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Understanding the role of microglial extracellular vesicles (EVs) in neuroinflammation spreading: an *in vitro* study

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ABBREVIATIONS

3´UTR 3'untranslated region
α-Syn α-Synuclein
AAM Alternative Activated Microglia
AAV Adeno-Associated Virus
Aβ Amyloid-β
AD Alzheimer's disease
AGO Argonaute
ALIX ALG-2-interacting Protein X
ALS Amyotrophic Lateral Sclerosis
AP-1 activator protein 1
APC Antigen Presenting Cell
APOE Apolipoprotein
APP Amyloid Precursor Protein
ARG1 Arginase 1
ASDs Autism Spectrum Disorders
ATP Adenosine Triphosphate
BBB Blod Brain Barrier
BDNF Brain-derived Neurotrophic Factor
C9orf72 chromosome 9 open reading frame 72
CAM Classical Activated Microglia
CCL Chemokine Ligand
CCR Chemokine Receptor
CD Cluster of Differentiation
CFH Complement Factor H
Chi3l3 Chitinase-3-Like-3
CNS Central Nervous System
CSF Cerebrospinal Fluid
COX 2 Cyclooxygenase 2
DAM Disease-Associated Microglia
DAMPs Danger-Associated Molecular Patterns
DAP12 DNAX activating protein of 12 kDa
DGCR8 Di George Syndrome Critical Region 8
DNA Deoxyribonucleic Acid
EAE Experimental Autoimmune Encephalomyelitis
EGF Epidermal Growth Factor
EMR1 EGF-like module-containing mucin-like hormone receptor-like 1
ESCRT Endosomal Sorting Complex Required for Transport
EVs Extracellular Vesicles
FIZZ1 Found in inflammatory zone 1
FUS Fused in Sarcoma
GABA Gamma-Aminobutyric Acid
GAPDH Glyceraldehyde-3-phosphate Dehydrogenase
Hexb Hexosaminidase subunit beta
HMC3 Human Microglia Clone 3
HNRNPA1 Heterogeneous Nuclear Ribonucleoprotein A1

HSC Heat Shock Cognate
HSP Heat Shock Protein
IBA1 Ionized calcium-binding adapter molecule 1
IFN Interferon
IGF1 Insulin-like growth factor 1
IL- Interleukin-
ILVs Intralumenal Endosomal Vesicles
iNOS inducible Nitric Oxide Synthase
IRAK-1 Interleukin 1 Receptor Associated Kinase 1
IRFs IFN Regulatory Factors
JNK c-Jun N-terminal kinases
LDLR Low Density Lipoprotein Receptor
LPS Lipopolysaccharides
MCSF Macrophage Colony-Stimulating Factor
MAPK Mitogen-activated protein kinase
MCPIP1 Monocyte Chemotactic Protein-Induced Protein 1
MCT1 Monocarboxylate transporter 1
MHC Major Histocompatibility Class
MIP Macrophage inflammatory protein
MiRNA MicroRNA
MMP Metalloproteinase
MRE miRNA Response Element
MVB Multivesicular Bodies
MVs Microvesicles
NADPH Nicotinamide Adenine DInucleotide Phosphate
NF-Kb Nuclear Factor kappa B
ND Neurodegenerative Diseases
NFT neurofibrillary Tangles
NGF Neural Growth Factor
NLR NOD-like receptors
NLRP3 Family Pyrin Domain Containing 3
NO Nitric Oxide
NP Nanoparticles
NTC No–template control
NTA Nanoparticles Tracking Analysis
P2RY Family of Purinergic G protein-coupled receptors
PAMPs Pathogen-associated molecular pattern
PBS Phosphate Buffered Saline
PCD Programmed Cell Death
PCR Polymerase Chain Reaction
PD Parkinson's disease
PDGF Platelet-Derived Growth Factor
PI3–K Phosphatidyl Inositol-3 kinase
PLP Proteolipoprotein
PNP Purine Nucleoside Phosphorylase
RAGE Receptor for Advanced Glycoxidation End-products
RISC RNA-induced silencing complex
RNA Ribonucleic Acid

RNS Reactive Nitrogen Species
ROS Reactive Oxigen Species
Sall1 Spalt-like transcription factor 1
Siglec-H Sialic acid-binding immunoglobulin-type lectin H
SHIP1 Src homology-2 domain-containing inositol 5 phosphatase1
SMAD Small Mother Against Decapentaplegic
SOCS Suppressor of Cytokine Signaling
SOD1 Superoxide Dismutase 1
TARDBP TAR DNA Binding Protein
TDP-43 Transactive Response DNA binding protein 43 kDa
TGF- β Transforming growth factor beta
TLR Toll-like Receptor
TMEM119 transmembrane protein 119
TNF Tumor Necrosis Factor
TREM2 Triggering Receptor Expressed on Myeloid cells
VEGF vascular endothelial growth factor
XPO Exportin

ABSTRACT

The paradigm of microglia activation has been increasingly studied in recent years, in several neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) with the aim to uncover the mechanisms underlying neuroinflammation (Hickman *et al*.,2018).

In fact, microglia, the immune cells of the brain, play a critical role in the inflammatory condition following the onset of neuropathology and their activation status is considered as a double-edged sword that executes both detrimental and beneficial effects; generally, proinflammatory microglia phenotype predominate at the injury site in the end stage of disease characterized by increasing accumulated inflammatory mediators, when the immunoresolution and repair process of anti-inflammatory microglia phenotype failed (Saitgareeva *et al*., 2020).

Taking these evidences together, the first aim of this thesis was to prove if activation could be transmitted among microglial cells. The microglial polarization has been pharmacologically induced in two different *in vitro* models: N9, microglial murine cell line, have been treated by using LPS towards a proinflammatory/neurotoxic phenotype or ATP towards antinflammatory/neuroprotective status; HMC3, human microglial cell line, have been activated using IFN-γ+ATP, in order to enhance the immune response releasing vesicles.

Considering that the phenotypic shift could be related to a change in the content of extracellular vesicles (EVs) involved in intercellular communication and, therefore, by the effect that the content of these vesicles has on surveying microglia and other cell types (Delpech *et al*., 2019; Lemaire *et al*., 2019), non-activated microglia have been treated with the conditioned medium by differentially activated microglia, as well as with the isolated exosomes.

Furthermore, by using the above-mentioned *in vitro* approaches, we investigated the expression profiles of microRNAs in N9 cells, identified as regulators of microglial activation; in particular, miRNA-155, miRNA-124, miRNA-34a, miRNA-125b that are known to be dysregulated in different pathological states (Christoforidou *et al*., 2020; Juźwik *et al*., 2019).

We can speculate that the increased expression of miRNA-34a observed in our model underline a possible contribution in the diffusion of proinflammatory activation of microglia. Thus, we tried to downregulate miR-34a expression using cleaving sequences of anti-mir-34a DNAzyme delivered by DNA nanostructures (Wu *et al*., 2020) aimed to confirm the involvement of miR-34a in this process and that its downregulation leads to a reduction of microglia polarization towards the neurotoxic phenotype.

In conclusion, this thesis work reveal a new inflammation spreading mechanism that involves release of vesicles containing specific cargos by donor polarized microglia, particularly miRNAs, able to influence the phenotypic shift in unpolarized microglia.

Given that evidence, the role of EVs miRNAs released by microglia deserves to be deeply investigated both as potential therapeutic targets and as biomarkers for neurodegenerative diseases.

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1. INTRODUCTION

1.1 NEUROINFLAMMATION: PHYSIOLOGICAL *VS* PATHOLOGICAL RESPONSE

Neuroinflammation is defined as the process whereby the innate immune system of Central Nervous System (CNS), in particular in the brain and spinal cord, is triggered following an inflammatory challenge, such as those caused by injury, infection, exposure to a toxin, neurodegenerative disease or aging to protect itself (Lyman *et al*., 2014).

Following activation of the immune response certain molecular and cellular mechanisms are recruited, which have various physiological, biochemical and behavioral consequences to reestablish the homeostasis (Di Sabato *et al*., 2016). Inflammatory response appears as a double-edged sword because immune signals could be supportive or destructive to the CNS, hence can promote both reparation and damaging of brain and spinal cord tissues (Shabab *et al*., 2017). Persistence of physiological mechanisms of protection or reparation results in an hyperproduction of certain neuroinflammatory mediators, which causes cells damages or death (Wyss-Coray *et al*., 2002).

These responses are mediated by two types of immune cells: the hematopoietic system cells (lymphocytes, monocytes and macrophages) and glial cells of the CNS, notably astrocytes and microglia (Stoll & Jander, 1999).

In response to harmful stimuli, glial cells are the first to be activated; within any scenario of immune-mediated brain injury, microglia qualify as the principal immune effector cells that respond by synthesizing and releasing powerful and appropriate mediators, such as cytokines, chemokines, interleukins, nitric oxide and reactive oxygen species, aimed to homeostasis restoration and limitation of tissue damage (Chan *et al*., 2019).

Neuroinflammation can be further distinguished in two main responses, during acute and chronic conditions. Basically, acute inflammation involves the early and defensive responses to an injurious agent that paves the way for restoring the physiological status in the damaged site; chronic inflammation results from immune-mediated responses that are persistent (Streit *et al*., 2004).

Acute reaction is composed by a rapid activation of glial cells that is commonly defined as “reactive gliosis" that refers to the expansion of active glial cells appearing immediately after

injury. When it persists, gliosis can become detrimental and limiting for functional recovery. The positive feedback loop and the cumulative effects of immunological glial activation contribute to expand the initial maladaptive reactions, thus worsening the unbalanced inflammatory process that become chronic and attempts the functional recovery. This chronicity may result also from Blood Brain Barrier (BBB) breakdown that allows cells of the peripheral immune cells to leave the blood stream and infiltrate the damage area (Pekny *et al*., 2016).

These mechanisms appear to be characterized by a dramatic increase in proinflammatory factors production, such as cytokines and chemokines that fuel the inflammatory cascade; hence, neuroinflammation biomarkers can be studied in any acute or chronic brain diseases, to distinguish between healthy and pathological state, but also to study a possible therapeutic strategy (Tansey *et al*., 2010).

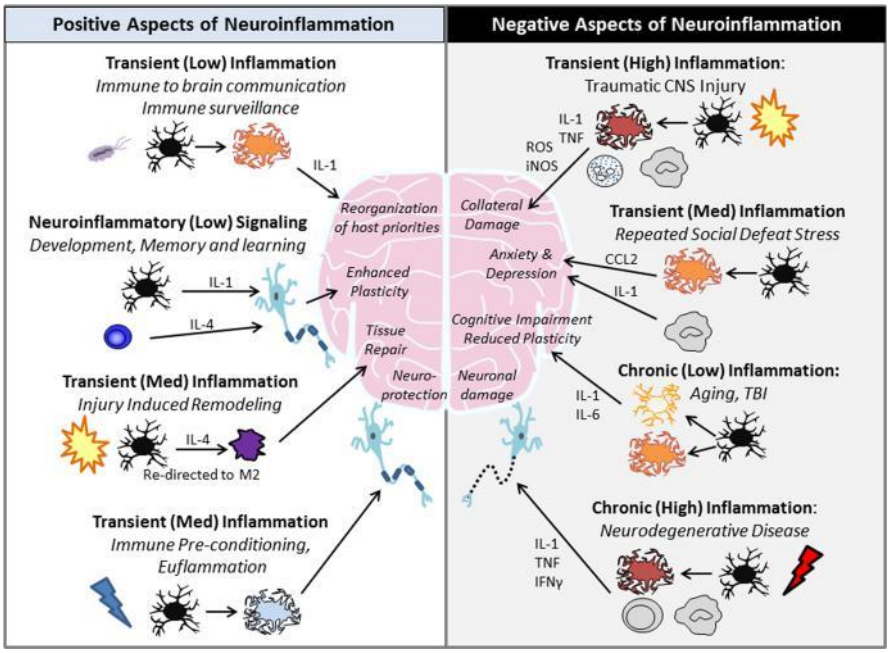


Figure 1. Positive and negative aspects of neuroinflammation. Reproduced from: Di Sabato *et al*., 2016

1.2 NEUROINFLAMMATION IN NEURODEGENERATIVE DISEASES

With the increase in life expectancy, the impact of neurodegenerative diseases (NDDs), including Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral

Sclerosis (ALS), as well as demyelinating disorders, such as multiple sclerosis (MS), is increasing considerably (Cova *et al*., 2017). However, the pathological mechanisms underlying NDDs are poorly understood, and with few exceptions, their causes are essentially unknown. The AD onset is characterized by impaired ability to remember recent events and recognition of people/object due to a decline in neurons localized in in the basal forebrain and hippocampus. AD is associated to extracellular deposition of amyloid-β (Aβ) plaques due to an abnormal production and ineffective phagocytosis of Aβ peptides derived from amyloid precursor protein (APP) cleavage and by neuronal accumulation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein (Bachiller *et al*., 2018). Mutations responsible for rare familial forms have been identified in three genes, the amyloid precursor protein (APP) gene on chromosome 21, the presenilin 1 (PSI) on chromosome 14, and the presenilin 2 (PS2) on chromosome 1, have been found in families with an autosomal dominant AD, besides an allelic variant of apolipoprotein-E (APOE) epsilon4 has been associated also with sporadic disease onset (Bettens *et al*., 2013).

PD, the second most common neurodegenerative disease, is characterized by the dopaminergic neuronal death in the substantia nigra and the aggregation of α-synuclein protein from monomers into fibrillary protein (Lewy bodies) (Sanchez-Guajardo *et al*., 2013). The first symptoms of PD are resting tremor bradykinesia, akinesia and muscle rigidy. At death, patients show dopaminergic cell loss within the substantia nigra (SN) pars compacta and resultant dysfunction of the basal ganglia and immunohistochemical studies have shown α-synuclein-positive accumulations within cortical neuronal processes, named Lewy bodies but they differ in distribution from those observed in AD, which are located near the amyloid plaque and neurofibrillary tangle. Several families from diverse backgrounds have been observed with Parkin mutations; SNCA (PARK1) and, less frequently DJ-1 (PARK6) and PINK1 (PARK7), cause the autosomal recessive forms associated with early onset (Shulman *et al*., 2011). The LRRK2 gene, which encodes for dardarine, can cause the dominant sporadic or familial forms. Exposure to environmental toxins may increase risk of developing PD; consistent findings indicate methyl-phenyl-tetrahydropyridine (MPTP) and the pesticide rotenone association with increased PD susceptibility (Van Der Mark *et al*., 2012).

Similar to other neurodegenerative conditions, ALS is caused by a combination of genetic (e.g. mutations in four main genes C9orf72, TARDBP, SOD1, and FUS), environmental and aging-related factors. Though most cases of ALS are sporadic, 10% of cases have a clear

Mendelian inheritance and high penetrance. Inheritance in familial ALS (fALS) is usually autosomal dominant, but some autosomal recessive inheritances have been described. To date, mutations in only one gene, called Cu/Zn superoxide dismutase (SOD1), lead to classical dominantly inherited ALS. The neuropathological hallmark of ALS is the progressive loss of upper and lower motor neurons and leads to muscle weakness (Masrori *et al*., 2020). Another important neurological disabling disease whose incidence is increasing worldwide is MS, classified as an organ-specific T-cell mediated autoimmune disease generating inflammatory lesions with infiltration of B-cells and plasma cells, leading to destruction of the myelin sheath. Epstein–Barr virus (EBV) infection, ultraviolet B light (UVB), smoking and vitamin D, combined with genetic background, play important roles in MS development.

The main genetic risk associated with MS resides in HLA-DRB1*15 because of the role in antigen presentation, but more than 150 single nucleotide polymorphisms associated with MS susceptibility, including include those within IL7R, IL2RA, TNFR1, BAFF and CYP2R1. MS can be mainly classified in two-stage disease relapsing–remitting disease and delayed neurodegeneration causing non-relapsing progression with a worsening of the symptoms including optic neuritis, mobility problems, muscle spasms. Based on these evidences, it is clear that the etiology of NDDs concerns the role of genetic and toxic environmental factors in the initiation of these diseases; some NDDs have a familial occurrence, suggesting a genetic basis, but, in the majority of patients, the initiation of the disease is sporadic (Forman *et al*., 2004). It means that any genetic contribution to the neurodegenerative process is minimal (Brown *et al*.,2005).

During the last decade growing evidence suggested a role of neuroinflammation in the progression and likely etiology of neurological disorders, including neurodegenerative ones (Przedborski *et al*. 2003). Several molecular mechanisms that incentive neuronal death may be triggered by inflammatory cells and their mediators at various phases of the neurodegenerative cascade (Amor *et al*., 2010).

Low-grade inflammatory responses are essential to clear injured/dead cells from the tissues; prolonged and increased inflammatory reaction with high levels of inflammatory mediators such as cytokines including TNF-α, IL-1β, IL-6, IL-8, TGF-β chemokines (e.g. CCL2, CCL5, CCL8, CX3CL1) matrix metalloproteinases (e.g. MMP2, MMP9), prostaglandin (e.g. PGE2), cyclooxygenase 2 (COX 2), ROS, RNS (Kempuraj *et al*., 2016; Cartier *et al*., 2004) and

activation of immune cells achieve opposite functions by mediating deleterious onset and progression of NDDs (Ransohoff, 2016).

In AD, the environment around Aβ plaques have been shown to contain increased levels of chemoattractant for microglia, including MCP-1, MIP-1α, MIP-1β, IL-8, and MCSF, leading to increased numbers of plaque- associated microglia. During Aβ accumulation, microglial cells have been shown to be activated by several sensing systems, including TLRs. It has been reported in vivo and in vitro that TLR2, TLR4 and TLR6 are engaged to promote neurotoxic inflammation and deficiency in CD36, TLR4 or TLR6 reduces the neurotoxic effects of Aβ and cytokine production (Stewart *et al*., 2010). AD-associated TREM2 (Triggering Receptor Expressed on Myeloid cells) mutation have been associated to increased TLR-induced TNF production with a signaling adapter DAP12 (Colonna *et al*., 2016; Hamerman *et al*., 2006). Another sensing system interacting with Aβ is the receptor for advanced glycoxidation end-products (RAGE) that is involved in glial cells activation and, in particular, in ApoE-mediated cellular signaling. ApoE4 allelic variant is associated with an elevated risk of AD, exhibiting low anti-inflammatory properties LDLR-mediated signaling that leads to an activation of microglia through JNK kinase activity. NOD-like receptors (NLRs) act as a sensor for cellular insult. NLRP3 in glial cells provide the recruitment of apoptosis-associated proteins and liberation of proinflammatory mediators, such as IL-1β and IL-18 (Butterfield *et al*., 2002).

The aggregation of α-Syn in PD can induce increased expression of pro-inflammatory cytokines by activated microglia, release of ROS after its internalization and activation of NADPH oxidase and the production of nitric oxide: these mechanisms perturb the balance of dopamine neuron survival and death of neighboring cells via various signaling pathways, such as p38 MAPK, NF-κB and TLRs pathways (Sampson *et al*., 2016). Mechanisms that act to regulate the inflammatory processes have been described. TREM2 has been reported to be involved in PD: down-regulation of TREM2 leads to the expression of pro-inflammatory factors and aggravates progression of PD (Zhang *et al*., 2018). In this sense, also Nurr1 (orphan nuclear receptor) participates to arrest the production of glial neurotoxic products and a reduced expression leads to and amplified immune response (Xu *et al*., 2021).

Similar to other neurodegenerative conditions, in ALS the activation of inflammatory pathways in combination with the prominent gliosis are the principal initiators of the cascade that culminates in the motor neuron death. Motor neuron expressing mutant SOD1 seems to be more sensitive to NADPH oxidase-dependent production of ROS, NO, peroxide

and FasL produced by surrounding glial cells (Raoul *et al*., 2006) and increased levels of IL-12, cytokine implicated in the stimulation of adaptive immune response, may lead chronicization of the response (Nguyen *et al*., 2004).

MS pathogenesis denotes the presence of autoreactive T cells and B lymphocytes, but evidences indicate the contribution of myeloid cells in the immunopathology of MS, such as monocytes, macrophages and microglia (Lucchinetti *et al*., 2000). The MS patients show a dysfunctional regulatory process in which high levels of secreted IL-6, IL-12, TNF-α and ROS are not contained due to low production of IL-10 secretion from Treg cells, Breg cells and anti-inflammatory myeloid cells, promoting persistence of inflamed parenchyma instead of remyelination processes and tissue repair. Moreover, the CD16+ monocyte promoted CD4+ T cell trafficking, contributing to the breakdown of BBB by promoting T cell entry into the CNS (Kouwenhoven *et al*., 2001).

Based on the plethora of the players that are involved in the neuroinflammation of AD, PD, ALS, MS and other neurodegenerative diseases, it is clear that the inducers and effectors of the inflammatory response are responsible for positive feedback that is gradually amplifying, aggravating the progression of the existing pathology.

1.3 ROLE OF MICROGLIA IN NEUROINFLAMMATION

1.3.1 THE ORIGIN OF MICROGLIA

Microglia are the resident mononuclear phagocytes of the CNS, belonging to the glial system of non-neuronal cells and are distributed throughout the brain and the spinal cord. Microglia were first described as a distinct glial cell type in 1919 by Pio del Rio Hortega (Pérez-Cerdá *et al*., 2015), but the origin of these cells was debated for a long time and multiple schools of thought have emerged. Nowadays, it is known that microglia originate from yolk sac erythro-myeloid progenitors, then infiltrating into the brain parenchyma during early embryogenesis for differentiation and maturation and renewing themselves throughout adulthood (Saijo & Glass, 2011). Microglia are long-lived cells with a relatively low turnover rate and locally self-renewing without any contribution from bone-marrow-derived cells at a steady-state, but from potential progenitor source within the CNS (Wurm *et al*., 2021). During the embryonic and fetal stages, myeloid progenitors migrate into the developing nervous system, where

they differentiate into parenchymal microglia, constituting one of two resident microglial populations that will continue to proliferate throughout life (Kettenmann *et al*., 2011). This migration occurs between the last half of the first trimester and the beginning of the second trimester of fetal life in humans, while in mouse models it occurs in the tenth to the nineteenth day of embryonic life (Rezaie, 2003).

On the other hand, a second microglial population becomes established in the CNS surrounding cerebral vasculature (Garaschuk *et al*., 2019). They differentiate into vessel-associated microglia (VAM) and perivascular macrophages (PVMs). These cells cross the vessel wall to invade the nervous system where they constitute the second resident microglial pool as a component of neuro-glia-vascular unit (Chen *et al*., 2023).

In physiological conditions, microglia originate from progenitors that have established themselves in the CNS and which maintain a proliferative capacity; in pathological conditions, following an increased permeability of the BBB, other immune cells, including monocytes derived from the bone marrow, which in the CNS differentiate into microglia and macrophages, infiltrate the nervous tissue (Tay *et al*.,2019).

Microglia are generally classified as macrophages and they express many macrophage-associated markers, such as CD11b, CD14 and EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1; known as F4/80 in mice). Microglial cells are labeled with many reagents, which also mark hematopoietic cells. The existence of markers recognizing mature microglia, but not embryonic ones, such as P2ry12 and Tmem119 (Bennett *et al*., 2016), indicate that the transcriptome of mature microglia is different from the one of immature microglia and other tissue macrophages (Li *et al*., 2019). Additionally derived from PU.1$^{-/-}$ mice experiments, PU.1 is a transcription factor expresses in cells of hematopoietic lineage; in fact, lack of PU.1 results in mice depletion of macrophages, eosinophils, B cells, and the impairment of the development of neutrophils and T lymphocytes. Interestingly, microglia in PU.1$^{-/-}$ mice were absent, confirming their hematopoietic origin (Walton *et al*., 2000). However, the two myeloid populations are genetically distinct and differ in their electrophysiological characteristics (Kierdorf *et al*., 2013). These differences imply different functions between infiltrating microglia and macrophages, which are highly evident in mouse models of neuropathology (Mildner *et al*., 2007; Ochocka & Kaminska, 2021).ù

1.3.2 FUNCTIONS AND MORPHOLOGY OF MICROGLIA

Microglia occupy a central position in the defense and maintenance of the homeostasis and account for 5–20% of the total glial cell population within the CNS parenchyma (Dos Santos *et al*., 2020; Perry *et al*., 2010). Due to the large variability in the number of microglial cells present in healthy and diseased brain, this quantification is only estimated. The density and morphology of microglia is region-specific, but it has not yet been elucidated whether these differences may be related to functional heterogeneity. Across brain regions, in white matter microglia show elongated soma and prolongation privileged along fiber tracts, besides, in the circumventricular organs, microglia exhibit compact morphology with short processes. Contrarily, in gray matter microglia show many elaborate radial branches (Shemer *et al*., 2015). The specific interactions between microglial cells and neuronal population in each circumstance may be prerequisites for the recruitment of proper microglial phenotypes that are required for the maintenance of homeostasis (Askew *et al*., 2017).

Microglia have an active role in immune surveillance and are sensitive to many stimuli or changes in the microenvironment of the CNS. Surveying microglia are not passive in the CNS, rather they scan the surrounding milieu of the CNS via receptors for CX3C-chemokine ligand 1 (CX3CL1 or fractalkine), CD47, CD200, and CD22 (Hanisch & Kettenmann, 2007).

In the healthy CNS, microglia have a ramified morphology described with a small soma, little perinuclear cytoplasm, and several branched processes. In response to an insult or any disturbance potentially danger for brain homeostasis, microglia rapidly change their shape from ramified cells, to arborized reactive cells with retracted processes (Ransohoff & Perry, 2009).

They migrate to the site of damage following chemotactic gradients, proliferate, and participate in the presentation of antigens (APC), phagocytosis of cellular debris and secretion of inflammatory compounds, including cytokines and proteases, which promote microglia motility and extracellular matrix remodeling. If the damage is persistent, microglia continue to change their morphology adopting an amoeboid shape with an expansion of the cell body and reduction of branching processes (Tremblay *et al*., 2010). Thus, four main phenotypes have been distinguished based on morphological and molecular criteria: ramified, primed, reactive and amoeboid that allowed the identification a multistep activation process, in which microglia transit from *surveying* state to an *activated* one that

primarily serve for protection of the functional and structural properties of the CNS (Carson et al., 2007).

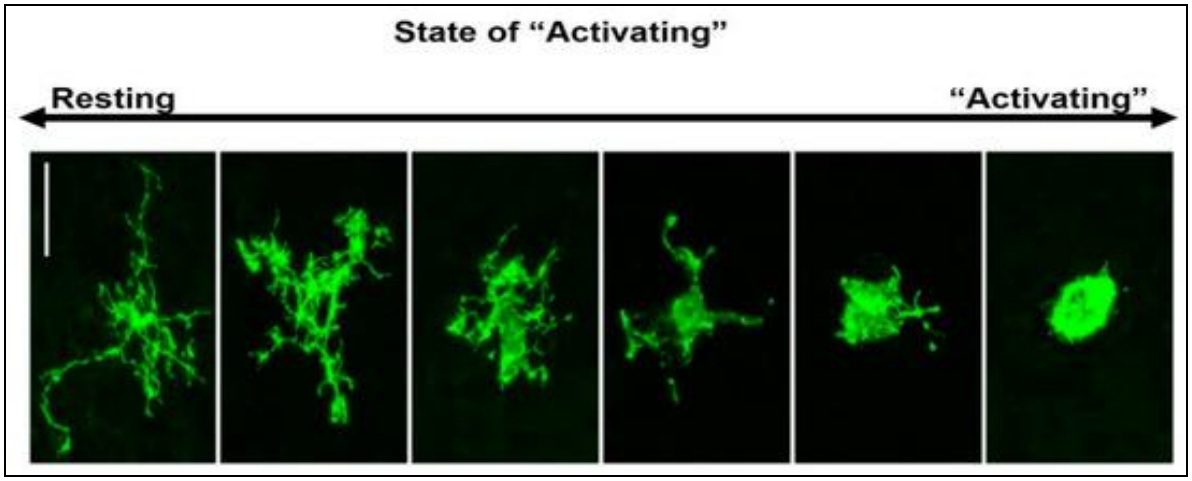


Figure 2. Morphological features of microglia. The morphology of Iba1+ cells from cells with a small soma and fine ramified in a steady state, to cells that progressively retract processes to acquire activated morphology. Reproduced from: Rawlinson *et al*., 2020

1.3.3 PHYSIOLOGICAL FUNCTIONS OF MICROGLIA

In the healthy homeostatic CNS, microglia comprise the predominant myeloid population, followed by non-parenchymal CNS macrophages (CNS- or border-associated macrophages), but only microglia distinctively express transmembrane protein 119 (TMEM119), P2Y G-protein-coupled 12 (P2RY12), hexosaminidase subunit beta (Hexb), sialic acid-binding immunoglobulin-type lectin H (Siglec-H), Spalt-like transcription factor 1 (Sall1) and low levels of CD45 (Spiteri *et al*., 2022).

Several studies reveal the physiological roles of microglia under normal conditions, not limited to defense and innate immunity; dysfunction of these normal functions can result in developmental disorders (Wake & Fields, 2011).

