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Microglia at the Crossroads of Neuroinflammation: Irisin

and

Chrysin-loaded extracellular vesicles as emerging CNS therapeutic strategies

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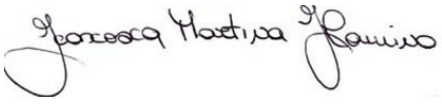

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ABSTRACT

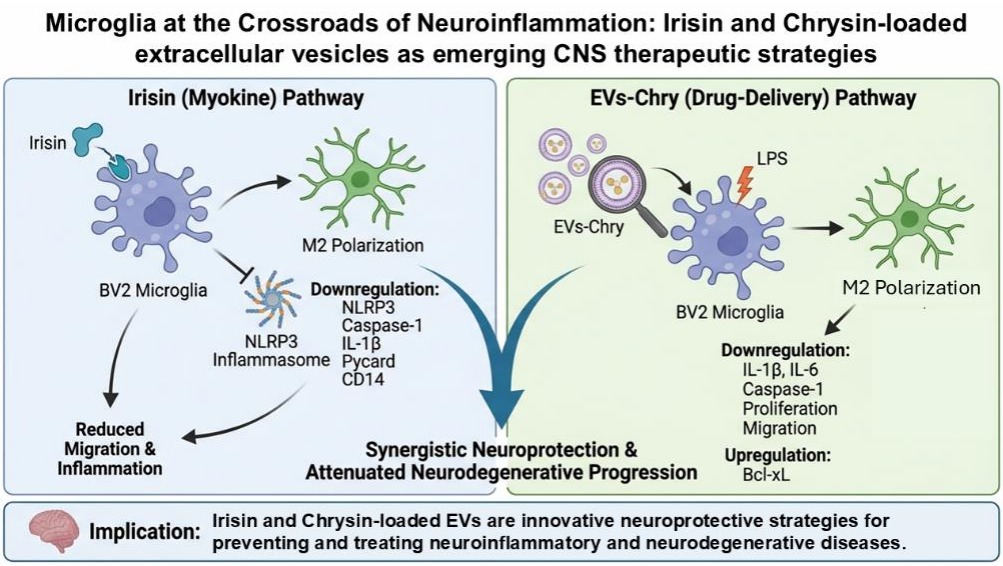
Neuroinflammation contributes to the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Microglia, the resident immune cells of the central nervous system, serve as critical mediators of neuroinflammation and tissue homeostasis. These cells can polarize into distinct phenotypes: the pro-inflammatory M1 phenotype, characterized by amoeboid morphology and release of inflammatory mediators, and the anti-inflammatory M2 phenotype, which promotes tissue repair and neuroprotection. the pro-inflammatory M1 phenotype, characterized by amoeboid morphology and release of cytokines such as IL-1β, IL-6, and TNF-α, and the anti-inflammatory M2 phenotype, which promotes tissue repair and neuroprotection through markers including CD206 and Arginase-1.

Extracellular vesicles (EVs) are nanoscale, membrane-bound particles released by all cell types that facilitate intercellular communication by transporting bioactive cargo including proteins, nucleic acids, and lipids. EVs emerging as key mediators capable of modulating inflammatory responses in both physiological and pathological CNS conditions. EVs are classified based on size into small EVs (30-100 nm), medium EVs (100-1000 nm), and large EVs (500 nm-5 μm), playing crucial roles in both physiological and pathological CNS conditions. The characterization and therapeutic application of EVs represent an innovative biotechnologies and regenerative medicine, offering precise drug delivery systems and novel medical devices for targeted interventions in neurodegenerative disorders.

During my PhD the research activity has been focalized in the discover of two novel neuroprotective strategies targeting microglial neuroinflammation using BV2 microglial cell models. In the first period, I have investigated the irisin, an exercise-induced myokine, demonstrated anti-inflammatory effects by modulating the NLRP3 inflammasome pathway. At physiological concentrations (5 nM), irisin reversed LPS-induced amoeboid morphology, reduced cell migration, and decreased CD14 expression. Importantly, irisin downregulated NLRP3 inflammasome components while upregulating Arginase-1, a marker of M2 polarization. Then I evaluated the anti-inflammatory potential of chrysin-loaded extracellular vesicles (EVs-Chry). EVs-Chry significantly attenuated lipopolysaccharide (LPS)-induced microglial activation by reducing cell proliferation, restoring resting morphology, and decreasing migratory capacity. Co-treatment with EVs-Chry and LPS reduced pro-inflammatory cytokines (IL-1β, IL-6) and caspase-1 expression while enhancing anti-apoptotic Bcl-xL levels, indicating a shift toward neuroprotective phenotypes.

These findings support the therapeutic potential of chrysin-loaded EVs and irisin as innovative neuroprotective strategies for preventing and treating neuroinflammatory and neurodegenerative disorders through targeted modulation of microglial activation states and inflammasome pathways. These results demonstrate that irisin and chrysin-loaded EVs effectively modulate microglial responses, contributing to the development of innovative biotechnological approaches for treating age-related diseases. This research highlights the potential for targeting the biological processes underlying aging and chronic degenerative disorders, while advancing strategies for healthy aging. The findings support the therapeutic promise of chrysin-loaded EVs and irisin as novel neuroprotective interventions that prevent and treat neuroinflammatory and neurodegenerative conditions through targeted modulation of microglial activation states and inflammasome pathways, directly addressing key priorities in aging research.

GRAPHICAL ABSTRACT



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INTRODUCTION

1.1 Microglial Cells

Microglia are a cell lineage resident in the central nervous system (CNS) that originates from the mesodermal system and are described as ramified macrophages [1]. Microglia are an essential component of innate immunity, playing a fundamental role in the development and maintenance of homeostasis by cooperating with other CNS cells. Microglia represent approximately 10% of adult CNS cells, and their density varies across different regions of the CNS. Different studies have showed that microglia cells originate from early erythromyeloid progenitors in the extraembryonic yolk sac [2]. These progenitors settle in the embryonic CNS and differentiate into mature microglia through a stepwise developmental program. Microglia self-renew throughout life, proliferating at a slow rate in balance with finely regulated apoptosis, maintaining homeostatic conditions. During development, they clear and phagocytose foreign materials that threaten the CNS, eliminate synapses in neural circuits, and maintain homeostasis. They produce and target neuroprotective factors in both physiological and pathological conditions, thus enhancing the neuroprotective phenotype of microglia [3]. Brain-resident microglia originate from yolk sac macrophages during embryogenesis. Unlike circulating monocytes, which have a blood-derived myeloid lineage, microglia and most tissue macrophages emerge exclusively from erythromyloid progenitors in the yolk sac (YS). These progenitors differentiate into YS macrophages during embryonic development. These YS precursor macrophages then migrate to and colonise the CNS parenchyma before the blood–brain

barrier matures, subsequently differentiating into microglia. Once established in the CNS, microglia maintain their population through self-renewal and are distributed throughout the brain in non-overlapping regions. They represent 0.5–16% and 5–12% of the total cell count in the human and mouse brain, respectively, with variation by anatomical region. They maintain a constant number of cells throughout life through a dynamic and finely tuned balance between local proliferation and apoptosis, independent of peripheral progenitors [1]. Under normal conditions, the morphology of microglia in a healthy brain is ramified, with numerous long, thin and highly ramified processes. Their highly mobile nature and unique ability to extend and retract enables them to actively sense and respond to environmental aberrations. These processes enable microglia to perform steady-state brain functions such as maintaining synaptic integrity and neuronal maturation and generally maintaining cellular homeostasis [4]. This is because they allow microglia to constantly interact with neurons and other glial cells, both directly and via secreted mediators. When sensing environmental changes, microglia can rapidly migrate towards the triggering stimuli via their ramified processes, guided by chemotactic signals. Microglial activation commonly involves a morphological transformation from a ramified state to an amoeboid state. This is characterised by an enlarged cell body, shorter processes and numerous cytoplasmic vacuoles [5]. This morphological transformation also accompanies functional responses such as migration, antigen presentation, and phagocytosis. In addition to the well-known branched and amoeboid morphologies, ultrastructural studies have characterised other microglial phenotypes. The bipolar/rod morphology is considered a

transitional state between the branched and amoeboid states. This phenotype exhibits distinct transcriptome profiles and a high proliferative and phagocytic capacity. Microglia with these morphological characteristics have primarily been characterised in the elderly brain and in neuropathological conditions, where they tightly align with and surround injured axons, generally performing neuroprotective functions. Furthermore, bipolar/rod microglia are involved in other essential functions, such as synaptic stripping, which facilitates the reconnection of neuronal circuits. Other reported morphologies include hypertrophic, dystrophic, satellite, gitter cell-like and obscure microglia [1]. Microglia maturing in the CNS display characteristics that distinguish them from tissue-resident macrophages, such as the downregulation of certain cell surface proteins, including CD45 and MHC class II molecules (MHCII) [6]. They also exhibit the expression of unique microglial signature genes, including TMEM119, P2Y12, and SALL1 [7]. The development, differentiation, and maintenance of microglia in the CNS are regulated by a variety of factors, such as the interaction between the microglial receptor CX3CR1 and the neuronal ligand CX3CL1, which regulates microglial proliferation and activation; CSF1R and the neuronal-secreted ligands IL-34 and CSF-1 are critical for microglial development and maintenance [8].

1.1.1 Neuroinflammation

Neuroinflammation is the infiltration of immune cells into the CNS, coupled with the activation of microglia and astrocytes. It is a complex network of interactions involving immune and non-immune cells as well as mediators of the immune response [9]. Microglia play

a key role in regulating neuroinflammation and maintaining homeostasis. Under normal physiological conditions, they exist as "homeostatic" cells, but in response to pathological stimuli, they shift to a "reactive" state. Neuroinflammation's roles include countering threats and repairing injuries. However, the damaging response of neuroinflammation, sustained over time, fuels neurodegeneration [10].

The CNS is not immune-privileged, but rather subject to immune surveillance in homeostasis. Upon injury to the CNS, an influx of different immune cell subsets occurs, each exerting distinct and interactive effects on the neural parenchyma. Neuroinflammation often evolves to become compartmentalized within the CNS, requiring a new set of therapies to cross the blood-brain barrier to act locally [11].

Neuroinflammation has similarities in neurological conditions with different etiologist: microglia activation, leukocyte infiltration, and T-cell–microglia/astrocyte interactions [12]. Microglial activity appears detrimental in multiple early phases, perhaps due to the early influx of T cells that rapidly subverts microglia; an early protective state that becomes uncontrolled characterizes microglia in other conditions. These varied roles of microglia require the ability to define their precise functional state when using immunotherapies such as TREM2 agonists or monoclonal antibodies against amyloid-β because inadvertent stimulation of already dysregulated proinflammatory microglia in advanced stages may increase their neurotoxicity [13].

1.1.2 Neuroinflammation in Neurodegenerative diseases

In neurodegenerative diseases, microglial activation is a central element, with dynamic transitions between neuroprotective and neurotoxic phenotypes occurring during disease progression [14]. The accumulation of disease-specific misfolded proteins serves as the primary trigger for innate immune activation. However, significant differences emerge in the timing and intensity of inflammatory responses, specific cytokine profiles, and the relative importance of different cell types [14].

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of protein inclusions called Lewy bodies. Neuroinflammation plays a key role in the pathogenesis of PD, as demonstrated by multiple lines of clinical and experimental evidence [15]. Incremental microglial activation has been documented in the substantia nigra of PD patients, and the degree of microglial activation correlates with the loss of dopaminergic terminals in early-onset PD. Aggregated α-synuclein plays a key role in triggering neuroinflammation in PD. Aggregates of α-synuclein released from dying dopaminergic neurons activate microglia toward a pro-inflammatory phenotype. Studies have shown that fibrillar α-synuclein and mutations associated with early-onset PD induce the most robust immune responses in microglial cells. Extracellular α-synuclein can act through TLR and inflammasome pathways in mononuclear cells, leading to the production of IL-1β [16]. NLRP3 inflammasome signaling in microglia is particularly relevant: this multiprotein complex is triggered by α-synuclein, and different α-

synuclein species lead to specific microglial NLRP3 inflammasome responses [17]. Interestingly, α-synuclein can also induce its own degradation through NLRP3 inflammasome-mediated mechanisms. Increased α-synuclein expression drives microglia toward a reactive pro-inflammatory phenotype, and cytokines such as TNF-α, NO, and IL-1β derived from pro-inflammatory microglia modulate the neuroinflammatory process in PD [17]. The cytokine profile in PD patients reflects a systemic pro-inflammatory environment. Elevated levels of the pro-inflammatory cytokines TNF, IFNγ, IL-1β, IL-6, IL-2, CXCL8, and CCL2 have been detected in the serum of PD patients and correlate with disease severity and disability [18] [16].

