencies result in increased inflammation and alterations in microglial phagocytosis patterns. Yet these are organelles with less scientific attention. It is established that CO can activate the PPAR-λ [53] in macrophages, which, as the name suggest, is related to peroxisome proliferation. So does CO regulate microglial inflammation and/or phagocytosis in a peroxisomal dependent manner? Thus, the second aim of this project is:

i) To assess the effect of defects in peroxisomal proteins in lipid peroxidation (as a measure of peroxisomal function) and;
ii) To evaluate if CO-mediated anti-inflammatory action on the microglia is mechanistically dependent on the peroxisomal function.

Mutant microglial cell line cultures were used – ACOX-1 $^{-/-}$ and ABCD1$^{-/-}$ABCD2$^{-/-}$ BV2 microglia. Along with the wildtype microglia, these mutants were pre-treated or not with ALF-826 and subjected to an inflammatory stimuli (LPS). Then, lipid peroxidation were measured using the thiobarbituric acid assay (TBARs).

Materials and Methods

1. Materials

The plastic material used in this thesis was acquired from Sarstedt (Germany) and Corning (NY).

2. Cell Culture

2.1. BV-2 cell line

The model used for the microglia was the BV2 murine microglial cell line. This is an immortalized cell line that exhibits phagocytic activity and can secrete cytokines upon stimulation. This cell line shows similar migration, cytokine and nitric oxide release as primary microglial cell cultures [5].

The BV2 murine microglial cell line was kindly supplied by Dr Ana Raquel Santiago (*Faculdade de Medicina da Universidade de Coimbra*). Knockout (KO) by CRISPR/Cas9 BV2 cell lines for ACOX-1$^{-/-}$ and double KO for ABCD1$^{-/-}$ and ABCD2$^{-/-}$, were supplied by Dr Stéphane Savary (*Université de Bourgogne Faculté des Sciences, France*).

2.1.1. Cell culture maintenance

Cells were cultured in RPMI-1640 medium with sodium bicarbonate (R0883, Sigma-Aldrich) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (10500-064, Gibco), 2% (v/v) L-glutamine at 200 mM (25030.024, Thermo-Fisher Scientific) and 1%(v/v) Penicillin/Streptomycin (P/S) (P07124, Sigma-Aldrich). Cell passage was done when 75% cell confluency was reached. The passage process consisted of cell scraping to detach them and then transferring ¼ of the scraped cells into a new culture vessel. Culture was maintained in an incubator with a humified atmosphere at 37 °C and 5% CO2.

2.2. CAD cell line

The model used for neuronal-like cells were the CAD (Cath.-a-differentiated) mouse catecholaminergic neuronal cell line. This cell line is a variant of the Cath-a cell line, which can be differentiated by removing the serum from the medium. This cell line has lost the oncogene but remains immortalized [79]. The CAD cell line expresses many neuron specific proteins (such as synaptic vesicle proteins, neurotransmitter enzymes and glial proteins) and is morphological similar to neurons [79,80], making it a useful *in vitro* model for this study. CAD catecholaminergic neuronal cell line was kindly supplied by Dr Federico Herrera (*Faculdade de Ciências da Universidade de Lisboa*).

2.2.1. Cell culture maintenance

These cells were cultured in DMEM-F12 medium with L-glutamine and 15 mM HEPES (L0093, Biowest) supplemented with 8% FBS and 2% P/S. Cell passage was performed when 70% confluency was reached. For the CAD cell line, passage was done by removing the old cell medium, pipetting fresh culture medium over the cells to detach them and then transferring ¼ of the cells to a new culture vessel. Culture was maintained in an incubator with a humified atmosphere at 37 °C and 5% CO2.

2.3. Cell culture freezing and thawing

Both BV-2 and CAD cell lines were frozen and thawed following the same protocol. After cell detachment, the cell containing medium was collected into a tube and centrifuged at 500*g* for 5min. The supernatant was carefully taken and discarded. The cell *pellet* was resuspended in a freezing solution that consisted of 90% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO) (D8418, Sigma-Aldrich) and added into 1 mL freezing vials. These vials were stored at -80°C into Styrofoam racks to freeze more slowly. Cell culture thawing consisted of taking the cell vials out of the -80°C ark, rapidly thawing them in 37ºC water bath, followed by transferring the cell solution to fresh medium. This suspension was centrifuged at 500g for 5min in order to eliminate the organic solvent DMSO present in the freezing solution. The supernatant was then taken and discarded, while the pellet was resuspended in fresh medium, which in turn was transferred to a culture vessel.

2.4. Cell culture plating and treatments

The cellular samples used for immunoblotting, the TBARs and Griess assays were obtained following the timeline in Figure 17. BV-2 microglial cells (WT, ACOX-1$^{-/-}$ and ABCD1$^{-/-}$ABCD2$^{-/-}$) were seeded onto 6 well plates at a concentration of 55 × 10^4 cells per well. The used LPS concentration was 0.5 ng/mL and ALF-826 concentration was 50 µM.

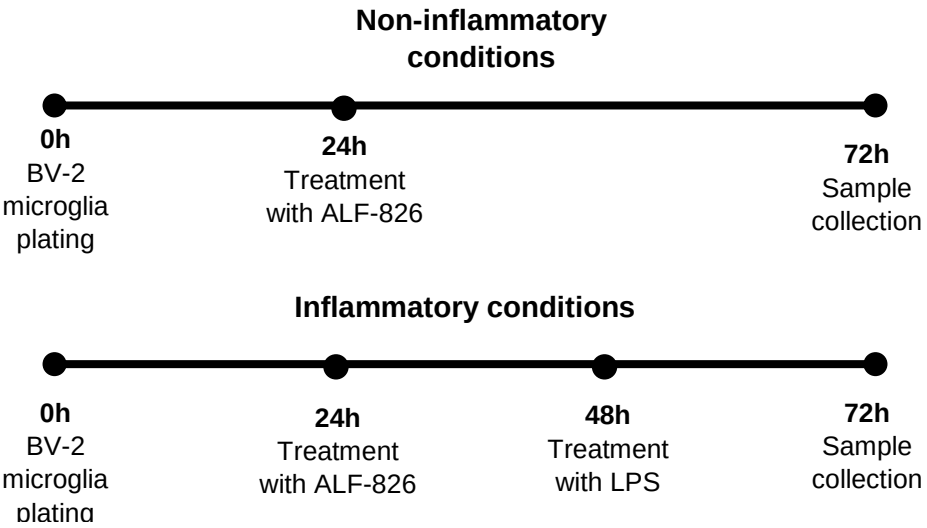

Figure 17 *– Timeline of cell plating and treatment for samples used in TBARs and Griess assays and Immunoblotting*

3. Reagent preparation

The CORM used in this project was ALF 826, which was supplied by Proterris Lda. A stock solution was prepared by dissolving the compound in DMSO followed by a 1:10 dilution in a $NaHCO_3$ solution (0.1 mM, pH 8.3). After reaching a final concentration of 2.5 mM, the ALF-826 solution was filtered, aliquoted and stored at -80°C.