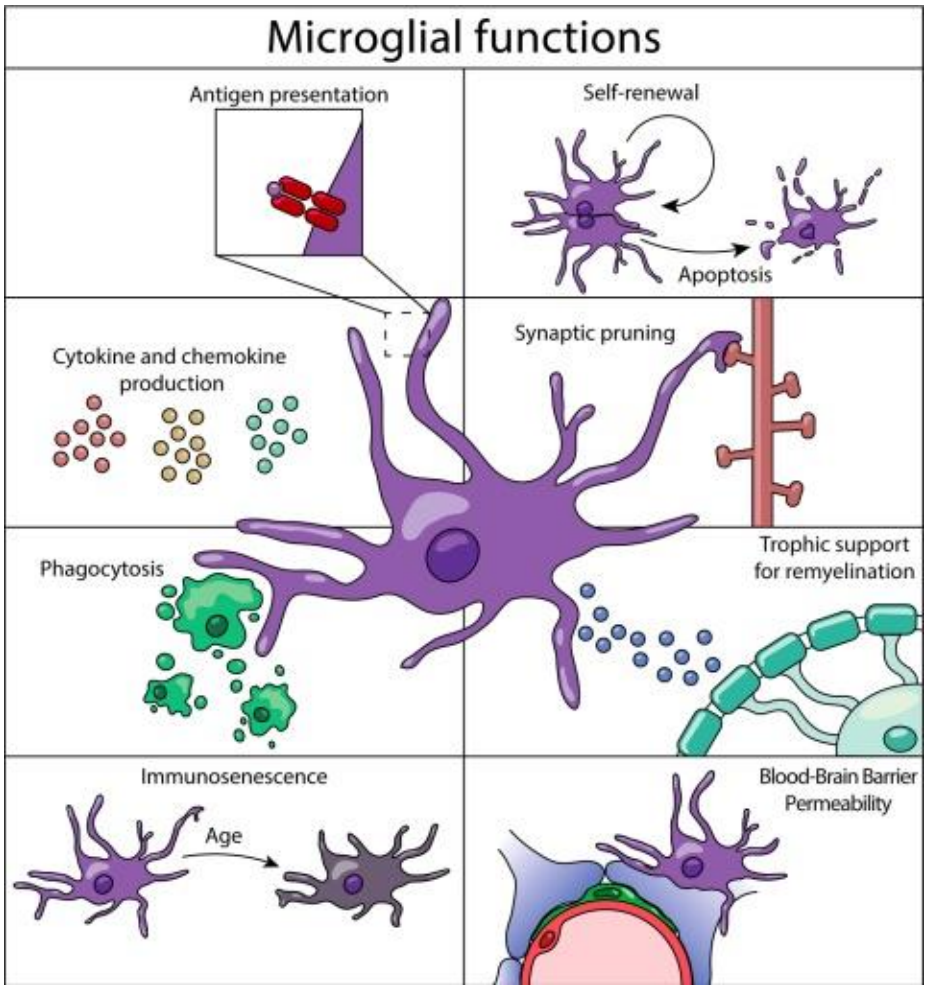


Figure 3. Microglial functions in the CNS. Microglia support maintainance and integrity of Central Nervous System (CNS) parenchyma. Reproduced from: Amor *et al*., 2021

Numerous elaborate studies suggest that microglia may influence neuronal circuit organization, not only during postnatal development, but also throughout the lifespan. During early neurodevelopment, neurons establish extranumerary synapses than persist in a mature brain; microglia are primarly implicated in the removal process called *synaptic pruning* (Schafer *et al*., 2011).

Microglia-mediated engulfment is mediated by an interaction between the phagocytic complement receptor expressed on the surface of microglia and its ligand C3, a complement protein that is expressed in developing synapses. In the pruning phase in mature brain, neurons upregulate the expression of the chemokine CX3CL1 and the recruitment to inactive synapsis of microglia expressing the receptor CX3CR1 (Harrison *et al*., 1998; Paolicelli *et al*., 2011).

The regulation of appropriate neuronal numbers is an indispensable process to establish the proper neural circuit formation. considering that at birth the number of neurons is greater than that which will be maintained in adults, following a principle of redundancy (De la Rosa & De Pablo, 2000). Supernumerary neurons are eliminated through PCD (Programmed Cell Death) during postnatal development; PCD may occur both in proliferating and post-mitotic neurons and it is a regular process of healthy development, rather than a dysfunctional phenomenon that can occur, for example, in autism spectrum disorders (ASDs) and other psychiatric diseases (Wake *et al*., 2009). In addition, microglia can regulate neurogenesis, proliferation of astrocytes and oligodendrocytes, modulates the differentiation of neurons and angiogenesis (Czeh *et al*., 2011).

For instance, in the hippocampus, the adult neurogenic and memory area, microglia release neurotrophic factors, such as insulin-like growth factor 1 (IGF1) and brain-derived neurotrophic factor (BDNF), that regulate the proliferation of neural progenitors, of which only a small part integrates and differentiates into mature neurons, the remaining part die by apoptosis and are phagocytosed (Choi *et al*., 2008).

Phagocytosis is the process by which microglia remove cellular debris which implies recognition, engulfment, and degradation of the different types of dead resident cells (apoptotic or necrotic), aggregated proteins or invading pathogens (Sierra *et al*., 2013). This is a pivotal mechanism required for the maintenance of tissue homeostasis that includes the increased production of TGFβ and neural growth factor (NGF) in basal conditions, as well as a decreased production of TNF-α, nitrite, and prostaglandin E2 (Fujita & Yamashita, 2021; Savill *et al*., 2002); on the other hand, an excessive microglial phagocytosis due to distinct phagocytic recognition systems depending on tissue, inflammatory state, and which *eat-me* signals are present on target cells, contributes to the failure of neuroprotection and can lead to neurodegeneration (Gabandé-Rodríguez *et al*., 2020).

Moreover, the physiological state of microglia relies on a system of receptors and signaling cascades that controls microglial reactivity and maintain cells in their surveilling state, which, if let loose, may cause considerable damage. Surveillant microglia possess a series of these receptors including many types of ionotropic and metabotropic receptors to glutamate, ATP, adenosine, acetylcholine, GABA, adrenaline and noradrenaline, serotonin and histamine. Microglia also employ ion channels in regulating ionic balance that is crucial for microglial functions (Timmerman *et al*., 2021; Verkhratsky *et al*., 2020). The major ion transporters

expressed by microglia include Na+/H+ exchanger 1 (NHE1) and Na+/Ca2+ exchangers (NCX), which are involved in regulation of intracellular pH and Ca2+ during signaling in microglial activation (Verkhratsky *et al*., 2021).

1.3.3 PATHOLOGICAL FUNCTIONS OF MICROGLIA

The immune responses are fine regulated by checkpoint mechanisms from initiation to resolution ensuring the counteraction of various stimuli, to keep tissue homeostasis. In the pathological condition, however, the immune responses are uncontrolled and correlate with cell dysfunction that occurred within the inflammatory processes (Goldmann *et al*., 2016).

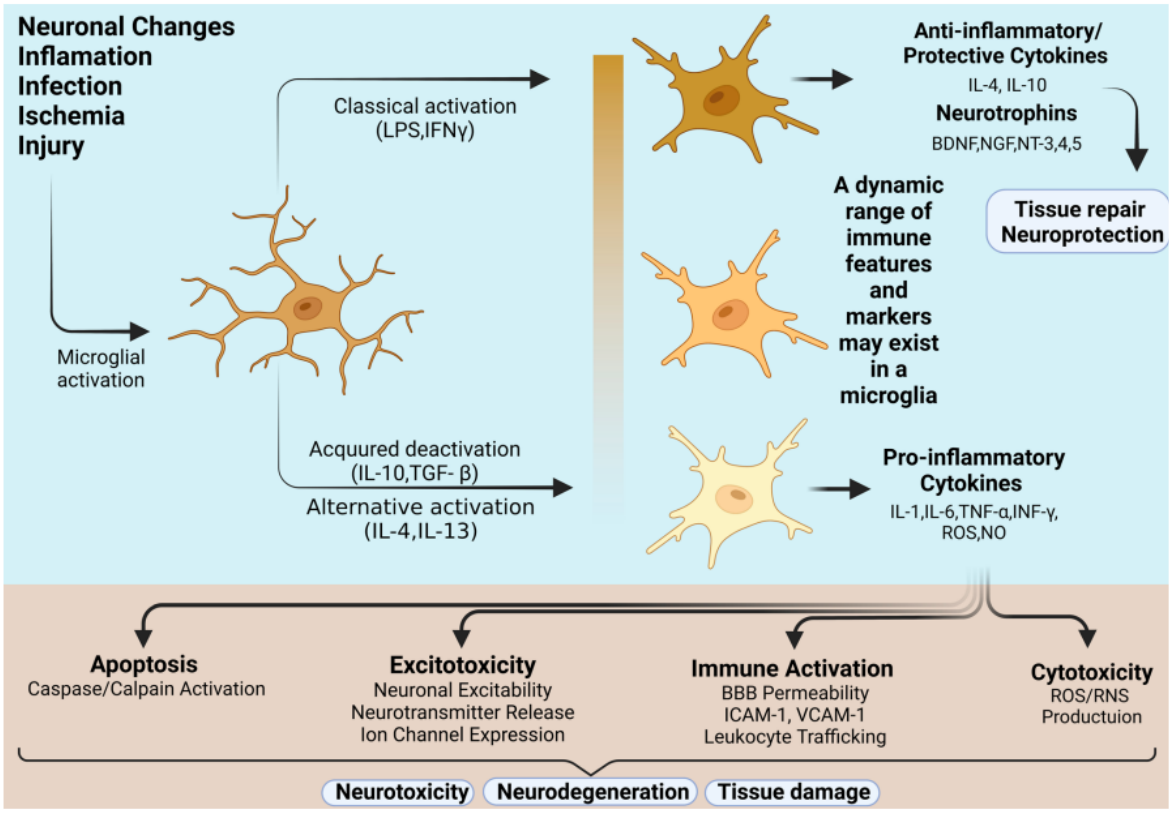


Figure 4. Microglia heterogeneity in the context of pathology. Microglia activation as a determinant key for diseases progression. Adapted from: Javanmehr *et al*., 2022.

Microglial cells dynamically shift their phenotype overlapping pro- and anti-inflammatory states, allowing them to adapt to circumstantial changes during the progression of brain disorders (Tang *et al*., 2016). Microglial receptors provide these cells to recognize danger-

and pathogen-associated molecular patterns (DAMPs and PAMPs) associated with pathology (Dasari *et al*., 2021).

The PAMP signals are directly associated with pathogens including fragments of bacterial cells, viral envelopes or DNAs/RNAs; the DAMP signals are molecules normally present in the CNS, but either absent or localized intracellularly (enzymes/proteins released from impaired cells) or molecules normally used for intercellular signaling but present in higher concentrations. For example, ATP in low concentrations prevents microglial activation, whereas higher concentration ATP stimulates microglia activation through G protein–coupled P2Y receptors (Davalos *et al*., 2005; Patro *et al*., 2016).

Microglia express both ionotropic (mainly P2X4 and P2X7) and metabotropic purinoceptors (P2Y2, P2Y6, P2Y12, and P2Y13). The motility is triggered by the activation of P2Y12 and operates in early defensive response (Mildner *et al*., 2017). In pathological conditions, massive recruitment of microglia with the upregulation of P2X7 receptors triggering microgliosis and secretion of pro-inflammatory factors (Monif *et al*., 2010). The P2X4 receptors are expressed in several subtypes of microglia and its activation through CCL2 promote microglial cells hyper-responsitivity to extracellular ATP (Toyomitsu *et al*., 2012).

Among pattern-recognition receptors (PRRs), the Toll-like receptors (or TLRs) are ubiquitously expressed in microglial cells (Lehnardt, 2010). The TLR1, TLR2 and TLR6, are activated by bacterial tri- and diacyl- lipopeptides, lipoteichoic acid and peptidoglycan, the TLR3, TLR7 and TLR8 are susceptible to viral RNA, whereas TLR 9 detects bacterial and viral unmethylated CpG DNA; TLR4 is the receptor for gram-negative bacterial cell wall component lipopolysaccharide (LPS) and TLR5 to bacterial flagellin (Aravalli *et al*., 2007). Furthermore, microglia express multiple receptors that influence phagocytosis such as Fc receptors, complement receptors and triggering receptor expressed on myeloid cells 2 (TREM2) and cytokine receptors including TNF-α receptors, interleukin receptors and receptors to interferon β and γ to face the pathogen or the damage (Norris et al., 2019). All these receptors are linked to various signaling cascades such as mitogen-activated protein kinase (MAPK) and activator protein 1 (AP-1), NF-κB or IFN regulatory factors (IRFs) inflammation and phosphatidyl inositol-3 kinase (PI3–K) that are master regulators of microglia reactivity (Kaminska *et al*., 2016).

All these signals produce many phenotypes of activated microglia, that are selected for the defense of the nervous system against insults. The defense conceptually requires balancing of two opposite arms, neurotoxic and neuroprotective.

A binary classification has been adopted to characterize microglia polarization. Under this simplistic system, *classical activated microglia* (CAM) represent the "detrimental phenotype" that lead to brain dysfunction through pro-inflammatory factors, such as predominantly IL-1β, IL-2, IFN-γ, TNF-α, and TNF-β nitric oxide (NO), superoxide free radicals that generate reactive oxygen species (ROS) and reactive nitrogen species (RNS); whereas the *alternative activated microglia* (AAM), the "beneficial phenotype", produce TGF-β, IL-4, IL-5, IL-6, IL-10, IL-13 and other neurotropic factors such as vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), platelet-derived growth factor (PDGF), to induce brain repair as they clear toxic cellular debris through phagocytosis and resolve inflammation (Lyu *et al*., 2021).

The activation status of microglia has been detected by signature markers: inducible nitric oxide synthase (iNOS), major histocompatibility class II (MHC II), COX2, CD16, CD32, and CD86 are widely used for identification of CAM; on the contrary, Arginase 1 (Arg1), Mannose receptor (CD206), Found in inflammatory zone 1 (FIZZ1), and Chitinase-3-Like-3 (Chi3l3) identify AAM. AAM microglia can be under classified in three subtypes depending on the function: a) contributing to the repair of damaged tissue by expressing neurotrophic factors, b) the deactivating phenotype related to immune modulation producing anti-inflammatory mediators; c) characterized by phagocytosis function and clearing out cell debris (Franco & Fernandez-Suarez, 2015). Recently, single-cell RNA analysis of CNS immune cells in neurodegenerative disorders discovered disease-associated microglia (DAM), a subset of microglia showing a unique transcriptional and functional signature. The transition from homeostatic microglia to first stage is TREM-independent, typified by downregulation of homeostatic microglia genes (Cx3cr1, P2ry12) and upregulation of the TREM2 regulators (Tyrobp and Apoe). The second step is TREM2-dependent signaling that involves upregulation of the lysosomal, phagocytic, and lipid metabolism pathways (such as Lpl, Cst7, and Axl) and have pro-proliferative and pro-survival function on microglia activation (Deczkowska *et al*., 2018; Paolicelli *et al*., 2022).

It is important to underline that microglia show overlapping phenotypes with co-expression of markers of both polarization states, demonstrating phenotypic variability and exhibit temporal and spatial heterogeneity (Masuda *et al*., 2019).

Microglia change their phenotype over time; in fact, microglia are rapidly activated after acute brain injury in response and express AAM phenotype markers aiming to resolve the insult, then gradually switch toward CAM markers and continue to worsen the impairment, establishing a cycle of neurotoxicity, which is self-perpetuating (Liao *et al*., 2012; Mathys *et al*., 2017).

1.4 INTERCELLULAR COMMUNICATION: ROLE OF EXTRACELLULAR VESICLES

In the CNS, crosstalk among glial cells and neurons is determining for a multiplicity of biological functions, starting from brain development to regulate differentiation, neural circuit maturation, homeostasis maintenance and repair after injury (Petralla *et al*., 2021). Glia cells not only orchestrate inflammatory responses upon diseases or invading pathogens, but they also provide continuous neurotrophic support, remodeling and pruning synapses.

In addition to the classical direct cell-to-cell contact and the paracrine action of secreted molecules, glia and neurons can communicate by exchanging EVs and the release and uptake of EVs in the nervous system provide a novel mechanism of intercellular communication which allows a coordinated regulation across long distances (Budnik *et al*., 2016).

EVs have been isolated from almost all body fluids emerging as potent participants in the progression of disease, serving as vehicles to spread misfolded proteins (amyloid β peptide and tau/phosphorylated tau in AD, α-synuclein in PD, and SOD1/mutant and TDP-43 in ALS) and propagating inflammatory signals in response to damage; because of their properties, EVs are being considered as a promising shuttle to target therapies (Verderio *et al*., 2012).

EVs are double membrane-enclosed vesicles released into the extracellular space that can be divided into at least three types according to size and origin: exosomes are small (30–150 nm) vesicles that are generated by the incorporation of intralumenal endosomal vesicles (ILVs) inside the multivesicular bodies (MVB) and shedding of from the cytosol to the cell surface; the second type of EV, termed microvesicles (MVs) or ectosomes (100–1000 nm), originate by direct outward budding of the plasma membrane into the extracellular space

and finally, the third type is termed apoptotic bodies that are released during cell death (500–2000 nm) (Sebaihi *et al*., 2017; Yuana *et al*., 2013).

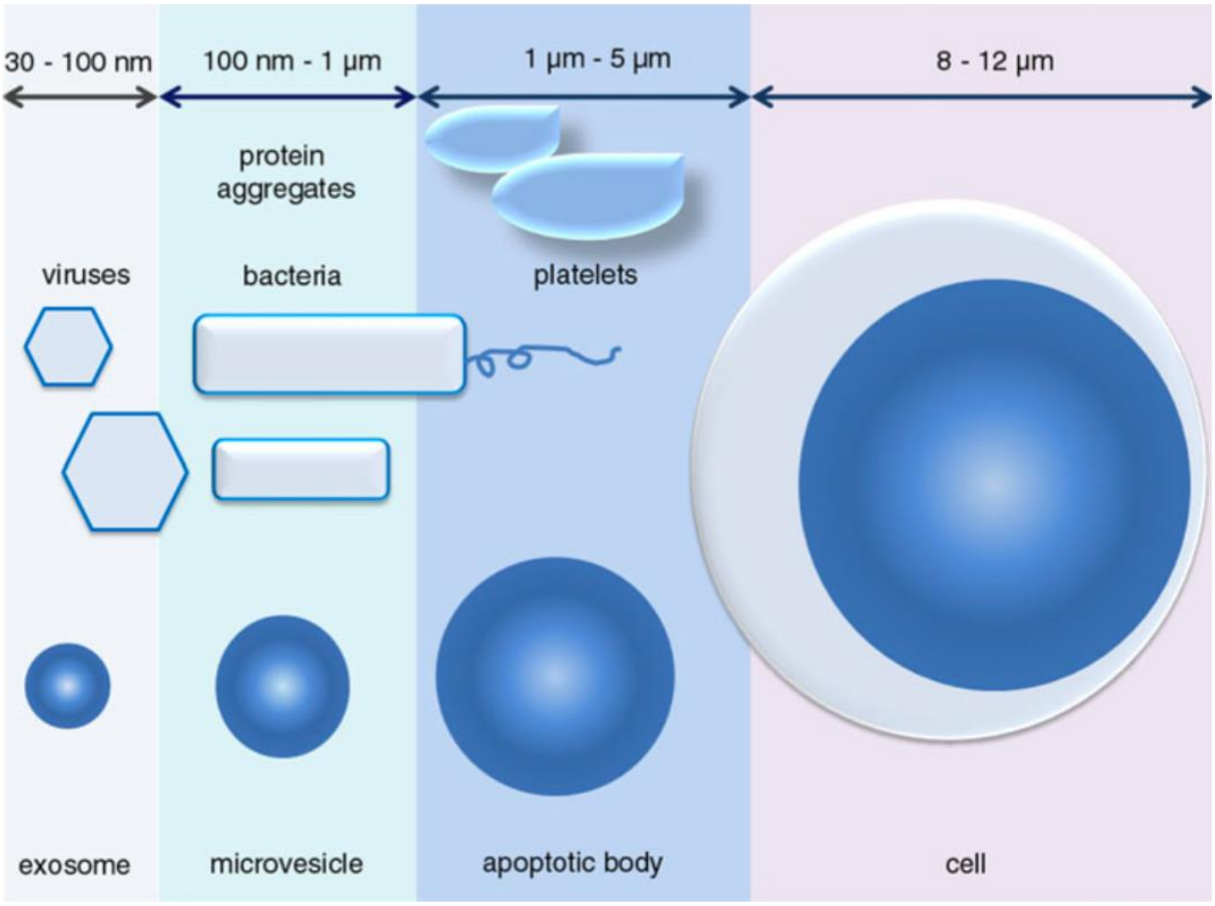


Figure 5. Size classification of extracellular vesicles. Reproduced from: György *et al*., 2011.

The outward budding of EVs, specifically of exosomes, is regulated by the endosomal sorting complex required for transport (ESCRT) and ESCRT-associated proteins (ALIX, TSG101, HSC70, and HSP90β) or sphingomyelinase (ESCRT-independent manner) mainly controlled by 1 heparanase and ARF6/PLD2, associated with the presence of syntenin-1, syndecan, and CD63 (Mir & Goettsch, 2020).

The release of exosomes could be reduced after the inhibition of neutral sphingomyelinase (nSMase), a protein responsible to produce ceramide and its derivatives abundant in exosomes such as proteolipoprotein (PLP), tetraspanins (CD63, CD9, CD81) and TSG101 (Doyle *et al*., 2019).

The EVs cargos vary with respect to biogenesis, donor cell type, and health conditions. In general, all EVs are loaded with various proteins (tetraspanins, heat shock proteins, cytoskeleton proteins ribosomal proteins etc.) lipids (sphingomyelin, phosphatidylcholine,

phosphatidylethanolamine, phosphatidylserine, ganglioside GM3 and phosphatidylinositol, prostaglandins, cholesterol) and nucleic acids (ssDNA, microRNAs ribosomal RNA, long non-coding RNA, etc.) (Zhang *et al*., 2019).

EVs can reach the recipient cells through a ligand-to-receptor interaction and the uptake can occur by fusion with the plasma membrane, by internalization through endocytosis, macropinocytosis or phagocytosis. Phagocytosis depend upon the phagocytic capabilities of the receiving cell as the membrane fusion requires a similar fluidity between the 2 fusing membranes; macropinocytosis may represent an alternative way through which EVs may transfer their content (Abels & Breakefield, 2016).

EVs can interact with target cells through a ligand-to-receptor interaction and functionality of the cargos depends on entry into the cytoplasm, and potentially even into the nucleus. Specific EV proteins such as MHC I and II, transferrin receptors and tetraspanins stimulate signaling cascade of selected cells by triggering, for example, integrins and calcium signaling, mitogen-activated protein kinase (MAPK) activation or some HSPs, such as HSP60 and HSP70, can bind membrane receptors present mainly on immune cells, such as CD14, CD91 and TLRs (Simons *et al*., 2009). In particular, microglia-derived EVs express specifically aminopeptidase CD13 and the lactate transporter MCT-1 (Potolicchio *et al*., 2005) and their shedding increases in response to extracellular ATP through the P2X7 receptors and cargos proteins are mostly implicated in extracellular matrix reorganization, autophago-lysosomal pathway and cellular metabolism (Drago *et al*., 2017). On the other hand, stimulation with LPS increases EVs release containing proinflammatory cytokines and inflamma-miRNAs (Kumar *et al*., 2017).

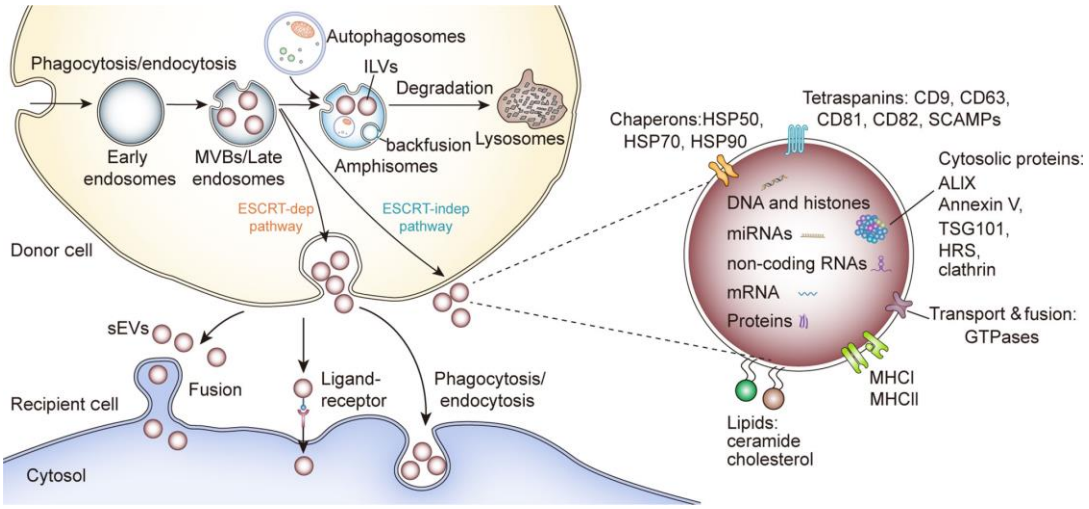


Figure 6. Schematic representation of Extracellular Vesicles (EVs): biogenesis, cargo contents and uptake. Reproduced from: Gao *et al*., 2021

1.5 MICRORNAS: MECHANISM AND FUNCTIONS

MicroRNAs (MiRNAs) were first identified in the nematode *Caenorhabditis elegans* in the early 1990s (Lee *et al*., 1993). Nowadays, more than 2500 mature microRNAs have been identified in the human genome and a significant fraction appears to be highly conserved in other animals (Kozomara *et al*., 2019). Considering that more than 95% of human cellular RNAs are "non-coding" RNAs, it is estimated that these non-coding RNAs regulate >30% of protein-coding genes involved in different biological processes (Hüttenhofer *et al*., 2005). Furthermore, a single miRNA can regulate hundreds of mRNA targets, while several miRNAs can regulate the same transcript, taking in account that miRNAs are temporally and differentially expressed among different tissues (Cloutier *et al*., 2015). MiRNAs-related research progresses in studying therapeutic approaches to either suppress or restore the expression of disease-associated miRNAs, since several studies implicate that aberrant miRNAs are linked with various human pathologies (Bandiera *et al*., 2010; Soifer *et al*., 2007).

1.5.1 ORIGIN AND BIOGENESIS

MiRNAs are class of endogenous, small (15-25 nucleotides), single-stranded non-coding RNAs that play an important role in regulation of gene expression on a post-transcriptional level by base-pairing with partial complementary sequences of the 3´untranslated region

(UTR) of target RNAs (messenger RNA, circular RNAs and long noncoding RNAs), termed the miRNA response element (MRE) (Nair *et al*., 2020). This interaction results in gene silencing with subsequent cleavage of the mRNA strand or destabilization of the mRNA through shortening of its polyA tail or inhibiting the translation of the mRNA into proteins (Lai *et al.*, 2002). MiRNA genes are located throughout the genome; commonly, miRNA genes are located in clusters through many different regions, most frequently within intergenic regions and introns of protein-coding genes (Lagos-Quintana *et al*., 2003). All miRNA classes follow a series of biogenesis steps that convert the primary miRNA transcript into the active one. There are two main miRNA processing pathways classified as canonical and non-canonical pathways (Ponomarev *et al*., 2013).

In the canonical pathway, the primary miRNAs (pri-miRNAs) are first transcribed by RNA polymerase II (Pol II), and then processed into single hairpins called precursor miRNAs (pre-miRNAs) by the "microprocessor complex", composed by ribonuclease III nuclear enzyme, DiGeorge Syndrome Critical Region 8 (DGCR8), an RNA binding protein and by Drosha. Subsequently, in both pathways, pre-miRNAs are exported to the cytoplasm by an exportin 5 (XPO5)/RanGTP complex and are further processed by Dicer, the RNase III endonuclease, giving rise to a mature miRNA duplex (Achkar *et al*., 2016).

The directionality of the miRNA strand determines the name of the mature miRNA form: the 5p strand derives from the 5' end of the pre-miRNA hairpin while the 3p strand originates from the 3' end. (O'Brien *et al*., 2018). Generally, the strand with lower 5' stability or 5' uracil is preferentially loaded into the Argonaute (AGO) protein, where one strain of the dsRNA is selected to bind mRNA of target genes (mature miRNA), the other remaining non-functional miRNA strain is destroyed. Proteins such as HnRNPA1, SMAD1 and SMAD5 have been shown to interact with miRNA precursors and regulate their subsequent processing to mature miRNA (O'Brien *et al*., 2022).

Non-canonical pathway miRNAs are independent of either the microprocessor complex or DICER to complete their maturation. The pre-miRNA produced from small introns as a result of splicing (mirtrons) are processed independently of the nuclear cleavage by the microprocessor and, thus, classified as DROSHA-independent; the same fate pertains to 7-methylguanosine (m7G)-capped pre-miRNA and small nucleolar RNA (snoRNA). An example of DICER-independent process concerns the endogenous short hairpin RNA (shRNA) that are

cleaved in too short pre-miRNA (~ 18 bp) and it is loaded directly into AGO2 (Ha & Kim, 2014).