Alzheimer's disease (AD) is characterized pathologically by the extracellular accumulation of plaques containing amyloid beta (Aβ) and the development of intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein [19]. Neuroinflammation plays a significant role in the pathogenesis of AD, with evidence suggesting that inflammatory responses occur early in the disease. Studies of inflammatory biomarkers have revealed a complex dynamic during AD progression. Some cytokines, including IL-1β, IL-6, and TNF-α, show slowly increasing levels from the early stage of the disease, while levels of other cytokines such as IL-18, MCP-1, and IP-10 peak at a certain stage of the disease [20]. A landmark study found higher levels of TNF-α and lower levels of TNF-β in the CSF of patients with mild cognitive impairment who progressed to AD, compared to controls who did not progress. The longitudinal study by Fan et al. provided crucial insights into microglial dynamics in AD, suggesting two peaks of microglial activation in the disease trajectory. The first and last peaks could be

neuroprotective and pro-inflammatory, respectively, as microglia are known to shift from neuroprotective to pro-inflammatory activation phenotypes during aging. Dysfunction of microglia and astrocyte metabolism can lead to the accumulation of Aβ [21]. In turn, Aβ aggregates activate microglia and astrocytes through TLRs to release neuroinflammatory mediators that promote neurodegeneration. Morphological changes in microglia and astrocytes surrounding senile plaques are indicative of a neuroinflammatory response [21]. Microglia can be neuroprotective by degrading and clearing Aβ and tau. However, the persistent interaction between Aβ and Aβ-induced pro-inflammatory cytokines overwhelms microglia's clearance capacity. Increases in the size and number of Aβ plaques during late-onset AD may reflect the decreased clearance capacity of microglia [22]. Pro-inflammatory microglia decrease phagocytic activity and further transform microglia into pro-inflammatory phenotypes. Furthermore, pro-inflammatory microglia increase tau phosphorylation and exacerbate tau pathology [23].

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive, adult-onset neurodegenerative disease in which motor neurons are selectively affected. Neuroinflammation is a common pathological mechanism in ALS patients with and without genetic mutations, characterized by the infiltration of activated microglia and astrocytes [24].

Activated microglia and astrocytes, which produce pro-inflammatory cytokines, are upregulated in postmortem tissues of ALS patients. Activated pro-inflammatory microglia and astrocytes produce toxic factors that cause initial damage and disease progression [25]. The G93A-SOD1 transgenic mouse model has

demonstrated the ability of microglia to switch from a neuroprotective to a pro-inflammatory phenotype from disease onset. SOD1 mutant microglia isolated from mice with early-stage ALS express higher levels of M2 microglial phenotype markers and lower levels of pro-inflammatory microglial markers, compared to SOD1 mutant microglia isolated from mice with end-stage ALS [26]. Overall, as ALS progresses, neuroprotective microglial function may decline and the proportion of pro-inflammatory phenotypes may increase. Peripheral inflammation appears to play a more prominent role in PD with evidence of systemic immune dysregulation, in AD, neuroinflammation is more closely associated with the central pathology of plaques and tangles. In ALS, non-autonomous cell toxicity mediated by microglia and astrocytes is particularly evident [27]. These differences underscore the need for personalized therapeutic approaches that consider not only common inflammatory mechanisms but also the specific characteristics of each disease, the disease stage at the time of surgery, and the predominant inflammatory phenotype in each patient [28].

1.1.3 Microglia Activation and Polarization

Microglial activation is often associated with neurodegeneration, a degenerative process underlying neurodegenerative diseases. Under normal physiological conditions, microglia are in a homeostatic state, morphologically presenting branches with extensive contact with other cells, including neurons, oligodendrocytes, and astrocytes, as well as with blood vessels. If the neurological system is compromised and microglia perceive pathological stimuli, this cell line transitions into a reactive state [29].

Microglia express various immune pattern recognition receptors (PRRs), including toll-like receptors (TLRs), NODs, and scavenger receptors. PRRs recognize exogenous pathogenic molecules known as pathogen-associated molecular patterns (PAMPs) or endogenous host-derived molecules known as damage-associated molecular patterns (DAMPs) [30]. PAMPs induce an antimicrobial response and inflammation in response to infection, while DAMPs stimulate sterile inflammation, a type of inflammatory response triggered by the activation of cell surface receptors that recognize signals released by damaged cells in response to CNS injuries such as trauma and hypoxia [31]. When microglial PRRs interact with PAMPs/DAMPs, several intracellular cascades, kinases, and downstream transcription factors are activated, ultimately leading to the synthesis of molecular mediators of inflammation and other cellular responses. Microglial activation is associated with changes in cell surface receptor expression, unique polarization responses, and the release of a variety of inflammatory mediators that contribute to both a protective tissue repair role and a damaging neurotoxic response. Microglial activation is frequently defined in terms of two broad polarization states: a classically activated M1-like phenotype or an alternatively activated M2-like phenotype [32]. Microglia, demonstrate remarkable morphological plasticity that reflects their functional state and role in CNS homeostasis. Microglia are characterized by their acute sensitivity to environmental variations, which triggers morphological transformations corresponding to distinct activation states. In the absence of stimulation, microglia exist in a resting state characterized by a branched morphology and reduced cytoplasm (Figure 1). This ramified configuration is not merely a passive state but actively

contributes to brain homeostasis by regulating synaptic remodelling and neurotransmission [33]. The extensive branching allows resting microglia to continuously survey their microenvironment and maintain CNS surveillance. The proinflammatory M1 phenotype exhibits a larger soma with reduced ramifications, adopting an amoeboid shape. This morphological transformation accompanies the production of inflammatory cytokines including TNF-α, IL-6, IL-1β, and IL-12, along with reactive oxygen species and nitric oxide through NADPH oxidase and inducible nitric oxide synthase expression. The amoeboid morphology of M1 microglia is associated with neurological damage and represents a potentially harmful activation state [2].

Conversely, the anti-inflammatory M2 phenotype maintains a branched morphology with a small soma, resembling the resting state architecture. This phenotypic state supports neuroprotective functions through the production of anti-inflammatory cytokines such as IL-10 and TGF-β, alongside neurotrophic factors including brain-derived neurotrophic factor (BDNF), NGF, and GDNF. M2 microglia promote phagocytosis of cellular debris and misfolded proteins while supporting neuronal survival and tissue repair [34].

Persistent M1 activation and the associated transition to amoeboid morphology contribute to chronic neuroinflammation and overproduction of proinflammatory cytokines, factors implicated in neurodegenerative diseases (NDs) including PD, AD (Figure 2) [35].

These polarization states differ in their triggers, the expression of phenotypic markers, and the secreted mediators, all of which determine the overall outcome. The classically activated M1 phenotype is associated with pro-inflammatory and neurotoxic

responses, while the M2 phenotype primarily mediates anti-inflammatory and neuroprotective functions. Potent activators of the M1 phenotype in microglia include α-syn aggregation in neurons, which subsequently causes α-syn accumulation outside neurons, leading to neuronal toxicity [36]. Extracellular α-syn (monomers, oligomers, and fibrils) have also been observed to regulate the inflammatory response of microglia. The clearance of excessive α-syn from the cytoplasm is primarily facilitated by activated microglia. Extracellular α-syn acts as a DAMP and activates several receptors, including TLR2, inducing innate immune responses on the surface of microglial cells. Extracellular α-syn induces the nuclear factor kappa B (NF-κB) signaling pathway in microglia, which is an important component of the microglial response to inflammation [37]. The pro-inflammatory M1 microglial phenotype can be induced *in vitro* by interferon-γ (IFNγ) and the bacterial endotoxin LPS. LPS has been shown to cause dopaminergic neuron death *in vivo* and *in vitro* by inducing M1 microglia via TLRs. LPS is normally activated by binding to Toll-like receptor 4 (TLR4) and Toll-like receptor 2 (TLR2) receptors on the microglial membrane. It then binds to myeloid differentiation protein 2, with the involvement of the co-receptors CD14 and LPS-binding protein [38]. Activation of TLR4 by LPS triggers downstream signaling molecules, such as myeloid primary response protein 88 (MyD88) and toll-domain-containing adaptor/TIR-1 containing interferon-inducing interferon-β, as well as transcription factors such as NF-κB, STAT5), and interferon regulatory factors [38]. This triggers a chain reaction that leads to the upregulation of M1-associated gene transcription, such as chemokines, cytokines, and genes expressed in pro-inflammatory

responses [39]. IFNγ is also a potent trigger for the activation of polarization toward the M1 phenotype. IFNγ is a cytokine that plays an essential role in both innate and adaptive immunity against infections by viruses, some bacteria, and protozoa [40]. Both the activation of macrophages/microglia and the induction of the production of MHC II molecules are significant functions of IFNγ. IFNγ acts on IFNγ receptors 1 and 2 and activates the JAK/STAT pathway, leading to the phosphorylation and nuclear translocation of STAT1 and other IRFs [40]. Transcription factors activated by M1-like microglia upregulate pro-inflammatory cell surface markers, such as MHC II and CD86. They also induce the production of a variety of pro-inflammatory mediators, including cytokines such as tumor necrosis factor-α (TNFα), interleukins IL-1β, IL-6, IL-12, IL-17, IL-18, IL-23, and chemokines such as CCL12 and CXCL10. and other pro-inflammatory mediators, including ROS and RNS, iNOS, and COX-2. M1-like microglia play an important role in triggering innate immune responses to combat foreign pathogens and initiating the adaptive immune response. However, chronic activation in pathological conditions contributes to neuroinflammation, oxidative stress, and neurotoxicity [41].

The anti-inflammatory M2 microglia phenotype is the result of "alternative activation" and is often associated with functions such as immune resolution and tissue repair through the secretion of anti-inflammatory and neurotrophic factors. When brain homeostasis is compromised due to brain injury or chronic stress, the CNS possesses endogenous defence mechanisms that promote tissue repair [14]. Various anti-inflammatory cytokines, growth factors, and hormones, such as glucocorticoids, are released by injured neurons and promote

the transformation of surrounding microglia into a protective M2-like phenotype. M2 microglia can be activated by four major anti-inflammatory cytokines: IL-4, IL-10, IL-13, and TGF-β. IL-4 and IL-13 promote the alternative activation state and generally act to antagonize pro-inflammatory M1 responses, such as the production of TNFα, IL-6, and iNOS. The multifunctional cytokine TGF-β plays a critical role in angiogenesis, immunoregulation, and tissue repair and, together with IL-10, induces the acquired deactivated state [42]. M2 phenotypes are subclassified into three states, M2a, M2b, and M2c, which have overlapping biochemical roles but differ in activating stimuli, marker expression, and mechanism of action. M2a is considered an anti-inflammatory, phagocytic, and wound-healing phenotype and is activated during stimulation with the cytokines IL-4 or IL-13. This is accompanied by activation of the JAK1/3-STAT6 pathway, leading to the upregulation of cell surface markers CD206, Arg1 [43]. This microglial phenotype is involved in immunity against parasites, collagen formation, and tissue repair. M2b microglia are generally characterized as an inflammation-regulating phenotype and are activated by the fusion of TLR and FCγ receptors, and their subsequent interaction with B-cell-derived IgG [44]. Polarized M2b microglia share similar characteristics to M1 microglia, as they can be activated *in vitro* by TLR agonists and are characterized by the expression of COX-2 and M1-associated cell surface proteins CD86 and MHCII. However, M2b microglia also exhibit different responses, such as the recruitment of regulatory T cells and the release of the anti-inflammatory cytokine IL-10. IL-10, along with TGF-β and glucocorticoids, triggers activation of the immunosuppressive M2c phenotype. Microglia of the M2c phenotype play a prominent

role in matrix remodelling, tissue repair, and immunoregulation [45]. This IL-10-induced M2c polarization is mediated by interaction with the IL-10R1 and IL-10R2 receptors and subsequent activation of the JAK1/STAT3 pathway. This leads to upregulation of the cell surface marker CD163, the cytokines TGF-β and IL-10, and the inhibition of M1-associated pro-inflammatory cytokines [46]. Only two categories are commonly used, but different types of activation states and different targets and receptors are continuously present to regulate the microglial response. A spectrum of microglial functional states exists, forming a continuum rather than discrete phenotypes. It is important to emphasize that in most cases the phenotype is transient rather than definitive [47]. Microglia are capable of dynamically shifting polarization states between M1-like and M2-like phenotypes, exhibiting a continuous spectrum of various activation phenotypes. Different phenotypic markers can coexist, displaying several intermediate phenotypes. The overall contribution of microglia in the normal and diseased brain and the transition between different phenotypic states depend on the activating mechanism, the duration of the signal, and the regulatory signaling molecules [2].

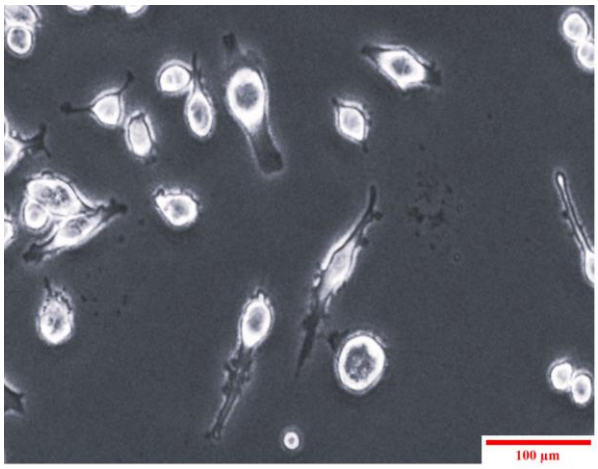

Figure 1. Morphological analysis of BV2 cells under control conditions. Plates were evaluated by Leica microscopy at 10× and 20× magnification.