Lipopolysaccharide (LPS) solution was used to simulate an inflammatory stimuli. This solution was prepared by dissolving LPS (1.09023, Millipore) in Phosphate-buffered saline (PBS, Table 2) for a final concentration of 1 mg/mL in aliquots. This process was done in a laminal flow chamber so as to preserve sterility. The aliquots were stored at -20°C.

Table 2 *– Composition of PBS which is used in washing processes and solution-making*

Reagent	Composition
PBS pH 7.2	1.54mM NaCl; 34 mM Na_2HPO_4; 20 mM KH_2PO_4

4. RNA silencing

BV2 microglia were seeded onto 24 well plates at a concentration of 6×10^4 cells *per* well (volume was 1 mL) and were cultured in the incubator for 4h to allow cell attachment into wells. Then the silencing protocol began. The expression of CD200R was knocked down by CD200R1 coding siRNA (181777, Invitrogen) prepared into liposomes. Liposomal solution was prepared by adding Lipofectamine RNAiMAX (13778-075, Invitrogen) volume/well and CD200R1 siRNA (15 pmol/well) in Opti-MEM medium (11058021, Gibco) vol/well followed by gentle agitation during 20 min to form liposomes. 50 µL of liposome solution was added to each well after its medium was replaced by antibiotic free RPMI-1640 medium (500 µL). The plates with liposome solution were cultured in the incubator for 4h. Then, 500 µL of RPMI-1640 medium with 2% P/S was added to each well so that the final % P/S (v/v) was 1%. After 24h of transfection, cells can be treated for the engulfment assay described in Section 5.

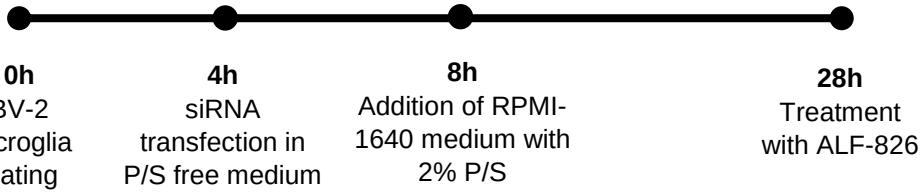

Figure 18 *– Timeline of RNA silencing*

5. Engulfment assay

The engulfment assay started with the seeding of BV-2 microglia onto 24 well plates at a concentration of 6 × 10^4 cells *per* well, followed by cell treatments as presented in Figure 19. The concentration of ALF-826 was 50 µM and the concentration of LPS was either 0.5 or 5 µg/mL. In control conditions microglia was not silenced, the transfection did not occur so the subsequent treatments were brought forward 4h.

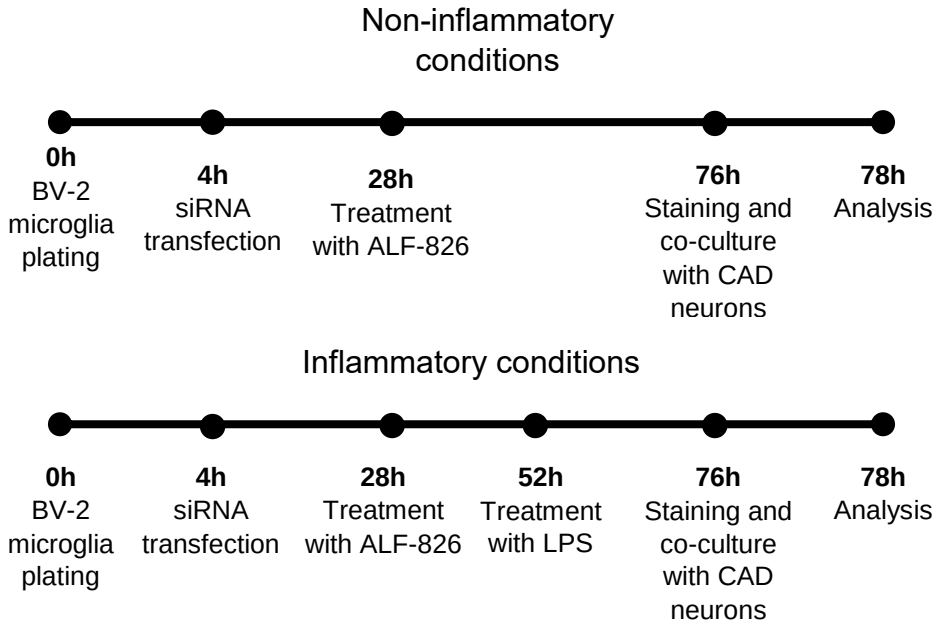

***Figure 19** – Timeline of plate treatment for samples used in engulfment assay with RNA silencing*

Following these treatments, BV-2 and CAD cells were counted to establish a 1 neuron: 2 microglia ratio cell co-culture. Of note, CAD cell line is cultured in t-flasks and the passage was done in the day of co-culture. Before establishing the co-culture, the individual cell lines were stained. CAD cells were stained with 25 µM of 5-(and-6)-carboxytetramethylrhodamine N-succinimidyl ester (5-TAMRA, 53048, Sigma-Aldrich) for 15min, then washed three times with PBS to eliminate the excess of dye, and resuspended in RPMI-1640 medium supplemented with 0.5% FBS. While BV-2 cell medium was replaced by RPMI-1640 medium supplemented with 0.5% FBS and then BV-2 cells were stained using microglia-specific Isolectin B4 – Alexa488 (I21411, Invitrogen). In order to co-culture, the stained CAD cell suspension was seeded on top of the microglia culture in the 24 well-plaque. The cells were maintained in co-culture for 2h in the incubator.

Then the co-culture was collected by scraping the plaque wells and collecting the medium with cells. The samples were then analyzed using flow cytometry for the co-localization of dyes indicating microglial engulfment of neurons. The cytometer used was an Attune Acoustic Focusing Cytometer (Applied Biosystems). The fluorescence was measured in BL1 channel for green and BL2 channel for red fluorescence, and a 488 nm laser line was used for excitation. Besides the analytical conditions, an unstained and single positive staining controls of monocultures were used to adjust gates, compensation settings and auto-fluorescence levels. In order to exclude cell debris,

unrelated events or single CAD cells from the analysis, BL1 negative events were excluded. The quantification of BL1 and BL2 fluorescence was used to assess microglial engulfment levels.

6. Measurement of lipid peroxidation

The interaction of oxygen with unsaturated lipids, known as lipid peroxidation, creates a wide range of oxidation products. Lipid hydro peroxides (LOOH) are the principal products of lipid peroxidation. Some of the secondary products of this reaction include malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) [81]. MDA is reported as the most mutagenic product of lipid peroxidation [81]. The thiobarbituric acid reactive substance (TBARs) assay measures the increase in absorbance at 532 nm resultant from the formation of adducts between MDA and thiobarbituric acid [82] (Figure 20).