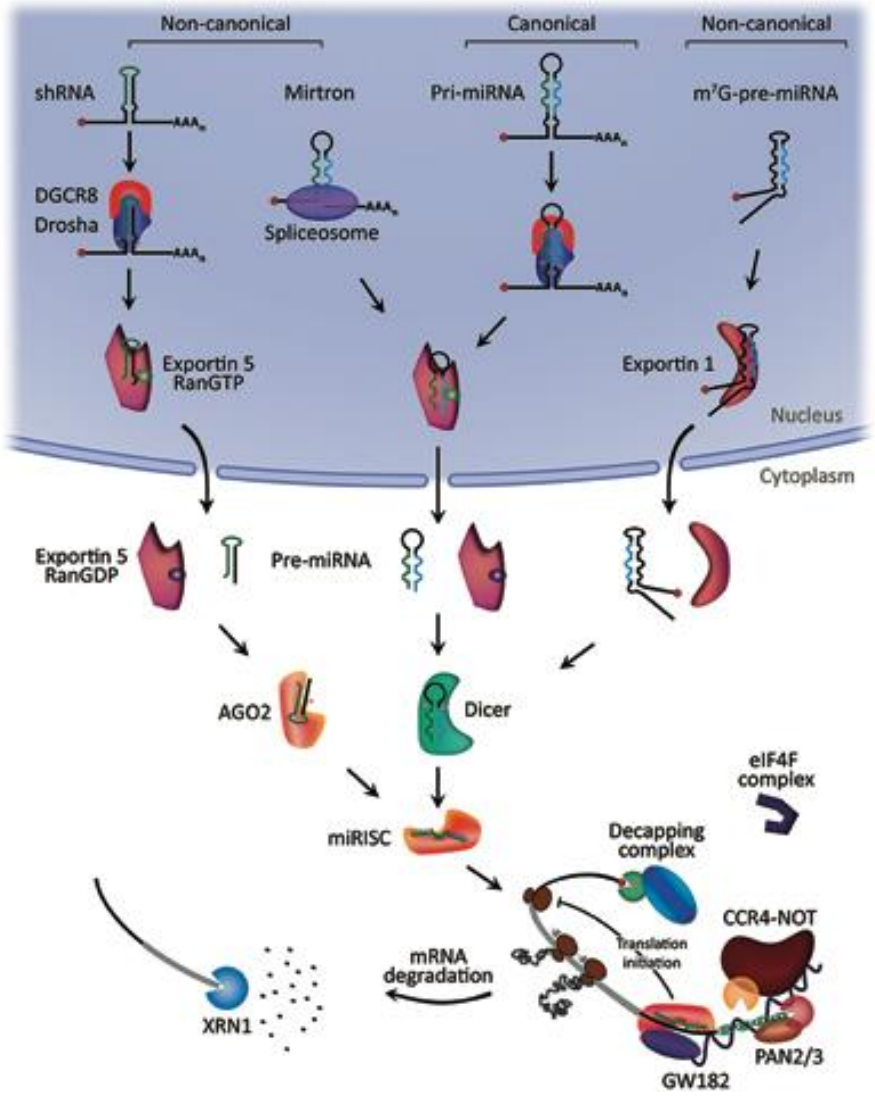


Figure 7. Synthesis of microRNA (miRNA) through different pathways. Reproduced from: O' Brien *et al*., 2018.

Based on relative stability, one strand of the duplex participates in miRNA-mediated modulation of translation remaining in the miRISC (miRNA-induced silencing complex) or miRNP (ribonucleoprotein complex). As mentioned, the intricacy of miRNA functions is accentuated by the fact that each miRNA binds an average of 200 RNAs, and individual RNAs can be bound by multiple miRNAs (Gebert *et al*., 2019).

MicroRNAs bind RNAs 3'UTR (less frequently 5' region or in coding areas) sequences complementary to the miRNAs 6–8 oligonucleotide "seed sequence" and the cause RNA target degradation or sequestration (Daugaard & Hansen, 2017).

In addition to their autocrine regulatory roles, miRNAs can also be released from parent cells to act over short or long distances in a paracrine way, selectively packaged into EVs or in association with a protective protein (for example Ago2 or high-density lipoprotein) in order to be stabilized for long periods in the extracellular space (Diehl *et al*., 2012).

1.5.2 MIRNAS IN MICROGLIA-MEDIATED INFLAMMATION

Neuroinflammation is among the many processes in which is involved miRNAs regulation and their expression is commonly altered during neurological disorders. The dysregulated expression of several inflammatory-associated miRNAs (inflamma-miRs) impacts not only in the cells of origin, but also in the neighboring ones, which are their recipient (Khan *et al*., 2022). Since miRNAs are known to regulate a multitude of genes leading to microglial phenotypic switches, the majority of research focus on identification of key regulatory pathways that are altered between different phenotypic states in microglia (Flynt & Lai, 2008).

Inflammatory processes may be enhanced or suppressed by miRNAs and the action of multiple miRNAs on these pathways may be either synergistic or antagonistic (Slota & Booth, 2019).

1.5.3 MIRNAS WITH PRO-INFLAMMATORY ACTIVITY

miRNA-155: one of the most important miRNAs associated with inflammation is miR-155 and the aberrant expression of miR-155 plays a fundamental role in the functions of immune response and has a wide variety of anti-inflammatory targets, such as Src homology-2 domain-containing inositol 5 phosphatase1 (SHIP1) and suppressor of cytokine signaling 1 (SOCS1) promoting the pro-inflammatory pathways, including the microglia switch into a pro-inflammatory profile. Interestingly, IL-10, a strong anti-inflammatory cytokine, inhibits miR-155 expression, increasing SHIP1 and SOCS1 expression and mitigating TLRs signaling (He *et al*., 2014).

After inflammatory stimuli, miR-155 levels increase supporting a key role of this miRNA in both innate and adaptive immunity and the inhibition of miR-155 reduces, NO production, iNOS expression and genes, such as TNF-α, IL-6 and IL-1β (Cardoso *et al*., 2012; Lukiw *et al*., 2012) also demonstrated that upregulation of miR-155, together with miR-145b in human neuronal–glial primary cocultures after stress treatment, lead to spread of AD inflammatory signaling. MiR-145b, was shown to directly bind to the 3'-UTR of the mRNA of nuclear receptor subfamily 4 group A member 2 (NR4A2; also known as nuclear receptor related 1 protein (Nurr1)), a member of the orphan nuclear receptor family. It was found that inhibits the expression of proinflammatory mediators in both microglia and astrocytes. The ablation of NR4A2-mediated activation of microglia results in an increase in pro-inflammatory factor expression, including TNF-α and IL-1β (Dong *et al*., 2018). According to another study, it was shown that NR4A2 operates in anti-inflammatory manner through downregulating CCL2 in both in vivo and in vitro PD models. In an MS murine model, the activation of NR4A2 signaling pathway reduces disease incidence as well as severity through an NF-κB-dependent pathway (Jakaria *et al*., 2019). Moreover, p53-mediated transcriptional activity is required for the induction of miR-155, miR-145 and miR-34a to modulate microglia behavior.

miRNA-34a: while miR-155 directly targets c-Maf mRNA, miR-145 and/or miR-34a target the transcriptional regulator Twist-2, resulting in downregulation of the anti-inflammatory transcription factor c-Maf, accompanied by increased expression of other pro-inflammatory marker genes (Su *et al*., 2014). MiR-34a has important implications for AD pathogenesis targeting TREM2, a microglial receptor important for mediating clearance of Aβ42-peptides via phagocytosis: up-regulation of the pro-inflammatory miRNA-34a and TREM2 down-regulation was also observed in reactive oxygen species (ROS)-, IL-1β- and TNF-α-treated microglia, an effect that could be counteract using anti-miRNA-34a therapeutic strategies (Bhattacharjee *et al*., 2016).

miRNA-9: MiR-9 also acts in microglia-mediated inflammatory responses targeting monocyte chemotactic protein-induced protein 1 (MCPIP1), a negative regulator of macrophage activation induced by various inflammatory stimuli including MCP-1 and LPS. (Yao *et al*., 2014) In another study, AD patients were compared to age-matched controls and was reported an upregulation in CSF of miR-9 together with miR-125b (Alexandrov *et al*., 2012).

miRNA-125b: miR-125b was shown to directly repress the ubiquitin-editing enzyme A20 and to consequently amplify NF-κB function in microglia in a P2X7 receptor-dependent manner,

leading to uncontrolled toxic CAM reactions. These results highlight an important role for miR-125b in ALS via modulation of microglial activation and motor neuron injury (Parisi *et al*., 2016).

1.5.4 MIRNAS WITH ANTI-INFLAMMATORY ACTIVITY

Other miRNAs, such as miR-124, miR-146a and miR-21, are more often associated with an anti-inflammatory response.

miRNA-146a: miR-146a induction was inversely correlated with the level of inflammation-related proteins, including CFH and IRAK-1. Pro-inflammatory signaling through the NF-κB transcriptional pathway leads to increased expression of miR-146a, which subsequently suppresses NF-κB transcriptional activity, serving as a negative feedback mediator of pro-inflammatory responses (Su *et al*., 2016).

miRNA-124: miR-124 is among the most well studied miRNAs that promoted the neuronal survival and regulation of microglia polarization. In normal conditions, it is poorly expressed in microglia, promoting their steady-state and it is directly related with a more anti-inflammatory phenotype, with rescued motility and phagocytosis profile (Sun *et al*., 2015). Conversely, other studies demonstrated that miRNA-124 is linked to symptomatic associated neuroinflammation in ALS patients and overexpression of miR-124 was detected in the brain of ALS mice at the late stages of the disease (Pinto *et al*., 2017). Furthermore, exosomes derived from mutant-SOD1 motor neurons are enriched in miR-124, which showed to activate microglia and nuclear factor-κB (NF-κB) signaling pathway, stimulating the release of numerous neurotoxic cytokines (Vaz *et al*., 2021).

1.5.5 MIRNAS AS THERAPEUTIC TARGETS: THE DNAZYME

Given the central role of miRNAs in regulating molecular range of biological phenomena in health and disease and they may serve as important therapeutic targets. By regulating the expression of multiple genes, miRNAs surge of interest, in particular about the pathogenesis of many neuroinflammatory-based disorders, in which many dysregulated pathways are shared. The ability to control the expression of miRNA in vitro and in vivo will serve as the basis for the development for miRNA-based therapies to reverse their dysregulated expression (Van Rooij *et al*., 2014).

In certain circumstances, miRNA expression reduction drives the disease; in this case miRNA mimics can be used to restore their expression and function. In contrast, anti-miRNAs (antagomiRs) and miRNA sponges can be exploited to restrain the activity of upregulated miRNAs responsible for disease or block its binding to endogenous mRNA targets (Li *et al*., 2014).

One of the main obstacles of miRNA-based therapies in the treatment of disorders in the CNS are the crossing of the BBB, the insufficient molecular stability, the potential toxicities and the off-target effects of miRNAs. Several encouraging studies are investigating to mediate the delivery to the CNS of such miRNA-based treatments, including lipid-based or polymeric nanoparticle-based delivery systems or, additionally, viral vectors such as adenovirus vectors and adeno-associated virus (AAV) vectors (Lu *et al*., 2019).

A promising avenue that is being explored focuses on DNAzyme-based nanotherapeutic platforms; DNA is particularly programmable and biocompatible with very low immunogenicity effect and, compared to RNA and proteins, it is more stable and cost-effective. Since the initial report in 1994 by Breaker, many different types of DNAzymes have been applied, catalyzing RNA/DNA cleavage and ligation, and detecting intracellular miRNAs (Breaker & Joyce, 1994; Silverman, 2016; Shao *et al*., 2015).

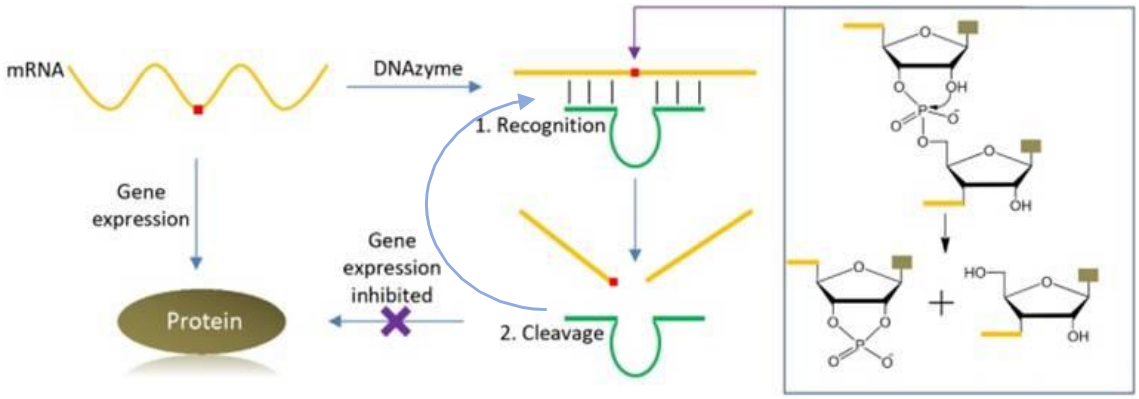


Figure 8. Schematic illustration of DNAzyme function. Adapted from: Zhou *et al*., 2017.

DNAzymes (deoxyribozymes) are in-vitro selected catalytic DNA oligonucleotides. Among the potentially interesting reactions catalyzed by certain DNAzymes is the cleavage of RNA linkages after their sequence-specific binding. Each DNAzyme has a catalytic core flanked by two recognition sides to secure more stability in binding its substrates by Watson-Crick basis pairing, in presence of specific metal ions such as Mg2+, Pb2+, Mn2+, Cu2+ and Na+ to bind a specific 2'-OH group of RNA to the adjacent phosphodiester linkage and could be incorporated into cells by spontaneous endocytosis, through a non-specific interaction with cell surface receptors (Fokina *et al*., 2012).

DNAzymes are often associated with nanomaterials, such as gold nanoparticles (AuNP) or tetrahedral nanostructures to amplify the signals of their catalytic function (Zhou *et al*., 2017).

A tetrahedral nanostructure contains four single-stranded DNA self-assembles spontaneously through designed oligonucleotides annealed together in solution and sticky ends produced by extending the oligonucleotides beyond the vertices interact to combine a three-dimensional tetrahedron (Yan *et al*., 2021). The catalytic functional units are inserted into the tetrahedral structure and, deriving from DNA, it enlarges permeability for cellular internalization and specificity towards the target to which they are directed (Goodman *et al*., 2004).

2. AIM OF THE STUDY

Microglia, the CNS-resident immune cells, provide to immune surveillance and inflammatory responses, along with other physiological functions, such as removal of cellular debris, presentation of antigen and synaptic pruning to maintain CNS homeostasis (Norris et al., 2019). Upon equilibrium disturbances, microglia rapidly change their morphology adopting activated profiles, which range from anti-inflammatory, alternatively activated microglia (AAM), to proinflammatory, classically-activated microglia (CAM), phenotypes. The CAM status results from microglial over-activation, releasing pro-inflammatory cytokines and neurotoxic factors contributing to neuronal damage.

The impact of chronic microglia activation might lead to neuroinflammation, which contribute to progression and worsening of neurodegenerative process. In addition, microglial cell reactivity occurs in a coordinated manner and propagates across the brain parenchyma spreading the neuroinflammatory signal from cell to cell (Orihuela *et al*., 2016).

EVs have recently emerged as a main promoter of intercellular communication among CNS cells, by the transfer of proteins, lipids, and especially non-coding RNAs (Brites & Fernandes, 2015), and microglia is known to release EVs, whose content changes depending on the activation state (Turola *et al*., 2012), but the role of activated microglial-released EVs in modulating the phenotype of control microglial cells has never been evaluated.

In the present study, we tested whether EVs released by pharmacologically activated microglial cells towards an AAM or a CAM treatment could influence control, unstimulated microglia, by using immortalized microglial cell lines, i.e. the widely used murine N9 and the human microglial cell line HMC3. Our in vitro studies aim to understand whether different types of activation could determine change in vesicles content, in particular on miRNAs, and whether this could influence the activation state of control microglial cells (Nuzziello & Liguori, 2019).

Furthermore, the role of specific microglial, exosomal miRNAs known to be involved in neuroinflammation, also named inflamma-miRNAs, has been evaluated. Several

dysregulated miRNAs have been found in NDDs and highlighted as relevant for the control of activation of microglia, among which miR-34a. The goal of this research is to propose a strategy to modulate the inflammatory state of microglia by targeting miR-34a using functionalized DNA nanostructures, i.e. DNAzymes, which are able to interfere with gene expression by their cleavage of intracellular mRNA after their sequence-specific binding with a sensitivity boost deriving from their intrinsic properties (Peng *et al*., 2019).

3. MATERIALS AND METHODS

3.1 CELL CULTURES AND TREATMENTS

3.1.1 IMMORTALIZED CELL LINES

In order to use immortalized cell line cultures, sterile instruments and solutions were used under a laminar flow hood.

N9 cells are common retroviral-immortalized murine microglia cell line maintaining the crucial properties of in vivo microglia; HMC3 are human microglial clone 3 cell line of human embryonic microglia SV40-dependent immortalized from human embryonic microglia. Cells were kept in culture in 10 cm Ø Petri dishes with 9 ml of Dulbecco's Modified Eagle Medium (DMEM, Euroclone) supplemented with 10% FBS and 1% Penicillin/Streptomycin solution at 37 °C incubator containing 5% CO2. The medium was changed every 2 days and cells were passed 2/3 times/week with 1:2 split ratio. For subsequent experiments, cells were trypsinized and plated at an initial density of 2×10^5 cells/35 mm diameter Petri dish and $1x10^5$ cells /100 mm diameter dish. 35 mm diameter Petri dishes were used for Western blot analysis, miRNAs extraction, nitrite detection and immunocytochemistry, while 100 mm diameter Petri dishes were used for NTA assay.

3.1.2 CELLS TREATMENTS

N9 activation: cells were plated into uncoated 35mm dishes at a density of 2×10^5 cells/dish; after seeding for 8 hours to in DMEM without serum to allow adhesion. Microglial cells were treated for 30 minutes with LPS 1 µg/ml was induced by bacterial endotoxin lipopolysaccharide (LPS) 1 µg/ml (Sigma-Aldrich, St. Louis, MO, United States), known to activate microglia towards a pro-inflammatory phenotype (Bagasra *et al*., 1995), increasing the production of pro-inflammatory cytokines and chemokines (Qin *et al*., 2004; Kong *et al*., 1997; Lieberman *et al*., 1989), as well as to induce the up-regulation of nitric oxide synthase (iNOS) and the consequent increase of nitric oxide (NO) secretion; ATP 200 µM (Sigma-

Aldrich, St. Louis, MO, United States), known to induce vesicles release (Du et al., 2018) and anti-inflammatory phenotype polarization.

HMC3 activation: cells were plated into uncoated 35mm dishes at a density of 2 x 10^5 cells/dish; after seeding for 2 hours to in DMEM with serum to allow adhesion. Since the classic activation stimulus LPS, commonly used in rodent cells, was not able to induce a pro-inflammatory activation of cultured HMC3. Thus, cells were treated with high glucose to the final concentration of 5g/l w/ or w/o IFN-γ (1µg/ml) (Sigma-Aldrich, St. Louis, MO, United States): higher concentration of glucose are known to reinforce immune responses through a boost in glycolysis (Cheng *et al*., 2014; Hung *et al*., 2022), as IFN-γ mediated activation through the JAK/STA1 pathway followed by the expression of metalloproteinases, IL-1β and the nitric oxide production (Nareika *et al*., 2009); to test the indirect polarization induced by exosomes, cells were treated for 3h and 30 minutes with IFN-γ 1 µg/ml diluted in DMEM without serum (Sigma-Aldrich, St. Louis, MO, United States) and then by adding to culture media ATP 500µM (Sigma-Aldrich, St. Louis, MO, United States).

EVs inhibition: N9 cells pre-treatment with GW4869 (Sigma-Aldrich, St. Louis, MO, United States) for 4h was used to mediate the inhibition of exosomes release. N9 cells were seeded into uncoated 35mm dishes at a density of 2,5x105 cells/dish in 1mL of DMEM w/o FBS and were allowed to settle at 37°C for 8h. Homeostatic N9 cells were treated with LPS/ATP for 30 min or 4h with GW4869 before replacement with fresh medium.

DNAzyme treatment on N9 cells: tetrahedral nanostructures were supplied by prof. Giampaolo Zuccheri (Department of Pharmacy and Biotechnology, University of Bologna, Italy). Designed DNA nanostructures can carry one copy of such DNAzyme and could be assembled modularly to be able to run efficient control experiments. Cleaving efficiency of the DNAzyme has been previously tested against target DNA-RNA hybrids and verified their functionality in conditions mimicking the intracellular milieu (low concentration of magnesium ions, presence of ATP). DNAzyme was diluted in DMEM without serum to a final concentration of 50 µM and treatment was performed on control microglia for 4h and, after a change of medium, left to incubate for 24h to condition the new medium.

Preparation of conditioned media: after pharmacological activation with or without blocking EVs release, microglial cells were left to incubate for 24h to condition the new medium for N9 cells or overnight (O/N) for HMC3.

For analysis of activation markers, cells were collected in lysis buffer (1%SDS, 50 mM Tris HCl pH 7.4, 1 mM EDTA, protease inhibitor cocktail 10 µl/ml) and stored at -80°C for western blot analysis. An equal volume of conditioned medium (500µl) was collected and concentrated by using Microcon-YM-3 (EMD Millipore Corporation, Billerica, MA, USA), resuspended in Loading buffer 4 X (1M Tris HCl pH 7.0 1 ml; 20%SDS 2 ml; Glycerol 2 ml; 20 mg of Bromophenol Blue, 1 M DTT 1:5) and stored at -20 ° C for western blot analysis.

3.2 PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR VESICLES FROM CONDITIONED MEDIUM

Serum-free medium was collected from cultures of microglia treated or not, filtered in a 0.22 µm pore filter (Sigma-Aldrich, St. Louis, MO, United States) and further centrifuged at 1,000xg for 5 min to remove cell debris. After that, the supernatant was centrifuged at 100,000xg at 4°C to pellet EVs (Ultracentrifuge Optima L-90K, Beckman).
EVs were characterized by evaluation of expression of proteins, through Western blot analysis as well as by evaluation of nucleic acids' content, through extraction and quantification of miRNAs, as explained below.
Isolated EVs were also used to treat microglial cells to evaluate their effect on activation both in N9 cells and HMC3. To this aim, vesicles collected as previously described, were resuspended in 1 mL of DMEM w/o FBS.

For Western Blot analysis: aliquots of the "clean" conditioned medium (500 µl) obtained after centrifugation and further concentrated by using Microcon-YM-3 (Millipore, Billerica, Mass., USA) or the parallel EVs pellet obtained after ultracentrifugation of 1mL of conditioned medium, were resuspended in Loading buffer 4 X (1M Tris HCl pH 7.0 1 ml; 20% SDS 2 ml; Glycerol 2 ml; 20 mg of Bromophenol Blue, DTT 1:5 of the total volume).

EVs characterization: EVs size and concentration were evaluated by Nanoparticle tracking analysis (NTA) using the Nanosight lodge at CPT, Centro Piattaforme Tecnologiche, University of Verona (model NS300, Malvern, United Kingdom). For NTA, EVs pellet was obtained by centrifuging 20mL of medium and resuspended in 500 µL phosphate-buffered saline (PBS, NaCl 137 mM, Na2HPO4 10 mM, KH2PO4 1,8 mM, pH7,4). Particles (EVs) were detected using a 488 nm laser and their concentration was adjusted by observing a particles/frame rate.

3.3 IMMUNOCYTOCHEMISTRY

Microglial cells were plated on 35 mm Petri dishes at the density of $1,5x10^5$, with or without stimulation.

N9 cells: ALIX (1:200, Santa Cruz Biotechnology) staining was performed on N9 cell, as biomarker of EVs (Kalra et al., 2012; Kristan et al., 2016; Van Niel et al., 2018). Cell cultures were fixed in 4% paraformaldehyde (PFA). After a quick wash in PBS, 4% PFA in PBS was added for 20 minutes. Following another wash in PBS, fixed cells were stored at 4°C. Fixed cells were permeabilized with PBS-0.1% Triton at RT by washing 3 x 10 minutes. Non-specific sites were saturated with PBS-0.1% Triton + 3% BSA (Sigma-Aldrich, St. Louis, MO, United States), for 60 min at RT. Cells were then incubated overnight at 4°C with primary antibodies diluted in PBS-Triton + 1% BSA. Cells were washed 3 x 5 minutes in PBS before the incubation with fluorescent secondary antibody Goat anti-Mouse IgG Alexafluor555 (Abcam Cambridge, United Kingdom) diluted 1:1000 in PBS-Triton + 1% BSA for 90 minutes at RT. To remove nonspecific signals, 3 x 5 minutes additional washes in PBS were performed. Nuclei were stained by incubating cells with Hoechst 33258 solution (2µg/ml), a dye that binds to chromatin. After a quick wash in PBS, Pro Long Gold AntifademReagent (Life Technologies) was used to mount fixed and stained cells. Furthermore, EVs were labeled with lipophilic dye to analyze their incorporation into microglial cells. In this case, EVs pellet after centrifugation was resuspended in 1mL of PBS and 0,25µL of lipophilic diI (ThermoFisher Scientific, MA, United States) (stock solution diluted in EtOH, final concentration 4mg/mL) was added and incubated at 37 °C for 1 hour in the dark. At the end of the incubation period, another centrifuge at 100,000 g at 4°C was performed and the pellet was resuspended in 1mL of

culture media before treating N9 cells in culture. Stained cells/EVs were photographed with a Nikon EZ-C1 confocal microscope (Eclipse TE 2000-S; Nikon, Tokyo, Japan).

HMC3 cells: 24h after treatments with IFN-γ (1µg/ml) plus 5g/l glucose, HMC3 cells were fixed with 4% paraformaldehyde for 20 min in cold methanol, followed by antigen retrieval in 10mM Sodium Citate pH 6. Non-specific sites were then blocked by incubation in PBS-0.1% Triton X-5% BSA, 22.52mg/ml glycine before the overnight incubation with primary antibodies IBA1 diluted (1:500, Abcam Cambridge, United Kingdom) in PBS-0.1% Triton X-2% goat serum (Sigma-Aldrich, St. Louis, MO, United States). Then, followed 3 washes in PBS-0.1% Triton, fixed cells were incubated with fluorophore-conjugated secondary antibodies (Donkey anti-Goat IgG Alexafluor488, Abcam Cambridge, United Kingdom) diluted 1:1000 in PBS-Triton + 1% BSA for 90 minutes at RT. Nuclei were stained with Hoechst 33258 and images acquired with a Nikon EZ-C1 confocal microscope with 100X oil immersion objective to quantify the mean fluorescence intensity of IBA1. The latter staining was also used to define the microglial morphology as function of cell size (area, perimeter, diameter) and roundness by using the ROI management plugin and measurement setting of Fiji (ImageJ) software (all reagents from Sigma-Aldrich).

3.4 NITRIC OXIDE DETECTION ASSAY

Accumulation of nitrite, as indirect measure of NO production, in cell culture medium was measured by the Griess method (Guevara et al., 1998). A nitrate standard curve was performed with $NaNO_2$ at known concentrations from 50 µM to 0.39 µM to quantify nitrite concentration in the medium. 5 mM sulfanilamide (Sigma-Aldrich) was added to culture media and the standard curve. Sulfanilamide reacts with nitrite under acid conditions to form a diazonium cation, which subsequently couples with N-1-naphthyl-ethylenediamine dihydrochloride (NEDA 40mM; Sigma-Aldrich) to produce a colored azo dye. After 15 minutes of incubation at RT in the dark, absorbance was read at 540 nm in a Multiplate Spectophotometric Reader (Bio-Rad Laboratories).

3.5 WESTERN BLOT

Western Blot allowed us to verify the activation status of microglia through the expression of proteins in cell lysates and the release of cytokines in the conditioned medium. 500 µl of conditioned medium were collected and concentrated using Microcon-YM-3 filters (EMD Millipore Corporation, Billerica, MA, USA) and resuspended in Loading buffer 4 X (1M Tris HCl pH 7.0 1 ml; 20% SDS 2 ml; Glycerol 2 ml; 20 mg of Bromophenol Blue, DTT 1:5 of the total volume), while parallel cell samples were collected in Lysis Buffer (1 % SDS, 50 mM Tris pH 7.4, 1 mM EDTA, 10 µl/ml protease inhibitor cocktail and 10 µl/ml phosphatase inhibitor cocktail) and sonicated. Protein content in each sample was quantified by the Lowry assay (Lowry et al. 1951), by using a BSA (Sigma-Aldrich 1,5mg/ml) standard curve at known protein concentrations. For this assay, solution 1 consisting of 98% of solution A (2% Na_2CO_3 in NaOH 0.1M), 1% of solution B ($CuSO_4 \cdot 5H_2O$ 0.5%) and 1% of solution C (1% Na-K tartrate) was added to each sample and to each point of the standard curve. Subsequently solution 2 (50% Folin and Ciocalteu's phenol reagent and 50% bidistilled water) was added and after 30 minutes absorbance was read in a spectrophotometer at 700 nm. Concentrated conditioned media and cell samples resuspended in Loading Buffer were boiled and loaded into a 10% or 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE; Bio-Rad). Acrylamide and protein markers were supplied by Bio-Rad, while the other reagents were from Sigma-Aldrich. Gel electrophoresis was run at constant 120 V in an electrophoretic Mini-Protean II cell (Bio-Rad) filled with 1X running buffer (H_2O bd; 25 mM Tris base; 192mM Glycine; 0.1% SDS). After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Amersham™ Protran®). Transfer from the cathode to the anode was carried out at constant 400 mA for 2 hours in the Mini Trans-Blot Cell system (Bio-Rad), with 1X transfer buffer (50 mM Tris, 200mm glycine, plus 20% methanol. Membranes were blocked for 1h with a blocking solution made of 4% nonfat dried milk (Bio-Rad)/0.1% Tween-20 in PBS (Sigma-Aldrich), pH 7.4 and incubated over night at 4 °C with primary antibodies in PBS-0.1% Tween 20. Membranes were washed 3 x 10 minutes with blocking solution and incubated with specific secondary antibodies conjugated to horseradish peroxidase diluted 1:5000 for 90 minutes at RT in PBS-0.1% Tween 20, pH 7.4. Membranes were then washed 3 x 10 minutes with PBS-0.1% Tween 20, pH 7.4 and 5 minutes with PBS. Labeled proteins were visualized by using the ClarityTM Western ECL Substrate (Bio-Rad) and detected using a ChemiDoc™ MP imaging system and Image Lab software (Bio-Rad).