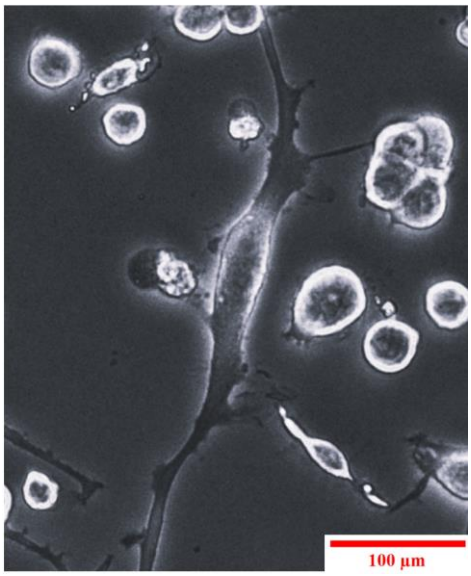

Figure 2. Morphological analysis of BV2 cells following 24-hour incubation with LPS. Plates were evaluated by Leica microscopy at 10× and 20× magnification.

1.1.4 Neuroinflammatory pathways as treatment targets

Chronic and prolonged microglial activation in neuroinflammation appears to be a major risk factor contributing to the pathogenesis of multiple neurodegenerative diseases, including AD, PD, and ALS.

The NLRP3 inflammasome belongs to the superfamily of PRRs, which recognize pathogen-associated molecular patterns [48]. The unique feature of NLRP3 activation is its two-step process: priming and inflammasome assembly. NLRP3 may serve as a hub integrating multiple signals to determine the intensity of microglial activation [49]. Numerous studies have demonstrated that the NLRP3 inflammasome plays a crucial role in the pathogenesis of neuroinflammation. NLRP3 is an important site in the innate immune system, recognizing both invading exogenous pathogens and detecting endogenous cellular damage. Activation of the NLRP3 inflammasome requires two steps. The first step is priming, which *in vitro* can be triggered by LPS or, more generally, DAMPs/PAMPs, recognized by TLR4 and inducing the expression of IL-1β, IL-18, and NLRP3 via activation of the NF-κB [50]. The second step is activation of the NLRP3 inflammasome, which is induced by various PAMPs and DAMPs and can activate multiple upstream signaling events. These include K+ efflux, Cl- efflux, lysosomal disruption, mitochondrial dysfunction, production of ROS, and release of oxidized mitochondrial DNA. Activation of NLRP3 stimulates the assembly and oligomerization of the NLRP3 inflammasome complex, which further activates caspase-1, leading to the cleavage of pro-IL-1β and pro-IL-18. GSDMD is also cleaved, and N-GSDMD inserts

into the membrane, forming pores and inducing pyroptosis while releasing inflammatory cytokines such as IL-1β and IL-18. Furthermore, activation of the NLRP3 inflammasome activates caspase-8 and caspase-3, cleaving GSDME, with the cleaved N-GSDME forming pores in the cell membrane and releasing IL-1β and IL-18 [51]. These inflammatory factors not only induce local inflammatory responses, but can also affect other organs through the bloodstream, further exacerbating systemic inflammation [52]. Chronic inflammation mediated by NLRP3 causes the prolonged release of cytokines, leading to neuronal death and further compromising CNS homeostasis. Excessive NLRP3 activation can trigger an inflammatory response in glial cells, with the release of numerous cytokines, and can cause neuronal damage and dysfunction. Indeed, proinflammatory cytokines not only support the progression of neuroinflammation but also inhibit neuronal survival and regeneration. Furthermore, abnormal NLRP3 activation is associated with pathological conditions such as stroke, trauma, and aging, where neuroinflammation further exacerbates disease progression [53]. Drugs targeting the NLRP3 inflammasome have shown promising results in mouse models of CNS diseases. For example, the NLRP3 inhibitor MCC950 has shown potential in ameliorating inflammation and related pathology in neurodegenerative animal models [54]. These findings highlight the NLRP3 inflammasome as a viable therapeutic target with the potential to alleviate the impact of neuroinflammation in various CNS diseases [55].

The NLRP3 inflammasome appears to play a crucial role in the pathogenesis of several CNS disorders, acting as a bridge between innate immunity and neuroinflammation. Neuroinflammation driven

by the NLRP3 inflammasome significantly contributes to the pathogenic development of multiple sclerosis (MS) [56]. Peripheral caspase-1 mRNA levels are associated with MS severity, and MS patients also show increased expression of caspase-1, IL-1β, and IL-18. In the pathogenesis of MS, the NLRP3 inflammasome plays a key role in inducing T cell migration to the CNS. In AD, the accumulation of amyloid-β plaques triggers activation of the NLRP3 inflammasome, resulting in increased production of IL-1β, which subsequently recruits immune cells and exacerbates neuronal damage. [57]. Similarly, in PD, the presence of α-synuclein aggregates is associated with NLRP3 activation, highlighting the role of the inflammasome in the inflammatory milieu surrounding pathological protein aggregation [58].

NF-κB comprises a family of five transcription factors involved in various cellular functions, particularly in mediating inflammatory responses. This family is composed of NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel. Activation of NF-κB promotes the transcription of target genes, many of which are proinflammatory [59]. NF-κB signaling is activated through two distinct pathways: the canonical and the non-canonical. In the canonical pathway, in an inactive state, p65/p50 dimers are sequestered in the cytoplasm by IκBα. Upon exposure to proinflammatory stimuli such as cytokines, pathogens, and danger-associated molecular patterns, these p65/p50 dimers are released from IκBα due to a phosphorylation cascade that results in proteasomal degradation of IκBα [60]. Subsequently, p65/p50 can translocate to the nucleus, where they bind to its cognate κB motif, leading to the activation and expression of NF-κB target genes. The non-canonical

activation pathway involves a subset of members of the tumour necrosis factor receptor superfamily, leading to the activation of NF-κB-inducing kinase [61]. NIK phosphorylates IKKα, which phosphorylates the C terminus of p100 to generate p52. After the phosphorylation cascade, p52/RelB translocate to the nucleus, triggering the expression of NF-κB target genes that play a role in immune cell development. TLRs primarily activate the canonical NF-κB signaling pathway, leading to the expression of proinflammatory factors. Microglial activation is one of the early events leading to the development of AD, given that the primary function of microglia in the brain is protection from pathogens and the removal of cellular debris, including the formation of amyloid beta Aβ plaque [62]. Therefore, activation of NF-κB signaling and the resulting release of cytokines and chemokines from microglia lead to the chronic inflammation observed in AD [63].

1.2 Extracellular Vesicles

According to the guidelines established by the International Society for Extracellular Vesicles (ISEV), the term "*extracellular vesicle*" identifies biological particles naturally secreted by all cell types [64]. EVs can be released by both prokaryotic and eukaryotic cells in response to specific signals. EVs are traditionally classified into three main subtypes based on their biogenesis and size: exosomes, microvesicles, and apoptotic bodies [65]. Exosomes range in size from 30 to 150 nm and are These entities are characterized by a lipid bilayer and lack a functional nucleus, which renders them incapable of replication. They can contain a variety of biomolecules, including proteins, lipids, and specific nucleic acids that reflect their

cellular origin. Exosomes typically exhibit a diameter between 30 and 150 nm and originate from the endosomal pathway. This process involves the invagination of the endosomal membrane, leading to the formation of multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) [66]. The subsequent fusion of MVBs with the plasma membrane results in the secretion of ILVs into the extracellular environment as exosomes. Their biogenesis is frequently mediated by the endosomal sorting complex required for transport (ESCRT), including Rab GTPases which is a subunit of ESCRT, although ESCRT-independent mechanisms, involving ceramides and tetraspanins such as CD9, CD63, and CD81, also regulate the loading of molecular cargo [66] [67]. Exosome biogenesis is composed of various protein complexes: syntetin-1, TSG101, ALIX, ceramide, sphingomyelinases, and tetraspanins, including clusters of differentiation CD9, CD63, and CD81, which also regulate cargo sorting into vesicles. Various studies show that exosome production in all brain cell types depends, at least in part, on an ESCRT-independent mechanism [68] [69]. Microvesicles, or microparticles, typically range in size from 100 to 1,000 nm and are released by direct budding from plasma membranes. Apoptotic bodies are EVs with a diameter ranging from 50 to 5,000 nm and are released by blebbing of plasma membranes during apoptosis. They also express specific apoptosis markers such as Annexin V and Histone H3. Size alone cannot be used to distinguish EV subtypes, as they often overlap in size or lack specific markers. Consequently, the ISEV has simplified the classification of EVs into two main size-based subtypes: small (<200 nm) and medium-large EVs (>200 nm) [70]. EVs can interact with recipient cells in a variety of ways. They can enter through

several mechanisms, including clathrin-dependent fusion, macropinocytosis, and lipid-mediated endocytosis. EVs can also activate the target cell's membrane protein. In clathrin-mediated endocytosis, EVs bind to the plasma membrane and are internalized [71]. Once inside cells, they can be degraded by lysosomes or escape to a lysosome and release their cargo into the cytoplasm. Macropinocytosis is the most commonly used; in this case, the plasma membrane undergoes actin-dependent disarray and internalizes the EVs. Another internalization mechanism is lipid-raft-mediated endocytosis, in which disruption of these structures, altering cholesterol dynamics, induces EV uptake [72]. Once internalized, EVs release their bioactive cargo, thereby modulating gene expression and signaling pathways within the recipient cell. These molecules activate various signaling pathways in target cells, altering gene expression through translation and post-translational modifications of the recipient cell's RNA [73].

The EV-TRACK (Transparent Reporting And Centralizing Knowledge in Extracellular Vesicle Research) Consortium has proposed a tool called EV-METRIC for more rigorous separation and characterization of EVs [74]. Various techniques and technologies, such as differential ultracentrifugation, immune capture, ultrafiltration, and size-exclusion chromatography, are widely used to identify and separate EVs from biological fluids. Structural analysis utilizes advanced techniques such as atomic force microscopy, flow cytometry, ELISA. Other imaging techniques, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM), are used in addition to electron microscopy to analyse all EVs. In the CNS, EVs have emerged as critical players in neurological

pathogenesis, intercellular communication, and as potential vehicles for drug or vaccine delivery, in addition to serving as circulating diagnostic biomarkers [75].

When EVs come into contact with target cells, they can influence their biological behaviour in various ways. As signaling complexes, EVs have the ability to directly activate target cells. EVs can also provide receptors and exchange bioactive lipids between cells. Furthermore, EVs can deliver cargo molecules involved in both physiological and pathological processes from parent cells to target cells, generating biological responses [76].

1.2.1 Extracellular Vesicles Crosstalk in Central Nervous System

Brain-derived vesicles are a heterogeneous population of CNS-derived EVs that are secreted into the extracellular space and can be recovered in the blood and cerebrospinal fluid [77]. EVs are known to mediate interactions between cells in the nervous system, as well as communication between peripheral organs and the CNS. Neurons, astrocytes, oligodendrocytes, and microglial cells release EVs and exchange signaling molecules through them [77].

EVs play an important role in promoting neural development, regulating the progression of inflammation, and modifying tumour characteristics. Alterations in EV signaling, particularly changes in miRNA expression, or in the movement of inflammatory molecules can induce changes in the physiological microenvironment, closely associated with the degree of CNS injury. EVs are able to cross the blood-brain barrier (BBB) to enter the brain from the periphery or

exit via circulating cerebrospinal fluid or the lymphatic system; For this reason, they can be studied as potential biomarkers or therapeutic carriers of CNS diseases [78]. The ability of EVs to traverse the BBB makes them ideal candidates for both biomarkers and therapeutic carriers. However, they play a dual role: while they assist cells in the clearance of toxic protein aggregates, they may also facilitate the propagation of pathology by transferring these cytotoxic loads to healthy cells. In the CNS, EVs have been suggested to be potential carriers in the intercellular delivery of misfolded proteins associated with neurodegenerative disorders, such as tau and amyloid β in AD, α-synuclein in PD, SOD1 in ALS, and in Huntington's disease [79]. Consequently, analysing EVs in peripheral blood offers a non-invasive method that may be more sensitive than a CSF biopsy for early diagnosis and prognostic monitoring. EVs carry a molecular "fingerprint" of their parent cell, including lipids, carbohydrates, and non-coding RNAs that act as extrinsic regulators of CNS function [80]. Specifically, microglia-derived EVs modulate synaptic transmission by inducing neuronal production of ceramide and sphingosine, and they influence presynaptic transmission through interactions with the endocannabinoid system [81]. Under both physiological and pathological conditions, microglia communicate simultaneously in multiple ways, including through the use of proteomic complexes that enable cell-to-cell communication, the release of soluble factors, and the exchange of biomolecules via secreted vesicles. Microglia-derived EVs have been shown to regulate synaptic transmission by inducing neuronal production of ceramide and sphingosine [82]. Furthermore, microglia-derived EVs modulate presynaptic transmission through the endocannabinoid system.

Increased sphingolipid metabolism enhances excitatory neurotransmission, confirming the role of microglia in the physiological modulation of synapses. Extracellular ATP acts as a potent stimulus for the release of microglial vesicles, which are enriched with IL-1β and GAPDH, thereby promoting the propagation of the neuroinflammatory response [83]. Since these vesicles were found to be loaded with functional P2X7 receptors, microglia may be able to reduce the amount of these receptors on the plasma membrane by releasing these vesicles, thereby reducing P2X7-mediated apoptosis [84]. Furthermore, in primary microglial cultures, as well as in the BV2 cell line, serotonin binding to specific serotonin receptors can promote exosome release through an increase in cytosolic Ca2+ [85]. Recombinant Wnt3 also induces exosome secretion through a GSK3-independent mechanism. Microglia-derived EVs are also implicated in neurodegenerative processes [86]. For example, treating the BV2 cell line with alpha-synuclein increases the secretion of EVs, which express high levels of MHC-II and TNF-α and induces neuronal apoptosis. Stimulation with LPS triggers the release of microvesicles loaded with the pro-inflammatory cytokine IL-1β and miR-155, amplifying the inflammatory reaction. These extracellular vesicles then intensify proinflammatory reactions [87]. Other stimuli that induce EV secretion from cultured microglia include proinflammatory cytokines, IL-4, capsaicin, ethanol, and manganese. In summary, microglial EVs serve as key vectors for the propagation of neuroinflammation within the CNS [88].