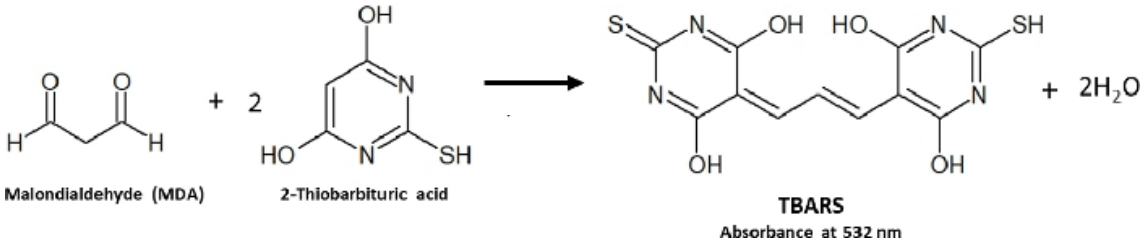

Figure 20 – *TBARs assay reaction*

The plating and treatment of BV-2 cell line used for this assay is described in Section 2.3 (Figure 17). At the end of the treatment, the cells were scraped and collected into *eppendorfs* tubes. The medium was centrifuged at 1000*g* for 5min, the cell containing *pellet* was kept for this assay and the supernatant taken for the Griess assay. 200 µl of the TBARs solution (Table 3) was added into the cellular pellet. Holes were poked in the tube caps and they were heated at 99ºC for 10min. Samples were cooled down, and then centrifuged at 3000*g* for 5min. In the end, 100 µL of the supernatant obtained was plated in duplicates onto a 96 well plate. The plates absorbance was read at 540 nm using Tecan.

Table 3 *– Composition of the TBARs reagent*

Reagent	Volume
H_2O Milli Q	1.6 mL
Trichloroacetic acid (131067.1608, AppliChem) 20% (m/v)	3 mL
Thiobarbituric acid (T5500, Sigma) 1% (m/v)	3 mL
Sodium dodecyl sulfate (7910.500, Omnipur) 8,1% (m/v)	400 µL

7. Indirect measurement of NO production

NO is a molecule involved in the inflammatory process. Measuring NO directly is challenging because the molecule is a gas, has a short life and is quickly metabolized to nitrite or nitrate in the presence of oxygen. Given this, to estimate NO production, the determination of nitrite and/or nitrate content can be used. The Griess assay is one of the most used techniques to ascertain nitrite content. This assay is based on the conversion of nitrite to a purple-coloured azo-dye which can be measured spectrophotometrically at a wavelength of ~540 nm [83].

The Griess assay was performed as follows. The plating and treatment of microglial cells was done alike to TBARs and is described in Section 2.3 (Figure 17). For this assay, the cellular supernatant was collected and centrifuged at 10000*g* for 5min with the purpose of removing any cells or debris remaining in the medium. After this, the supernatant was plated in triplicates into a 96 well plate at a volume of 50 µl *per* well. To this volume, 50 µl of the Griess reagent was added. A blank was made by adding cell medium and Griess reagent in the same volumes. The plate was shaken for 10 minutes and then its absorbance was read at 540 nm using Tecan.

8. Immunoblotting

The cellular extracts used for immunoblotting were obtained as shown in Figure 17 of Section 2.3. At the end of the treatment, the cell medium was eliminated, the cells were washed twice with 600 µL of PBS and diluted in 200 µL of Loading Buffer (Table 4) to lyse the cells. Protein extract was heated at 100°C for 5 min.

Table 4 *– Composition of the buffers used in immunoblotting*

Buffers	Composition
Running Buffer 10X pH 8,3	0.4 M Tris base; 3.2 M Glycine; 57.6 mM SDS
Running Buffer 1X pH 8,3	900 mL H_2O milliQ; 100 mL Running Buffer 10X
Transfer Buffer 10X	25 mM Glycine; 192 mM Tris base
Transfer Buffer 1X	700 mL H_2O milliQ; 100 mL Transfer Buffer 10X; 200 mL Ethanol
Stacking Buffer pH 6,8	0.5 M Tris-HCl; 0.4% (m/v) SDS; 1L H_2O milliQ
Separating Buffer pH 8,8	1.5 M Tris-HCl; 0.4% (m/v) SDS; 1L H_2O milliQ
T-TBS 10X	3 M NaCl; 1M Tris-HCl
Loading Buffer pH 6.8	0.05 M Tris-HCL, 2% (m/v) SDS, 8% (m/v) Glycerol, 0.1% (v/v) Bromophenol blue, 3% β-Mercaptoethanol

A volume of 20 µL of cell extract was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, for 2h, along with the NZYColour Protein Marker II (NZYTech). The proteins were then transferred onto a PVDF membrane (IPVH0010, Millipore) for 1h40min, blocked with 5% non-fat milk in T-TBS solution, and incubated overnight with the primary antibody, rabbit anti-ACOX-1 (kindly supplied by Dr Savary) dil. 1/200 in 1% non-fat milk, at RT. The

membrane was then incubated for 2 hours at RT with the secondary antibody, donkey ECL™ Anti-Rabbit IgG HRP conjugated (NA934V, Cytiva) dil. 1/5000 in 1% non-fat milk. The membranes were rinsed three times with T-TBS for 10 minutes between blocking and incubation with both antibodies. The electrophoresis and transfer success were evaluated using Rouge Ponceau (Biotium). The last step was the exposure of the membranes to Clarity Western ECL Substract (170-5061, Bio-Rad) for 1min, then to X-ray film (10742837, Cytiva) resulting in the revelation of bands. This was all done in a dark chamber.

9. Statistical analysis

The data presented is the mean ± SEM. The two-tailed and unpaired Student's t-test (GraphPad) was used to compare different groups of conditions, with p-values under 0.05 being statistically significant.

Results and discussion

1. Engulfment assay

1.1. Gate definition

Before proceeding to the analysis of engulfment using flow cytometry, it was necessary to adjust the gates to i) exclude unnecessary elements to the analysis (unmarked cells and cell debris) and ii) select the signal that corresponds only to microglial engulfment of neurons. So, in all experiments as well as the conditions tested, an unstained control, a stained microglia monoculture and a stained neuron monoculture were analysed in the cytometer. These samples allowed to discern which were the BL1 positive events and the BL2 positive events (Figure 21).