Primary Antibody	Dilution	Secondary Antibody
iNOS (Cell Signaling, Massachusetts)	1:1000	**Anti-Rabbit** (Jackson ImmunoResearch)
TREM2 (Cell Signaling, Massachusetts)	1:1000	
NFkβ (Cell Signaling, Massachusetts)	1:1000	
pNFkβ (Cell Signaling, Massachusetts)	1:1000	
IL-1β (Cell Signaling, Massachusetts)	1:1000	
PNP (Santa Cruz Biotechnology)	1:1000	
ALIX (Santa Cruz Biotechnology)	1:500	**Anti-Mouse** (Jackson ImmunoResearch)
GAPDH (Santa Cruz Biotechnology)	1:20 000	

Table 1. Primary and secondary antibodies and relative dilutions used in Western blot analysis.

3.6 CELL VIABILITY ASSAY

MTT is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. (Meerloo et al., 2011). N9 microglial cells were seeded at a density of 10×10^3 cells/well in 96-well plates in 200 µl of DMEM medium with 10% FBS. The cells then received 200 µl of DMEM medium without FBS plus various concentrations of GW4869 (1, 3, 5 and 10 µM) suggested in literature (Asai *et al*., 2015; Tsutsumi et al., 2019) incubated for 24h. The cells were then incubated with MTT (5 mg/mL) in a culture medium for 1h at 37°C. The medium was discarded and cells were lysated in 100 µl of Tris 0,1M pH 7,5 + Triton 5%. The optical absorbance at 570 nm was determined with a Microplate Reader (Bio-Rad): the darker of the solution indicates the greater number of viable and metabolically active cells.

3.7 MICRORNAS ISOLATION AND RT-qPCR

Cellular and vesicles miRNAs were isolated using miRVana miRNA Isolation kit (Invitrogen, Massachusetts, USA). Cel-miR 39 (5'- UCACCGGGUGUAAAUCAGCUUG- 3') 10pM was added to lysate samples as a spike-in exogenous control. The sample is first lysed in a denaturing lysis solution which stabilizes RNA and inactivates RNases. The lysate is are subjected to Acid-Phenol:Chloroform extraction which removes most of the other cellular components. This is further purified over a glass-fiber filter to yield about 200 bases fraction enriched in miRNAs. Small RNAs quantification were performed using Nanodrop 2000 (ThermoFisher Scientific, MA, United States). Real Time PCR (qRT-PCR) is a technique that allows DNA quantification by measuring DNA amplification during the exponential phase of the polymerase chain reaction. For gene expression, 10ng of small RNAs per 15µL RT reaction (100mM dNTPs, MultiScribe™ Reverse

Transcriptase, 50 U/µL, 10x Reverse Transcription Buffer, RNase Inhibitor, 20 U/µL, 5x RT Primer, nuclease-free water) were converted to complementary DNA (cDNA) using TaqMan MicroRNA Assays (ThermoFisher Scientific, MA, United States). The reaction tubes were placed into a thermal cycler with the following settings: 16°C x 30 mins, 42°C x 30 mins, 85°C x 5mins. Reverse transcriptase quantitative PCR (qPCR) was accomplished using TaqMan Universal Master Mix II (ThermoFisher Scientific, MA, United States). 1,33 µL of cDNA template or nuclease-free water for No–template controls (NTCs) were added to PCR Reaction Mix (20x TaqMan Small RNA Assay PCR Master Mix, Nuclease–free water), transfer into 96–well optical reaction plate (BioRad).

Expression of miRNA-155 (5' – CUCCUACAUAUUAGCAUUAACA – 3'), miRNA-124 (5' – CGUGUUCACAGCGGACCUUGAU – 3'), miRNA-34a (5' – UGGCAGUGUCUUAGCUGGUUGU – 3') and miRNA-125b (5' – UCCCUGAGACCCUAACUUGUGA – 3') were assessed in cells and EVs by RT-qPCR. Spike-in cel-miR 39 was used as exogenous controls to normalize the expression levels of miRNAs in different conditions. RT- qPCR was performed by CFX96 Touch Real-Time PCR Detection System (BioRad) with the following settings: 95°C x 10 mins, 95°C x 15 seconds, 60°C x 60 seconds (40 Cycles). Relative miRNA concentrations were calculated using the in 2-ΔΔCt method, where Ct represents the cycle threshold. ΔCt values were calculated as difference between the target genes and the expression of the housekeeping gene. ΔΔCt values were calculated as differences to control sample; ΔΔCt values were expressed as percentage of values measured for controls.

3.8 STATISTICAL ANALYSIS

Each experiment in this study was performed at least three times. Results were presented as mean values ± SE. Since we always compared different treatments, we determined the differences between them by using Student's *t* test. Statistical analysis was performed using GraphPad PRISM 5.0 (GraphPad Software, San Diego, CA, United States) $p < 0.05$ was considered as statistically significant.

4. RESULTS

4.1 MURINE MICROGLIA

4.1.1 ACTIVATION OF N9 MICROGLIA CELL LINE

Based on the results of previous studies, several stimuli are known to activate microglia, among which LPS, a pro-inflammatory stimulus and ATP able to induce neuroprotective status promoting the vesicles shedding (Cunha *et al*., 2016; Guo *et al*., 2022).

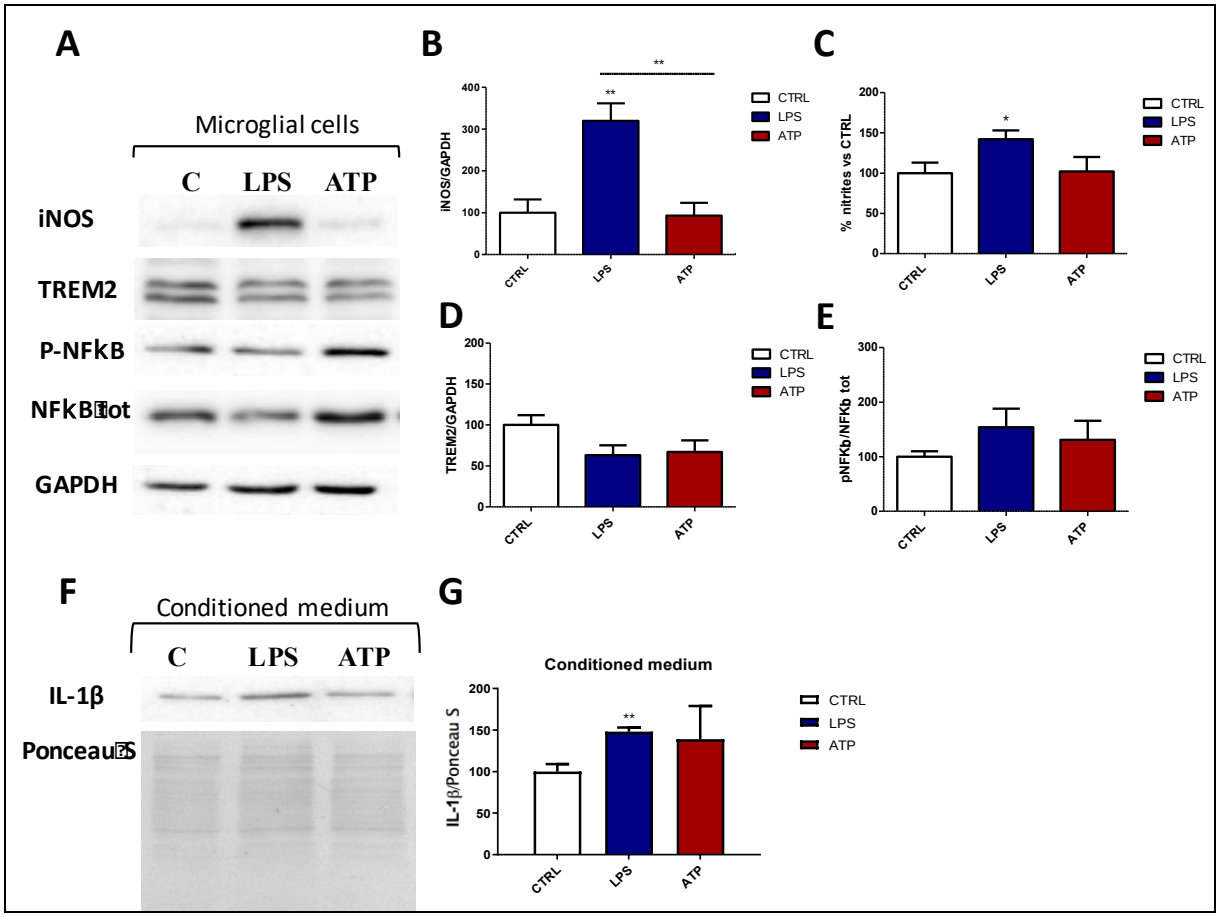


Fig.1 Pharmacological activation of N9 microglial cells. Phenotypic characterization of N9 microglial cells activation 24h after a 30 minutes exposure to LPS (1µg/ml) or ATP (200µM) or control medium by Western Blot analysis (A) and relative quantification of iNOS (B), TREM2 (D) vs the reference protein GAPDH, as well as the ratio of phosphorilated NFκB vs total NFκB (E). Griess method analysis of nitrites accumulation in the 24h conditioned medium derived from microglial cells pharmacologically activated. (C) Western Blot analysis (F) and relative quantification (G) of IL-1β in conditioned medium normalized vs Ponceau S. Results are mean ± s.e. of at least 3 independent experiments. Student's *t* test between control and LPS or ATP treated cells, *p<0.05 and **p<0.001.

We have established a culture protocol to pharmacologically activate N9 microglial cells towards neurotoxic or neuroprotective phenotype through a short-term (30minutes) LPS (1µg/ml) or ATP (200µM) treatment respectively, with untreated cells as controls, followed by a 24 hours medium conditioning. We analyzed two different inflammatory markers as specific for characterizing activation states: iNOS (inducible nitric oxide synthase), which is widely considered a marker of CAM microglia, and the microglial immunoreceptor TREM2 (Triggering receptor expressed on myeloid cells 2), which is involved in regulating microglial phagocytosis and commonly used to mark AAM microglia (Liu *et al*., 2020). iNOS expression was upregulated 3-fold in LPS-treated microglia compared to control conditions, thus indicating a CAM phenotype in line with the literature data. In contrast, iNOS was unchanged compared to the control state when cells were treated with ATP (Fig. 1a,b). In parallel to iNOS induction, the release of NO, measured through nitrite content in conditioned media, further confirms the upregulation of iNOS following LPS treatment only (Fig.1c). Moreover, analyzing the conditioned media, we detect a statistically significant increase in LPS-mediated IL-1β release normalized using Ponceau S (Fig.1f, g). On the other hand, neither LPS nor ATP administration showed significant variation in the expression level of TREM2 protein (Andreasson *et al*., 2016) (Fig.1a, d), an important innate immune receptor uniquely expressed on the microglia and is involved in downregulating neuroinflammation in the CNS. Despite it was reported that the increased glycolytic rate in microglia stimulated proinflammatory response mediated by TLR/NF-kB pathway (Shen *et al*., 2017) and because the activation of NF-κB by LPS induced the expression of pro-inflammatory cytokines (Zhang *et al*., 2019), we evaluated the phosphorylation of the Serine 536 on the NF-kB (p65); we observed only a slight non-significant increase in the phosphorylation of the Serine 536 on the NF-kB (p65) domain in cells treated with LPS (Fig.1a,1e).

4.1.2 EFFECT OF MEDIA CONDITIONED BY ACTIVATED MICROGLIAL CELLS ON CONTROL MICROGLIA PHENOTYPIC STATE

After confirming the typical marker of microglia polarization in N9 cells following pharmacological treatments, to evaluate whether activated microglia could release factors able to modulate the phenotypic state of control microglial cells, the indirect treatment through 24h conditioned media derived from microglial cells previously unstimulated or

stimulated by the above described LPS or ATP treatments have been investigated (Fig. 2a).

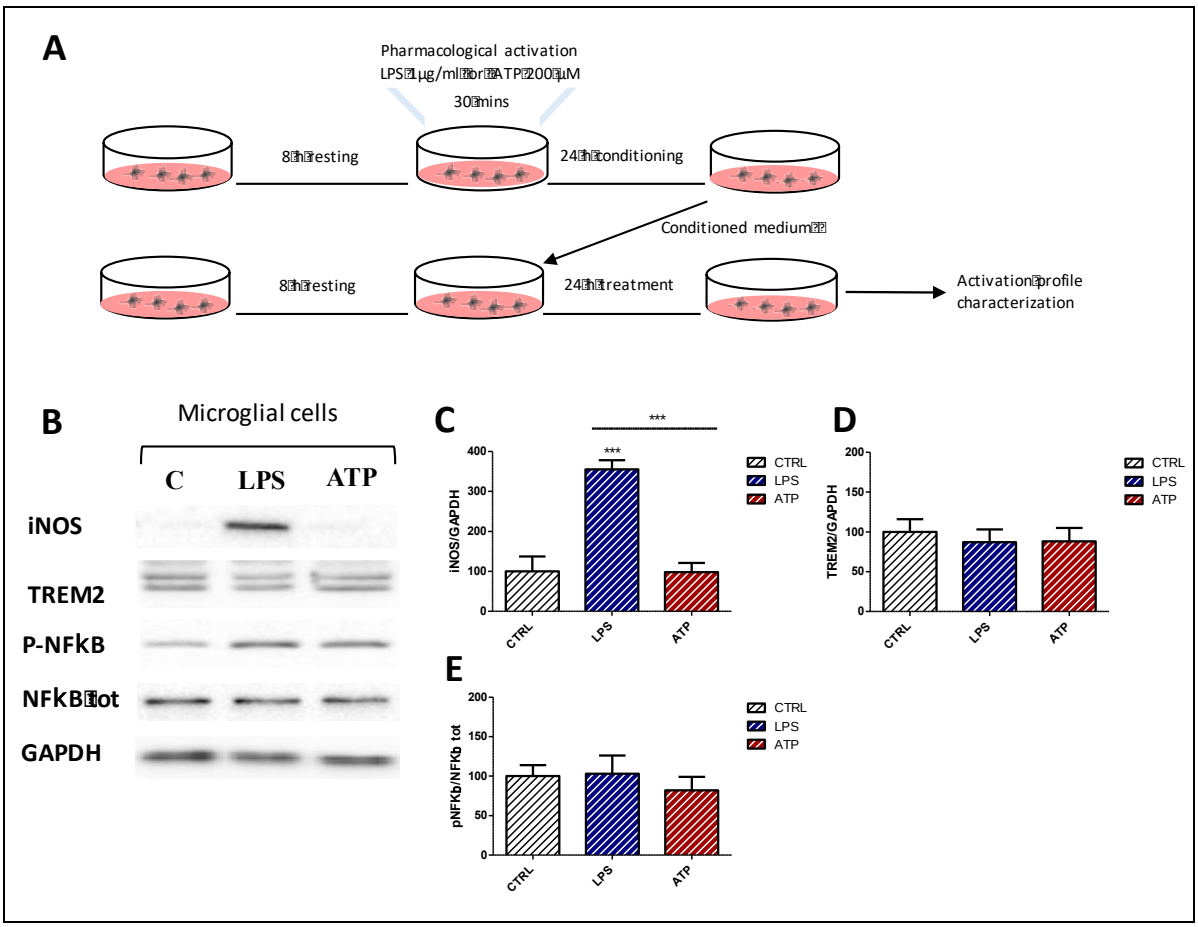


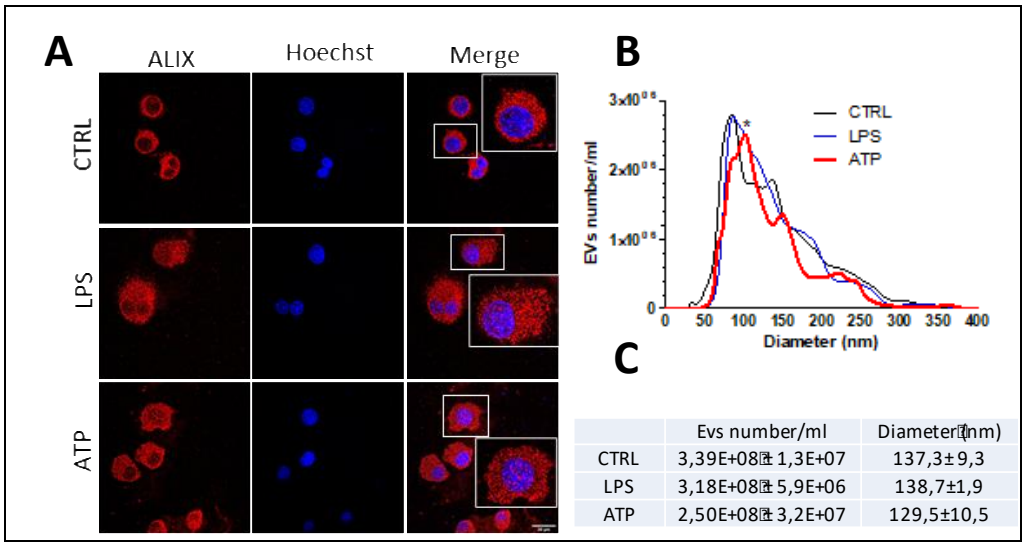
Fig.2 Activation of N9 cells via conditioned medium from pharmacologically activated microglial cells. Experimental scheme of microglia treatment with 24h-conditioned medium derived from microglial cells previously treated with LPS (1µg/ml) or ATP (200 µM) for 30 minutes (A). Western Blot analysis of cell lysates (B) and relative quantification of iNOS (C), TREM2 (D) expression vs the reference protein GAPDH, as well as phosphorylated NFκB vs total NFκB (E). Results are the mean ± s.e. of at least 3 independent experiments. Student's *t* test between control and LPS or ATP treated cells, *p<0.05, **p<0.01, ***p<0.001.

As shown in figure 2, microglial cells exposed to media conditioned for 24h by microglial cells previously activated through a 30 minutes exposure to LPS (1µg/ml), but not to ATP (200 µM) or control medium, show a statistically significant induction of iNOS expression, thus indicating that CAM cells are able to induce a switch of the phenotype in another cell culture through the release of proinflammatory factors in the culture medium. As expected, medium conditioned by cells treated with ATP did not promote a polarization in a proinflammatory status associated with no changes of iNOS expression comparing to the control (Fig. 2b,d). No changes were observed evaluating the expression level of TREM2 and the ratio NF-kB(p65)/NF- kB.

4.1.3 ANALYSIS OF MICROGLIA EXTRACELLULAR VESICLES IN CONDITIONED MEDIA DERIVED FROM ACTIVATED MICROGLIAL CELLS ACTIVATED

It is reported in the literature that microglial cells release EVs and that their composition changes depending on the phenotypic state. Therefore, it could be hypothesized that EVs released by pharmacologically activated microglial cells could be involved in the activation effect of the medium conditioned by LPS-treated microglia. In order test this hypothesis, here we isolated the EVs from the above-described conditioned media by sequential ultracentrifugation and we characterized them by using different approaches.

First, we evaluated the expression of the exosomal characteristic marker ALIX by immunofluorescent analysis (Fig. 3b) in untreated or LPS- or ATP-treated microglial cells. No differences were found in the ALIX expression and in the localization of EVs in the cells.



	Evs number/ml	Diameter (nm)
CTRL	3,39E+08 ± 1,3E+07	137,3 ± 9,3
LPS	3,18E+08 ± 5,9E+06	138,7 ± 1,9
ATP	2,50E+08 ± 3,2E+07	129,5 ± 10,5

Fig.3 Analysis of extracellular vesicles derived from pharmacologically activated N9 microglial cells. Immunofluorescence analysis of N9 cells pharmacologically activated with LPS 1µg/ml or ATP 200µM indicates the presence of exosomal marker proteins, Alix (A). Size-distribution of EVs isolated from the extracellular media of N9 cells, after treatment with LPS 1µg/ml or ATP 200µM. Evaluation of the EVs size and density by NTA indicates that the majority of vesicles from MNs have diameter ~100 nm, with differences in number between control and ATP treated cells (B,C). Results are the mean ± s.e. of at least 3 independent experiments. Student's *t* test between control and LPS or ATP treated cells, *p<0.05.

Then, we confirmed the EVs size (diameter) and number by using NTA (Fig. 3b, c): interestingly, the two different pharmacological stimuli seem not alter the size of the vesicles comparing to the control, which is around 100nm corresponding with the exosomal size (Raposo, et al., 2013). NTA analysis showed the presence of larger vesicles, but these

were relatively rare compared to the bulk of the population closer to the 100 nm. In contrast, despite ATP is known to induce the shedding of vesicles, in our model it was shown a slight decrease in the number of released exosomes: this could be explained because the most of vesicles release occurs in the first hours post treatment, but for our experimental needs, i.e. the timing parallelism with the conditioned medium, we isolated the vesicles after 24 hours (O'Brien et al., 2020), that is anyway compatible. After the demonstration that EVs with dimensions compatible with exosomes and expressing the ALIX exosomal marker are present in very similar number in media conditioned by different activated microglia, as well as by control cells, and considering that exosomes are known to be involved in neuroinflammation (Ceccarelli, *et al*., 2021), we decided to test whether the exosomal fraction released in the medium by activated microglia could be involved in the previously observed in vitro activation spreading.

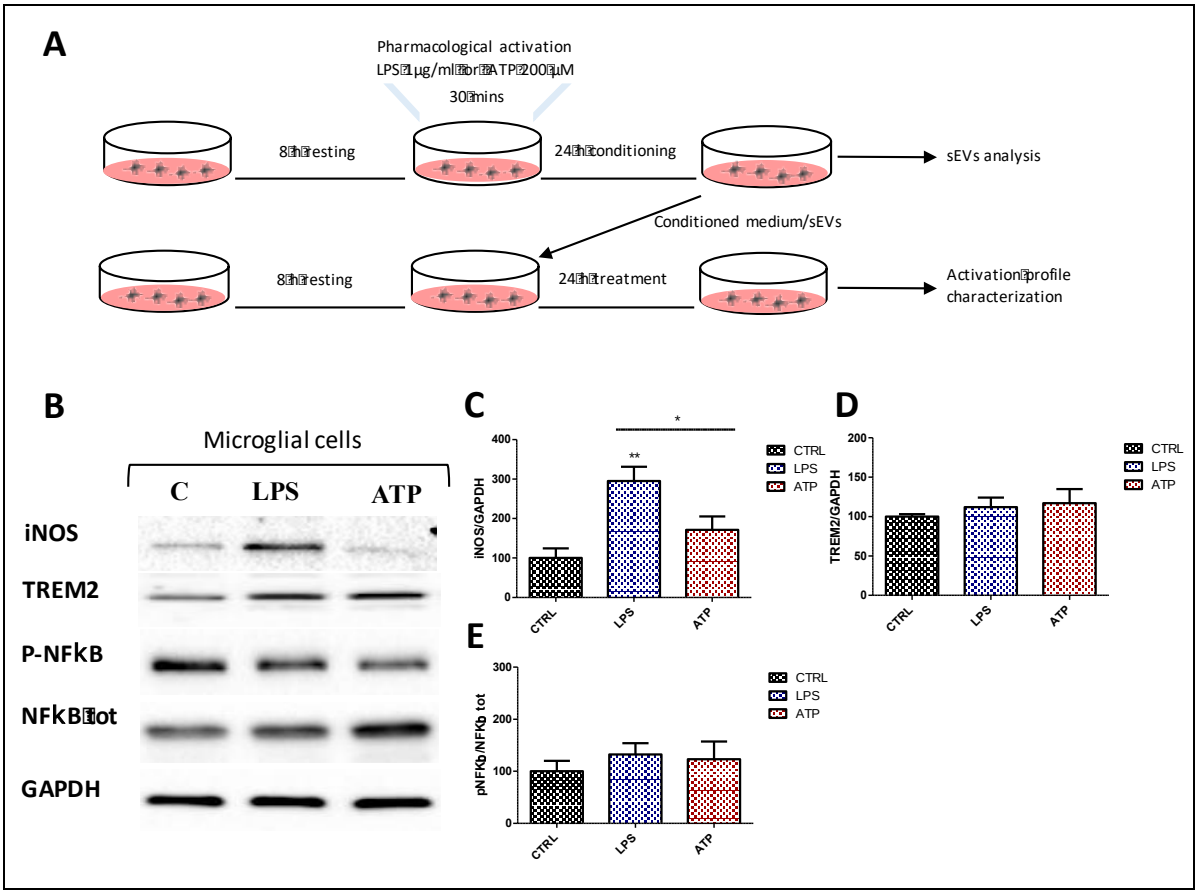


Fig.4 Extracellular vesicles derived from LPS-activated microglial cells induce activation in control microglia. Experimental scheme of microglia activation through the treatment with sEVs derived from microglial cells previously treated with LPS (1µg/ml) or ATP (200 µM) (A). Western Blot analysis (B) and relative quantification of iNOS (C), TREM2 (D), vs the reference protein GAPDH, as well as ratio of phosphorylated NFκB vs total NFκB (E). Results are the mean ± s.e. of at least 3 independent experiments. Student's *t* test between control and LPS or ATP treated cells, *p<0.05, **p<0.01.

To do that, we isolated exosomes by ultracentrifugation from 24h conditioned media by LPS- or ATP-treated or control cells and, after washings, we resuspended these exosomes in an equal volume of unconditioned medium, we exposed control microglial cells to these exosomal medium for further 24h and then we evaluated their activation phenotypes through the WB analysis inflammation markers (see Figure 4a for a representative scheme).
LPS-stimulated microglia-derived EVs induce a statistically significant increase of iNOS expression in recipient cells (Fig.4b, c), which does not change in cells treated with EVs by control or ATP-treated cells, while none of the treatments determine significant alterations in TREM2 level (Fig.4b,4d) and do not significantly influence the phosphorylation of the p65 (Ser536) of NF-κB in our model (Fig. 4b, e).
These data strongly indicate that pharmacologically LPS-activated microglia, but not ATP or control cells, can further activate control microglial cells through EVs and therefore suggest the central role of microglial released vesicles in the spreading of activation, at least in our in vitro model.