1.2.2 Role of EVs in Neuroinflammation

EVs have emerged as pivotal players in neuroinflammatory processes, exhibiting both pro-inflammatory and anti-inflammatory properties depending on the cellular context, timing, and nature of the inflammatory stimulus [89]. During neuroinflammation, oxidative stress increases substantially, inducing the release of EVs that serve as key regulators of inflammation and contribute to pathological processes such as vascular calcification. Multiple inflammatory stimuli, including LPS, ATP, and pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ, activate microglia and dramatically alter their EV secretion profile, promoting the release of vesicles with a distinct cargo composition that includes pro-inflammatory cytokines, inflammatory proteins, specific microRNA, DAMPs, thereby highlighting the crucial role of microglia-derived EVs as propagators and amplifiers of neuroinflammatory responses [90]. When microglia become activated by these inflammatory triggers, they undergo phenotypic transformation toward a pro-inflammatory M1 state and release EVs with dramatically altered molecular cargo that can initiate and perpetuate inflammatory cascades in recipient cells. These M1 microglia-derived EVs carry diverse biomolecules including DNA fragments, various types of RNA, proteins with enzymatic and signaling functions, bioactive lipids, DAMPs that serve as endogenous danger signals, and an array of cytokines and chemokines that orchestrate immune cell recruitment and activation [91]. The cargo composition reflects not only the activation state of the parent microglia but also the specific inflammatory context, with different stimuli producing EVs with distinct molecular signatures and

functional properties. The mechanisms through which EVs modulate neuroinflammatory responses involve complex interactions with multiple pattern recognition receptors and signaling pathways in recipient cells. EVs can bind to TLRs, thereby initiating the activation of downstream signaling cascades. Upon EV-TLR interaction, adaptor proteins including MyD88, TRAF6 and IκBα become activated, forming a signaling complex that ultimately leads to the translocation of NF-κB to the nucleus and the transcription of pro-inflammatory genes [92]. Furthermore, EVs can bind to the receptor for advanced glycation end products, a multi-ligand receptor expressed on microglia, astrocytes, and neurons, which activates the Ras and MAPK signaling pathways [92]. These pathways subsequently trigger the phosphorylation and degradation of IκBα, allowing NF-κB nuclear translocation and resulting in the generation of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, which further amplify the inflammatory response and contribute to neuronal damage. During neuroinflammation, EVs also interact with inflammasome complexes, particularly NLRP3 inflammasome and AIM2 inflammasome [93]. The interaction between EVs and these inflammasome components promotes the assembly and activation of the inflammasome complex, leading to the recruitment and activation of caspase-1, a cysteine protease that serves as the central executioner of inflammasome-mediated inflammation. Activated caspase-1 cleaves several pro-inflammatory substrates, including pro-IL-1β and pro-interleukin-18, converting them into their mature, biologically active forms that are subsequently secreted and propagate inflammatory signalling to neighbouring cells. Additionally, caspase-1 cleaves pro-gasdermin D, generating an N-terminal fragment that

oligomerizes and forms pores in the plasma membrane, leading to a lytic form of cell death called pyroptosis [94]. Pyroptotic cell death results in the rapid release of intracellular contents, including additional pro-inflammatory molecules such as IL-1β, IL-18, ATP, and DAMPs, which further activate surrounding immune cells and perpetuate the inflammatory cycle. This EV-inflammasome axis represents a critical mechanism by which microglia-derived EVs can amplify and sustain neuroinflammatory responses, contributing to chronic inflammation and progressive neuronal damage in neurodegenerative diseases. Studies have demonstrated that ATP stimulation of microglia triggers the release of EVs enriched in IL-1β through acid sphingomyelinase activation, creating a feed-forward inflammatory loop that involves the continuous release of pro-inflammatory EVs and the activation of additional microglial cells [95]. Furthermore, inflammatory microglia-derived EVs carry specific microRNA signatures, particularly miR-146a-5p, which can be transferred to neurons where they target synaptic proteins including neuroligin-1 and synaptic scaffolding proteins, resulting in decreased dendritic spine density and loss of excitatory synapses [96]. This EV-mediated transfer of inflammatory signals represents a mechanism by which microglial activation can directly compromise synaptic integrity and neuronal function, providing a molecular link between neuroinflammation and synaptic dysfunction observed in neurodegenerative diseases. LPS-primed microglia exposed to additional inflammatory stimuli show enhanced EV secretion containing inflammasome components such as apoptosis-associated speck-like protein containing a CARD (ASC), which can be transferred to recipient microglia, increasing the expression of

NLRP3 and pro-IL-1β and priming these cells for enhanced inflammasome activation, thereby propagating the inflammatory response across the microglial network [97]. Similarly, astrocytes activated by inflammatory stimuli, particularly IL-1β and TNF-α, release EVs with altered protein and microRNA content that can reduce neuronal viability, decrease neurite outgrowth and branching, and suppress neuronal firing activity [97]. These activated astrocyte-derived EVs contain microRNAs that target components of neurotrophic signaling pathways, thereby interfering with pro-survival signals in neurons and contributing to neurodegeneration. The inflammasome-EV crosstalk has also gained attention, with evidence showing that EVs can serve as vehicles for inflammasome components and their products, facilitating the spread of inflammatory responses throughout the CNS and even across the blood-brain barrier to peripheral tissues [98]. However, it is crucial to recognize that EVs in neuroinflammation function as a double-edged sword as they can also mediate anti-inflammatory and neuroprotective effects depending on the phenotype of their parent cells and the temporal dynamics of the inflammatory response [99]. Microglia can also adopt an anti-inflammatory M2 phenotype in response to stimuli such as IL-10, TGF-β, and IL-4, and these M2 microglia release EVs with cargo compositions that promote inflammation resolution, tissue repair, and neuroprotection. M2 microglia-derived EVs have been shown to contain anti-inflammatory cytokines, neurotrophic factors such as BDNF and IGF-1, and specific microRNAs such as miR-124 that can modulate recipient cell phenotypes toward anti-inflammatory states and enhance neuronal survival [100]. Studies have demonstrated that M2 microglia-derived

EVs carrying miR-124 can promote the polarization of other microglial cells toward the M2 phenotype, reduce the expression of pro-inflammatory mediators, increase the production of neurotrophic factors, and ameliorate neuronal damage in models of ischemic brain injury and traumatic brain injury (TBI) [100]. Furthermore, these neuroprotective EVs can activate signaling pathways such as the PI3K/Akt pathway and enhance the expression of anti-apoptotic proteins, thereby protecting neurons from oxidative stress, excitotoxicity, and apoptotic cell death [101]. The balance between pro-inflammatory and anti-inflammatory EV populations in the CNS microenvironment likely represents a critical determinant of disease progression in neurological disorders [102]. Understanding the factors that govern the switch between beneficial and detrimental EV phenotypes, including the nature and duration of inflammatory stimuli, the metabolic state of parent cells, the local cytokine milieu, and cell-cell interactions within the neurovascular unit, will be essential for developing therapeutic strategies that can harness the neuroprotective potential of EVs while suppressing their pathology-promoting activities [103]. This dual nature of EVs in neuroinflammation underscores both the complexity of EV-mediated communication in the diseased brain and the therapeutic opportunities that may arise from precisely modulating EV production, cargo loading, and recipient cell targeting to restore homeostatic balance and promote neuroprotection in the setting of chronic neuroinflammation [104].

1.2.3 Use of EVs as delivery system of compounds

The wide range of contents present in EVs and released from cells under physiological conditions has many potential applications in brain therapy. Indeed, EVs have transient effects on target cells, and in many cases, their use may therefore be safer than using their progenitor cells [105]. Additional benefits can be achieved by loading unmodified EVs with molecules of interest. Effectively loading EVs with therapeutic products is one of the greatest challenges in the field. While synthetic carriers, such as lipid-based nanoparticles, are often loaded during the manufacturing process, simplifying the process, EVs must be loaded both during their biogenesis and after isolation. Loading EVs during biogenesis requires hijacking the progenitor cells' endogenous machinery to produce EVs containing specific molecules previously internalized by the cells. Internalization could be achieved by incubating the progenitor cells with the desired molecule [106].

Loading EVs with therapeutics after their isolation is a much more common strategy, not only due to the simplicity of the process but also due to the inefficient loading of cell packaging during EV biogenesis. The simplest method is passive incubation of isolated EVs with the tested molecules, followed by purification [107]. For example, exosomes have been loaded by incubating them at 22°C for 5 minutes with the hydrophobic drug curcumin, resulting in efficient delivery to the brain via the intranasal route and a reduction in inflammation, thanks to selective delivery to microglial cells [108].

For more complex molecules, active strategies such as electroporation are employed, which create temporary pores in the EV

lipid membrane to facilitate cargo entry. This technique applies electrical pulses to create transient pores, which facilitate the migration of molecules across membranes [109]. The content and composition of EVs released by producing cells depend on their physiological state. EVs released by brain cells can alter the surrounding microenvironment and contribute to the progression of brain diseases, or reach distant tissues via biological fluids. EVs also hold promise as a therapeutic tool due to their natural ability to transport nucleic acids and therapeutic molecules intercellularly, their low immunogenicity, and their biocompatibility, among other characteristics. As endogenous vesicles, EVs offer stability in the bloodstream, cloaking the immune system, and even the ability to cross the BBB and target specific cells [110].

1.2.4 Role of EVs in Gut-Brain Communication

Traumatic brain injury (TBI) represents a substantial global health crisis, contributing significantly to long-term morbidity and mortality. While the primary mechanical insult causes immediate damage to cellular membranes and ionic homeostasis, it is the secondary injury phase—characterized by neuroinflammation, oxidative stress, and BBB disruption—that dictates the long-term clinical trajectory [111]. Emerging evidence identifies the gut–brain axis (GBA) as a master regulator of this secondary phase. Central to this bidirectional communication are microbiota-derived extracellular vesicles (MEVs), lipid-bilayer enclosed nanoparticles, typically ranging from 40 to 400 nm in diameter, that act as stable mediators of inter-kingdom signaling [112]. MEVs facilitate the transfer of microbial signals that influence neuronal survival, synaptic plasticity,

and the host's immune landscape post-trauma. The burgeoning field of neuro-microbiology has identified MEVs as critical, stable mediators of inter-kingdom communication within the gut–brain axis (GBA), particularly following TBI [113]. These lipid-bilayer enclosed nanoparticles serve as sophisticated signaling vectors that transport bioactive molecular cargo—including proteins, lipids, and nucleic acids—from the gastrointestinal tract to the CNS. The structural and functional diversity of MEVs is fundamentally rooted in the cell wall architecture of the parent bacteria. Gram-negative Bacteria possess a complex double-plasma membrane. They generate several types of MEVs: Outer Membrane Vesicles (OMVs), produced via membrane blebbing; Inner–Outer Membrane Vesicles (O-IMVs); and Explosive Outer Membrane Vesicles (E-OMVs). OMVs are characterized by some outer leaflet rich in LPS and an inner leaflet of phospholipids [114]. Blebbing is often driven by lipid asymmetry, reduced protein cross-linking (involving genes like OmpA and TolA), or turgor pressure from periplasmic misfolded proteins. Lytic processes, such as those triggered by phage-derived endolysins, lead to the formation of E-OMVs laden with cytoplasmic material, including chromosomal DNA. In contrast, Gram-positive (G+) bacteria generate cytoplasmic membrane vesicles (CMVs) via processes such as "bubble cell death," which, while lacking LPS, are enriched in peptidoglycans and lipoteichoic acid [115] [116].

The functional repertoire of MEVs is determined by their cargo, which is protected from enzymatic degradation by the vesicle's lipid bilayer. MEVs carry structural components, porins (OmpA, OmpC), enzymes (proteases, glycosidases), and virulence factors. Probiotic-derived MEVs contain proteins that promote

immunomodulation and beneficial host interactions. The lipid profile is strain-dependent and sensitive to environmental shifts [117]. Common lipids include glycerophospholipids, cardiolipin, and sphingolipids, which influence membrane fluidity and host cell interaction. MEVs act as vectors for genetic material, including DNA associated with antibiotic resistance and virulence. Crucially, they carry small non-coding RNAs that regulate host gene expression post-transcriptionally, influencing immune phenotypes. The clinical relevance of MEVs becomes most apparent during the secondary phase of TBI, a period characterized by systemic immune activation and a compromised intestinal barrier, often described as a "leaky gut". TBI-induced dysbiosis alters the composition and abundance of gut microbiota, facilitating the translocation of MEVs across the intestinal epithelium into the systemic circulation. Crucially, these vesicles possess significant neuroinvasive potential, demonstrating the ability to traverse the BBB to interact directly with resident CNS cells, such as microglia and astrocytes. Within the neural environment, MEVs exert a dual influence on neuroinflammation based on their specific molecular signatures [118]. In the pathological context of TBI, the intestinal barrier's integrity is compromised, a state often referred to as "leaky gut". MEVs cross the intestinal epithelium via paracellular (between cells) or transcellular (through cells) routes. Once in the lamina propria, they are phagocytosed by dendritic cells and macrophages, subsequently entering the lymphatic and circulatory systems. MEVs exhibit neuroinvasive potential. They can traverse the BBB via systemic circulation, although the precise mechanisms remain under investigation. Once inside the CNS, MEVs interact with microglia and astrocytes to modulate neuroinflammatory pathways.