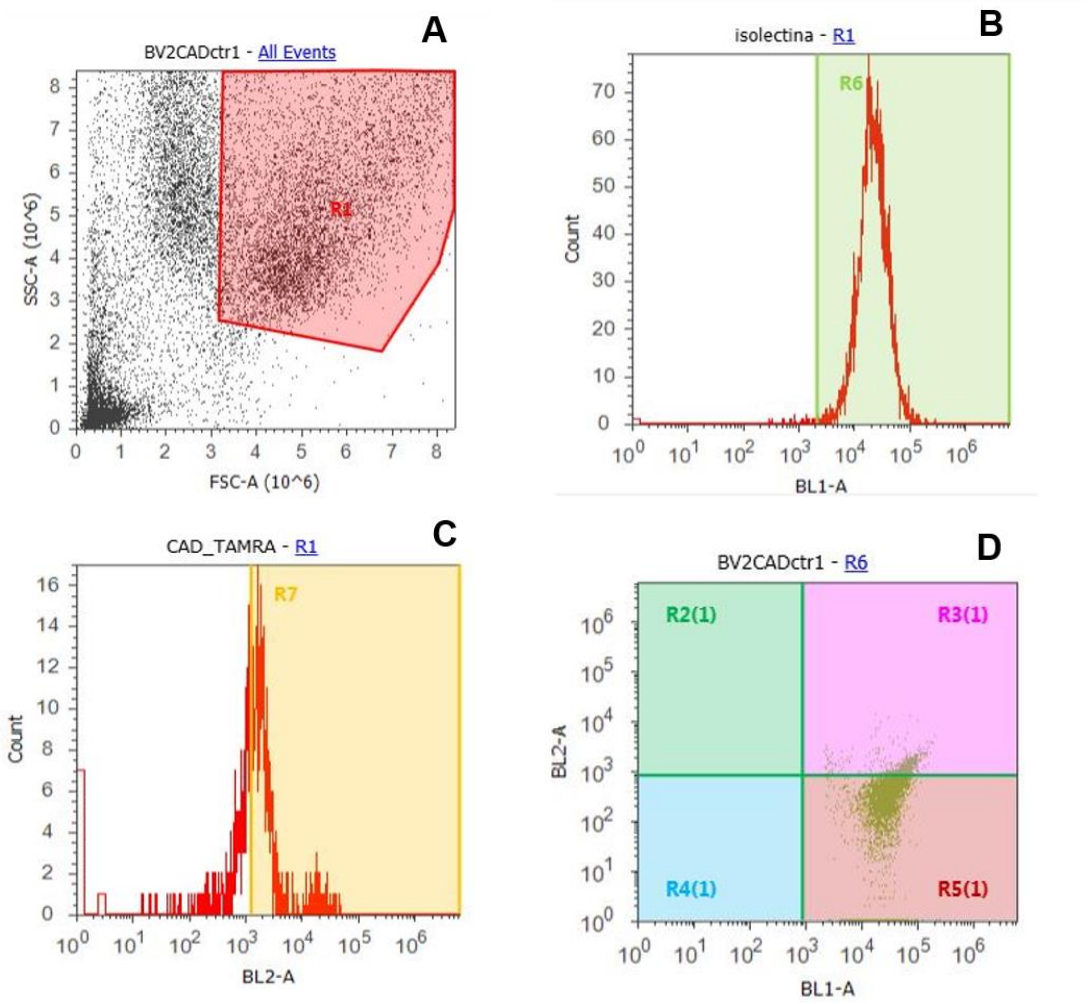

Figure 21 – Gates established using flow cytometry data to assess levels of microglial engulfment by live neurons. *Graphs (A) and (D) correspond to co-culture of stained microglia and neurons on the control conditions. Histograms in (B) and (C) correspond to monoculture of stained microglia and neurons respectively. (A) The gate defined in the FSC vs SSC graph allowed to remove any events that did not correspond to cells all events outside the red box R1; (B) Isolectin B4 – Alexa488's histogram. Events concerning to microglia are BL1 positive; (C) 5'TAMRA's histogram. Events concerning neurons are BL2 positive; (D) The quadrants defined in this graph were determined using histogram (B) and (C). Events corresponding to microglia or neurons were identified by drawing quadrants on the BL1 (green) versus BL2 (red) graph. Events within the R3(1) quadrant were considered as concerning microglial engulfment neurons given these are both BL1 and BL2 positive (show both green and red fluorescence).*

1.2. Optimizing the conditions

With the gates defined, it is now possible to discriminate which events are microglial engulfment of neurons. BV-2 microglia were plated and four different conditions tested: i) the untreated control; ii) treatment with the CORM ALF-826 for 48h; iii) LPS treatment for 24h to recreate inflammatory stimulation of the microglia and iv) ALF-826 pre-treatment followed by LPS stimulation. The BL1+ and BL2+ population was noted for each condition and normalized to the control (100%).

1.2.1. Treatment with LPS 0.5 µg/mL and non-differentiated neurons

In previous studies about engulfment of dead neurons, it was observed a decrease of phagocytosis under inflammation, which was reverted but CO treatment. It indicates that microglial clearance of dead neurons is not optimal under inflammatory conditions [77]. Therefore, it was decided to assess whether CO regulates the engulfment of live neurons under inflammatory conditions.

Firstly, 0.5 µg/mL of LPS the concentration previously used by our research group [21] was applied to stimulate inflammation in microglia (Figure 22).

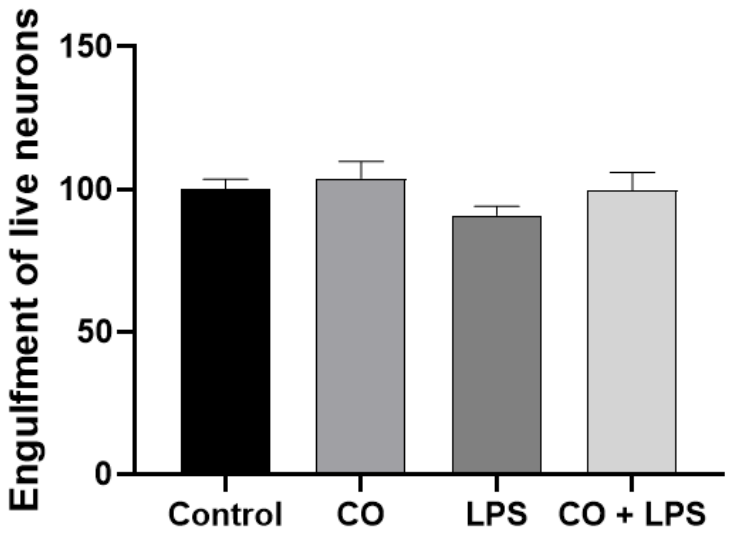

Figure 22 – Treatment with LPS at 0.5 µg/mL does not increase the microglial engulfment of live neurons. *BV-2 microglia were plated, then treated with CO at 50 µM for 24 hours and subsequently treated with LPS at 0.5 µg/mL for another 24 hours. The microglia were then labelled in green (Alexa Fluor-488 conjugated Isolectin B4), CAD neurons in red (5-TAMRA), and the two types of cells co-cultured for 2h. Flow cytometry was used to determine engulfment levels. Results are given as a percentage compared to control conditions. n=2 (6 technical replicates), error bars represent mean ± SEM.*

In BV-2 microglia treated with 0.5 µg/mL of LPS there was no stimulation of microglial engulfment of live neurons. Likewise, CO treatment also did not show any effect in the engulfment.

1.2.2. Treatment with LPS 0.5 µg/mL and differentiated neurons

The lack of increased microglial engulfment of live neurons under inflammatory conditions could be due to the fact that CAD cell line is not differentiated into neurons. The CAD neuronal cell line used is an immortalized cell line that when cultured in serum-free media stops dividing and undergoes morphological differentiation [80]. CAD cells after differentiation are morphologically and biochemically closer to neurons, with long processes and expression of neuron-specific proteins [79,80]. The available differentiation protocol consisted of growing neuronal CAD cell culture in FBS-free medium for 48 hours.