4.1.4 INHIBITION OF EVS RELEASE WITH GW4869 REDUCES THE LPS CONDITIONED MEDIUM ACTIVATION STIMULUS

To further demonstrate the functional role of exosomes in the spreading of microglial activation, we tested the effect on microglial phenotypes of media conditioned by microglial cells treated with LPS or untreated, in presence or absence of GW4869, which strongly reduces the exosomal release. In fact, the inhibitor GW4869 is known to act on nSMase (Neutral Sphingomyelinase), a family of enzymes able to convert sphingomyelin in ceramides that permit the formation of vesicles by ESCRT-independent pathway (Catalano *et al*., 2019).
First, we identified the most suitable concentration of the inhibitor by testing the effect of increasing concentrations of GW4869 (3uM, 5uM, 10uM) on microglial cells viability though MTT assay (Fig. 5d): 10uM shows a non-significant reduction of cells viability. Furthermore, by immunofluorescence analysis of vesicles stained with the lipophilic dye DiI (Zhang, *et al*., 2021), we confirmed the lower capacity of microglial cells to shed vesicles after 5µM GW4869 treatment for 4 hours (Fig. 5b, 5c). This effect was further confirmed by the WB analysis of vesicles released by N9 microglia activated by LPS for 30 minutes after a 4 hours treatment in presence of absence of 5µM GW4869, as depicted in the scheme (Fig. 5a). We

observed a significant reduction of EVs, evaluated through ALIX expression, when media derived from cells treated with the inhibitor compared to LPS-activated cells, thus demonstrating the successful action of the vesicles 'release inhibitor (Fig. 5e)

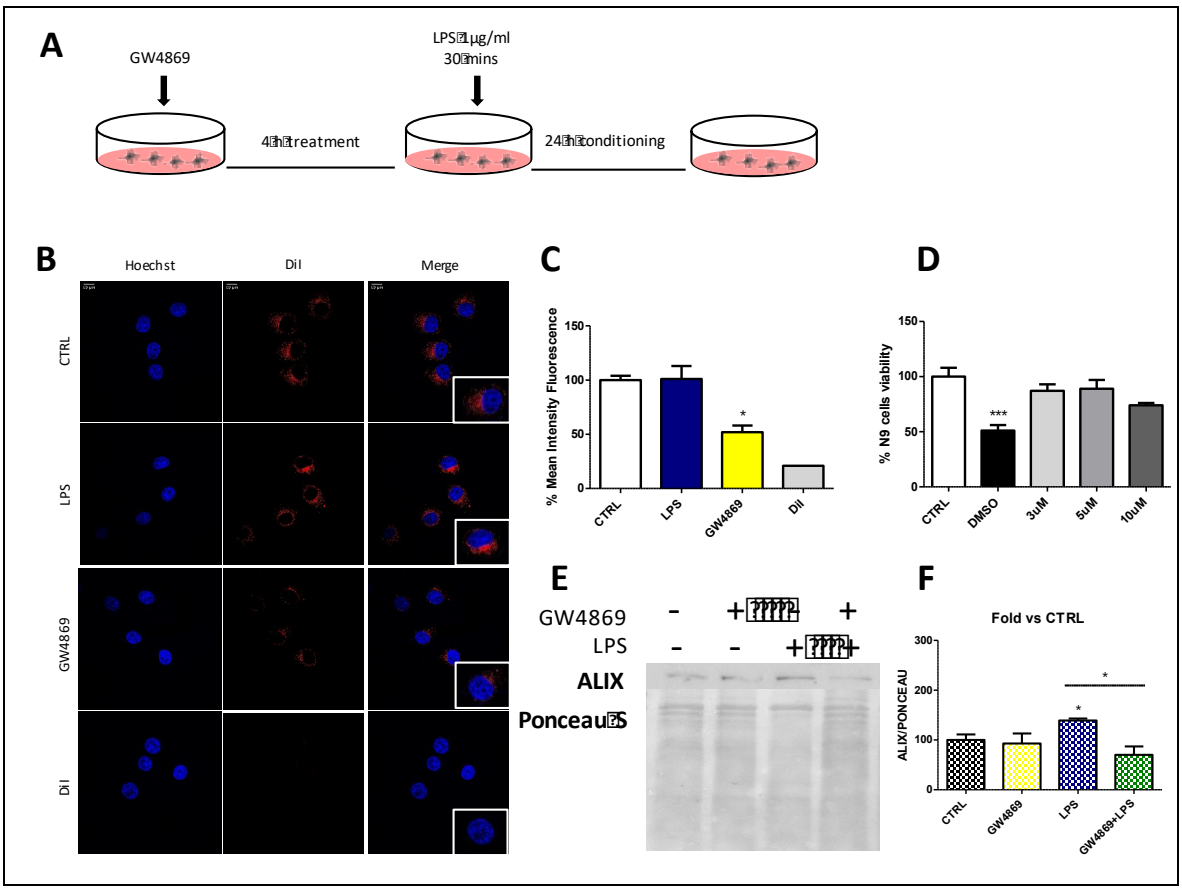


Fig.5 GW4869 effect on N9 microglia cells viability and exosomal release. Experimental scheme of microglia treatment with GW4869 (5µM) in presence/absence of LPS (1µg/ml) (A). Representative images (B) and quantification (C) of immunofluorescence analysis indicated the effect of the inhibitor GW4869 on the vesicles shedding. MTT Assay shows the GW4869 concentration (3uM, 5uM, 10uM) tested evaluating N9 cells viability compared to the control condition. (D) Western Blot analysis conditioned media derived from N9 cells treated with different time point of GW4869 using the exosomal marker proteins, Alix (E) and the relative quantification vs the Ponceau S (F) on 24h conditioned media. For each experimental condition 3 different fields of immunofluorescence were analyzed; results are the mean ± s.e. of at least 3 independent experiments. Student's *t* test between control and LPS or ATP treated cells, *p<0.05.

Subsequently, these media were used to treat parallel N9 cultures: as observed in Fig. 6a, b, c, levels of iNOS were lower in cells treated with media conditioned by cells treated with GW4869 compared to those treated with LPS only, thus confirming the role of EVs in the spreading of activation among microglial cells.

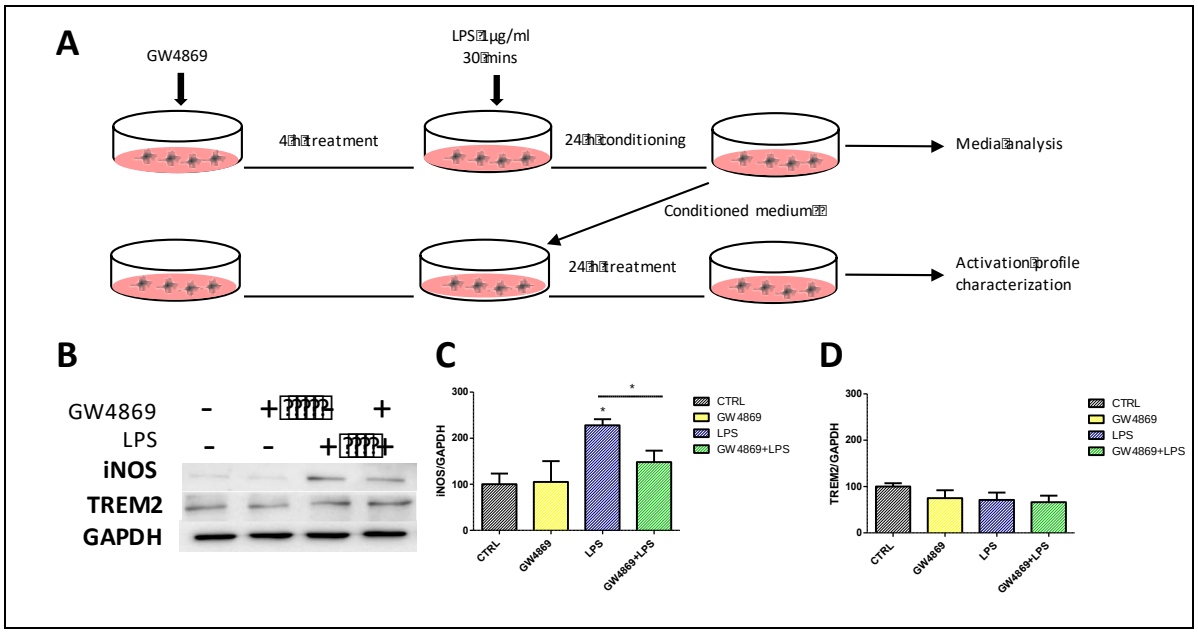


Fig.6 Activation of N9 cells via conditioned media from microglial cells treated with GW4869. Experimental scheme of microglia treatment with GW4869 (5µM) in presence/absence of LPS (1µg/ml) and N9 treatment through 24h conditioned medium. (A) Western Blot analysis (B) and relative quantification of iNOS (C), TREM2 (D) on N9 cells vs the reference protein GAPDH. Results are mean ± s.e. of 3 independent experiments. Student's *t* test between cells treated with conditioned medium of microglial cells previously treated with LPS+GW4869 and cells treated with conditioned medium derived from microglial cells treated only with LPS (*p<0,05).

4.1.5 NEUROINFLAMMATION-RELATED MICRORNAS COULD BE INVOLVED IN THE ACTIVATION SPREADING MEDIATED BY MICROGLIAL RELEASED EXOSOMES

As microglia activation is a hallmark of neurodegenerative diseases and given the evidence that microglial activation and miRNAs dysregulation in inflammatory process co-occur, we explored the expression of altered miRNAs that could be involved in the progress of inflammation. (Brites, 2020; Essandoh *et al*., 2016; Petralla, *et al*., 2021) To verify this assumption, we identify 4 miRNAs that are already known to be upregulated in neurodegenerative diseases and associated to inflammation pathway in microglial cells: miR-155, miR-124, miR-125b and miR-34a. In general, MiR-155, miR-125b and miR-34a are considered to preferentially drive a pro-inflammatory response, while miRNA-124 is more associated with anti-inflammatory response.

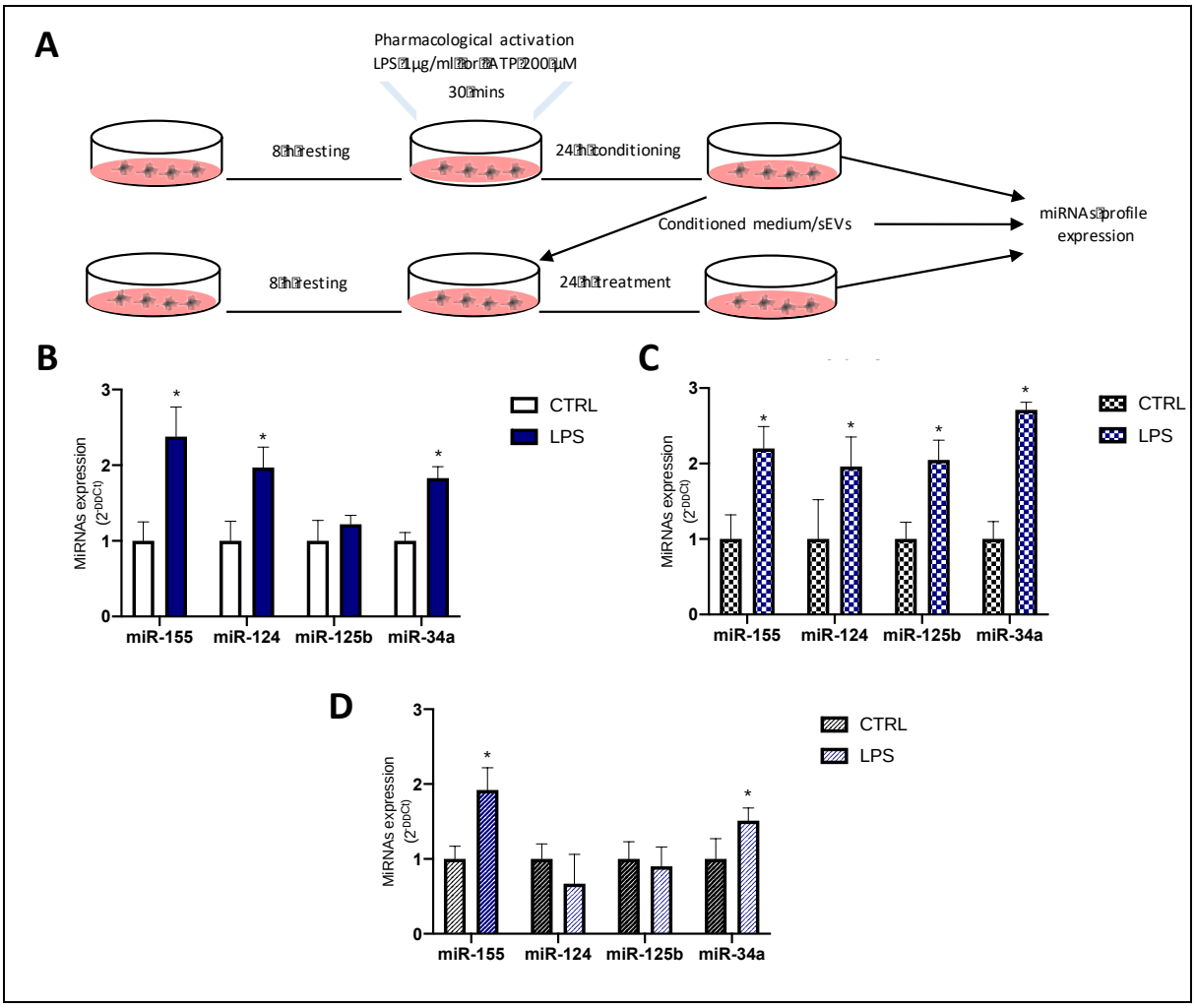


Fig.7 qRT-PCR gene expression analysis of selected microRNAs. Experimental scheme of microglial cells treatments to analyze microRNAs profile expression (A). qRT-PCR gene expression analysis of microRNAs extracted from cells (B), from EVs isolated from microglial 24h-conditioned media (C) and extracted from cells treated with conditioned medium derived from cells previously activated with LPS and from non-treated microglial cells (D). Student's *t* test between control and treated cells with direct or indirect treatment and EVs derived from control cells microglial cells previously treated with LPS (*p<0,05).

Intriguingly, all the selected microRNAs analyzed in microglial cells 24h after the 30 minutes treatment are significantly upregulated in cells treated with LPS (1µg/ml) comparing to control condition, except for miR-125b that was non-significant. (Fig.7b) Because of this trend, we wondered whether this profile was reflected also in the microglial cells that received the treatment with conditioned medium (Fig.7d), where no changes were observed for miR-124 and miR125b; however, the elevated level of miR-34a and increased expression of miR-155 suggest an effect due to inflammatory mediators delivered by media. Accordingly, we analyzed the miRNAs profile extracted from EVs (Fig.7c) and we found a strong alteration of all microRNAs content in the EVs that have an effect on indirect

polarization of microglial cells. These data underline an involvement of different inflammatory pathways modulated by different inflame-miRNAs determining distinct microglia activation.

To better evaluate the role of miRNAs in the switch of microglia status, in addition we examined the expression level of the considered miRNAs in antinflammatory condition using ATP treatment or conditioned media/EVs treated-microglia derived.

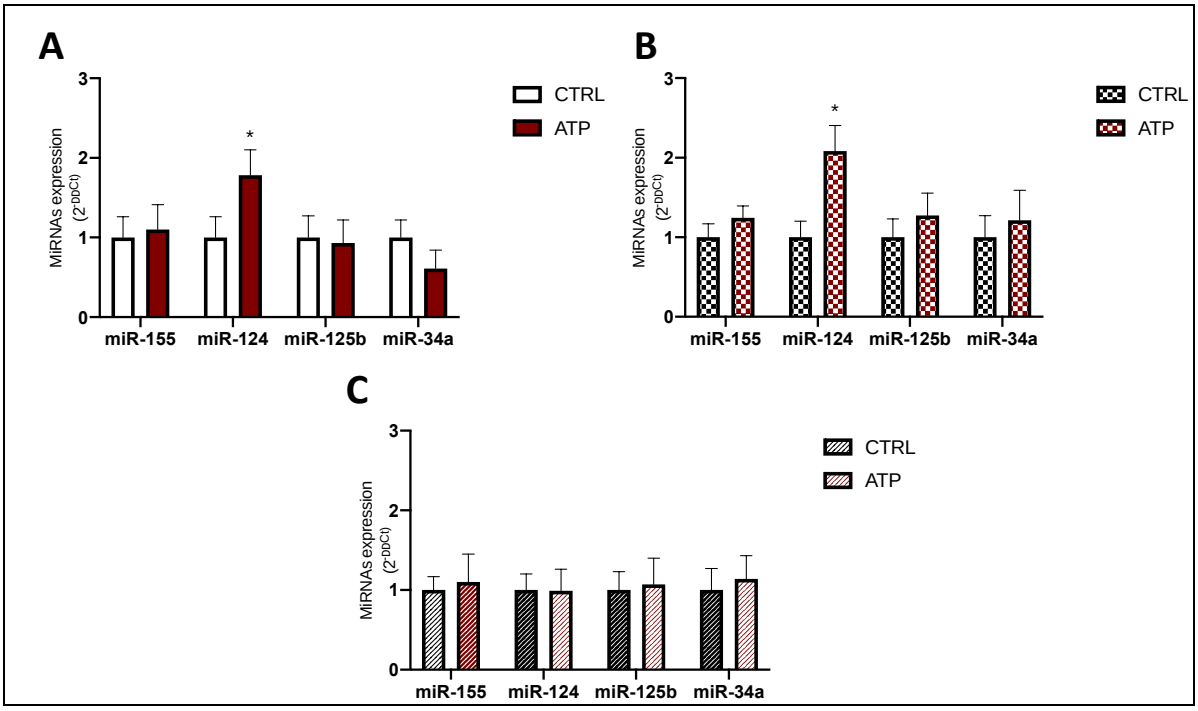


Fig.8 qRT-PCR gene expression analysis of selected microRNAs. qRT-PCR gene expression analysis of microRNAs extracted from cells (A), from EVs isolated from conditioned media after pharmacological activation (B) and extracted from cells treated with conditioned medium derived from cells previously activated with ATP and from non-treated microglial cells (C). Student's *t* test between control and treated cells with direct or indirect treatment and EVs derived from control cells microglial cells previously treated with ATP (*p<0,05).

MiR-124 expression is enhanced during the direct treatment with ATP, and on the other hand, we detected a slight decrease in miR-34a which is a scenario that seems to be in line with the induced anti-inflammatory phenotype (Fig. 8a). MiR-124 expression result upregulated in the EVs content (Fig.8b), whereas the treatment with conditioned medium did not fully modify the status of receiving cells (Fig.8c).

Altogether, these data strongly suggest that miRNA-155 and miRNA-34a could be involved in the spreading of proinflammatory activation in microglial cells through exosomes, as their

level of expression is increased by LPS treatment in both cells and exosomes, as well as in microglia indirectly activated by LPS-induced microglia conditioned media. In agreement with that, ATP does not induce any change in these miRNAs, while it increases the level of miRNAs-124 in both microglial cells and exosomes collected 24h after a 30 minutes stimulation by ATP. Furthermore, the unchanged level of miRNA-124 in cells treated with media conditioned by ATP-stimulated AAM microglia strongly correlates with the fact that neither these conditioned media nor the exosomes isolated from them are able to transfer this activation in control microglial cells.

4.1.6 INHIBITION OF MIR-34a EXPRESSION BY USING DNAZYME TREATMENT

To clarify the mechanism responsible for the diffusion of activation among microglia cells and to demonstrate a functional role of inflamma-miRNAs identified in CAM released exosomes in this neuroinflammation spreading process, we focused our attention on miRNA-34a, which is already known to be involved in proinflammatory microglial activation in NDDs (Bhattacharjee *et al*., 2016). To this aim, we decided to try to reduce the level of miRNA-34a in donor LPS-activated microglial cells, by using an innovative approach, i.e. the use of a specific DNAzyme, thanks to the collaboration with Dr. Giampaolo Zuccheri (Dept. Pharmacy and biotechnology, University of Bologna, Italy). We selected this methodology to avoid transfection and infection of microglial cells, which become activated following these procedures, while DNAzymes are nanostructured nucleic acids that are spontaneously phagocyted by microglial cells. Dr. Zuccheri lab designed, produced and chemically characterized the DNAzyme directed towards miRNA-34a (data not shown), while we examined the effect of this DNAzyme miR-34a in microglial cells.

Firstly, we checked whether the treatment itself with DNAzyme could cause a counterproductive activation of the microglia. As shown in figure 9, WB analysis of iNOS expression does not show any significant change in cells 4h treated with the DNAzyme (50μM) as it remains comparable to the control, using microglial cells 30 minutes treated with LPS (1μg/ml) as a positive control (Fig. 9).

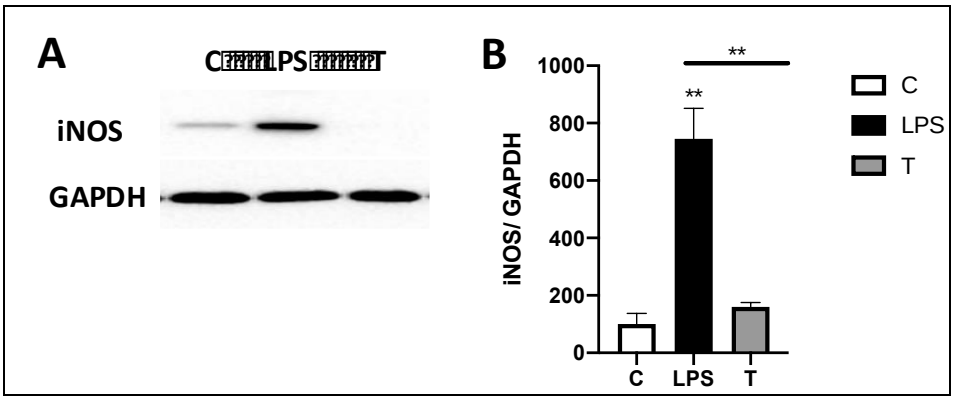


Fig.9 Effect of DNAzyme treatment on N9 microglial cells activation. Western Blot analysis (A) and relative quantification of iNOS (B) vs the reference protein GAPDH of N) cells treated or untreated with DNAzyme (50μM) or LPS (1μg/ml) as a positive control. Results are the mean ± s.e. of at least 3 independent experiments. Student's *t* test between control and LPS or T treated cells, **p<0.01.

Once observed that the treatment with DNAzyme does not influence microglial activation, as expected, we decided to test the effect of this molecule on LPS-activated microglia and the effect on the spreading through exosomes. To this aim, we performed a 4 hours pre-treatment on N9 cells with DNAzyme (50μM) before 30 minutes LPS activation and then we tested the effect of a further 24 hours conditioned medium on control microglia, as depicted in figure 10a, by analyzing the expression of iNOS and TREM2 in both cell cultures.

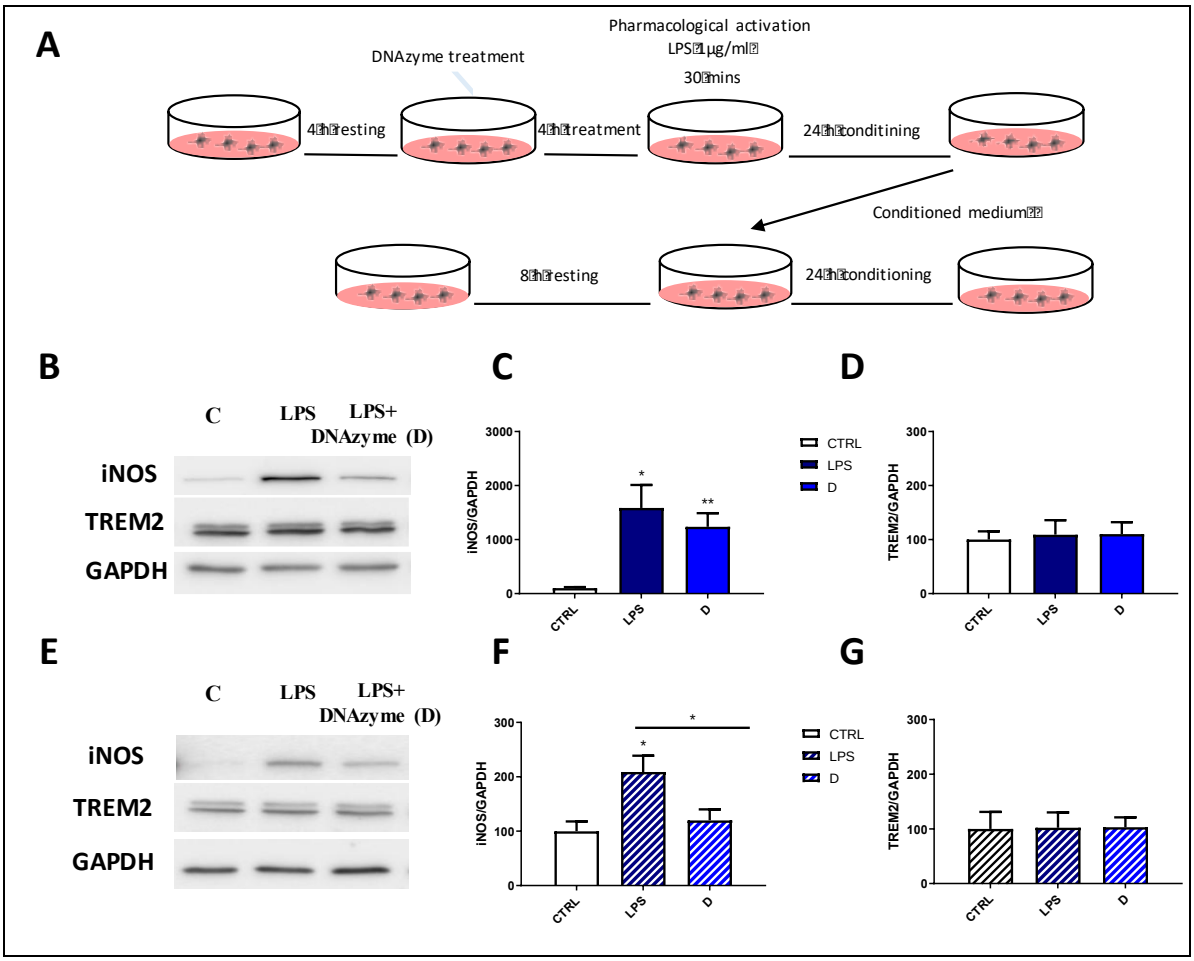


Fig.10 Treatment of N9 cells via conditioned medium from microglial cells pre-treated with DNAzymes (50µM) and activated with LPS (1µg/ml). Experimental scheme of microglia treated with 24h conditioned medium derived from microglia pre-treated for 4h with DNAzyme (50µM) and activated for 30 minutes with LPS (1µg/mL) (A). Western Blot analysis (B) and relative quantification of iNOS (C), TREM2 (D), vs the reference protein GAPDH in microglia pharmacologically activated and treated or not with DNAzyme. Western Blot analysis (E) and relative quantification of iNOS (F), TREM2 (G), vs the reference protein GAPDH of microglia treated with media conditioned for 24 hours after the 4 hours treatment with the DNAzyme and the following activation through 30 minutes LPS treatment. Results are the mean ± s.e. of 4 independent experiments. Student's *t* test between cells treated with conditioned medium from microglial cells previously treated with LPS vs cells treated with medium derived from non-treated microglial cells and between cells treated with DNAzyme *vs* non-treated cells. (*p<0.05).

As demonstrated in Figure 10 b, c, the DNAzyme treatment promoted a slight, but statistically significant reduction of iNOS expression and, consequently, of neurotoxic activation on microglial cells. Furthermore, a significant and even more evident decrease in iNOS expression was found in cells exposed to conditioned medium deriving from CAM previously treated for 4 hours with the DNAzyme, compared to them activated with LPS only (Fig. 10 e, f), thus indicating the diminution of the spreading of microglia pro-inflammatory activation. However, the cells retained the unchanged expression of TREM2 in all treatment

conditions (Fig. 10 b, d, e, g). Because of these results, we wondered whether the DNAzyme treatment could act in a similar manner when given directly on microglial cells receiving LPS-activated cells derived conditioned medium, as depicted in the scheme (Fig.11a).

To this aim, primarily we confirmed the microglia activation status 24 hours after the 30 minutes LPS treatment, by the significant increased level of iNOS observed in WB analysis (Fig. 11 b, c). Then, 24h conditioned medium by these cells was used to treat N9 cells previously exposed for 4 hours to DNAzyme, in which activation was evaluated through the WB analysis of iNOS and TREM2 markers. As shown in figure 11, cells that received the DNAzyme treatment showed a significant decreased level of iNOS comparing to those receiving directly the derived LPS-activated microglia conditioned medium (Fig.11 d, e), while TREM2 expression was unchanged (Fig.11 d, f). This immunomodulatory effect of the DNAzyme on microglial activation spreading seems to be related to the DNAzyme effect on miR-34a, that appeared less expressed compared to the level in microglia activated by LPS-conditioned medium only.

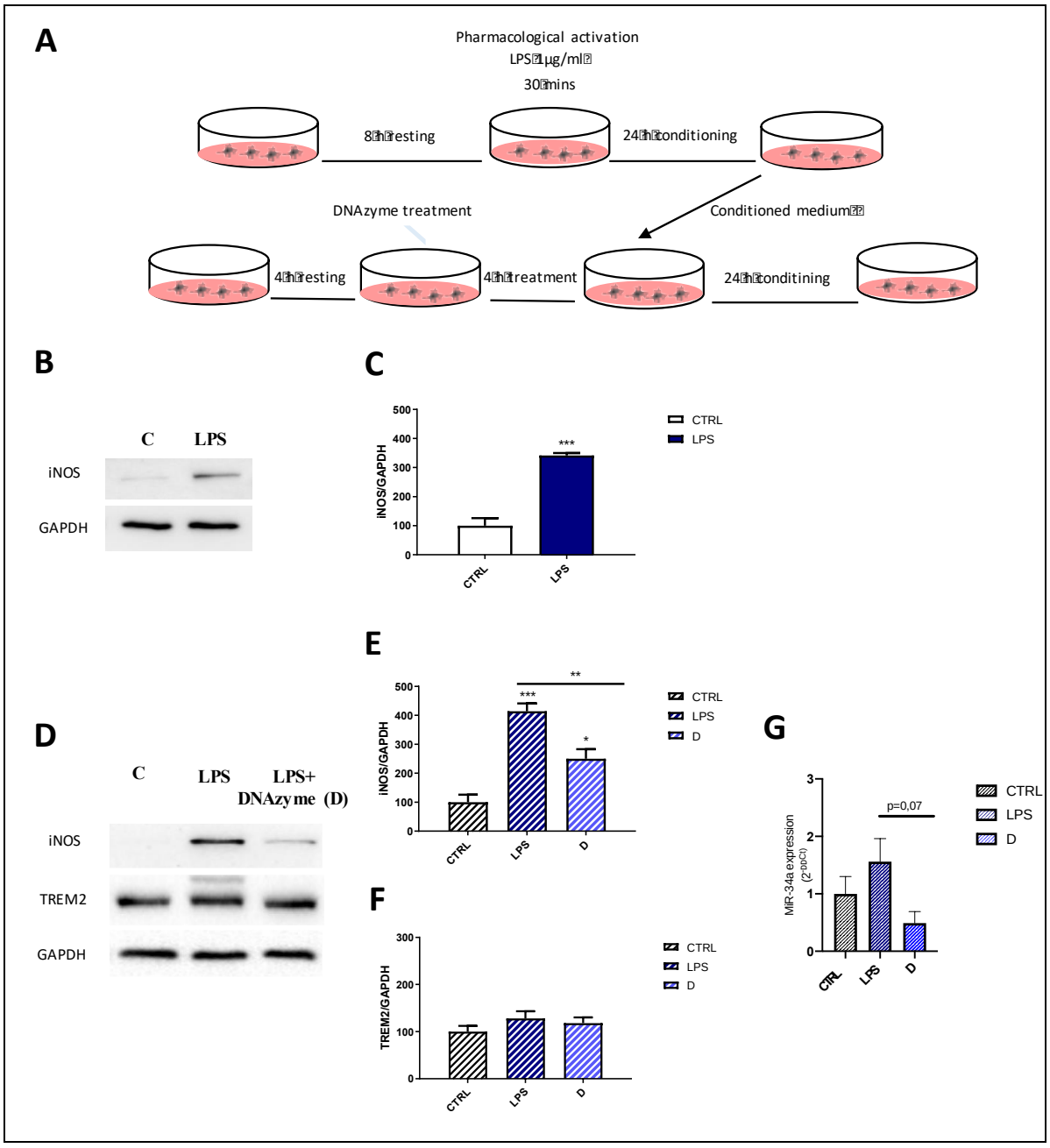


Fig.11 Effect of DNAzymes treatment (50µM) and microglia activated with conditioned medium derived from microglial cells previously treated with LPS (1µg/ml). Experimental scheme of microglia treatment with DNAzyme (50µM) followed by activation through conditioned medium from microglial cells previously treated with LPS (1µg/ml) (A). Western Blot analysis (B, D) and relative quantification of iNOS (C, E), TREM2 (F), vs the reference protein GAPDH. qRT-PCR gene expression analysis of miR-34a of cells receiving conditioned medium from LPS-activated cells and treated or not with DNAzyme (G). Results are the mean ± s.e. of 3 independent experiments. Student's *t* test between cells treated with conditioned medium from microglial cells previously treated with LPS *vs* cells treated with medium derived from non-treated microglial cells and between cells treated with DNAzyme *vs* non-treated cells. (*p<0.05, ** p< 0,01, and ***p<0.001).