MEVs also communicate through the autonomic nervous system, specifically the vagus nerve, where they interact with receptors to stimulate signaling molecules like short-chain fatty acids (SCFAs) [119]. MEVs exert a dual effect, deleterious or neuroprotective, depending on their molecular signature and origin. Pathogenic or dysbiosis-derived MEVs can activate TLRs, particularly TLR4, and the NLRP3 inflammasome [120]. This leads to the release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), exacerbating neuroinflammation and endothelial dysfunction. Chronic microglial activation, a hallmark of secondary TBI, can persist for years and is linked to the development of AD and PD. Commensal-derived MEVs from strains like Lacticaseibacillus rhamnosus and Bacteroides fragilis promote neuroprotection. They can drive regulatory T cell (Treg) differentiation, suppress NF-κB signaling, and promote neuronal regeneration. For example, L. johnsonii-derived EVs have been shown to suppress neurotoxic bile acid production, offering a protective mechanism against AD-like pathology [121].

Current diagnostic modalities, such as CT and MRI, often lack the sensitivity to detect subtle neuropathological changes in mild TBI. Conventional neuroimaging often fails to detect the subtle neuropathological shifts associated with mild TBI, whereas the molecular cargo of MEVs—which reflects the physiological state of the parent microbiota—can serve as a stable and sensitive biomarker for early detection and severity classification [122]. MEVs present a transformative alternative as minimally invasive "liquid biopsies". Due to their lipid bilayer, MEVs remain stable in peripheral fluids (blood, urine), and their cargo reflects the physiological state of the parental microbiota. Temporal shifts in the molecular cargo of MEVs

(e.g., miRNAs or pathognomonic proteins like tau and amyloid-β) allow for real-time monitoring of injury progression, severity classification, and prognosis. The therapeutic potential of MEVs is being harnessed through advanced biotechnology. Administration of probiotic-derived EVs can reverse gut dysbiosis and attenuate neuroinflammation in a dose-dependent manner [123]. Furthermore, the therapeutic landscape is being redefined by bioengineered MEVs. Through techniques such as genetic modification of bacterial strains or post-isolation loading via electroporation, these vesicles can be tailored to deliver neuroprotective agents with high specificity, bypassing the risks associated with live bacterial administration. To enhance specificity, MEVs can be engineered using CRISPR/Cas9 genetic modification of the parent strain or post-isolation methods like electroporation to load specific neuroprotective agents or miRNAs [124]. These engineered vesicles serve as versatile, non-cell-based delivery systems that can bypass the risks associated with live bacterial administration. MEVs work in concert with host-derived EVs (e.g., from mesenchymal stem cells or astrocytes) to promote hippocampal neurogenesis and synaptic preservation. The complexity of TBI necessitates a systems-level approach. The integration of MEV profiling with multi-omics, data genomics, transcriptomics, proteomics, and metabolomics, allows for a deeper understanding of the molecular subtypes of injury. The future of precision medicine in neurotrauma care lies in the systematic integration of MEV profiling with high-dimensional multi-omics data, including genomics, proteomics, and metabolomics. The application of artificial intelligence (AI) and machine learning to these vast datasets allows for the identification of biological subtypes of TBI and the prediction

of long-term outcomes, such as the risk of developing chronic traumatic encephalopathy or AD [125]. Artificial intelligence and machine learning algorithms can analyse these vast datasets to identify predictive biomarkers and identify individuals at risk for long-term neurodegeneration, such as chronic traumatic encephalopathy [126]. This holistic framework facilitates the development of personalized diagnostic and therapeutic strategies based on an individual's unique microbiome profile and immune status. Microbiota-derived extracellular vesicles represent a transformative nexus between microbiology, neuroscience, and precision medicine. Their role as systemic mediators of the gut–brain axis offers unprecedented opportunities for the diagnosis and treatment of TBI. However, clinical translation requires the standardization of isolation protocols and large-scale longitudinal validation studies. By leveraging AI and multi-omics integration, MEVs are poised to refine our ability to decode gut–brain communication, moving neurotrauma care toward individualized and highly effective patient interventions. By decoding the complex gut–EV–brain interactions, clinicians may soon be able to implement real-time, personalized interventions tailored to an individual's unique microbiome profile and immune status, ultimately improving the long-term cognitive and neurological recovery of patients affected by TBI (Figure 3) [127].

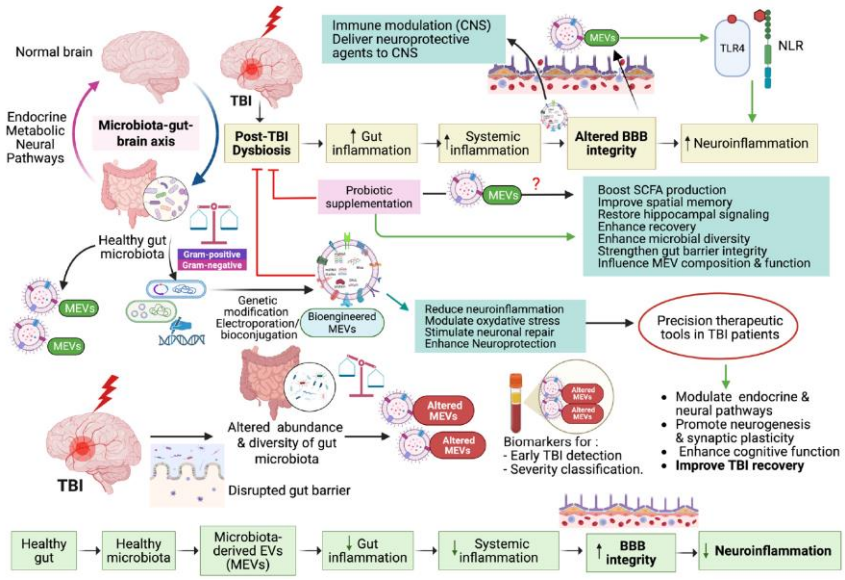

Figure 3. The potential role of MEVs in the bidirectional communication via the GMBA in TBI. Benameur T, Hasan A, Toufig H, Panaro MA, Filannino FM, Porro C. *Microbiota-Derived Extracellular Vesicles as Potential Mediators of Gut-Brain Communication in Traumatic Brain Injury: Mechanisms, Biomarkers, and Therapeutic Implications.* Biomolecules. **2025** Sep 30;15(10):1398. doi: 10.3390/biom15101398. PMID: 41154627; PMCID: PMC12564496.

1.3 Endogenous and Exogenous Compounds

Bioactive natural substances represent a rapidly expanding field of research for the treatment and prevention of various CNS diseases, from NDs to brain tumors such as glioblastoma [128]. These compounds, derived primarily from plants, offer a multitargeted therapeutic approach characterized by a generally favorable safety profile and the ability to simultaneously modulate multiple pathological mechanisms. Polyphenols represent a broad class of antioxidant compounds found in many plant foods that exert

immunomodulatory effects through their ability to regulate redox balance and inflammatory signaling networks [129].

Resveratrol, a stilbene polyphenol found in grapes, berries, and red wine, activates sirtuins, proteins involved in cellular regulation, and has antioxidant and anti-inflammatory properties. This compound reduces inflammation and free radicals, facilitates the elimination of beta-amyloid peptides, inhibits the GSK-3 enzyme, decreases the amount of phosphorylated tau protein in the brain, enhances cholinergic neurotransmission, and increases levels of BDNF. Preclinical studies have shown that resveratrol improves mitochondrial function and reduces beta-amyloid levels, suggesting therapeutic potential in the management of AD [130].

Vitamins and minerals are essential nutrients with potential benefits for cognitive health. Vitamin E serves as a powerful free radical scavenger, protecting neurons from the oxidative damage that contributes to neurodegenerative diseases. Clinical studies have shown that high doses of vitamin E can slow the progression of AD in certain patient populations [131]. Zinc plays a crucial role in various enzymatic processes and has antioxidant properties. Adequate zinc levels can help modulate the metabolism of beta-amyloid peptide and potentially reduce the risk of AD [132].

Lactoferrin is an iron-binding glycoprotein present in biological fluids such as milk, saliva, tears, and nasal and bronchial secretions. It is also found in the secondary granules of neutrophils and is released in response to inflammatory stimuli. Chemically, lactoferrin is a protein of approximately 80 kDa with the ability to bind two ferric ions per molecule, performing antimicrobial, antiviral, antioxidant, and immunomodulatory functions. In the CNS,

lactoferrin readily crosses the blood-brain barrier via receptor-mediated transcytosis and is locally released by macrophages and astrocytes in response to inflammatory triggers. The study "*Lactoferrin Attenuates Pro-Inflammatory Response and Promotes the Conversion into Neuronal Lineages in the Astrocytes*" demonstrates that lactoferrin exerts anti-inflammatory effects on DI-TNC1 astrocytes stimulated with LPS. Astrocytes are glial cells essential for neuronal support, maintenance of the blood-brain barrier, and modulation of synaptic activity. At the molecular level, lactoferrin acts on multiple pathways. It significantly reduces the expression of TLR4, a key receptor in the inflammatory response that recognizes pathogen-associated molecular patterns. It also decreases the expression of glial fibrillary acidic protein (GFAP), a marker of astroglial reactivity, and the pro-inflammatory cytokine IL-6. Simultaneously, it upregulates the expression of the transcription factor NRF2, involved in regulating antioxidant and anti-inflammatory responses, and increases levels of interleukin-10 (IL-10), an anti-inflammatory cytokine. A particularly innovative aspect of the study concerns lactoferrin's ability to promote the reprogramming of reactive astrocytes into proliferative neuroblasts. This effect is mediated by overexpression of the transcription factor SOX2, known for its role in cellular reprogramming. SOX2 can convert astrocytes into highly proliferative neuroblasts, which can subsequently differentiate into mature neurons when provided with neurotrophic factors. The study demonstrates that pretreatment with lactoferrin induces elevated SOX2 expression as early as 2 days after treatment, resulting in increased cell proliferation. Subsequently, after 9 days, expression of doublecortin (DCX), a neuroblast-specific

marker, and TUJ-1, a marker of early postmitotic neurons, is observed. At 16 days, cells pretreated with lactoferrin show a significant reduction in astroglial markers (GFAP and S100β) and an increase in the expression of neuronal markers such as neurofilament 68 and NeuN, acquiring neuron-like morphology. The observed effects suggest that lactoferrin not only attenuates neuroinflammation by reducing astroglial reactivity but also promotes neurogenesis through cellular reprogramming. This mechanism could represent a promising therapeutic strategy for neurodegenerative diseases, where progressive neuronal loss is accompanied by chronic neuroinflammation. Furthermore, lactoferrin does not exhibit significant tumorigenic potential, as demonstrated by comparison with U-87 MG glioblastoma cells, which exhibit significantly different doubling times and specific growth rates. These findings open new perspectives for the application of lactoferrin as a neuroprotective and pro-neurogenic agent in neurodegenerative diseases [133].

Curcumin is a natural polyphenolic compound extracted from the rhizome of Curcuma longa Linn, a perennial plant of the Zingiberaceae family. Chemically, curcumin has the formula $C_{21}H_{20}O_6$ and a molecular weight of 368.38 g/mol, with the IUPAC name (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. It is characterized by a chromanol ring and methoxy and hydroxyl groups that give it potent antioxidant properties, allowing it to neutralize reactive oxygen and nitrogen species. The primary mechanism of action of curcumin in the nervous system involves inhibition of the NLRP3 inflammasome, a multiprotein complex expressed in the cytoplasm of various immune and non-immune cells.

The inflammasome is composed of the protein NLRP3, the adaptor protein ASC, and pro-caspase-1. Its activation requires two signals: the first (priming) is triggered by the activation of TLRs or TNF signaling, which leads to the transcriptional activation of NF-κB and the production of precursor forms of IL-1β and IL-18. The second signal is induced by various PAMPs and DAMPs, including extracellular ATP, pore-forming toxins, RNA viruses, and particulate matter. Curcumin acts at multiple points in this pathway. It inhibits the NF-κB signaling pathway, reducing the transcription of inflammasome components. It blocks the activation of TLR4 and subsequent MyD88/NF-κB signaling, preventing the priming phase. It reduces oxidative stress and the production of reactive oxygen species (ROS), which are triggers for inflammasome activation. It also modulates calcium signaling and prevents mitochondrial dysfunction, cellular events proposed to trigger NLRP3 activation. In the brain, curcumin has demonstrated neuroprotective effects in several disease models. In kainic acid-induced epilepsy, it reduces the expression of NLRP3, activated caspase-1, and IL-1β in the hippocampus, decreasing neuronal loss and improving spatial learning and memory. In ischemic brain injury, curcumin attenuates microglial pyroptosis (inflammasome-dependent pro-inflammatory cell death) through suppression of NF-κB signaling and direct inhibition of NLRP3, reducing post-stroke white matter damage. In AD, nanoparticles containing curcumin and superparamagnetic iron oxide reduce β-amyloid plaques in APP/PS1 transgenic mice, attenuate microglial activation, and decrease inflammation associated with amyloid plaques by directly targeting NLRP3. Curcumin also reduces IL-18, CD68 (a marker of activated microglia), and NLRP3

itself, suggesting a specific action on inflammasomes associated with microglia and neurons. In neuropathic pain, curcumin reduces NALP1 inflammasome activation in spinal astrocytes, suppressing IL-1β production and alleviating pain sensitization. Despite its potent biological activity, curcumin has low bioavailability due to its poor aqueous solubility, rapid metabolism, and limited chemical stability, which limit its therapeutic use. To overcome these problems, nanocarrier systems such as nanoparticles, liposomes, and micelles have been developed that significantly improve the bioavailability and therapeutic efficacy of curcumin in the CNS. In AD, curcumin has been shown to reduce the accumulation of beta-amyloid plaques, decrease inflammation, and reduce oxidative stress. Animal studies have shown that curcumin can improve cognitive function and reduce the amount of amyloid plaques, although its low bioavailability represents a significant limitation for clinical application. In glioblastoma, curcumin exhibits radiosensitizing effects in combination with irradiation and temozolomide in cell lines, reducing proliferation and increasing G2/M arrest and apoptosis at comparatively low micromolar levels. These radiosensitizing effects have been replicated with different radiation modalities and are consistently attributed to interference with survival pathways intersecting NF-κB and related nodes [134].