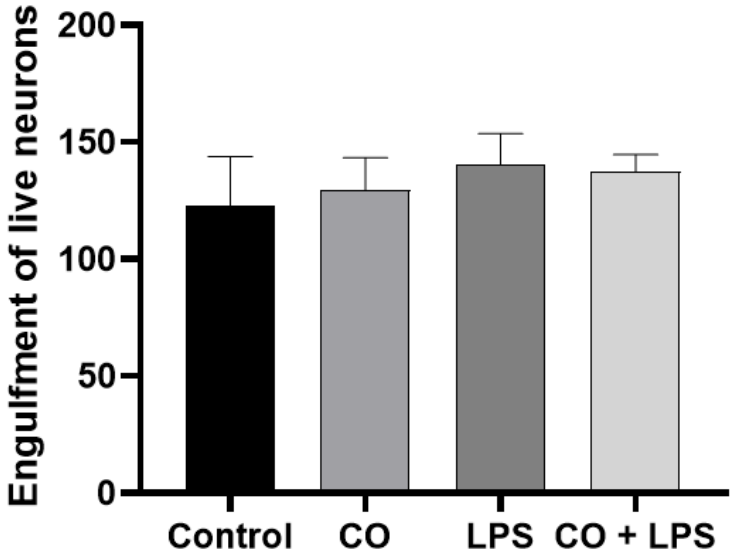

Figure 23 – Treatment with LPS at 0.5 µg/mL does not increase the microglial engulfment of live differentiated neurons. *BV-2 microglia were plated, then treated with CO at 50 µM for 24 hours and subsequently treated with LPS at 0.5 µg/mL for another 24 hours. 48 hours before staining CAD neurons were cultured in FBS free medium to initiate differentiation. The microglia were then labelled in green (Alexa Fluor-488 conjugated Isolectin B4), CAD neurons in red (5-TAMRA), and the two types of cells co-cultured for 2h. Flow cytometry was used to determine engulfment levels. Results are given as a percentage compared to control conditions. n=2 (6 technical replicates), error bars represent mean ± SEM.*

Neuron differentiation process does not seem to alter the microglial engulfment response under all conditions (Figure 23). Nevertheless, at the end of the differentiation protocol it should be noted that differentiated CAD neurons demonstrated morphology identical to the undifferentiated ones. In the papers describing CAD neuron differentiation [79,80] both authors left the neuron culture in serum-free medium for 5 days, observing the large processes present in neurons. Thus, a longer period of differentiation could be used.

1.2.3. Treatment with 5 µg/mL LPS and non-differentiated neurons

Based on the fact that both differentiated and non-differentiated cultures treated with 0.5 µg/mL LPS did not change microglial engulfment of neurons under inflammation, we decided to increase of LPS concentration to better mimic acute inflammation.

Thus, experiments were done with an increased LPS concentration of 5 µg/mL (Figure 24). Cell culture observation at the microscope revealed that BV-2 cells were alive and that 5 µg/mL of LPS did not trigger a noteworthy quantity of dead cells.

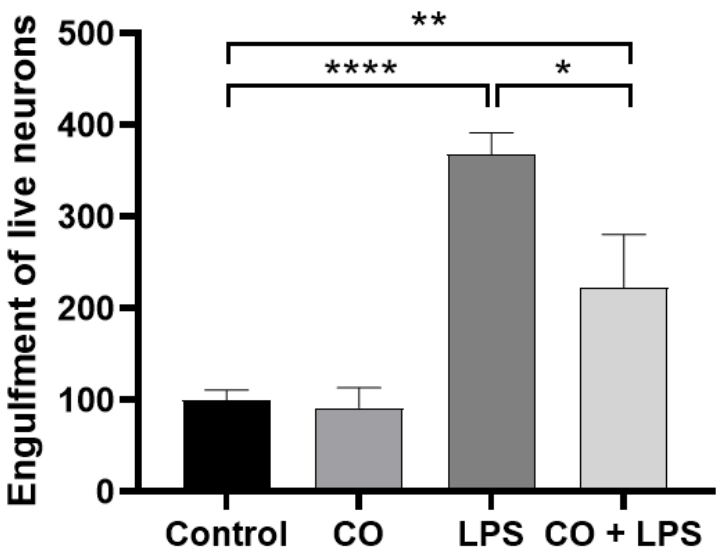

Figure 24 – CO treatment reverts microglial clearance of live neurons under inflammatory conditions. *BV-2 microglia were plated, treated with CO at 50 µM for 24 hours and subsequently treated with LPS at 5 µg/mL for another 24 hours. The microglia were then labelled in green (Alexa Fluor-488 conjugated Isolectin B4), CAD neurons in red (5-TAMRA), and the two types of cells co-cultured for 2h. Flow cytometry was used to determine engulfment levels. Results are given as a percentage compared to control conditions (100%). n=4 (13 technical replicates), error bars represent mean ± SEM, *p<0.05, **p<0.01, ****p<0.0001 by unpaired two-tailored t-test.*

In non-inflammatory conditions, CO pre-treatment had no effect on microglial engulfment of live neurons. This is consistent with previous data [21,77] and shows that CO by itself does not alter microglial engulfment. The engulfment of live neurons was increased by LPS treatment at a concentration of 5 µg/mL, demonstrating that inflammation can cause the microglial intake of live neurons. CO pre-treatment partially reversed LPS's impact by reducing microglial engulfment of live neurons. Even though CO pre-treatment reverted engulfment under inflammation, it is still high when compared to control. CO can modulate microglial phagoptosis as well phagocytosis, but it cannot revert engulfment levels to basal levels. This finding might be attributable to the high concentration of inflammatory stimulation employed.

There are a few factors to account concerning these findings. Firstly, the inflammatory stimuli employed was LPS, a molecule found in the membrane of Gram-negative bacteria that targets primarily TLR-4. Most inflammatory processes in the brain are started by endogenously generated cytokines such as IFN-γ. With this in mind, it would be interesting to assess if the inflammatory reaction would be the same with endogenous inflammatory stimuli. Secondly, the concentration of LPS employed is

unusually high, so it would be valuable to define whether inflammatory stimuli may occur under physiopathological conditions. Further studies could be done using other concentrations of such as 1 µg/mL, which has been used to stimulate BV-2 microglia.

1.2.4. Treatment with 5 µg/mL LPS and differentiated neurons

Because an increased concentration of LPS resulted in increased engulfment in non-differentiated neurons, it was evaluated if the same occurred in differentiated neurons (Figure 25). Again, the differentiation was done by removing serum from the culture medium for 48h.

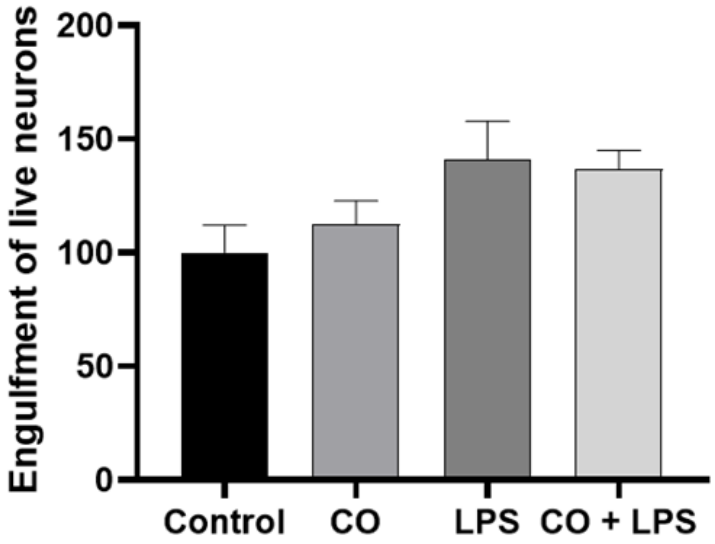

Figure 25 – Treatment with LPS at 5 µg/mL increases slightly the microglial engulfment of live differentiated neurons. *BV-2 microglia were plated, treated with CO at 50 µM for 24 hours and subsequently treated with LPS at 5 µg/mL for another 24 hours. 48 hours before staining CAD neurons were cultured in FBS free medium to initiate differentiation. The microglia were then labelled in green (Alexa Fluor-488 conjugated Isolectin B4), CAD neurons in red (5-TAMRA), and the two types of cells co-cultured for 2h. Flow cytometry was used to determine engulfment levels. Results are given as a percentage compared to control conditions (100%). n=2 (6 technical replicates), error bars represent mean ± SEM.*