4.2 HUMAN MICROGLIA

Microglial patho/physiology have been extensively studied in experimental mouse models (Lin *et al*., 2017; Figuera-Losada *et al*., 2014), however the characterization of these cells in humans is limited by the lack of sources of primary microglia, including aborted fetal tissues, biopsies from epileptic patients, post-mortem brain tissues, or healthy tissue from tumor excisions. Given the important differences that the two species present from a biochemical, genetical and pharmacological point of view, it is necessary to find strategies that allow the confirmation of data obtained in murine microglia (Dello Russo *et al*., 2018). HMC3 have been characterized for the expression of several myeloid lineage markers, including CD68, CD11b, CD14 (Peudenier *et al*., 1991) and IBA1 (Etemad *et al*., 2012). These cells are able to react to inflammatory stimuli, regulating the expression of typical activation markers of microglia (Gosselin *et al*., 2017).

4.2.1 ACTIVATION AND CHARACTERIZATION OF HMC3 MICROGLIA CELL LINE

As reported in Figure 12, treatment with increasing concentration of LPS (0, 1, 10 µg/ml) for 24h in serum free medium did not induce any modulation of microglial phenotype based on the markers observed in our experimental condition.

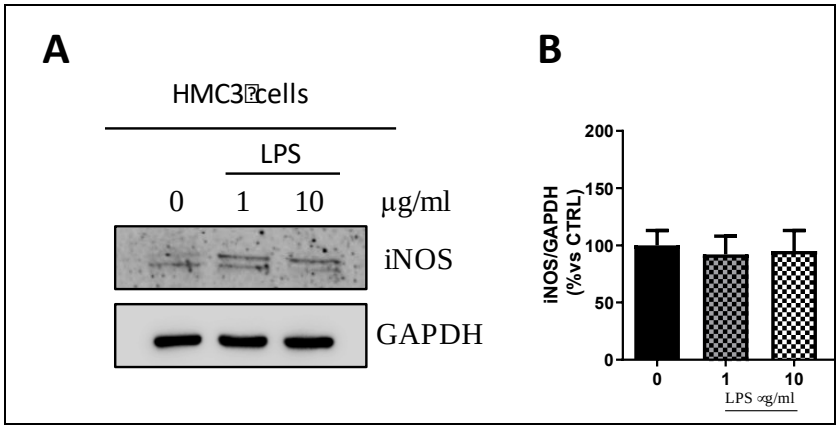


Fig.12 Human microglial clone 3 cell line (HMC3) activation upon treatment with increasing concentration of LPS. Western blot analysis and relative densitometry of iNOS *vs* the reference protein GAPDH in HMC3 cells treated with increasing concentration of LPS (A, B) (0,1,10 µg/ml) for 24h in serum free medium (4,5 g/L glucose). N=3 ± s.e *vs* % CTRL.

Since the classic activation with LPS stimulus, commonly used in rodent cells, was not able to induce a pro-inflammatory activation of cultured HMC3 cells, we stimulated the HMC3 cells with IFN-γ increasing the amount of glucose to the final concentration of 5 g/l, in line with the metabolic inducement reported along with the phenotypic shift from quiescent to activated-microglia (Ghosh *et al*., 2018).

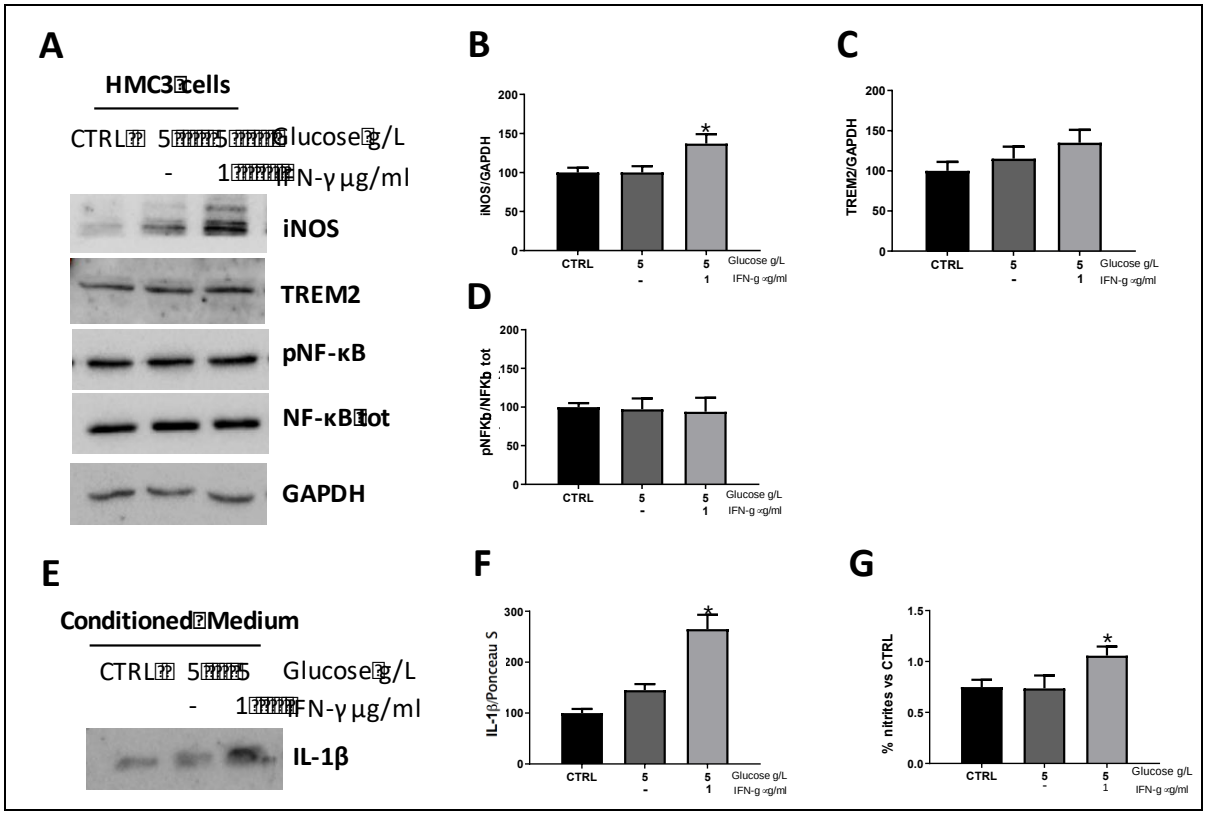


Fig.13 Human microglial clone 3 cell line (HMC3) activation and characterization. Western blot analysis and relative densitometry of iNOS (A, B), TREM2 (A, C), pNF-κB/ NF-κB tot (A, D), in lysed HMC3 cells treated with glucose (final concentration 5g/l) or IFN-γ 1µg/ml +glucose (final concentration 5g/l) for 24h in serum free medium vs the reference protein GAPDH. IL-1β expression (E, F) and nitrite quantification (G) in conditioned medium of HMC3 cell treated with glucose (final concentration 5g/l) or IFN-γ 1µg/ml +glucose (final concentration 5g/l) for 24h. Results are the mean ± s.e. of 3 independent experiments. One-way ANOVA, followed by Dunnett's test *p<0,05 *vs* CTRL (glucose 4,5g/l).

Moreover, the high glucose co-treatment with IFN-γ increased the nitrite quantification in the conditioned medium and the amount of released IL-1β (Figure 13 e, f, g). Taken together these data leading to assume that in our experimental conditions the inflammatory response was evident as to indicate a phenotypic shift, towards the pro-inflammatory one.

Lastly, the increased fluorescence intensity of IBA1 in IFN-γ +glucose cells together with the reduced cell size accompanied by increased roundness, thus indicated a reduced branching

respect to the control cells, and therefore activation while preserving ameboid morphology (Fig. 14 a, b, c), supporting an early phenotypic shift toward the pro-inflammatory status.

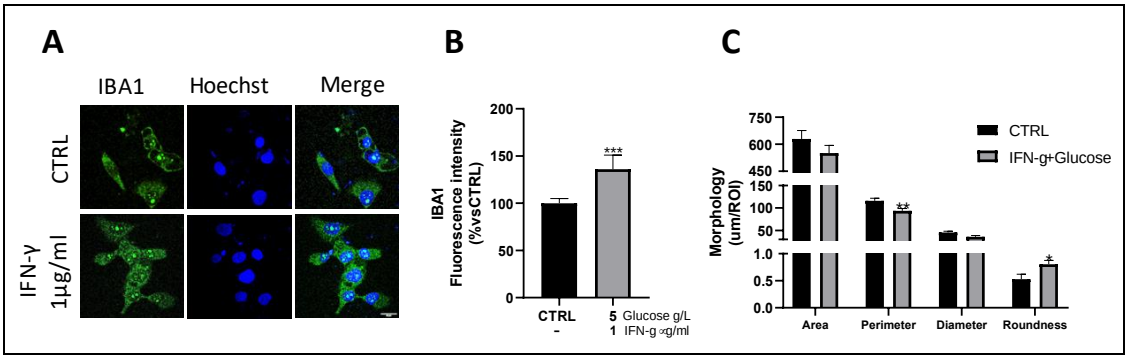


Fig.14 Phenotypic marker IBA1 expression in human microglial clone 3 cell line (HMC3) treated with IFN-γ + glucose. Representative immunofluorescence and relative quantification of fluorescence intensity of IBA1 (A,B) and morphology analysis of IBA1+ cells (A, C) in HMC3 cells treated with IFN-γ 1µg/ml +glucose (final concentration 5g/l) for 24h. N=3. Scale bar=20µM. One-way ANOVA, followed by Dunnett's test *p<0,05, **p<0.01, ***p<0.001 *vs* CTRL (glucose 4,5g/l).

4.2.2 EFFECT OF EXTRACELLULAR VESICLES IN CONDITIONED MEDIA DERIVED FROM ACTIVATED HMC3

In the perspective to step forward in understanding the neuroinflammation spreading and after characterizing the HMC3 activation profile, we developed an experimental scheme (Fig. 15a) that would allow us to replicate and compare the results obtained on N9 cells on human cells *in vitro* model.

To assess whether HMC3 release vesicles in our plan, we treated cells with IFN-γ (1µg/ml) w/wo ATP (500µM) and after 4h in total, we changed media. We collect media following the O/N conditioning to analyze the vesicles shedding through ALIX expression.

Data shown in Fig. 15 b, c suggest that the release of vesicles is associated to an activation of microglia through IFN-γ treatment but could be amplified by using ATP which acts through the family of P2X receptors.

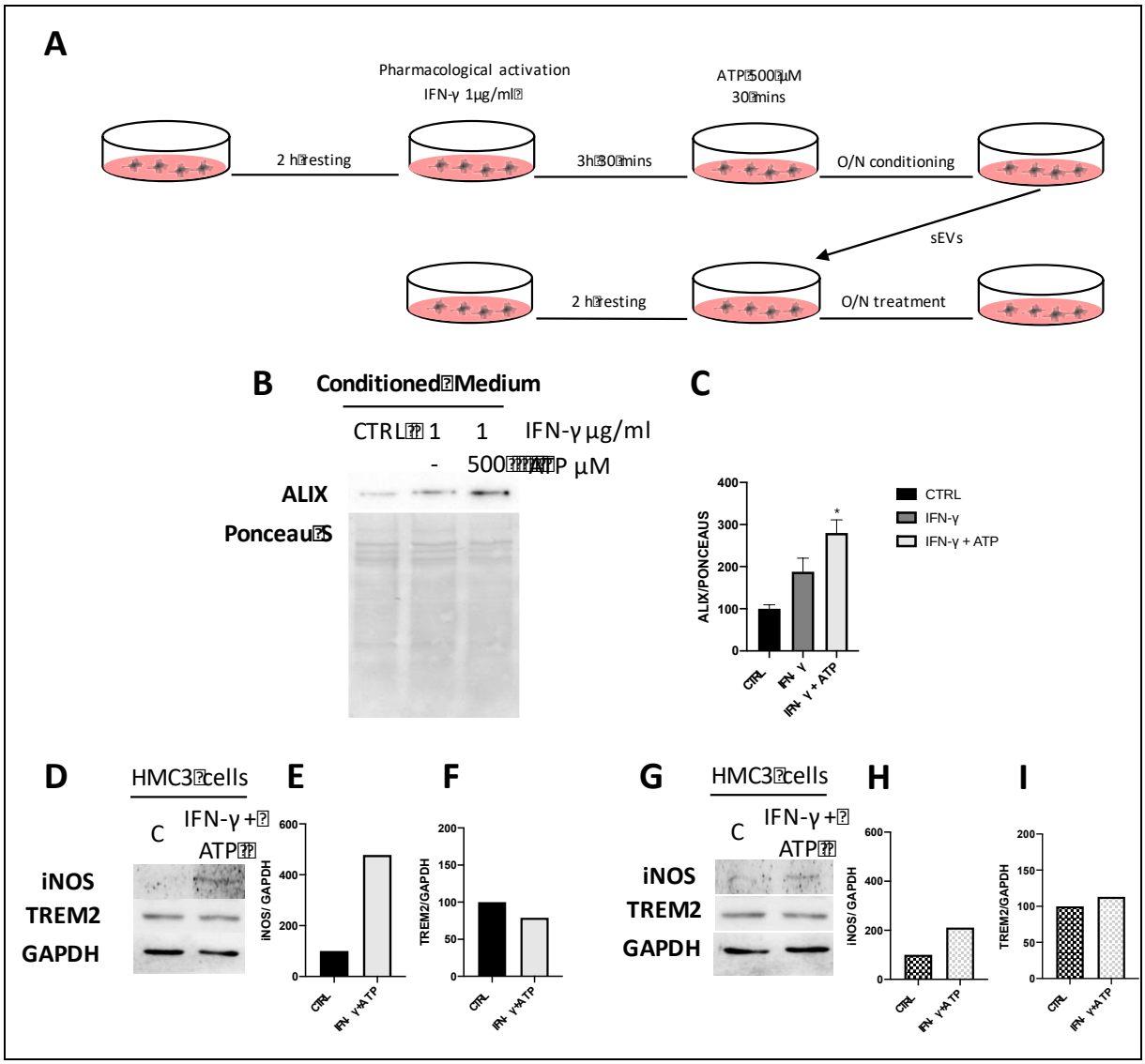


Fig.15 HMC3 activation treated with IFN-γ 1µg/ml + ATP 500µM and via EVs isolated from O/N conditioned medium. Experimental scheme of direct and indirect treatment on HMC3 to analyze activation profile markers (A). Western Blot analysis (D, G) and relative quantification of iNOS (E, H), TREM2 (F, I), vs the reference protein GAPDH on HMC3 treated with IFN-γ 1µg/ml + ATP 500µM or EVs extracted from activated/non-activated HMC3 conditioned medium. Results are the mean ± s.e. of 2 independent experiments for ALIX, One-way ANOVA, followed by Dunnett's test *p<0,05 and N=1 for iNOS and TREM2.

In this sense, we next assessed if the sEVs isolated from IFN-γ + ATP activated cells can expand the activation to other steady-state microglia, as proved on N9 cells. When directly activated with IFN-γ + ATP, HMC3 showed increased levels of iNOS compared to the control untreated cells (Fig. 15 d, e); on the contrary, TREM2 remains unchanged (Fig. 15 d, f). Once the exosomes were isolated from the treated/untreated cells, we used them as a treatment for other plated HMC3 cells. Similarly, to our findings on the effects produced by the EVs on N9 microglial cells, HMC3 showed increased protein expression of iNOS when exosomes

added derived from IFN-γ + ATP activated cells and no changes in TREM2. Data highlight the role of EVs in enhancing microglia proinflammatory polarization when released from activated donor cells. We couldn't, however, confirm these trends because further experiments should be done.

5. DISCUSSION

In this thesis, we showed data on a possible mechanism about the propagation of inflammatory activation among microglial cells in CNS by using *in vitro* model. Neuroinflammation is mainly mediated by microglia, the resident immune cells of the CNS able to sense pathogens, toxins, or injury. The inflammatory response to harmful insult is initially preservative for CNS homeostatic state, but excessive response is counterproductive for repair and beneficial resolution; on the contrary, prolonged inflammation results in a collateral brain damage provoking onset and progression of neurodegenerative diseases such as AD, PD, ALS and MS (Hickman *et al*., 2018).

The main goal of this project was to demonstrate that intercellular communication among microglial cells involving the shedding of EVs, in particular exosomes, enable to induce a rearrangement of the activation state of recipient microglia in a context-specific manner due to a change in their cargos, such as lipids, proteins, nucleic acids but principally in microRNAs.

We performed most of our studies on N9 immortalized murine microglial cells, a widely-used cell culture model to study microglia in vitro (Timmerman, 2018; Liu *et al*., 2020; Sarkar *et al.*, 2018) that exhibit plasticity to mimic *in vivo* microglial polarization; in order to confirm the translational relevance of our data, we also performed preliminary experiments by using also HMC3, human microglial cell line, gaining visibility in recent years although in the literature there are still few research articles on them (Dello Russo *et al*., 2018).

Depending on the milieu, microglia switch them phenotype from a surveillant to a reactive phenotype to protect CNS from dangerous stimuli in order to reestablish the healthy status (Colton *et al*., 2009; Spiteri *et al*., 2022). In CNS inflammatory-associated diseases, CAM phenotype is hyperactivated concomitantly with a suppression of the anti-inflammatory phenotype, leading to an imbalanced inflammation (Joseph & Venero, 2013). First, in order to characterized N9 cells activation analyzing inflammatory-associated markers, we pharmacologically treated N9 microglia using 1 μg/ml of LPS or 200μM of ATP to reproduce two different activation pathways: LPS was used to investigate the proinflammatory

response based on the recognition of PAMP by TLRs, specifically TLR4 signal transduction pathways resulting in activation of the NF-κB pathway and production of various innate immune and neurotoxic factors, and ATP which exert an anti-inflammatory role by enhancing phagocytosis. ATP is released into the extracellular environment in a context of neuronal damage and induces a variety of responses by binding purinergic receptors P2X, P2Y, P1127 (Illes *et al*., 2020). It was already demonstrated the acquisition of an ATP-mediated anti-inflammatory phenotype by stimulation of the ATP-sensitive Kir6.1 potassium channel (Kir6.1 / K-ATP) (Du *et al*., 2018). In our model, the increased level of iNOS after 30 minutes LPS exposure and the unchanged levels of TREM2 compared to untreated control indicate a predominantly polarization of microglia by promoting the classical activation without compromise the phagocytic properties of these cells, that remain stable. As claimed by others, phagocytosis could have different outcomes independently from the activation state of microglia (Sierra *et al*., 2014). The confirmation of activation status also came from the release of proinflammatory cytokines, such as IL-1β (Zhang *et al*., 2012), and the measurement of NO accumulation: LPS-treated microglial cells showed higher level of nitrites concentration in the extracellular medium, suggesting that the level of NO is concomitant with iNOS expression (Sierra *et al*., 2013). After assessing that LPS induces CAM in N9 microglia cells, as demonstrated by iNOS induction, while ATP maintains TREM2 levels, with no changes in iNOS expression, we decided to test whether the LPS-activated microglia conditioned medium was able to induce further activation in unstimulated cells. The experimental plan avoided possible LPS contamination, as the pharmacological treatment of microglia was followed by washings to eliminate remnant LPS, before changing the medium to be conditioned. We observed that media conditioned by microglia for 24h previously activated with LPS, but not with ATP or in untreated conditions, are able to induce proinflammatory polarization in homeostatic cells that have never been exposed to the pharmacological treatments: this strongly suggests that activated microglial can release mediators, which can activate other microglial cells, thus inducing the spreading of microglia activation.

In this thesis, we tried to define the activation profile of HMC3 to validate the use of human cellular model to analyze the mechanisms underlying the process of diffusion of inflammation among microglial cells and it is necessary to confirm the data obtained in

murine microglia. Numerous advantages derive from the use of immortalized human cells since species show multiple differences from the genetic point of view, of the immune and neurological mechanisms and allow to limit the use of animals necessary to obtain primary cultures (Smith & Dragunow, 2014). The most well characterized stimulus for activating microglia towards the CAM phenotype, both in mouse and primary rat cell lines, is treatment with LPS (Polazzi & Contestabile, 2006; Orihuela *et al*., 2016; Jurga, *et al*., 2020). However, previous studies have shown that HMC3 are less responsive to LPS than both rodent and human primary cells from which they derive (Smith & Dragunow, 2014; Wolf *et al*., 2017). Genes expression changes in mouse models of human inflammatory conditions do not necessarily correlate with human genomic changes, thus explaining the failure of human trials (Seok *et al*., 2013). In fact, while there are genes and proteins similarly expressed in microglia across different species, like the Ionized calcium-binding adaptor molecule 1 (IBA1) or the transcription factor PU.1 (Smith *et al*., 2013b), other are not: this is the case of the TLR 4, whose expression level in human microglia is lower compared to rodent cells (Jurga *et al*., 2020; Parajuli *et al*., 2012). Although the expression of TLR1, 2 and 6 was detected in HMC3 in basal conditions, it was observed that both HMC3 and embryonic microglia do not express CD14, a TLR4 co-receptor required to recognize the LPS (Janeway & Medzhitov, 2002). The described low expression of CD14 in HMC3 cells, and upregulated by IFN-γ, might take advantage to the priming role of IFN-γ that emphasizes the responsiveness to other inflammatory markers (Wang *et al*., 2021). Moreover, it was already evident from literature that treatment of HMC3s with IFNγ induces an increase in the expression of the MHCII transcript (Dello Russo *et al*., 2018; Peudenier *et al*., 1991), the CXCR3 receptor and the CCR3 chemokine, detected flow cytometric analysis (Flynn *et al*., 2003; Etemad *et al*., 2012; Gosselin *et al*., 2017).

Thus, we enrolled IFN-γ to activate HMC3 cells as function of pro-inflammatory markers since IFN-γ has a priming role in the process of microglia activation (Dello Russo *et al*., 2018; Polazzi & Monti, 2010; Monzón-Sandoval *et al*., 2022; Ta *et al*., 2019). In order to reinforce the activation of microglia, we acted also on glucose metabolic pathway: an increased glycolytic flux elevates cytosolic NADH levels (Zhang *et al*., 2002) and promoting transcription of pro-inflammatory genes (Shen *et al*., 2017) and the increase of immune reactivity (De Chirico *et al*., 2022; Galván-Peña & O'Neill, 2014). We demonstrated upon IFN-γ plus high glucose stimulation the CAM polarization through the increased expression

of iNOS accompanied by secretion of IL-1β and the augmented concentration of NO in the medium. Nevertheless, the co-treatment did not influence the phosphorylation of the p65 (Ser536) of NF-κB in our cells and TREM2-dependent modulation of the inflammatory response, meaning the preserving of the phagocytic properties of microglia (Zhang *et al*., 2018). Finally, the microglia status of activation upon IFN-γ plus high glucose stimulation was confirmed by increased fluorescence intensity measured through IBA1 staining, a microglial specific marker (Ito *et al*., 1998; Korzhevskii *et al*., 2016). In addition, activated cells still retaining the ameboid morphology of myeloid cells and supported by the reduced size and increased roundness of microglial cells, leading us to speculate about an intermediated polarized phenotype toward the pro-inflammatory one (Leyh *et al*., 2021; Rai *et al*., 2020).

A peculiarity of microglial cells is the ability to establish communication with other cells that inhabit the CNS parenchyma. Microglia can communicate with other cells by cell-to-cell contact, by secretion of soluble factors and by shedding a large number of EVs (Ceccarelli *et al.*, 2021; Frühbeis *et al*., 2013). Growing evidences indicate that EVs are an advantageous way to transfer functional elements over long range distances (Prada *et al*., 2013). In particular, in the context of neuroinflammation, microglia-derived EVs raise large interest because of their controversial roles; if, on one hand, EVs released by microglia have been shown beneficial effect in resolving excessive neurotoxic response, on the other hand they could contribute to overstimulate inflammatory stimuli (Paolicelli *et al*., 2019; Trotta *et al*., 2018). In this sense, we evaluated whether microglial released EVs could play a role in the activation effect of LPS-treated microglial conditioned medium.

In what concern N9 cells, according to the NTA analysis, EVs released by microglia in the conditioned medium after LPS stimulation show dimensions comparable to exosomes and appear similar to the untreated control concerning the number and the size (Midekessa *et al.*, 2021; Murgoci *et al*., 2020). Therefore, here we collected exosomes from equal volumes (1mL) of 24h-conditioned medium by 30 minutes LPS- or ATP-treated microglia or control cells, through ultracentrifugation and we resuspended the vesicles in fresh medium to be given to untreated microglial cells. By the analysis of activation markers, here we provide new evidence of the acquisition of CAM phenotype without pharmacological treatment of direct contact with previously activated cells, but only by the exposure to exosomes released

by LPS-treated microglia. Therefore, this procedure showed an indirect mechanism strictly related with the capacity of different donor cells to release EVs containing different cargos that have a key role in modulating microglial function, modifying their inflammatory profile (Yáñez-Mó *et al*., 2015). In BV2 microglial cells model, it has already been studied that LPS-derived EVs can activate microglia in a manner similar to that of LPS alone; on the contrary, EVs derived from control cells cannot polarize microglia towards a pro-inflammatory state (La Torre *et al*., 2022).

This evidence was supported by the translation of these experiments on HMC3 cells. First, we demonstrated that adding the ATP in association with IFN-γ in culture medium, the concentration of EVs was enhanced, revealed by the significant overexpression of ALIX through Western Blot analysis of conditioned medium.

Moreover, we can speculate that CAM polarized HMC3 cell-derived EVs would influence activation of steady-state microglia, confirming on human cell line the same response seen in murine model. Despite our observations being currently limited to the analysis of one representative experiment, our work could open the way to a new model to reveal analogies/differences in murine and human in vitro models, in particular on the role of EVs and their cargos (Ceccarelli *et al*., 2022).

To further support the EVs role in the cell communication and the consequent phenotypic shift, we enrolled on N9 cells GW4869, a commercially available nSMase inhibitor that have emerged as pharmacological agents for preventing EVs release (Asai *et al*., 2015). We exposed microglia to GW4869 for 4 hours and later to 30 minutes with LPS, we observed, through ALIX expression and DiI EVs staining, that the inhibitor significantly reduces the secretion of EVs, thus confirming the inhibitory effect observed in other microglia models (Kumar *et al*., 2019). By testing the media conditioned by LPS-treated or control microglia previously exposed to GW4869, we observed that the reduction of EVs release significantly reduces the proinflammatory activation effect of microglia conditioned medium. Therefore, from these data, we can speculate that the content of LPS-derived EVs may act as signaling molecules to evoke the switching towards the proinflammatory phenotype (Aires *et al*., 2021; Essandoh *et al*., 2015). The fact that microglia-derived EVs where founded increased in cerebrospinal fluid (CSF) of Multiple Sclerosis (MS) and Experimental Autoimmune Encephalomyelitis (EAE) patients compared with healthy controls links the proinflammatory

signals propagation with the progression and the severity of neuropathology (Verderio *et al*., 2012). Additionally, it was found that microglia-derived EVs may also provide novel biomarker during the chronic stages of ischemic stroke (Ollen-Bittle *et al*., 2022).