Alpha-tocopherol, commonly known as vitamin E, belongs to a family of fat-soluble compounds that includes four tocopherols (α, β, γ, and δ) and four tocotrienols. Structurally, vitamin E is characterized by a chromanol ring and an isoprenoid or phytyl side chain. Tocopherols have a long, saturated side chain, while tocotrienols differ in the presence of unsaturated double bonds on the

side chain, giving the latter a greater affinity for lipid membranes. Alpha-tocopherol (DL-α-tocopheryl acetate) is the most biologically active form and is the only one that prevents ataxia with vitamin E deficiency (AVED), a rare inherited neurodegenerative disease. Vitamin E is naturally present in plant-based foods such as cereals, legumes, seeds, and seed oils, and in some animal-based foods such as milk, dairy products, butter, egg yolk, and some fish products. In the CNS, vitamin E plays a key role as a fat-soluble antioxidant, protecting cell membranes from oxidative damage caused by free radicals. The brain, with its high oxygen consumption and abundance of lipids, is particularly vulnerable to oxidative stress, making vitamin E essential for neuronal protection. This study demonstrates that α-tocopherol exerts neuroprotective and anti-inflammatory effects on BV2 microglial cells activated with LPS. Microglia are the resident macrophages of the CNS and the main mediators of the neuroinflammatory response. Under physiological conditions, microglia exhibit a ramified morphology with a small central cell body and numerous extensions. In response to pro-inflammatory stimuli such as LPS, microglia are activated, assuming an amoeboid morphology with an enlarged soma and reduced elongations, characteristic of the pro-inflammatory M1 phenotype. At the molecular level, α-tocopherol acts on multiple inflammatory pathways. It significantly reduces the expression of TLR4, the main LPS receptor that triggers the inflammatory cascade. This leads to a decrease in the activation of the PI3K/Akt signaling pathway, a crucial pathway for cell survival and the production of inflammatory mediators. α-Tocopherol also reduces the expression of CD40, a molecule from the TNFR family expressed primarily by

macrophages, microglia, and dendritic cells, which mediates pro-inflammatory immune responses through interaction with its ligand. The anti-inflammatory effects of vitamin E are also manifested by reducing the production of pro-inflammatory cytokines such as TNF-α and increasing the production of IL-10, an anti-inflammatory cytokine responsible for modulating and regulating the immune response. Simultaneously, α-tocopherol increases the expression of CD206 (mannose receptor), a marker of the anti-inflammatory M2 phenotype of microglia, suggesting reprogramming toward an anti-inflammatory and reparative state. α-Tocopherol preserves the typical branched morphology of microglia in the physiological state, prevents the transition to the amoeboid morphology induced by LPS, and significantly reduces the migratory capacity of activated microglia. These morphological and functional effects are correlated with the reduction of oxidative stress, as vitamin E neutralizes free radicals by donating hydrogen atoms and stabilizing them, thus protecting cell membranes from peroxidative damage. The results suggest that α-tocopherol may have therapeutic applications in NDs characterized by chronic neuroinflammation, such as AD and PD. Modulating microglial activation represents a promising strategy for preventing neuronal damage secondary to persistent inflammation. However, the study has limitations related to the use of an *in vitro* model with immortalized cells, requiring further research in primary cultures or animal models to confirm these findings and evaluate the therapeutic efficacy of vitamin E *in vivo* [135].

These interventions have not been presented as stand-alone treatments but rather as adjuvant and chemopreventive agents that complement surgery, radiotherapy, and chemotherapy with

comparatively low toxicity. In conclusion, bioactive natural substances represent a promising and multifaceted approach to address CNS pathologies, from neurodegeneration to brain tumors. Their ability to simultaneously modulate multiple pathological pathways, combined with a generally favorable safety profile, positions them as low-toxicity complements to standard therapies. However, successful clinical translation requires optimization of dosing, formulation, and identification of patients who could benefit most from these approaches, as well as rigorously designed clinical trials that confirm the efficacy observed in preclinical models [136].

1.3.1 Irisin

Irisin is a myokine derived from the proteolytic processing of the transmembrane protein FNDC5, encoded by the FNDC5 gene located on the short arm of chromosome 1 [137]. It is primarily known for its metabolic functions, such as stimulating the browning of white adipose tissue, which promotes increased energy expenditure and improved insulin resistance in obesity. FNDC5 has a complex structure with an N-terminal signal peptide (amino acids 1–28), an FNIII domain (amino acids 33–124), a transmembrane domain (150–170), and a cytoplasmic tail (171–209) [137]. Its maturation requires two proteolytic cleavages: one between the signal peptide and the ectodomain, and the other between the ectodomain and the transmembrane domain, probably mediated by ADAM10 in muscle cells [138]. Irisin itself is a 112-amino acid peptide with a mass of 12.6 kDa, characterized by an FNIII-like domain with a beta-sandwich structure composed of three beta-strands on one side and four on the other, which gives it stability and the ability to interact

with receptors such as integrin αV/β5 in astrocytes and microglia [138]. Its secretion is primarily induced by aerobic exercise, which activates PGC-1α in skeletal muscle, the main regulator of FNDC5, although plasma levels vary between species and measurement methods, with gold-standard values by mass spectrometry of 3–4 ng/mL in humans and 0.3 ng/mL in mice, while ELISA shows heterogeneity due to nonspecific binding. FNDC5 and irisin are expressed in various tissues, including the heart, muscle, kidney, and brain (hypothalamus, cerebral cortex, cerebellum, and olfactory bulbs). Immunodetection is found in the cytoplasm of Purkinje cells in the cerebellum, pituitary, and pineal gland, confirming their intrinsic presence in the CNS [139].

In neuroinflammation, irisin exerts neuroprotective effects by modulating key inflammatory pathways and reducing the activation of microglia and astrocytes, the primary mediators of the immune response in the neuronal microenvironment. Neuroinflammation involves the secretion of proinflammatory cytokines such as IL-1β, IL-6, IL-33, and TNF-α from activated microglia, which adopt an amoeboid morphology with retracted branches, as well as astrogliosis with increased extracellular matrix proteins, triggering cascades such as NF-κB via IL-1R1 or pathological pathways like JAK-STAT, PI3K-Akt, MAPK, and TLR [140]. Irisin counteracts this by inhibiting NF-κB and TLR4/MyD88 in cerebral ischemia models, reducing phosphorylation and the release of proinflammatory cytokines, as shown in pretreatments with 100 nM on LPS-treated macrophages or in oxygen-glucose-deprived PC12 cells, where it decreases ROS, IL-1β, IL-18, and apoptosis via ROS-NLRP3 inhibition. In LPS-treated BV2 microglia or FNDC5 knockout models, it promotes the anti-

inflammatory M2 phenotype, reducing Iba1 and proinflammatory polarization via integrin αV/β5-AMPK, as seen in intracerebral haemorrhage, where it increases p-AMPK and Bcl-2 while reducing IL-1β, TNF-α, MPO, and Bax [141].On astrocytes, irisin pretreatment protects neurons from Aβ toxicity by reducing IκB expression and phosphorylation, thereby preventing NF-κB release, and it inhibits propofol-induced IL-1β/IL-6 cytokines or, in 3D AD models, increases neprilysin (NEP) for Aβ degradation via downregulation of ERK-STAT3 [142].

These mechanisms extend to antioxidant and pro-neurotrophic pathways: it activates Nrf2 to suppress ferroptosis and oxidative stress in septic encephalopathy, cAMP-PKA-CREB for BDNF and synaptic plasticity in hippocampal slices (25 nM in humans, 75 pmol in rodents), improving memory in AD models, and PI3K-Akt-mTOR or Akt-ERK1/2 to reduce infarction and apoptosis in middle cerebral artery occlusion [143]. It also inhibits STAT3-p38 in murine diabetes, reducing hippocampal GFAP, IL-6/IL-1β, and MMP-9 in ischemia-reperfusion while preserving the blood-brain barrier. In PD, it regulates Akt-ERK1/2 via integrins to promote mitochondrial biogenesis, reducing apoptosis and phosphorylated α-synuclein, while improving nigral TH+ neuron counts and motor performance in PFF-MPTP models [144]. Exercise, which elevates irisin, treadmill 10–18 m/min or swimming 1 h/5 d/week, increases hippocampal BDNF/FNDC5, neuronal proliferation, and reduces immobility in depressive behavioural tests via the ERRα-PGC1-FNDC5-BDNF pathway. Despite controversies over human FNDC5 translation (ATA versus conserved ATG codon in exon 1), irisin crosses the BBB, offering therapeutic opportunities for AD, PD, ischemia, diabetes, and

early stress, though challenges remain in standardizing doses, human exercise protocols, and reliable measurements [145].

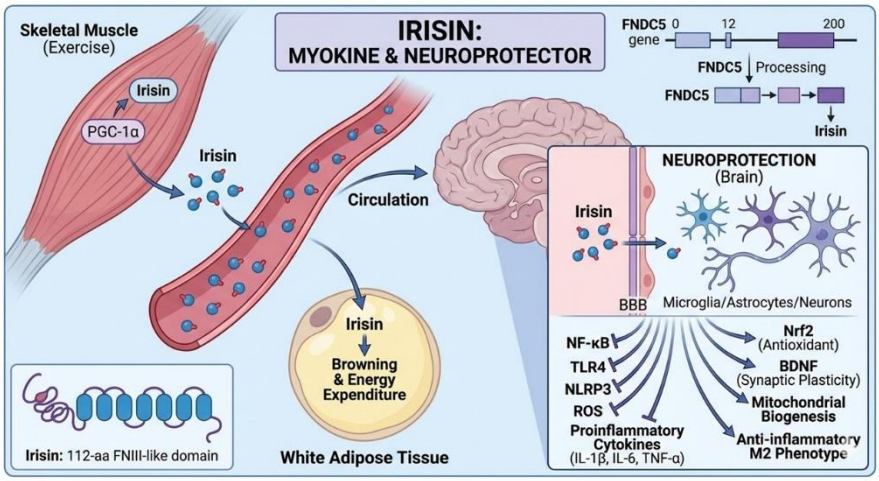

Figure 4. Irisin in CNS

1.3.2 Chrysin

Chrysin, scientifically known as 5,7-dihydroxy-2-phenyl-4H-chromen-4-one, is a natural flavonoid belonging to the flavone class, characterized by a typical chemical structure that includes two fused benzene rings (rings A and C) connected by a pyrone bridge and a third phenyl ring (B) in position 2 [146]. Its molecular formula is C15H10O4, with a molecular weight of approximately 254.24 g/mol, and it is distinguished by the two hydroxyl groups in positions 5 and 7 on ring A, which confer high polarity and reactivity, making it soluble in organic solvents such as DMSO but poorly water-soluble, a limitation that affects its bioavailability *in vivo* [146]. This structural configuration allows it to interact with multiple biochemical pathways, exerting antioxidant effects thanks to its ability to donate

electrons and neutralize ROS, as well as anti-inflammatory properties that make it promising in the context of neuroinflammation, a central pathological process in NDs such as AD, PD, and MS, as well as in CNS lesions [147]. Originally isolated from the flowers of Passiflora caerulea and incarnata, chrysin is abundant in acacia honey, up to 50% of secondary metabolites, propolis, and mushrooms such as Pleurotus ostreatus, as well as in plants such as Robinia pseudoacacia. Neuroinflammation, mediated by the hyperactivation of glial cells, such as microglia and astrocytes, leads to the release of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, activation of iNOS and COX-2 enzymes, and accumulation of ROS, culminating in neuronal damage, oxidative stress, and apoptosis [148]. Chrysin counteracts these events by primarily inhibiting the NF-κB pathway, a key transcription factor that, once activated by stimuli such as LPS or trauma, translocate to the nucleus, inducing gene expression of inflammatory mediators. In models of cerebral ischemia, spinal cord injury, TBI, and PD, oral doses of 10–200 mg/kg significantly reduce levels of NF-κB p65, TNF-α, IL-1β, IL-6, and IL-17, preserving inflammatory homeostasis without altering anti-inflammatory cytokines such as IL-10 in some contexts. A key mechanism emerges in the modulation of kinase pathways: chrysin suppresses the activation of MAPK, p38, JNK, ERK, PI3K/Akt/mTOR, and JAK/STAT, reducing the phosphorylation of IKK-β, which degrades IκBα, maintaining inactive NF-κB in the cytoplasm [149]. In BV2 microglial cells exposed to LPS/IFN-γ, concentrations of 1–20 μM inhibit NO, iNOS, COX-2, and cytokines by blocking p-JNK and p-p38. In *in vivo* models of experimental MS, 20 mg/kg for 25 days attenuates CD4+ inflammatory infiltrates in the

spinal cord, reducing IFN-γ and TNF-α in the hippocampus, prefrontal cortex, and spleen. Similarly, in hepatic encephalopathy caused by thioacetamide or chronic stress, it normalizes GFAP, NF-κB, and cytokines, improving cognitive and behavioural functions. Its antioxidant effect amplifies these benefits: it increases SOD, GPx, catalase, and GSH, reducing MDA and lipid peroxidation, while activating Nrf2/HO-1 to counteract ROS in ischemia-reperfusion injury [150].