In the case of differentiated neurons, CO pre-treatment under non-inflammatory conditions did not alter significantly engulfment compared to the control. Under inflammatory conditions engulfment appears to increase slightly and CO pre-treatment did not have any effect on this increase. Considering that it LPS treatment seems to increase slightly engulfment, it could be interesting to conduct further neuron differentiation tests. As discussed in the section 1.2.2. time of differentiation could be increased.

1.3. CD200R1 silencing effect on engulfment

So far CO has shown to be involved in microglial engulfment with both dead [77] and live neurons (Figure 16). Thus, one could ask if the CD200/CD200R pair is implicated in live neuron engulfment as it is in dead neuron engulfment [77]. To determine this, knockdown of the CD200R1 gene in microglia using siRNA was done. The silenced microglia were subjected to the same treatments as the non-silenced (Figure 26). Microglial engulfment of non-differentiated live neurons was assessed using flow cytometry using the same gates defined in Figure 21.

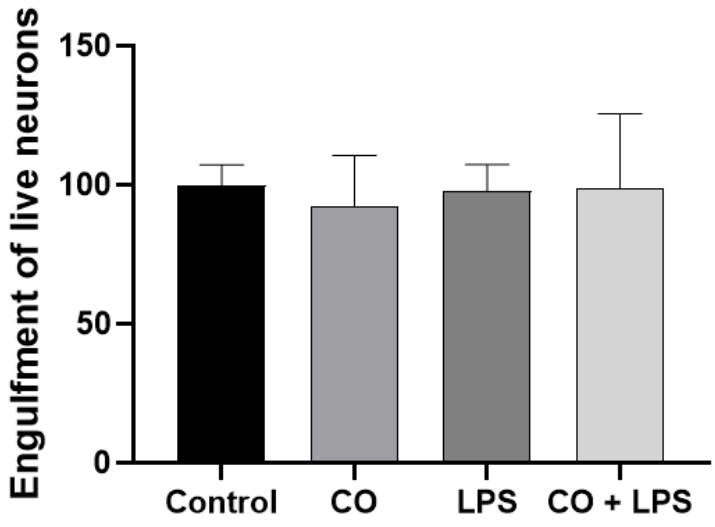

Figure 26 – Microglial engulfment of live neurons under inflammatory conditions depends on CD200R1 gene expression. *BV-2 microglia were transfected with CD200R1 siRNA. Following 24h after transfection, microglia cells were treated with CO at 50 µM for 24 hours and subsequently treated with LPS at 5 µg/mL for another 24 hours. The cells were then labelled in green (Alexa Fluor-488 conjugated Isolectin B4), CAD neurons in red (5-TAMRA), and the two types of cells co-cultured for 2h. Flow cytometry was used to determine engulfment levels. Results are given as a percentage compared to control conditions. n=3 (9 technical replicates), error bars represent mean ± SEM.*

Intriguingly, inflammatory conditions show different patterns between non-silenced and silenced microglia. LPS treatment at 5 µg/mL does not induce an increase in live neuron engulfment, as the levels of engulfment are the same as the control. Moreover, CO pre-treatment does not change engulfment under control and inflammatory conditions, presenting the same levels of engulfment as the control. This could indicate that CD200/CD200R controls phagoptosis since its' knocked down expression reverts phagocytosis of live neurons. Likewise, as phagocytosis of live neurons is harmful since it leads to neuronal loss, it is possible that the silencing in this instance had a cytoprotective role. Accordingly, Lyons et al [40] reported an increase of phagocytosis of amyloid-β plaques that in microglia obtained from $CD200^{-/-}$ mice, thereby it is not unreasonable to consider that the absence of CD200/CD200R communication may result in beneficial effects in a neuroinflammation context. However, given the lack of statistical significance, further n's would be required before drawing a firm conclusion regarding the influence of CD200/CD200R on phagoptosis.

2. Effect of peroxisomal defect in inflammation and lipid peroxidation

Inflammation can be regulated by ROS. In fact, ROS signalling can activate transcription factors like MAPK and NF-κB, which in turn increase the expression of pro-inflammatory cytokines [84]. Along with mitochondria, peroxisomes are one of the main sources of endogenous ROS generation [81]. Thus, the role of peroxisomes on inflammatory response and lipid peroxidation was assessed. Likewise, the relevance of peroxisomes on CO-mediated anti-inflammatory effect was also studied.

The effect of peroxisomal deficiency on inflammation and lipid peroxidation was determined, and five independent experiments were performed. However, out of those five biological n's, only one set was considered valid to be analysed (n = 1) since LPS induced nitrite production in only one biological n. In fact, it is well established that all strains of BV-2 microglia (WT, ABCD1$^{-/-}$ ABCD2$^{-/-}$ and ACOX-1$^{-/-}$) increase NO production following LPS-induced inflammatory stimuli [21,78]. Thus, any experiment without an increase of nitrite production under LPS treatment had to be discarded. The data is normalized to the control condition (100%). The Griess assay is an indirect measure of NO production through the colorimetric measure of nitrite [83].