EVs, including exosomes, have been reported to be vehicles to deliver miRNAs to other target cells, especially in the refined communication present in the nervous system (Xue *et al*., 2021). In particular, it has been already demonstrated that microglia release miRNAs through exosomes involved in a wide range of biological function and, for that reason, miRNAs dysfunction are clearly involved in many different neuropathologies (Guedes *et al*., 2013). Thus, we hypothesized that EVs miRNAs-enriched released by microglia cells could have direct relevance in proinflammatory immune response amplification among cells (Slota *et al*., 2019). To test our hypothesis, we focused our attention on four miRNAs already known to be involved with neuroinflammation, also called inflamma-miRs: miR-155, miR-124, miR-125b and miR-34a. All these miRNAs have been related to microglial polarization, enhancing the inflammatory response. In this regard, miR-155 is an important mediator known to regulate in the inflammatory pathogenesis of neurodegenerative disorders including ALS (Koval *et al*., 2013), PD (Thome *et al*., 2016), and AD (Aloi *et al*., 2021). In microglial cells miR-155 ia able to target anti-inflammatory proteins, such as the suppressor of cytokine signaling 1 (SOCS-1) and IL-10 increasing the expression of IFN- β, leading to the upregulation of neurotoxic characteristic mediators of the CAM phenotype, including iNOS, IL-6, and TNF-α (Zingale *et al*., 2022). Moreover, miR-155 expression could be also related to another pathway that includes the activation of p53 that, through Twist2 and c-Maf downregulation, promote inflammation along with contribution of miR-34a (Su *et al*., 2013). MiR-34a up-regulated expression have been consistently associated with various brain pathology (Chua *et al*., 2019): increased levels were found in cerebrospinal fluid and plasma of AD patients and animal models (Sarkar *et al*., 2019; Wang *et al*., 2009). Specifically, the downregulation of TREM2 has been linked to an up-regulation of miRNA-34a impairing the phagocytic functions of microglia, compromising its beneficial effects on counteract inflammation (Bhattacharjee *et al*., 2016). Together with these miRNAs, miR-125b directs microglia toward a preponderant CAM phenotype increasing TNF-α transcription by interfering with the STAT3 pathway, but also on the direct suppression of anti-inflammatory parameters such as IL4R, Arg1, and BDNF in ALS microglia (Parisi *et al*., 2016). Taken

together, the results showed a significant increase of all chosen miRNAs when microglia were activated with LPS. In line with our studies, because EVs derive their cargo from the contents of the cells that produce them, data reinforced the idea that the intercellular communication among microglial cells by EVs shedding could be involved in the propagation of neuroinflammation through the delivery of altered microRNAs, together with other inflammation factors (Yang *et al*., 2018). Intriguingly, the microglial cells that receive EVs from LPS-treated cells present a different miRNAs profile. Recipient cells treated with a pool of vesicles carrying dysregulated levels of miRNAs appear to shift their phenotype by presenting a mixed population with upregulated levels of miR-155 and miR-34a, unchanged levels of miR-125b and a downregulation of miR-124, consequences of persistent inflammation dampening through transition of microglia to a proinflammatory phenotype (Nuzziello *et al*., 2019).

In what concern miR-124, it was known to characterize the AAM phenotype, supporting tissue repair and regeneration through anti-inflammatory cytokines production by targeting MEKK3/ NF-κB pathway such as TGF-β1, IL-10, arginase-1, and FIZZ1 (Zhao *et al*., 2021). We may hypothesize that, in our model, the up-regulation of miR-124 is due to a compensation mechanism 24h after LPS treatment, but also to a mixture of phenotypes as being a heterogeneous culture, thus reinforcing its protective effects (Hirbec *et al*., 2019). In fact, cultured cells directly treated with ATP revealed a significant overexpression only in miR-124 and the contents of vesicles released from these cells showed the same expression profile reflecting the anti-inflammatory activation status: as it became evident, the microglia responded differently to distinct stimuli with an established role of specific miRNAs (Brites *et al*., 2015). Taken together, these findings suggest that LPS induces a strong proinflammatory stimulus on microglia that may reproduce the leading mechanism of long-lasting neuroinflammation in neurodegenerative disorders. Overall, our model identifies as a propagation mechanism the transport through EVs of dysregulated miRNAs that impact on microglia activation.

We believe that dampening inflammation may be a novel therapeutic approach for neurodegenerative diseases, providing a basis for future studies. In our cellular assays, we could observe a detectable effect of miRNAs altered profile on microglia polarization. Our data suggest that miR-34a is involved in the CAM commitment in response to inflammatory

stimulus both with LPS direct treatment and through the administration of exosomes proinflammatory miRNA-enriched. Among the unbalanced miRNAs network, we next investigated whether inhibiting miR-34a could alleviate the propagation of inflammation signals in microglia cells. In collaboration with Prof. Giampaolo Zuccheri, we tested a synthetic RNA-cleaving deoxyribozyme (DNAzyme) through which RNA target is recognized by complementary Watson–Crick base pairing characterized by chemical modifications with the goal to improve the affinity for miRNA-34a (Fan *et al*., 2017; Schubert *et al*., 2003). In our model, the data indicate an important modulation of microglia neurotoxic phenotypic shift highlighted by significantly reduced level of inflammatory marker employing 4h DNAzyme pretreatment followed by microglia activation using both directly LPS and conditioned medium. We can speculate that the decrease is not solidly significant because DNAzyme can act as a miRNA competitor, sequestering target miRNA and could represent another strategy to modulate miRNA activity for loss-of-function studies. In this sense, our study represents a first step in developing miRNA-based treatments, although more in depth studies are needed to corroborate these evidences.

6. FINAL CONSIDERATIONS

In this study, first we optimized and defined the experimental conditions for activation of microglia in vitro models (N9 and HMC3 cell lines) in order to perform the in vitro experiments to study microglia activation spreading; then, we explored how proinflammatory microglia-derived conditioned medium and, specifically, EVs, can favor phenotypic shift of steady state microglia. Aside from this, we found no signs of activation induced by AAM-derived exosomes, but, on the contrary, we observed that CAM-derived exosomes affect the polarization of receiving microglia. To prove the effective involvement of the vesicles in the diffusion mechanism of microglial activation, we reduced the shedding of microglial vesicles, by using GW4869, a N-Smase inhibitor.

Among the central regulators of these process, there are the small noncoding miRNAs, also delivered by vesicles, which become deregulated; we evaluated the expression of specific miRNAs, already know to be related to neurodegeneration, such as miRNA-34a, which down regulate TREM2 expression, or miRNA-155 and -125b, which is known to play a pro-inflammatory role, or miRNA-124, which is anti-inflammatory. Our interest was about miR-34a, since its expression was dysregulated in N9 cells in both types of treatments (pharmacological and via conditioned media) and in vesicular content.

Therefore, we wondered whether the inhibition of upregulated miR-34a may contribute to minimize the activation spreading. To reach this aim, we introduce the DNAzyme treatment directed against miR-34a, thanks to the complementarity of the sequences. Suppressing the miR-34a, by cleavage or sequestration, we modulated the proinflammatory activation, restoring the normal conditions. Considering the role of neuroinflammation spreading during neurodegeneration mediated by intercellular communication in brain parenchyma, this study contributes to add relevant findings regarding the functions of the exosomal content, especially of miRNAs, in the progression of the diseases. Importantly, this study might serve as a base for future studies to highlight mechanisms underlying the phenotypic changes and activation of microglia that lead to excessive and toxic immune response.

Future experiments should be performed to confirm data on the DNAzyme treatment: optimization in the vehiculation and effects could be tested on different disease in *in vitro* model (e.g. microglial cells derived from iPScells from healthy controls and patients) in order to verify possible side effects, increased shelf life and cross-BBB delivery efficiency. It would be of interest to expand the study of the same strategy towards modulating the intracellular expression of other inflamma-miRNAs and compare the effects of exosomes derived from treated/untreated cells.

In conclusion, if exosomal cargos are able to influence the environment, their cells of origin and miRNAs analyses may be helpful in understanding the patho/physiological function of microglia in neurodegeneration and, consequently, exosomal miRNA-based manipulation of signal pathways in the target cell and modification of inflammatory factors could undoubtedly lead to important insights about development for efficient therapeutic strategies.

7. BIBLIOGRAPHY

1. Abels, E.R., Breakefield, X.O. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. Cell Mol Neurobiol 36, 301–312 (2016).

2. Achkar, N. P., Cambiagno, D. A., & Manavella, P. A. (2016). miRNA biogenesis: a dynamic pathway. Trends in Plant Science, 21(12), 1034-1044.

3. Aires ID, Ribeiro-Rodrigues T, Boia R, Ferreira-Rodrigues M, Girão H, Ambrósio AF, Santiago AR. Microglial Extracellular Vesicles as Vehicles for Neurodegeneration Spreading. Biomolecules. 2021 May 21;11(6):770.

4. Alexandrov, P. N., Dua, P., Hill, J. M., Bhattacharjee, S., Zhao, Y., and Lukiw, W. J. (2012). microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). Int. J. Biochem. Mol. Biol. 3, 365–373.

5. Aloi, M. S., Prater, K. E., Sopher, B., Davidson, S., Jayadev, S., & Garden, G. A. (2021). The pro-inflammatory microRNA miR-155 influences fibrillar β-Amyloid1-42 catabolism by microglia. Glia, 69(7), 1736-1748.

6. Amor, S., McNamara, N. B., Gerrits, E., Marzin, M. C., Kooistra, S. M., Miron, V. E., & Nutma, E. (2021). White matter microglia heterogeneity in the CNS. Acta Neuropathologica, 1-17.

7. Amor S, Puentes F, Baker D, van der Valk P. Inflammation in neurodegenerative diseases. Immunology. 2010 Feb;129(2):154-69.

8. Andreasson, K. I., Bachstetter, A. D., Colonna, M., Ginhoux, F., Holmes, C., Lamb, B., ... & Van Eldik, L. J. (2016). Targeting innate immunity for neurodegenerative disorders of the central nervous system. Journal of neurochemistry, 138(5), 653-693.

9. Aravalli, R. N., Peterson, P. K., & Lokensgard, J. R. (2007). Toll-like receptors in defense and damage of the central nervous system. Journal of neuroimmune pharmacology, 2(4), 297-312.

10. Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., ... & Ikezu, T. (2015). Depletion of microglia and inhibition of exosome synthesis halt tau propagation. Nature neuroscience, 18(11), 1584-1593.

11. Askew, K., Li, K., Olmos-Alonso, A., Garcia-Moreno, F., Liang, Y., Richardson, P., ... & Gomez-Nicola, D. (2017). Coupled proliferation and apoptosis maintain the rapid turnover of microglia in the adult brain. Cell reports, 18(2), 391-405.

12. Bachiller, S., Jiménez-Ferrer, I., Paulus, A., Yang, Y., Swanberg, M., Deierborg, T., & Boza-Serrano, A. (2018). Microglia in neurological diseases: a road map to brain-disease dependent-inflammatory response. Frontiers in cellular neuroscience, 12, 488.

13. Bandiera, S., Hatem, E., Lyonnet, S., & Henrion-Caude, A. (2010). microRNAs in diseases: from candidate to modifier genes. Clinical genetics, 77(4), 306-313.

14. Bagasra, O., Michaels, F. H., Zheng, Y. M., Bobroski, L. E., Spitsin, S. V., Fu, Z. F., ... & Koprowski, H. (1995). Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. Proceedings of the National Academy of Sciences, 92(26), 12041-12045.

15. Bhattacharjee S, Zhao Y, Dua P, Rogaev EI, Lukiw WJ. microRNA-34a-Mediated Down-Regulation of the Microglial-Enriched Triggering Receptor and Phagocytosis-Sensor TREM2 in Age-Related Macular Degeneration. PLoS One. 2016 Mar 7;11(3):e0150211.

16. Bettens, K., Sleegers, K., & Van Broeckhoven, C. (2013). Genetic insights in Alzheimer's disease. The Lancet Neurology, 12(1), 92-104.

17. Breaker RR, Joyce GF. A DNA enzyme that cleaves RNA. Chem Biol. 1994; 1: 223-9.

18. Brites, D. (2020). Regulatory function of microRNAs in microglia. Glia, 68(8), 1631-1642.

19. Brites, D., & Fernandes, A. (2015). Neuroinflammation and depression: microglia activation, extracellular microvesicles and microRNA dysregulation. Frontiers in cellular neuroscience, 9, 476.

20. Brown, R. C., Lockwood, A. H., & Sonawane, B. R. (2005). Neurodegenerative diseases: an overview of environmental risk factors. Environmental health perspectives, 113(9), 1250-1256.

21. Budnik, V., Ruiz-Cañada, C., & Wendler, F. (2016). Extracellular vesicles round off communication in the nervous system. Nature Reviews Neuroscience, 17(3), 160-172.

22. Butterfield, D. A., Griffin, S., Munch, G., & Pasinetti, G. M. (2002). Amyloid β-peptide and amyloid pathology are central to the oxidative stress and inflammatory cascades under which Alzheimer's disease brain exists. Journal of Alzheimer's Disease, 4(3), 193-201.

23. Cardoso AL, Guedes JR, Pereira de Almeida L, Pedroso de Lima MC. 2012. miR- 155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. Immunology 135:73-88.

24. Carson, M. J., Bilousova, T. V., Puntambekar, S. S., Melchior, B., Doose, J. M., & Ethell, I. M. (2007). A rose by any other name? The potential consequences of microglial heterogeneity during CNS health and disease. Neurotherapeutics, 4(4), 571-579.

25. Cartier, L., Hartley, O., Dubois-Dauphin, M., & Krause, K. H. (2005). Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. Brain Research Reviews, 48(1), 16-42

26. Ceccarelli, L., Marchetti, L., Giacomelli, C., & Martini, C. (2021). Advances in microglia cellular models: focus on extracellular vesicle production. Biochemical Society Transactions, 49(4), 1791-1802.

27. Ceccarelli, L., Marchetti, L., Rizzo, M., Moscardini, A., Cappello, V., Da Pozzo, E., ... & Martini, C. (2022). Human Microglia Extracellular Vesicles Derived from Different Microglia Cell Lines: Similarities and Differences. ACS omega, 7(27), 23127-23137.

28. Chan, E. W., Yeo, E. T. Y., Wong, K. W. L., See, M. L., Wong, K. Y., & Gan, S. Y. Piper sarmentosum roxb. Root extracts confer neuroprotection by attenuating beta amyloid-induced pro-inflammatory cytokines released from microglial cells. Current Alzheimer Research, (2019) 16(3), 251-260.

29. Chen, S., Li, J., Meng, S., He, T., Shi, Z., Wang, C., ... & Gao, Y. (2023). Microglia and macrophages in the neuro-glia-vascular unit: From identity to functions. Neurobiology of Disease, 179, 106066.

30. Cheng, S. C., Quintin, J., Cramer, R. A., Shepardson, K. M., Saeed, S., Kumar, V., ... & Netea, M. G. (2014). mTOR-and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity. Science, 345(6204), 1250684.

31. Christoforidou, E., Joilin, G., & Hafezparast, M. (2020). Potential of activated microglia as a source of dysregulated extracellular microRNAs contributing to neurodegeneration in amyotrophic lateral sclerosis. Journal of neuroinflammation, 17(1), 1-15.

32. Choi SH, Veeraraghavalu K, Lazarov O, Marler S, Ransohoff RM, Ramirez JM, Sisodia SS. Non-cell-autonomous effects of presenilin 1 variants on enrichment-mediated hippocampal progenitor cell proliferation and differentiation. Neuron. 2008 Aug 28;59(4):568-80.

33. Cloutier F, Marrero A, O'Connell C, Morin P, Jr. 2015. MicroRNAs as potential circulating biomarkers for amyotrophic lateral sclerosis. J Mol Neurosci

34. Colonna M, Wang Y. TREM2 variants: new keys to decipher Alzheimer disease pathogenesis. Nat Rev Neurosci. 2016; 17:201–207.

35. Colton, C. A. (2009). Heterogeneity of microglial activation in the innate immune response in the brain. Journal of neuroimmune pharmacology, 4(4), 399-418.

36. Cova I, Markova A, Campini I, Grande G, Mariani C, Pomati S. Worldwide trends in the prevalence of dementia. J Neurol Sci. (2017); 379:259–60.

37. Cunha C, Gomes C, Vaz AR, Brites D. Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization. Mediators Inflamm. 2016; 2016:6986175. Epub 2016 Dec 21.

38. Czeh M, Gressens P, Kaindl AM. The yin and yang of microglia. Dev Neurosci. 2011;33(3-4):199-209.

39. Dasari, R., Bonsack, F., & Sukumari-Ramesh, S. (2021). Brain injury and repair after intracerebral hemorrhage: The role of microglia and brain-infiltrating macrophages. Neurochemistry international, 142, 104923.

40. Daugaard, I., & Hansen, T. B. (2017). Biogenesis and function of ago-associated RNAs. Trends in genetics, 33(3), 208-219.

41. Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., ... & Gan, W. B. (2005). ATP mediates rapid microglial response to local brain injury in vivo. Nature neuroscience, 8(6), 752-758.

42. Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., & Amit, I. (2018). Disease-associated microglia: a universal immune sensor of neurodegeneration. Cell, 173(5), 1073-1081.

43. De Chirico, F., Poeta, E., Babini, G., Piccolino, I., Monti, B., & Massenzio, F. (2022). New models of Parkinson's like neuroinflammation in human microglia clone 3: Activation profiles induced by INF-γ plus high glucose and mitochondrial inhibitors. Frontiers in Cellular Neuroscience.

44. De la Rosa, E.J. and de Pablo, F. (2000) Cell death in early neuraldevelopment: beyond the neurotrophic theory. Trends Neurosci. 23,454–458

45. Dello Russo, C., Cappoli, N., Coletta, I., Mezzogori, D., Paciello, F., Pozzoli, G., ... & Battaglia, A. (2018). The human microglial HMC3 cell line: where do we stand? A systematic literature review. Journal of Neuroinflammation, 15(1), 1-24.

46. Delpech, J. C., Herron, S., Botros, M. B., & Ikezu, T. (2019). Neuroimmune crosstalk through extracellular vesicles in health and disease. Trends in Neurosciences, 42(5), 361-372.

47. Diehl, P., Fricke, A., Sander, L., Stamm, J., Bassler, N., Htun, N., ... & Peter, K. (2012). Microparticles: major transport vehicles for distinct microRNAs in circulation. Cardiovascular research, 93(4), 633-644.

48. Di Sabato DJ, Quan N, Godbout JP. Neuroinflammation: the devil is in the details. J Neurochem. (2016) Oct;139 Suppl 2(Suppl 2):136-153.

49. Dos Santos, S. E., Medeiros, M., Porfirio, J., Tavares, W., Pessôa, L., Grinberg, L., ... & Herculano-Houzel, S. (2020). Similar microglial cell densities across brain structures and mammalian species: implications for brain tissue function. Journal of Neuroscience, 40(24), 4622-4643.

50. Doyle LM, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. Cells. 2019 Jul 15;8(7):727.

51. Drago, F., Lombardi, M., Prada, I., Gabrielli, M., Joshi, P., Cojoc, D., ... & Verderio, C. (2017). ATP modifies the proteome of extracellular vesicles released by microglia and influences their action on astrocytes. Frontiers in pharmacology, 8, 910.

52. Du, R.-H., Sun, H.-B., Hu, Z.-L., Lu, M., Ding, J.-H., & Hu, G. (2018). Kir6.1/K-ATP channel modulates microglia phenotypes: implication in Parkinson's disease. Cell Death & Disease, 9(3).

53. Essandoh, K., Li, Y., Huo, J., & Fan, G. C. (2016). MiRNA-mediated macrophage polarization and its potential role in the regulation of inflammatory response. Shock (Augusta, Ga.), 46(2), 122.

54. Essandoh K., L. Yang, X. Wang, W. Huang, D. Qin, J. Hao, Y. Wang, B. Zingarelli, T. Peng, G.C. FanBlockade of exosome generation with GW4869 dampens the sepsis-induced inflammation and cardiac dysfunction Biochim. Biophys. Acta, 1852 (2015), pp. 2362-2371

55. Etemad, S., Zamin, R. M., Ruitenberg, M. J., & Filgueira, L. (2012). A novel in vitro human microglia model: characterization of human monocyte-derived microglia. Journal of neuroscience methods, 209(1), 79-89.

56. Fan, H., Zhang, X., & Lu, Y. (2017). Recent advances in DNAzyme-based gene silencing. Science China Chemistry, 60(5), 591-601.

57. Figuera-Losada, M., Rojas, C., & Slusher, B. S. (2014). Inhibition of microglia activation as a phenotypic assay in early drug discovery. Journal of biomolecular screening, 19(1), 17-31.

58. Flynt, A. S., & Lai, E. C. (2008). Biological principles of microRNA-mediated regulation: shared themes amid diversity. Nature Reviews Genetics, 9(11), 831-842.

59. Fokina AA, Meschaninova MI, Durfort T, Venyaminova AG, Francois JC (2012) Targeting insulin-like growth factor I with 10-23 DNAzymes: 2'-O-methyl modifications in the catalytic core enhance mRNA cleavage. Biochemistry 51(11):2181–2191

60. Forman, M. S., Trojanowski, J. Q., & Lee, V. M. (2004). Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs. Nature medicine, 10(10), 1055-1063.

61. Franco, R., & Fernandez-Suarez, D. (2015). Alternatively activated microglia and macrophages in the central nervous system. Progress in neurobiology, 131, 65-86.

62. Frühbeis, C., Fröhlich, D., Kuo, W. P., & Krämer-Albers, E. M. (2013). Extracellular vesicles as mediators of neuron-glia communication. Frontiers in cellular neuroscience, 7, 182.

63. Fujita, Y., & Yamashita, T. (2021). Neuroprotective function of microglia in the developing brain. Neuronal signaling, 5(1).

64. Gabandé-Rodríguez, E., Keane, L., & Capasso, M. (2020). Microglial phagocytosis in aging and Alzheimer's disease. Journal of neuroscience research, 98(2), 284-298.

65. Galván-Peña, S., & O'Neill, L. A. (2014). Metabolic reprograming in macrophage polarization. Frontiers in immunology, 5, 420.

66. Gao, Y., Qin, Y., Wan, C., Sun, Y., Meng, J., Huang, J., ... & Yang, K. (2021). Small extracellular vesicles: a novel avenue for cancer management. Frontiers in Oncology, 11, 638357.

67. Garaschuk, O., & Verkhratsky, A. (2019). Physiology of microglia. Microglia, 27-40.

68. Gebert, L. F., & MacRae, I. J. (2019). Regulation of microRNA function in animals. Nature reviews Molecular cell biology, 20(1), 21-37.

69. Ghosh, S., Castillo, E., Frias, E. S. & Swanson, R. A. (2018b) Bioenergetic regulation of microglia. Glia, 66(6), 1200-1212. 10.1002/glia.23271.

70. Goldmann, T., Wieghofer, P., Jordão, M. J. C., Prutek, F., Hagemeyer, N., Frenzel, K., ... & Prinz, M. (2016). Origin, fate and dynamics of macrophages at central nervous system interfaces. Nature immunology, 17(7), 797-805.

71. Goodman, R. P., Berry, R. M., & Turberfield, A. J. (2004). The single-step synthesis of a DNA tetrahedron. Chemical Communications, (12), 1372-1373.

72. Gosselin, D., Skola, D., Coufal, N. G., Holtman, I. R., Schlachetzki, J. C., Sajti, E., ... & Glass, C. K. (2017). An environment-dependent transcriptional network specifies human microglia identity. Science, 356(6344), eaal3222.

73. Guedes, J., Cardoso, A. L. C., & Pedroso de Lima, M. C. (2013). Involvement of microRNA in microglia-mediated immune response. Clinical and Developmental Immunology, 2013.

74. Guevara, Ibeth, et al. "Determination of nitrite/nitrate in human biological material by the simple Griess reaction." Clinica chimica acta 274.2 (1998): 177-188.

75. Guo S, Wang H, Yin Y. Microglia Polarization From M1 to M2 in Neurodegenerative Diseases. Front Aging Neurosci. 2022 Feb 16;14:815347.

76. György, B., Szabó, T. G., Pásztói, M., Pál, Z., Misják, P., Aradi, B., ... & Buzás, E. I. (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cellular and molecular life sciences, 68(16), 2667-2688.

77. Ha M, Kim VN. 2014. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15:509-24.

78. Hamerman, J. A., Jarjoura, J. R., Humphrey, M. B., Nakamura, M. C., Seaman, W. E., & Lanier, L. L. (2006). Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. The Journal of Immunology, 177(4), 2051-2055.

79. Hanisch, U. K., & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nature neuroscience, 10(11), 1387-1394.

80. Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., ... & Feng, L. (1998). Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. Proceedings of the National Academy of Sciences, 95(18), 10896-10901.

81. He X, Jing Z, Cheng G. 2014. MicroRNAs: new regulators of Toll-like receptor signalling pathways. Biomed Res Int 2014:945169.

82. Hickman, S., Izzy, S., Sen, P., Morsett, L., & El Khoury, J. (2018). Microglia in neurodegeneration. Nature neuroscience, 21(10), 1359-1369.

83. Hirbec, H., Rassendren, F., & Audinat, E. (2019). Microglia reactivity: heterogeneous pathological phenotypes. Microglia, 41-55.

84. Hung, H. C., Tsai, S. F., Sie, S. R. & Kuo, Y. M. (2022) High glucose enhances lipopolysaccharide-induced inflammation in cultured BV2 microglial cell line. Immun Inflamm Dis, 10(5), e610.

85. Hüttenhofer, Alexander, Peter Schattner, and Norbert Polacek. "Non-coding RNAs: hope or hype?." TRENDS in Genetics 21, no. 5 (2005): 289-297

86. Illes P, Rubini P, Ulrich H, Zhao Y, Tang Y. Regulation of Microglial Functions by Purinergic Mechanisms in the Healthy and Diseased CNS. Cells. 2020 Apr 29;9(5):1108.

87. Ito, D., Imai, Y., Ohsawa, K., Nakajima, K., Fukuuchi, Y., & Kohsaka, S. (1998). Microglia-specific localisation of a novel calcium binding protein, Iba1. Molecular brain research, 57(1), 1-9.

88. Jakaria, M., Haque, M.E., Cho, DY. et al. Molecular Insights into NR4A2(Nurr1): an Emerging Target for Neuroprotective Therapy Against Neuroinflammation and Neuronal Cell Death. Mol Neurobiol 56, 5799–5814 (2019).

89. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197-216.

90. Javanmehr, N., Saleki, K., Alijanizadeh, P., & Rezaei, N. (2022). Microglia dynamics in aging-related neurobehavioral and neuroinflammatory diseases. Journal of Neuroinflammation, 19(1), 1-20.

91. Joseph, B., & Venero, J. L. (2013). A brief overview of multitalented microglia. Microglia, 3-8.

92. Jurga, A. M., Paleczna, M., & Kuter, K. Z. (2020). Overview of general and discriminating markers of differential microglia phenotypes. Frontiers in cellular neuroscience, 14, 198.

93. Juźwik, C. A., Drake, S. S., Zhang, Y., Paradis-Isler, N., Sylvester, A., Amar-Zifkin, A., ... & Fournier, A. E. (2019). microRNA dysregulation in neurodegenerative diseases: A systematic review. Progress in neurobiology, 182, 101664.

94. Kalra, H., Simpson, R. J., Ji, H., Aikawa, E., Altevogt, P., Askenase, P., ... & Mathivanan, S. (2012). Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS biology, 10(12), e1001450.

95. Kaminska, B., Mota, M., & Pizzi, M. (2016). Signal transduction and epigenetic mechanisms in the control of microglia activation during neuroinflammation. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1862(3), 339-351.

96. Kempuraj D, Thangavel R, Natteru PA, Selvakumar GP, Saeed D, Zahoor H, Zaheer S, Iyer SS, Zaheer A. Neuroinflammation Induces Neurodegeneration. J Neurol Neurosurg Spine. 2016;1(1):1003. Epub 2016 Nov 18

97. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev. 2011 Apr;91(2):461-553.

98. Khan, I., Preeti, K., Fernandes, V., Khatri, D. K., & Singh, S. B. (2022). Role of MicroRNAs, aptamers in neuroinflammation and neurodegenerative disorders. Cellular and Molecular Neurobiology, 42(7), 2075-2095.

99. Kierdorf K., Erny D., Goldmann T., Sander V., Schulz C., Perdiguero E. G., et al.. (2013a). Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat. Neurosci. 16, 273–280. 10.1038/nn.3318

100. Korzhevskii, D. E., & Kirik, O. V. (2016). Brain microglia and microglial markers. Neuroscience and Behavioral Physiology, 46(3), 284-290.

101. Kouwenhoven, M., Teleshova, N., Özenci, V., Press, R., & Link, H. (2001). Monocytes in multiple sclerosis: phenotype and cytokine profile. Journal of neuroimmunology, 112(1-2), 197-205.

102. Koval, E. D., Shaner, C., Zhang, P., Du Maine, X., Fischer, K., Tay, J., ... & Miller, T. M. (2013). Method for widespread microRNA-155 inhibition prolongs survival in ALS-model mice. Human molecular genetics, 22(20), 4127-4135.

103. Kozomara, A., Birgaoanu, M., & Griffiths-Jones, S. (2019). miRBase: from microRNA sequences to function. Nucleic acids research, 47(D1), D155-D162.

104. Kristan E. van der Vos, Erik R. Abels, Xuan Zhang, Charles Lai, Esteban Carrizosa, Derek Oakley, Shilpa Prabhakar, Osama Mardini, Matheus H. W. Crommentuijn, Johan Skog, Anna M. Krichevsky, Anat Stemmer-Rachamimov, Thorsten R. Mempel, Joseph El Khoury, Suzanne E. Hickman, Xandra O. Breakefield, Directly visualized glioblastoma-derived extracellular vesicles transfer RNA to microglia/macrophages in the brain, Neuro-Oncology, Volume 18, Issue 1, January 2016, Pages 58–69.