From an anti-apoptotic perspective, chrysin rebalances Bax/Bcl-2, downregulates caspase-3, and upregulates BDNF/NGF, preventing neuronal death in PD and D-galactose-induced aging. Studies on diabetic rats with MCAO show a reduction in MPO and an increase in IL-10 post-reperfusion. Despite efficacy in 21 *in vivo* and 4 *in vitro* models, the review highlights gaps: poor bioavailability, with limited solubility, rapid hepatic metabolism, lack of human clinical studies, and data on chronic toxicity [151]. Typical doses range from 5–250 mg/kg orally (92% intragastric), delivered in DMSO/PBS, PG, or CMC, with effects observed in the hippocampus, striatum, SNpc, and spinal cord. Chrysin also mediates estrogen dependence (via ERα/β), as seen in IR where ER antagonists negate the benefits on IL-1/TNF-α [152]. Chrysin emerges as a promising neuroprotective agent among natural anti-inflammatory compounds due to its role in countering chronic glial neuroinflammation, a common driver of NDs and CNS lesions, positioning it as a candidate for adjuvant therapies. Despite these encouraging results, poor bioavailability, due to hydrophobic solubility and rapid hepatic metabolism, reduces effective plasma concentrations, requiring

advanced formulations such as PLGA nanoparticles or liposomes to improve absorption [153].

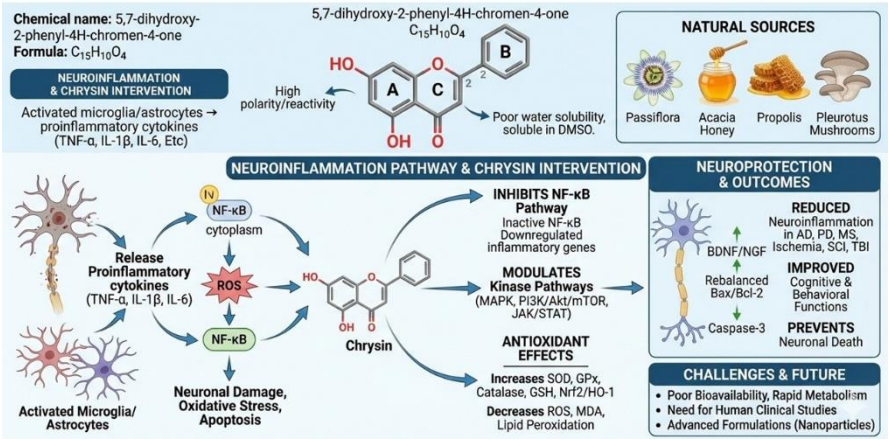

Figure 5. Chrysin in CNS

2. AIMS

This study investigates innovative strategies for modulating microglia-mediated neuroinflammation, exploring two complementary approaches with potential therapeutic applications in NDs in order to contrast aging.

2.1 Preventive medicine using a myokine with anti-inflammasome effects at physiological doses, combining a non-pharmacological intervention such as physical exercise with direct therapeutic options

The first objective focuses on evaluating the neuroprotective and anti-inflammatory effects of irisin, a myokine released during exercise, with particular emphasis on its ability to modulate the NLRP3 inflammasome. The aim of the study is to demonstrate that irisin, even at physiological concentrations, can reduce LPS-induced microglial activation by analyzing the expression of components of the inflammatory NLRP3 protein, pro-inflammatory cytokines, and promoting polarization toward the anti-inflammatory M2 phenotype. The study aims to provide scientific evidence on the mechanisms through which physical activity protects against neurodegenerative diseases, outlining a non-pharmacological preventive approach. Exercise is a safe and accessible intervention to reduce the risk of dementia and neurodegeneration, addressing multiple therapeutic targets as irisin can be used directly as a therapeutic agent at low physiological doses.

2.2 Biological nanotechnology using a natural delivery system with a high anti-inflammatory load

The second objective aims to develop and characterize chrysin-loaded extracellular vesicles (Chry-EVs) derived from BV2 microglial cells, to overcome the bioavailability limitations of this natural flavonoid. Chrysin, despite having demonstrated anti-inflammatory properties, has extremely low oral bioavailability (0.003-0.02%) and requires high doses that could be toxic. The aim is to evaluate the ability of Chry-EVs to attenuate LPS-induced neuroinflammation by analyzing the effects on cell morphology, proliferation, migration, and expression of pro-inflammatory cytokines. Particular attention will be paid to modulating inflammatory activity and regulating anti-apoptotic proteins, aiming to demonstrate the neuroprotective potential of this innovative delivery system. The research uses microglia-derived extracellular vesicles instead of synthetic nanoparticles, leveraging the natural targeting capabilities of EVs. Furthermore, EVs can cross the BBB more efficiently than synthetic systems. Furthermore, Chry-EVs not only deliver the chrysin but also contain endogenous anti-inflammatory molecules that enhance the therapeutic effect.

These studies significantly contribute to the understanding of the neuroinflammation and offer innovative strategies for the treatment or prevention of neurodegenerative diseases, with particular emphasis on approaches that exploit natural biological mechanisms.

3. METHODS

3.1 Microglial Cell Culture

In this study, the BV2 murine microglial cell line (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Euroclone), 100 U/mL penicillin, 100 µg/mL streptomycin, (penicillin–streptomycin; Euroclone), and 2 mM glutamine (glutamine; Euroclone). Cultures were incubated at 37 °C in a humidified incubator with 5% CO2. Adherent BV2 cells were detached using Trypsin-EDTA (1X in PBS; Euroclone) and replated at the desired density. For the cell migration and morphological assays, cells were seeded into six-well plates. For viability assay, cells were seeded into 24-well plates and incubated for 24 h. Following incubation, they were divided into experimental groups.

3.2 Irisin Solution Preparation

Irisin (Purity ≥ 95% (HPLC); Phoenix Pharmaceuticals Inc., Burlingame, CA, USA; CAS: 067-16) was initially prepared by dissolving it in DMSO (dimethyl sulfoxide, cell culture reagent; MP Biomedicals) to achieve a 1 M concentration. For the subsequent experiments, irisin was diluted in DMEM to prepare different dilutions from the stock solution.

3.3 Cell Culture and Treatments with Irisin

The cells were divided into experimental groups: one group of cells was treated with 5 nM irisin, while another with 1 µg/mL LPS from Escherichia coli O128: B12 (Sigma-Aldrich, St. Louis, MI,

USA). A third group of cells was pre-incubated with irisin and LPS for 1 h. The cells were grown and collected after 24 h.

3.4 Chrysin Solution Preparation

Chrysin (Purity ≥ 99.4% (HPLC); MP Biomedicals) was initially dissolved in DMSO (dimethyl sulfoxide, cell culture reagent; MP Biomedicals) to prepare 1M stock solution. For experimental purposes, this stock solution of chrysin was diluted in DMEM to prepare the desired working concentrations. To assess the *in vitro* effects of chrysin, cells were pre-incubated with chrysin diluted in DMEM for up to 24 h.

3.5 EVs Production with Chrysin and Isolation

The murine BV2 microglial cell line was used for the production of EVs. Cells were seeded and cultured to approximately 80% confluence as previously described, then treated with Chrysin at concentrations of 5 μM and 20 μM for 24 h. Following incubation, culture supernatants from both treated and untreated cells were collected and centrifuged at 1500× g for 10 min to remove cells and large debris. EVs were then isolated through successive centrifugation at 14,000× g for 45 min. The obtained EV pellets were washed and resuspended in 400 μL of (0.9% *w*/*v*) NaCl solution. The final supernatant from the washing step, which was free of EVs, served as the control (vehicle). EV concentration was determined by quantifying the total EV-associated proteins using the Qubit Protein Assay Kit with the Qubit® 2.0 Fluorometer (Life Technologies, Eugene, OR, USA). (Figure 6).

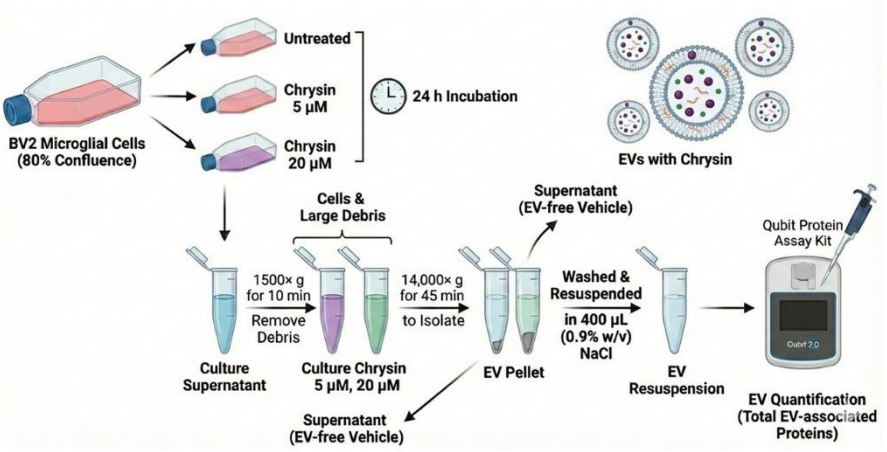

Figure 6. EVs production and isolation protocol.

3.6 EVs Analysis

The isolated exosomes were characterized by nanoparticle tracking analysis (NTA) using the ZetaView system (Particle Metrix, Meerbusch, Germany). The particle size distribution and concentration were analysed using the ZetaView instrument and its accompanying software. The system measures particle diameters within a range of 0–500 nm. Samples were diluted 1:1 with 1 × PBS to loading the measurement chamber. Data were collected from 11 distinct positions within the cell, with two measurement cycles performed for each sample. Nanoparticle tracking analysis (NTA) was conducted under standardized conditions: a temperature of 23 °C, sensitivity set to 85.0, a frame rate of 30 frames per second, and a shutter speed of 100. The total vesicle protein content was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

3.7 Cell Culture and Treatments EVs-loaded Chrysin

The BV2 cells were divided into six experimental groups: CTR without treatment substance, 1 μg/mL LPS from Escherichia coli O128: B12 (Sigma-Aldrich, St. Louis, MI, USA), 20 μM ,Chrysin, EVs-Chry (10 μg/mL), EVs-CTR (10 μg/mL), and group pre-incubated with EVs-Chry (10 μg/mL) and LPS for 1 h. The cells were grown and collected after 24 h.

3.8 Cell Proliferation Assay

The cytotoxic effects of chrysin on BV2 cells were evaluated using the CyQUANT® Cell Proliferation Assay (Thermo Fisher), a fluorescence-based method for quantifying cells and assessing cell proliferation and cytotoxicity. The BV2 cells were seeded in 96-well plates at a density of 5×10^4 cells per well and incubated at 37 °C in a humidified atmosphere with 5% CO2 for 24 h. The initial chrysin concentrations used to assess cytotoxicity were 5 μM, 10 μM, 20 μM and 50 μM. Then the CyQUANT® Assay was conducted using 5 μM and 20 μM of chrysin, both in the absence and presence of LPS, for 24 h. Fluorescence was measured using CLARIOstar® Plus plate reader (BMG LABTECH SARL, Champigny s/Marne, France) with excitation/emission filters set to ~480 nm and ~520 nm, respectively.

3.9 Cell Viability Assay

The cytotoxic effects of irisin on BV2 cells was assessed using an MTT assay, with Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich (CAS: 298-93-1)). The BV2 cells were seeded in 24-well plates at a density of 2 × 105 cells and maintained at 37 °C with 5%

CO2 for 24 h. The initial irisin concentrations used to assess cytotoxicity were 5 nM, 10 nM, and 20 nM. Based on the results, the lowest effective concentration was selected to further investigate the potential protective effects of irisin. The MTT assay was then conducted using 5 nM of irisin, both in the absence and presence of 1 µg/mL LPS, for 24 h. The cells were then treated with different stimuli for 24 h, including EVs-CTR (10 µg/mL) and LPS (1 µg/mL) for 24 h. Absorbance was measured using a spectrophotometer (Filter Max F5 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA, USA) set to a wavelength of 595 nm. The results are expressed as a percentage (%) of cell viability relative to the control condition (Figure 7).