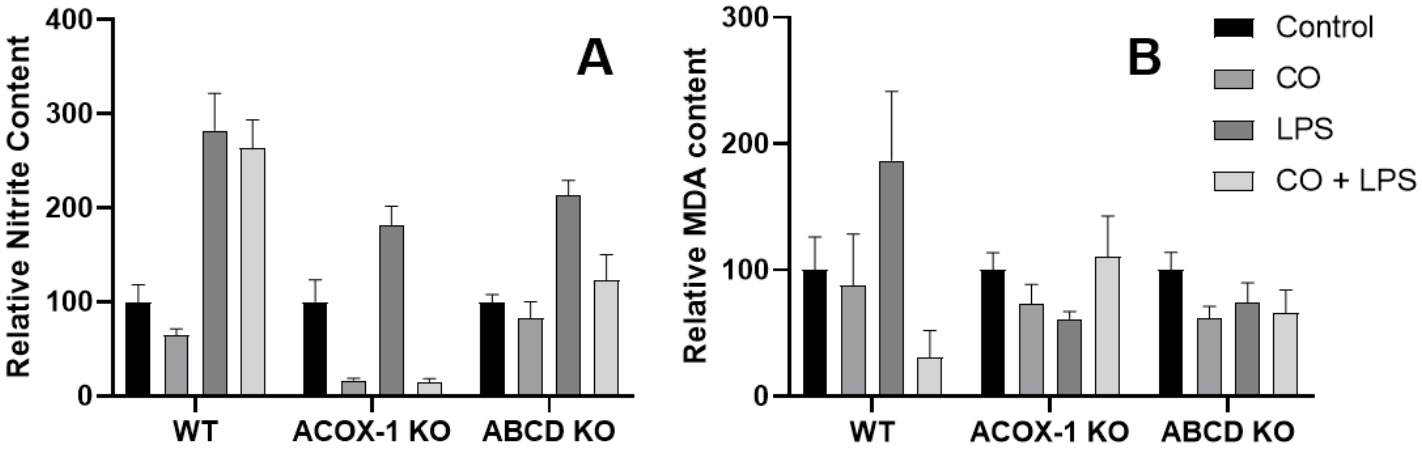

Figure 27 – Under inflammatory conditions, WT microglia appear to show an increase of both lipid peroxidation and NO production, ABCD1 $^{-/-}$ABCD2 $^{-/-}$ does not show an increase of lipid peroxidation, while NO production seems to increase and ACOX-1 $^{-/-}$ does not show an increase of lipid peroxidation, while NO production seems to increase. *BV-2 microglia cells were treated with CO at 50 µM for 24 hours, subsequently treated with LPS at 5 µg/mL for another 24 hours. (**A)** Cellular supernatant was added to an equal volume of Griess and absorbance was measured at 540 nm for nitrite measurement. (**B)** TBARS solution was added to cellular pellet, which was then heated for 10min at 99ºC, and the absorbance at 540 nm was measured to assess lipid peroxidation. Results were normalized to control conditions (100%). n=1 (4 technical replicates), error bars represent mean ± SEM.*

As expected, in all strains LPS treatment increased nitrite production. CO appears to (partially or totally) reverse NO generation, which indicate its anti-inflammatory property. However, given that this data corresponds to a single biological n, no statistical analysis could be done and further experiments are needed to determine the role of peroxisomes in CO-mediated anti-inflammation.

Regarding WT microglial cell line, Soares et al [21] have already showed that CO indeed reverts LPS induced NO production, which supports this preliminary data (Figure 27A). In ACOX-1$^{-/-}$ microglia cell line, CO treatment, appears to decrease nitrite content under normal and inflammatory conditions (Figure 27A). One could speculate that knocking out ACOX-1 combined with CO treatment results in a decreased basal activity of nitric oxide synthase. While CO treatment in ABCD1$^{-/-}$ABCD2$^{-/-}$ BV-2 cell line does not change basal levels of NO production (Figure 27A). Because CO can still revert the LPS-induced inflammatory response in the absence of ACOX-1 or of ABCD1 and ABCD2, it indicates that the CO-mediated anti-inflammatory effect may be not dependent on peroxisome.

During inflammation, there is an increase of lipid content in the cytosol, as well as a higher levels of ROS [78,84]. Accordingly, an increase in lipid peroxidation was observed in WT BV-2 cell line following LPS treatment (Figure 27B). While, CO seems to revert this increase by decreasing lipid peroxidation levels. However, as stated before, further data is required to conclude that CO reverts its effect.

Dysfunctional peroxisomes generated by knocking out genes presented an increased inflammatory response [75,78] and accumulation of VLC-FAs in the cytosol [67]. In contrast, it can also be speculated that dysfunctional peroxisomes present less oxidative activity leading to lower levels of lipid peroxidation. In fact, under inflammatory conditions, ABCD1$^{-/-}$ABCD2$^{-/-}$ and ACOX-1$^{-/-}$ cell lines presented lower levels of lipid peroxidation (Figure 27B). CO pre-treatment in ACOX-1$^{-/-}$ microglial cell line seems to revert the decreased lipid peroxidation induced by LPS treatment. While in ABCD1$^{-/-}$ ABCD2$^{-/-}$ microglial cell line, CO pre-treatment seems no to have any effect on the LPS-mediated decrease of lipid peroxidation levels.

Lipid peroxidation was measured using the TBARs assay, which is based on the colorimetric measurement of an adduct comprising of MDA and TBA [82]. MDA is one of the main products of lipid peroxidation, resulting from decomposition of arachidonic acid and larger polyunsaturated FAs [81]. Still, MDA is not the only secondary product of lipid peroxidation, with another major secondary product called 4-hydroxynonenal (4-HNE) [81,82]. 4-HNE is a very reactive molecule produced in large amounts as a result of membrane lipids' peroxidation, thus being considered one of the most toxic products of peroxidation [81]. Important to the scope of this work, 4-HNE also has a role in regulating transcription factors related to cell stress and inflammation such as NF- κB, and PPAR [81]. Therefore, assessing 4-HNE production could also be useful to determine the degree of lipid peroxidation in microglia.

For further work, lipid peroxidation levels should be normalized by the total amount of PU-FAs for each microglial cell line. This preliminary data suggests that peroxidation appears to decrease in the mutant strains. Nevertheless, this decrease could be associated with a reduced lipid production or a reduced levels of proteins involved in lipid β-oxidation.

The TBARs assay was used for its simple nature and low cost. Nonetheless, another factor to have in account is that while this assay can give a notion about peroxisome integrity, lipid peroxidation can occur in mitochondria, consequently this assay is not peroxisome specific. To further assess peroxisomal integrity, specific peroxisomal assays must be done. Catalase is an enzyme mostly restricted to the peroxisome, which can be quantified through western blotting [85] as a measure of peroxisomal population.

There is only one biological replicate and four technical replicates and this lack of biological n's does not allow any conclusions with statistical significance. It was difficult to pipette the samples onto the plate to be measured without dragging pellet, which affected the absorbance increasing it. The pH of the TBARs solution is also important to the reading of this results [82] and this factor was not controlled. The protocol of the assay needs to be optimized in the future to avoid such issues.

3. Quantification of ACOX-1 under inflammatory conditions

To further assess if the peroxisome has an important function during inflammation, ACOX-1 expression was analysed by immunoblotting. ACOX-1 is an enzyme that catalyses the first step of peroxisomal β-oxidation [66] and has a molecular weight of 74 kDa. Deficiencies in ACOX-1 expression in humans lead to neuroinflammatory [67,68,71,75,86] diseases and ACOX-1$^{-/-}$ BV-2 microglia express higher levels of pro-inflammatory cytokines IL-1β and IL-6 [68]. Protein samples were collected from WT BV-2 cell line under three conditions: control, with inflammatory stimuli (0.5 µg/mL LPS) and pre-treated with 50 µM of CORM ALF-826 and then inflammatory stimuli. Ponceau Red was used to estimate if all samples had similar levels of total protein.