105. Kumar, A., Henry, R. J., Stoica, B. A., Loane, D. J., Abulwerdi, G., Bhat, S. A., & Faden, A. I. (2019). Neutral Sphingomyelinase Inhibition Alleviates LPS-Induced Microglia Activation and Neuroinflammation after Experimental Traumatic Brain Injury. The Journal of pharmacology and experimental therapeutics, 368(3), 338–352.

106. Kumar, A., Stoica, B. A., Loane, D. J., Yang, M., Abulwerdi, G., Khan, N., ... & Faden, A. I. (2017). Microglial-derived microparticles mediate neuroinflammation after traumatic brain injury. Journal of neuroinflammation, 14(1), 1-17.

107. Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., & Tuschl, T. (2003). New microRNAs from mouse and human. Rna, 9(2), 175-179.

108. Lai, E. C. (2002). Micro RNAs are complementary to 3’ UTR sequence motifs that mediate negative post-transcriptional regulation. Nature genetics, 30(4), 363-364.

109. Landry, R. P., Jacobs, V. L., Romero-Sandoval, E. A., & DeLeo, J. A. (2012). Propentofylline, a CNS glial modulator does not decrease pain in post-herpetic neuralgia patients: in vitro evidence for differential responses in human and rodent microglia and macrophages. Experimental neurology, 234(2), 340-350.

110. La Torre, M.E.; Panaro, M.A.; Ruggiero, M.; Polito, R.; Cianciulli, A.; Filannino, F.M.; Lofrumento, D.D.; Antonucci, L.; Benameur, T.; Monda, V.; Monda, M.; Porro, C.; Messina, G. Extracellular Vesicles Cargo in Modulating Microglia Functional Responses. Biology 2022, 11, 1426.

111. Lemaire, Q., Raffo-Romero, A., Arab, T., Van Camp, C., Drago, F., Forte, S., ... & Lefebvre, C. (2019). Isolation of microglia-derived extracellular vesicles: towards miRNA signatures and neuroprotection. Journal of nanobiotechnology, 17(1), 1-21.

112. Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. cell, 75(5), 843-854.

113. Lehnardt, S. (2010). Innate immunity and neuroinflammation in the CNS: The role of microglia in Toll-like receptor-mediated neuronal injury. Glia, 58(3), 253-263.

114. Leyh, J., Paeschke, S., Mages, B., Michalski, D., Nowicki, M., Bechmann, I., & Winter, K. (2021). Classification of microglial morphological phenotypes using machine learning. Frontiers in cellular neuroscience, 241.

115. Li Q., Cheng Z., Zhou L., Darmanis S., Neff N. F., Okamoto J., et al.. (2019). Developmental heterogeneity of microglia and brain myeloid cells revealed by deep single-cell RNA sequencing. Neuron 101, 207–223.e10.

116. Li, Z., & Rana, T. M. (2014). Therapeutic targeting of microRNAs: current status and future challenges. Nature reviews Drug discovery, 13(8), 622-638.

117. Liao, B., Zhao, W., Beers, D. R., Henkel, J. S., & Appel, S. H. (2012). Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. Experimental neurology, 237(1), 147-152.

118. Lin, L., Desai, R., Wang, X., Lo, E. H., & Xing, C. (2017). Characteristics of primary rat microglia isolated from mixed cultures using two different methods. Journal of neuroinflammation, 14(1), 1-10.

119. Liu W, Taso O, Wang R, Bayram S, Graham AC, Garcia-Reitboeck P, Mallach A, Andrews WD, Piers TM, Botia JA, Pocock JM, Cummings DM, Hardy J, Edwards FA, Salih DA. Trem2 promotes anti-inflammatory responses in microglia and is suppressed under pro-inflammatory conditions. Hum Mol Genet. 2020 Nov 25;29(19):3224-3248.

120. Lu, Q., Wu, R., Zhao, M., Garcia-Gomez, A., & Ballestar, E. (2019). miRNAs as therapeutic targets in inflammatory disease. Trends in Pharmacological Sciences, 40(11), 853-865.

121. Lyman, M., Lloyd, D. G., Ji, X., Vizcaychipi, M. P., & Ma, D. (2014). Neuroinflammation: the role and consequences. Neuroscience research, 79, 1-12.

122. Lucchinetti, C., Brück, W., Parisi, J., Scheithauer, B., Rodriguez, M., & Lassmann, H. (2000). Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 47(6), 707-717.

123. Lyu, J., Jiang, X., Leak, R.K. et al. Microglial Responses to Brain Injury and Disease: Functional Diversity and New Opportunities. Transl. Stroke Res. 12, 474–495 (2021).

124. Masrori, P., & Van Damme, P. (2020). Amyotrophic lateral sclerosis: a clinical review. European journal of neurology, 27(10), 1918-1929.

125. Masuda, T., Sankowski, R., Staszewski, O., Böttcher, C., Amann, L., Scheiwe, C., ... & Prinz, M. (2019). Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. Nature, 566(7744), 388-392.

126. Mathys, H., Adaikkan, C., Gao, F., Young, J. Z., Manet, E., Hemberg, M., ... & Tsai, L. H. (2017). Temporal tracking of microglia activation in neurodegeneration at single-cell resolution. Cell reports, 21(2), 366-380.

127. Meerloo, J. V., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. In Cancer cell culture (pp. 237-245). Humana Press.

128. Midekessa, G., Godakumara, K., Dissanayake, K., Hasan, M. M., Reshi, Q. U. A., Rinken, T., & Fazeli, A. (2021). Characterization of Extracellular Vesicles Labelled with a Lipophilic Dye Using Fluorescence Nanoparticle Tracking Analysis. Membranes, 11(10), 779.

129. Mildner, A., Huang, H., Radke, J., Stenzel, W., & Priller, J. (2017). P2Y12 receptor is expressed on human microglia under physiological conditions throughout development and is sensitive to neuroinflammatory diseases. Glia, 65(2), 375-387.

130. Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, Heikenwalder M, Brück W, Priller J, Prinz M. Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. Nat Neurosci. 2007 Dec;10(12):1544-53.

131. Mir, B., & Goettsch, C. (2020). Extracellular vesicles as delivery vehicles of specific cellular cargo. Cells, 9(7), 1601.

132. Monif, M., Burnstock, G., & Williams, D. A. (2010). Microglia: proliferation and activation driven by the P2X7 receptor. The international journal of biochemistry & cell biology, 42(11), 1753-1756.

133. Monzón-Sandoval, J., Burlacu, E., Agarwal, D., Handel, A. E., Wei, L., Davis, J., ... & Webber, C. (2022). Lipopolysaccharide distinctively alters human microglia transcriptomes to resemble microglia from Alzheimer's disease mouse models. Disease Models & Mechanisms, 15(10), dmm049349.

134. Murgoci, A. N., Duhamel, M., Raffo-Romero, A., Mallah, K., Aboulouard, S., Lefebvre, C., ... & Salzet, M. (2020). Location of neonatal microglia drives small extracellular vesicles content and biological functions in vitro. Journal of extracellular vesicles, 9(1), 1727637.

135. Nair, A. A., Tang, X., Thompson, K. J., Vedell, P. T., Kalari, K. R., & Subramanian, S. (2020). Frequency of MicroRNA Response Elements Identifies Pathologically Relevant Signaling Pathways in Triple-Negative Breast Cancer. Iscience, 23(6), 101249.

136. Nareika, A., Sundararaj, K. P., Im, Y. B., Game, B. A., Lopes-Virella, M. F., & Huang, Y. (2009). High glucose and interferon gamma synergistically stimulate MMP-1 expression in U937 macrophages by increasing transcription factor STAT1 activity. Atherosclerosis, 202(2), 363-371.

137. Nguyen, M. D., D'Aigle, T., Gowing, G., Julien, J. P., & Rivest, S. (2004). Exacerbation of motor neuron disease by chronic stimulation of innate immunity in a mouse model of amyotrophic lateral sclerosis. Journal of Neuroscience, 24(6), 1340-1349.

138. Norris, G. T., & Kipnis, J. (2019). Immune cells and CNS physiology: microglia and beyond. Journal of experimental medicine, 216(1), 60-70.

139. Nuzziello, N., & Liguori, M. (2019). The MicroRNA centrism in the orchestration of neuroinflammation in neurodegenerative diseases. Cells, 8(10), 1193.

140. O'Brien J, Hayder H, Zayed Y, Peng C. 2018. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol (Lausanne) 9:402.

141. O'Brien, J. A., Nguyen, V. H. L., & Peng, C. (2022). Overview on miRNA classification, biogenesis, and functions. In MicroRNA in Human Malignancies (pp. 3-20). Academic Press.

142. Ochocka N, Kaminska B. Microglia Diversity in Healthy and Diseased Brain: Insights from Single-Cell Omics. Int J Mol Sci. 2021 Mar 16;22(6):3

143. Ollen-Bittle, N., Roseborough, A. D., Wang, W., Wu, J. L. D., & Whitehead, S. N. (2022). Mechanisms and biomarker potential of extracellular vesicles in stroke. Biology, 11(8), 1231.

144. Orihuela, R., McPherson, C. A., & Harry, G. J. (2016). Microglial M1/M2 polarization and metabolic states. British journal of pharmacology, 173(4), 649-665.

145. Paolicelli RC, Bergamini G, Rajendran L. Cell-to-cell Communication by Extracellular Vesicles: Focus on Microglia. Neuroscience. 2019 May; 405:148-157.

146. Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., ... & Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. science, 333(6048), 1456-1458.

147. Paolicelli, R. C., Sierra, A., Stevens, B., Tremblay, M. E., Aguzzi, A., Ajami, B., ... & Wyss-Coray, T. (2022). Microglia states and nomenclature: A field at its crossroads. Neuron, 110(21), 3458-3483.

148. Parajuli, B., Sonobe, Y., Kawanokuchi, J., Doi, Y., Noda, M., Takeuchi, H., ... & Suzumura, A. (2012). GM-CSF increases LPS-induced production of proinflammatory mediators via upregulation of TLR4 and CD14 in murine microglia. Journal of neuroinflammation, 9, 1-12.

149. Parisi C, Napoli G, Amadio S, Spalloni A, Apolloni S, Longone P, Volonte C. 2016. MicroRNA-125b regulates microglia activation and motor neuron death in ALS. Cell Death Differ 23:531-41.

150. Patro, I., Nagayach, A., Sinha, S., & Patro, N. (2016). General physiology and pathophysiology of microglia during neuroinflammation. Inflammation: the Common Link in Brain Pathologies, 17-42.

151. Pekny, M, and Marcela, P "Reactive gliosis in the pathogenesis of CNS diseases." Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1862.3 (2016): 483-491.

152. Peng, H., Newbigging, A. M., Wang, Z., Tao, J., Deng, W., Le, X. C. and Zhang, H. (2018). "DNAzyme-Mediated Assays for Amplified Detection of Nucleic Acids and Proteins." Anal Chem 90(1): 190-207.

153. Pérez-Cerdá, F., Sánchez-Gómez, M. V., & Matute, C. (2015). Pío del Río Hortega and the discovery of the oligodendrocytes. Frontiers in Neuroanatomy, 9, 92.

154. Perry, V. H., Nicoll, J. A., and Holmes, C. (2010). Microglia in neurodegenerative disease. Nat. Rev. Neurol. 6, 193–201.

155. Petralla, S., De Chirico, F., Miti, A., Tartagni, O., Massenzio, F., Poeta, E., ... & Monti, B. (2021). Epigenetics and Communication Mechanisms in Microglia Activation with a View on Technological Approaches. Biomolecules, 11(2), 306.

156. Peudenier, S., Hery, C., Montagnier, L., & Tardieu, M. (1991). Human microglial cells: characterization in cerebral tissue and in primary culture, and study of their susceptibility to HIV-1 infection. Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 29(2), 152-161.

157. Pinto S, Cunha C, Barbosa M, Vaz AR, Brites D. 2017. Exosomes from NSC-34 Cells Transfected with hSOD1-G93A Are Enriched in miR-124 and Drive Alterations in Microglia Phenotype. Front Neurosci 11:273.

158. Polazzi, E., & Contestabile, A. (2006). Overactivation of LPS-stimulated microglial cells by co-cultured neurons or neuron-conditioned medium. Journal of neuroimmunology, 172(1-2), 104-111.

159. Polazzi, E., & Monti, B. (2010). Microglia and neuroprotection: from in vitro studies to therapeutic applications. Progress in neurobiology, 92(3), 293-315.

160. Ponomarev ED, Veremeyko T, Weiner HL. 2013. MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. Glia 61:91-103.

161. Potolicchio, I., Carven, G. J., Xu, X., Stipp, C., Riese, R. J., Stern, L. J., & Santambrogio, L. (2005). Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. The Journal of Immunology, 175(4), 2237-2243.

162. Prada, I., Furlan, R., Matteoli, M., & Verderio, C. (2013). Classical and unconventional pathways of vesicular release in microglia. Glia, 61(7), 1003-1017.

163. Przedborski S, Vila M, Jackson-Lewis V. Neurodegeneration: what is it and where are we? J Clin Invest. (2003) Jan;111(1):3-10.

164. Rai, M. A., Hammonds, J., Pujato, M., Mayhew, C., Roskin, K. & Spearman, P. (2020) Comparative analysis of human microglial models for studies of HIV replication and pathogenesis. Retrovirology, 17(1), 35.

165. Ransohoff, R. M. (2016). How neuroinflammation contributes to neurodegeneration. Science, 353(6301), 777-783.

166. Ransohoff, R. M., & Perry, V. H. (2009). Microglial physiology: unique stimuli, specialized responses. Annual review of immunology, 27, 119-145.

167. Raoul, C., Buhler, E., Sadeghi, C., Jacquier, A., Aebischer, P., Pettmann, B., ... & Haase, G. (2006). Chronic activation in presymptomatic amyotrophic lateral sclerosis (ALS) mice of a feedback loop involving Fas, Daxx, and FasL. Proceedings of the National Academy of Sciences, 103(15), 6007-6012.

168. Rawlinson C, Jenkins S, Thei L, Dallas ML, Chen R. Post-Ischaemic Immunological Response in the Brain: Targeting Microglia in Ischaemic Stroke Therapy. Brain Sciences. 2020; 10(3):159.

169. Rezaie P, Microglia in the Human Nervous System during Development. Neuroembryology 2003; 2:18-31.

170. Saijo, K., & Glass, C. K. (2011). Microglial cell origin and phenotypes in health and disease. Nature Reviews. Immunology, 11(11), 775–787.

171. Saitgareeva, A. R., Bulygin, K. V., Gareev, I. F., Beylerli, O. A., & Akhmadeeva, L. R. (2020). The role of microglia in the development of neurodegeneration. Neurological Sciences, 41(12), 3609-3615.

172. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, Challis C, Schretter CE, Rocha S, Gradinaru V, Chesselet MF, Keshavarzian A, Shannon KM, Krajmalnik-Brown R, Wittung-Stafshede P, Knight R, Mazmanian SK. Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. Cell. 2016; 167:1469–1480e12.

173. Sanchez-Guajardo V, Barnum CJ, Tansey MG, Romero-Ramos M. Neuroimmunological processes in Parkinson's disease and their relation to á-synuclein: microglia as the referee between neuronal processes and peripheral immunity. ASN Neuro. 2013; 5:113–139.

174. Sarkar, S., Engler-Chiurazzi, E. B., Cavendish, J. Z., Povroznik, J. M., Russell, A. E., Quintana, D. D., ... & Simpkins, J. W. (2019). Over-expression of miR-34a induces rapid cognitive impairment and Alzheimer's disease-like pathology. Brain research, 1721, 146327.

175. Sarkar, S., Malovic, E., Sarda, D., Lawana, V., Rokad, D., Jin, H., ... & Kanthasamy, A. G. (2018). Characterization and comparative analysis of a new mouse microglial cell model for studying neuroinflammatory mechanisms during neurotoxic insults. Neurotoxicology, 67, 129-140.

176. Savill, J., Dransfield, I., Gregory, C., & Haslett, C. (2002). A blast from the past: clearance of apoptotic cells regulates immune responses. Nature Reviews Immunology, 2(12), 965-975.

177. Schafer DP, Lehrman EK, Kautzman AK, Koyama R, Mardinly AR, Barres BA, Stevens B. Microglia shape neural circuits in the developing brain. Soc Neurosci Abstr. 2011; 37:663–03.

178. Schubert, S., GuÈl, D. C., Grunert, H. P., Zeichhardt, H., Erdmann, V. A., & Kurreck, J. (2003). RNA cleaving '10-23'DNAzymes with enhanced stability and activity. Nucleic acids research, 31(20), 5982-5992.

179. Sebaihi, N., De Boeck, B., Yuana, Y., Nieuwland, R., & Pétry, J. (2017). Dimensional characterization of extracellular vesicles using atomic force microscopy. Measurement science and technology, 28(3), 034006.

180. Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., ... & Wong, W. H. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. Proceedings of the National Academy of Sciences, 110(9), 3507-3512.

181. Shabab, T., Khanabdali, R., Moghadamtousi, S. Z., Kadir, H. A., & Mohan, G. (2017). Neuroinflammation pathways: a general review. International Journal of Neuroscience, 127(7), 624-633.

182. Shao G, Ji S, Wu A, Liu C, Wang M, Zhang P, Jiao Q, Kang Y. DNAzyme-based probe for circulating microRNA detection in peripheral blood. Drug Des Devel Ther. 2015 Nov 16;9:6109-17.

183. Shemer, A., Erny, D., Jung, S., & Prinz, M. (2015). Microglia plasticity during health and disease: an immunological perspective. Trends in immunology, 36(10), 614-624.

184. Shen, Y., Kapfhamer, D., Minnella, A. M., Kim, J. E., Won, S. J., Chen, Y., Huang, Y., Low, L. H., Massa, S. M. & Swanson, R. A. (2017) Bioenergetic state regulates innate inflammatory responses through the transcriptional co-repressor CtBP. Nat Commun, 8(1), 624.

185. Shulman, J. M., De Jager, P. L., & Feany, M. B. (2011). Parkinson's disease: genetics and pathogenesis. Annual Review of Pathology: Mechanisms of Disease, 6, 193-222.

186. Sierra, A., Abiega, O., Shahraz, A., & Neumann, H. (2013). Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. Frontiers in cellular neuroscience, 7, 6.

187. Sierra A, Navascués J, Cuadros MA, Calvente R, Martín-Oliva D, Ferrer-Martín RM, Martín-Estebané M, Carrasco MC, Marín-Teva JL. Expression of inducible nitric oxide synthase (iNOS) in microglia of the developing quail retina. PLoS One. 2014 Aug 29;9(8):e106048.

188. Silverman SK. Catalytic DNA: scope, applications, and biochemistry of deoxyribozymes. Trends Biochem Sci. 2016; 41: 595–609.

189. Simons, M. and Raposo, G., 2009. Exosomes–vesicular carriers for intercellular communication. Current opinion in cell biology, 21(4), pp.575-581.

190. Slota JA, Booth SA. MicroRNAs in Neuroinflammation: Implications in Disease Pathogenesis, Biomarker Discovery and Therapeutic Applications. Non-Coding RNA. 2019; 5(2):35.

191. Smith, A. M., & Dragunow, M. (2014). The human side of microglia. Trends in neurosciences, 37(3), 125-135.

192. Soifer, H. S., Rossi, J. J., & Sætrom, P. (2007). MicroRNAs in disease and potential therapeutic applications. Molecular therapy, 15(12), 2070-2079.

193. Spiteri, A.G., Wishart, C.L., Pamphlett, R. et al. Microglia and monocytes in inflammatory CNS disease: integrating phenotype and function. Acta Neuropathol 143, 179–224 (2022). https://doi.org/10.1007/s00401-021-02384-2

194. Stewart, C. R., Stuart, L. M., Wilkinson, K., Van Gils, J. M., Deng, J., Halle, A., ... & Moore, K. J. (2010). CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimers. Nature immunology, 11(2), 155-161

195. Stoll, G., and Jander, S. (1999). The role of microglia and macrophages in the pathophysiology of the CNS. Prog. Neurobiol. 58, 233–247.

196. Streit, Wolfgang J., Robert E. Mrak, and W. Sue T. Griffin. "Microglia and neuroinflammation: a pathological perspective." Journal of neuroinflammation 1.1 (2004): 1-4.

197. Su, W., Aloi, M. S., & Garden, G. A. (2016). MicroRNAs mediating CNS inflammation: small regulators with powerful potential. Brain, behavior, and immunity, 52, 1-8.

198. Su, W., Hopkins, S., Nesser, N. K., Sopher, B., Silvestroni, A., Ammanuel, S., ... & Garden, G. A. (2014). The p53 transcription factor modulates microglia behavior through microRNA-dependent regulation of c-Maf. The Journal of Immunology, 192(1), 358-366.

199. Sun Y, Luo ZM, Guo XM, Su DF, Liu X. 2015. An updated role of microRNA-124 in central nervous system disorders: a review. Front Cell Neurosci 9:193.

200. Ta, T. T., Dikmen, H. O., Schilling, S., Chausse, B., Lewen, A., Hollnagel, J. O., & Kann, O. (2019). Priming of microglia with IFN-γ slows neuronal gamma oscillations in situ. Proceedings of the National Academy of Sciences, 116(10), 4637-4642.

201. Tang, Y., Le, W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol 53, 1181–1194 (2016).

202. Tay, T. L., Carrier, M., & Tremblay, M. È. (2019). Physiology of microglia. Neuroglia in Neurodegenerative Diseases, 129-148.

203. Tansey, M. G., & Goldberg, M. S. Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention. Neurobiology of disease, (2010 37(3), 510-518.

204. Thome, A. D., Harms, A. S., Volpicelli-Daley, L. A., & Standaert, D. G. (2016). microRNA-155 regulates alpha-synuclein-induced inflammatory responses in models of Parkinson disease. Journal of Neuroscience, 36(8), 2383-2390.

205. Timmerman, R., Burm, S. M., & Bajramovic, J. J. (2021). Tissue-specific features of microglial innate immune responses. Neurochemistry International, 142, 104924.

206. Toyomitsu, E., Tsuda, M., Yamashita, T. et al. CCL2 promotes P2X4 receptor trafficking to the cell surface of microglia. Purinergic Signalling 8, 301–310 (2012).

207. Tremblay, M. È., Lowery, R. L., & Majewska, A. K. (2010). Microglial interactions with synapses are modulated by visual experience. PLoS biology, 8(11), e1000527.

208. Trotta, T., Panaro, M. A., Cianciulli, A., Mori, G., Di Benedetto, A., & Porro, C. (2018). Microglia-derived extracellular vesicles in Alzheimer's Disease: A double-edged sword. Biochemical pharmacology, 148, 184-192.

209. Tsutsumi, R., Hori, Y., Seki, T., Kurauchi, Y., Sato, M., Oshima, M., ... & Katsuki, H. (2019). Involvement of exosomes in dopaminergic neurodegeneration by microglial activation in midbrain slice cultures. Biochemical and biophysical research communications, 511(2), 427-433.

210. Turola, E., Furlan, R., Bianco, F., Matteoli, M., & Verderio, C. (2012). Microglial microvesicle secretion and intercellular signaling. Frontiers in physiology, 3, 149.

211. Van Der Mark, M., Brouwer, M., Kromhout, H., Nijssen, P., Huss, A., & Vermeulen, R. (2012). Is pesticide use related to Parkinson disease? Some clues to heterogeneity in study results. Environmental health perspectives, 120(3), 340-347.

212. Van Niel, G., d'Angelo, G., & Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. Nature reviews Molecular cell biology, 19(4), 213-228.

213. Van Rooij, E., & Kauppinen, S. (2014). Development of micro RNA therapeutics is coming of age. EMBO molecular medicine, 6(7), 851-864.

214. Vaz AR, Vizinha D, Morais H, Colaco AR, Loch-Neckel G, Barbosa M, Brites D. 2021. Overexpression of miR-124 in Motor Neurons Plays a Key Role in ALS Pathological Processes. Int J Mol Sci 22:10.

215. Verderio, C., Muzio, L., Turola, E., Bergami, A., Novellino, L., Ruffini, F., ... & Furlan, R. (2012). Myeloid microvesicles are a marker and therapeutic target for neuroinflammation. Annals of neurology, 72(4), 610-624.

216. Verkhratsky, A., Semyanov, A., & Zorec, R. (2020). Physiology of astroglial excitability. Function, 1(2), zqaa016.

217. Verkhratsky, A., Sun, D., & Tanaka, J. (2021). Snapshot of microglial physiological functions. Neurochemistry International, 144, 104960.

218. Wake, H., & Fields, R. (2011). Physiological function of microglia. Neuron Glia Biology, 7(1), 1-3.

219. Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci. 2009 Apr 1;29(13):3974-80.

220. Walton MR, Gibbons H, MacGibbon GA, Sirimanne E, Saura J, Gluckman PD, Dragunow M. PU.1 expression in microglia. J Neuroimmunol. 2000 May 1;104(2):109-15.

221. Wang, X., Liu, P., Zhu, H., Xu, Y., Ma, C., Dai, X., ... & Qin, C. (2009). miR-34a, a microRNA up-regulated in a double transgenic mouse model of Alzheimer's disease, inhibits bcl2 translation. Brain research bulletin, 80(4-5), 268-273.

222. Wang, Y. J., Monteagudo, A., Downey, M. A., Ashton-Rickardt, P. G., & Elmaleh, D. R. (2021). Cromolyn inhibits the secretion of inflammatory cytokines by human microglia (HMC3). Scientific reports, 11(1), 1-11.

223. Wendimu, M. Y., & Hooks, S. B. (2022). Microglia phenotypes in aging and neurodegenerative diseases. Cells, 11(13), 2091.

224. Wu, Y., Li, J., Quan, K., Meng, X., Yang, X., Huang, J., & Wang, K. (2020). A DNAzyme cascade for amplified detection of intracellular miRNA. Chemical Communications, 56(70), 10163-10166.

225. Wurm J, Konttinen H, Andressen C, Malm T, Spittau B. Microglia Development and Maturation and Its Implications for Induction of Microglia-Like Cells from Human iPSCs. Int J Mol Sci. 2021 Mar 17;22(6):3088.

226. Wyss-Coray, T, and Lennart M. "Inflammation in neurodegenerative disease—a double-edged sword." Neuron 35.3 (2002): 419-432.

227. Xu Y, Jin MZ, Yang ZY, Jin WL. Microglia in neurodegenerative diseases. Neural Regen Res. 2021 Feb;16(2):270-280.

228. Xue Jiang, Saini Yi, Qin Liu, Jinqiang Zhang. The secretome of M1 and M2 microglia differently regulate proliferation, differentiation and survival of adult neural stem/progenitor cell. bioRxiv 2021.09.15.460424

229. Yan, J., Zhan, X., Zhang, Z. et al. Tetrahedral DNA nanostructures for effective treatment of cancer: advances and prospects. J Nanobiotechnol 19, 412 (2021).

230. Yáñez-Mó, M., Siljander, P. R. M., Andreu, Z., Bedina Zavec, A., Borràs, F. E., Buzas, E. I., ... & De Wever, O. (2015). Biological properties of extracellular vesicles and their physiological functions. Journal of extracellular vesicles, 4(1), 27066.

231. Yang, Y., Boza-Serrano, A., Dunning, C.J.R. et al. Inflammation leads to distinct populations of extracellular vesicles from microglia. J Neuroinflammation 15, 168 (2018).

232. Yao, H., Ma, R., Yang, L. et al. MiR-9 promotes microglial activation by targeting MCPIP1. Nat Commun 5, 4386 (2014).

233. Yuana, Y., Sturk, A., & Nieuwland, R. (2013). Extracellular vesicles in physiological and pathological conditions. Blood reviews, 27(1), 31-39.

234. Zhang, G., He, J., Xie, X., & Yu, C. (2012). LPS-induced iNOS expression in N9 microglial cells is suppressed by geniposide via ERK, p38 and nuclear factor-κB signaling pathways. International Journal of Molecular Medicine, 30, 561-568.

235. Zhang, J., Zheng, Y., Luo, Y., Du, Y., Zhang, X., & Fu, J. (2019). Curcumin inhibits LPS-induced neuroinflammation by promoting microglial M2 polarization via TREM2/TLR4/NF-κB pathways in BV2 cells. Molecular immunology, 116, 29-37.

236. Zhang, Q., Piston, D. W., & Goodman, R. H. (2002). Regulation of corepressor function by nuclear NADH. Science, 295(5561), 1895-1897.

237. Zhang Y, Feng S, Nie K, Li Y, Gao Y, Gan R, Wang L, Li B, Sun X, Wang L, Zhang Y. TREM2 modulates microglia phenotypes in the neuroinflammation of Parkinson's disease. Biochem Biophys Res Commun. 2018; 499:797–802.

238. Zhang, Y., Liu, Y., Liu, H., & Tang, W. H. (2019). Exosomes: biogenesis, biologic function and clinical potential. Cell & bioscience, 9(1), 1-18.

239. Zhao, J., He, Z., & Wang, J. (2021). MicroRNA-124: A Key Player in Microglia-Mediated Inflammation in Neurological Diseases. Frontiers in cellular neuroscience, 15.

240. Zhou, W., Ding, J., & Liu, J. (2017). Theranostic dnazymes. Theranostics, 7(4), 1010.

241. Zingale VD, Gugliandolo A, Mazzon E. MiR-155: An Important Regulator of Neuroinflammation. International Journal of Molecular Sciences. 2022; 23(1):90