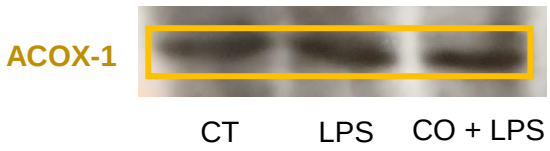

Figure 28 – LPS treatment does not change ACOX-1 expression. *WT BV-2 cells were treated with 50 µM ALF-826 for 48h and then with 0.5 µg/mL for 24h. Protein extracts were obtained through loading buffer lysis. Protein was separated using SDS-PAGE on a 10% polyacrylamide gel. Rabbit anti-ACOX-1 (1/200) was the primary antibody used, while the secondary was donkey anti-rabbit (1/5000).*

All conditions have the same expression of ACOX-1 (Figure 28). This indicates that inflammation does not affect ACOX-1 expression and that CO's anti-inflammatory effect on microglia is independent of ACOX-1. Kong et al [87] observed that in macrophages of large yellow croaker stimulated by palmitate for an inflammatory response, mRNA expression of ACOX-1 was upregulated, with elevated pro-inflammatory gene expression and ROS levels. Eventually, a different inflammatory stimuli may be needed to upregulate ACOX-1 expression. Nevertheless, results should be normalized using a loading control like the conserved protein beta-actin to assure that all samples have similar total protein expression.

Discussion and Conclusion

Neuroinflammation is a hallmark of many neurological problems, such as Alzheimer's disease or stroke. Microglia are the cell population that initiate inflammation in the CNS, so the study of their regulation and activation is significant. Microglia have a phagocytic function, which allows for the clearance of pathogenic cells and toxic cell debris. Phagocytosis was often thought as consequence of cell death. However, a new concept of cell death is being proposed – cell death by phagocytosis or phagoptosis. Despite occurring naturally, in pathological circumstances phagoptosis can be harmful because it results in an excessive death of viable neurons.

CO is an endogenous gas that has been found to be a modulator of inflammation and it decreases the secretion of pro-inflammatory cytokines *in vitro* and *in vivo*. Previous work from our group has also pointed out CO as a modulator of microglial phagocytosis of dead neurons, polarizing microglia from a pro-inflammatory response to an anti-inflammatory response. CO treatment has shown to increase the expression of the anti-inflammatory ligand-receptor pair CD200/CD200R.

Considering this, this work set out to evaluate if CO's pro-phagocytic potential applies to phagoptosis and if the ligand-receptor pair CD200/CD200R is mechanistically involved in CO's action. BV-2 microglia and CAD neuronal cell lines were used. Microglial engulfment was measured using co-localization of microglia and neurons via flow cytometry. Microglia were treated using ALF-826, a CORM that has been proven as anti-inflammatory, and subjected to LPS as an inflammatory stimuli. To assess the function of the CD200/CD200R pair, microglia had the CD200R1 gene knocked down by siRNA.

Firstly and surprisingly, the concentration of LPS previously used by our research group (0.5 µg/mL) did not induce phagocytosis of live neurons. This could indicate that phagocytosis of live neurons may occur under a scenario of acute inflammation. Stimulation with a higher concentration of LPS (5 µg/mL) increased microglial engulfment of live neurons. CO pre-treatment of microglia partially reverted inflammatory effect by decreasing engulfment. So, CO has a neuroprotective effect by limiting pro-phagocytic effect of microglia on live neurons under acute inflammation.

In order to test a more representative model, CAD cell line was differentiated into neuron like cells. Differentiated neurons did not get more phagocytized by microglia treated with 0.5 µg/mL LPS. On the other hand, differentiated neurons showed a slightly increased phagocytosis by microglia treated with 5 µg/mL LPS. CO pre-treatment did not influence this increase. So, further studies with differentiated neurons must be done. Considering that differentiated and non-differentiated neurons showed similar morphologies, extending the period of differentiation or changing the differentiation protocol may also be useful to improve neuronal differentiation and to assess engulfment of neurons with an *in vitro* model that mostly resembles live neurons.

Silencing CD200R had unexpected effects as all conditions showed similar engulfment levels. Differently from what was observed with non-silenced microglia, LPS stimulation did not increase live neuronal engulfment, which could indicate that CD200/CD200R communication can be harmful cases of acute inflammation. Therefore, silencing CD200R under these circumstances would have a cytoprotective effect. It has been reported that microglia from CD200 deficient mice show increased phagocytosis of

amyloid-β plaques [40]. For this reason, it is not impossible that a lack of CD200/CD200R communication might have positive results regarding phagocytosis. Considering that the reveal of phosphatidylserine on the membrane is a main signal associated with phagoptosis [31], it could be useful to evaluate the effect of inflammatory stimuli and CD200R silencing on neuronal expression and exposition of this phosphatidylserine.

Another potential regulator of inflammation is the peroxisome. These are small organelles ubiquitously present in mammal cells. Peroxisomes catalyse ROS metabolism and are exclusively responsible for β-oxidation of VLC-FAs. As both lipids and ROS are important in the inflammatory response, and disorders associated with peroxisome functioning often result in excessive neuroinflammation, this organelle may be important in microglia polarization. The role of peroxisomal disorders in inflammation was evaluated. Mutant BV-2 microglia cell lines with peroxisomal protein genes knocked out (ACOX-1$^{-/-}$ and ABCD1$^{-/-}$ ABCD2$^{-/-}$) were used. Peroxisomal function was assessed indirectly using the TBARs assay. ACOX-1 was quantified from protein extracts of WT BV-2 microglia stimulated with LPS.

WT microglia showed an increased lipid peroxidation under inflammation that was decreased when pre-treated with CO. In fact, the peroxisome deficient cell lines demonstrated decreased levels of lipid peroxidation under inflammatory circumstances. In ACOX-1$^{-/-}$ microglial cell line the LPS-induced decreased lipid peroxidation appears to be reversed by CO pre-treatment. Whereas CO pre-treatment appears to have no effect on the LPS-mediated lowering of lipid peroxidation levels in the ABCD1$^{-/-}$ABCD2$^{-/-}$ microglial cell line. Thus, it may indicate that CO anti-inflammatory effect is not dependent on peroxisomal function. More data is needed to evaluate whether CO's control of phagocytosis depends on peroxisomal function. Peroxisomal deficiencies are associated with higher inflammation and lipid accumulation in the cytosol. Considering that lipid peroxidation results from the reaction of lipids with ROS, and inflammation leads to increase of cellular ROS, one would expect that peroxisomal deficient microglia showed higher lipid peroxidation under inflammation. These results could also be interpreted that as peroxisomal deficient microglia show less oxidative activity, lipid peroxidation is lower.

To validate these results with statistical significance, more biological replicates must be done. The TBARs assay measures MDA, a product of lipid peroxidation. There are other products of lipid peroxidation, like HNE, that could be measured to evaluate peroxisome function. Besides, lipid peroxidation also occurs in the mitochondria, so a peroxisome function assay that is organelle specific could be employed. One example of such assay would be to measure catalase activity. In order to have more accurate results, lipid peroxidation should also be normalized by the total amount of cellular lipids.

Having in mind that ACOX-1$^{-/-}$ microglia are oriented towards phagocytosis [68], it would be intriguing to examine if CO modulates phagocytosis in a peroxisomal dependent manner. It is well established that the ligand-receptor CD200/CD200R modulates phagocytosis as well [40,42–44] and CO's pro-phagocytic effect involves this pair in phagocytosis of dead neurons [77]. Herein, it was shown that CO limits microglial phagocytosis of live neurons in a CD200/CD200R independent manner. For future studies, further experiments should be conducted to evaluate whether peroxisomal function controls phagocytosis. For example, engulfment assays based on flow cytometry could be performed on the KO BV-2 cell lines.

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