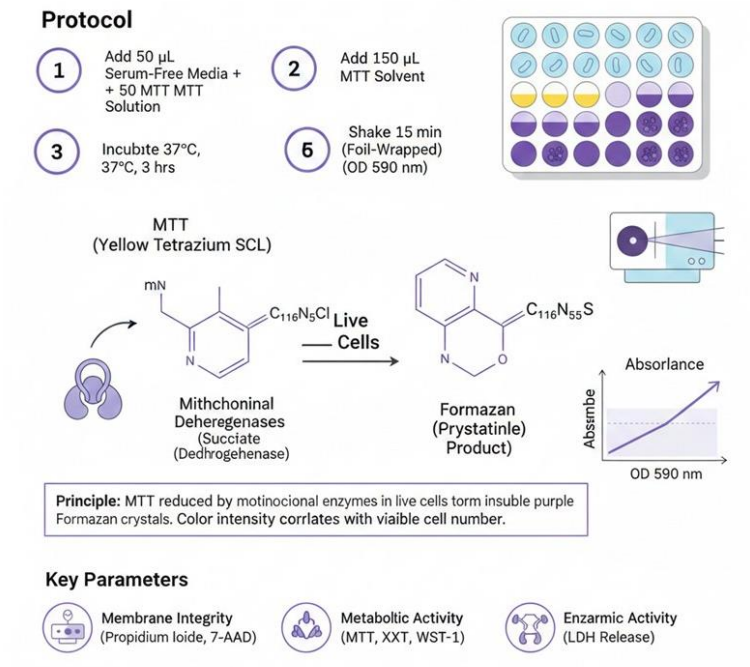

Figure 7. The MTT assay is a standard colorimetric assay for measuring the activity of enzymes that reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazan, giving the substance a blue/purple

colour. The mitochondrial enzyme succinatedehydrogenase (reductase) is active only in living cells and its function is to cleave the tetrazolium ring of MTT(yellow substance), thus forming formazan (blue).

3.10 Cell Morphology

Microglial morphology was analysed by measuring the cell body area to determine the effect of EVs-Chry or Irisin on BV2 cells' activation, both in the absence and presence of the pro-inflammatory stimulus LPS. Approximately 5×10^5 cells were seeded into six-well plates. All morphological analyses were performed in triplicate. The results are presented as the mean cell body area, calculated from five independent experiments, with three cells analysed per sample in each experiment. Morphological assessments were performed using a Leica DM IRB microscopy system (Leica Microsystems GmbH, Wetzlar, Germany) at 10× and 20× magnifications. Cell body areas (μm2) were quantified using ImageJ software version 1.8.0 with 64-bit Java 8 support.

3.11 Wound Healing Assay

To evaluate two-dimensional cell migration, a wound healing assay was performed using BV2 cells. A total of (1×10^6) cells were seeded into six-well plates and cultured until reaching confluence. A uniform scratch was created in the cell monolayer using a sterile scraper. Following PBS washing and replacement with fresh DMEM, the remaining cells were incubated for 24 h under various conditions: chrysin, EVs-Chry or Irisin or LPS treatment. All migration assays were conducted in triplicate. After 24 h, three representative images were captured per wound area for each condition using phase-contrast microscopy. Wound repair was quantified using ImageJ software and

expressed as the percentage of the initial wound area covered by migrating cells, relative to the initial wound size at time zero (the start of the assay).

3.12 Protein Extraction and Western Blot Analysis

Following the aforementioned treatments, the BV2 cells were detached from the plate by gentle scraping and collected after centrifugation at 2000 rpm for 10 min at 4 °C. The cells were then lysed using an ice-cold lysis buffer consisting of 50 nM Tris-HCl (pH8), 1% (v/v) Triton X-100, 1.5 M NaCl, 0.1% SDS, 1 μM leupeptin hemisulfate salt, 100 μM phenylmethylsulfonyl fluoride (PMSF), and 4 U/mL aprotinin (Sigma Aldrich).The lysates were collected by centrifugation at 12,000 rpm for 30 min at 4 °C after eight cycles of freezing and thawing.

A Western blot was performed as previously described, with the following primary antibodies: CD14 (1:500), IL-1β (1:500), caspase-1 (1:500), Arginase 1 (1:500), and β-actin (1:500) (Santa Cruz Biotechnology, Inc. Heidelberg, Germany). β-actin was used to normalize the protein expression levels. Another set of primary antibodies was used; this included anti-NLRP3 (EPR23094-1, (1:1000)) and anti-TMS1/ASC (EPR10403, 1:1000) (Abcam); CD40 (1:500), p-Akt (1:500), CD206 (1:500), TLR4 (1:500), and p-IKBα (1:500) (all from Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The membranes were incubated with the primary antibodies for 1 h at room temperature and then overnight at 4 °C before being incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, (1:10,000)) for 1 h at room temperature. Immunoreactive bands were

detected using chemiluminescence (BioRad Laboratories, Hercules, CA, USA). The obtained bands were subjected to densitometric analysis using the ImageJ software. The results are expressed in arbitrary units.

3.13 ELISA Quantification of Cytokines

An ELISA test was performed on culture supernatants collected from the morphological assay. After 24 h of incubation, the culture medium was carefully aspirated and centrifuged at 300 *G* for 10 min at 4 °C to remove residual cellular debris. The levels of the pro-inflammatory cytokines IL-6 and IL-1β, TNF-α and anti-inflammatory cytokines such as IL-10 along with the anti-apoptotic protein Bcl-xL, were determined using an ELISA kit, following the manufacturer's protocol (R&D system, a biotech brand; USA). Optical density (OD) was measured at 450 nm using a CLARIOstar® Plus microplate reader (BMG Labtech, Ortenberg, Germany).

3.14 Statistical Analysis

Statistical analyses were carried out using Statgraphics Centurion (Statgraphics Technologies Inc., The Plains, VA, USA). To analyze the data, we used two-sample comparison, one-way ANOVA, and Tukey's post hoc tests. Data from five independent experiments are presented as means ± SDs, with all assays performed in triplicate. Statistical significance was set at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

4. RESULTS

4.1 Irisin Attenuates Neuroinflammation

Irisin Attenuates Neuroinflammation Targeting the NLRP3 Inflammasome.

Filannino FM, Ruggiero M, Panaro MA, Lofrumento DD, Trotta T, Benameur T, Cianciulli A, Calvello R, Zoila F, Porro C. Molecules. 2024 Nov 28;29(23):5623. doi: 10.3390/molecules29235623. PMID: 39683782; PMCID: PMC11643767. Figure 8.

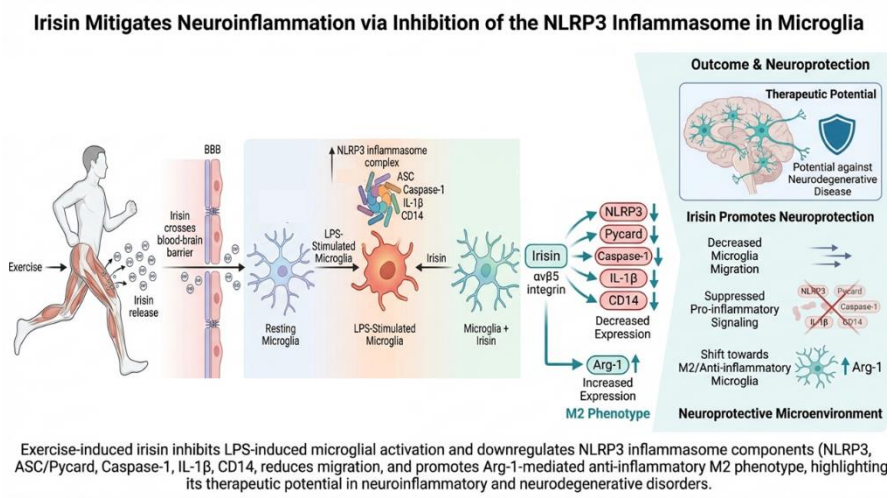

Figure 8. Irisin mitigates Neuroinflammation

4.1.1 Influence of Irisin on BV2 Cells

As shown in (Figure 9A), no cytotoxic effects were detected at all tested concentrations (5–20 nM) of irisin. At concentrations starting of 5 nM, irisin was able to stimulate proliferation. Therefore, the MTT assay was repeated at 24 h with an irisin 24 h with an irisin concentration of 5 nM in the presence or absence of 1 μg/mL LPS

(Figure 9B). Pretreatment with irisin and LPS for 24 h did not significantly affect the viability of BV-2 microglial.

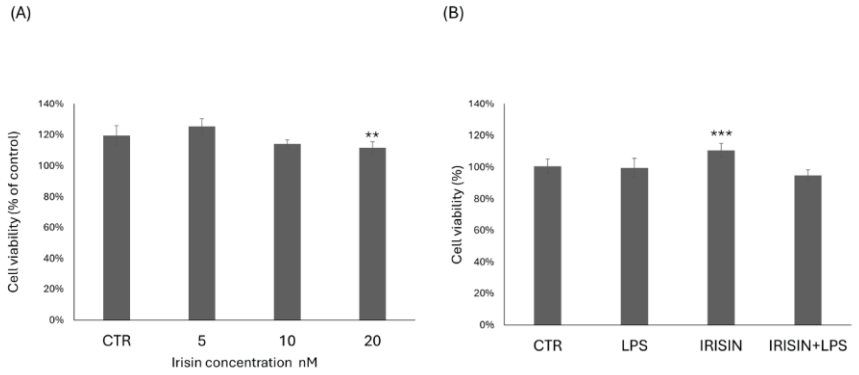

Figure 9. Effect of irisin on cell viability (MTT assay). BV-2 cells were incubated with irisin at concentrations spanning a dose–response curve, ranging from 5 nM to 20 nM (A). Irisin at a concentration of 5 nM was used to treat cells, either in the absence or presence of 1 µg/mL LPS (B). Data are reported as percentages compared to control values and are expressed as means ± SDs. ** $p < 0.01$ compared to the control. *** $p < 0.001$ compared to the control.

4.1.2 Irisin Induces Variability in the Morphology of BV2 Cells

Microglial cells are nervous system-specific immune cells that serve as tissue-resident macrophages. They are able to polarize into different phenotypes and retain the ability to switch functions to maintain tissue homeostasis. The morphological development of microglial cells is characterized by the progressive development of processes and cellular soma. Figure 10A shows that untreated cells, corresponding to the control condition, exhibit a typical microglial morphology in the quiescent state. This includes a small central body and numerous elongated processes. As expected, treatment with LPS (1 µg/mL) (Figure 10B) resulted in BV2 cells acquiring an amoeboid

morphology, which is linked to a pro-inflammatory condition. This was characterized by an increase in their soma and a reduction in their prolongation, highlighting an amoeboid morphology. Treatment with irisin (Figure 10C) did not affect cell morphology with respect to the control. The cells exhibited a branched morphology with a small central soma, which is associated with the resting state. Similarly, in cells treated with irisin and LPS (Figure 10D), irisin was observed to reverse the typical amoeboid phenotype induced by LPS, resulting in greater branching of the distal branches. The analysis of cellular areas (Figure 10E) confirmed the results, revealing that LPS treatment increased the size of BV2 cells, characteristic of the amoeboid form. The cellular area values were significantly higher compared to the control condition. However, when the cells were treated with irisin in the presence of LPS (1 µg/mL), irisin significantly reduced the LPS-induced increase in cellular area.

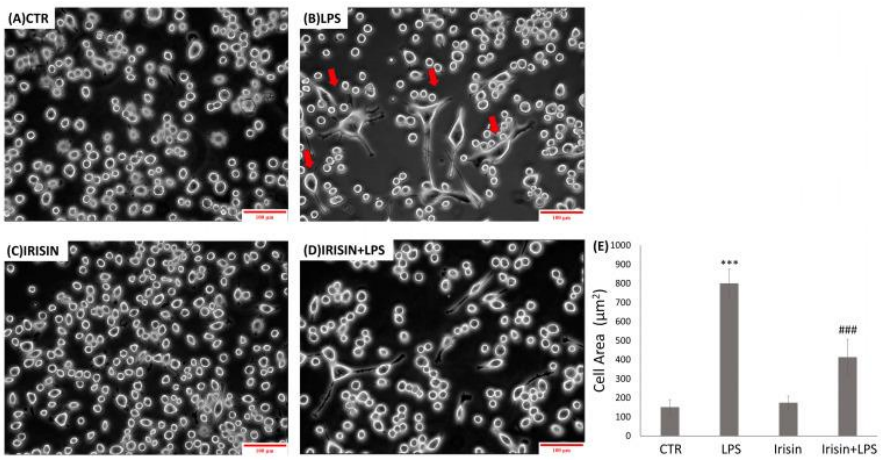

Figure 10. Morphological analysis of the BV2 cells following the administration of irisin, either alone or after LPS stimulation. The morphological analysis was conducted on BV2 cells in the control condition (A) and following the administration of 1 µg/mL LPS (B), 5 nM irisin (C), or 5 nM irisin in the presence of 1 µg/mL LPS (D). The scale bar is 100 µm (10× objective). The arrows in the

images indicate cells that have undergone a morphological change. The cell areas (μm2) were quantified using the ImageJ 1.8.0 software, which was bound with Java 8 64-bit (E). The data are expressed as means ± standard deviations. A significant difference can be observed between the control and LPS groups (*** $p < 0.001$), as well as between the IRISIN + LPS and LPS groups (### $p < 0.001$).

4.1.3 Effect of Irisin on Reducing Cell Migration

A wound closure assay was used to assess the effect on microglial motility after irisin treatment. For the assay, a scratch was made with a scraper, and BV2 cells were allowed to migrate within the scratch during a 24 h incubation period. The fixed and free cell areas were then measured to determine whether irisin stimulation, in the presence or absence of LPS, resulted in wound closure. The results of this experiment are shown in Figure 11. The data indicate that stimulation with LPS resulted in a significantly greater potential for cell migration compared to the control cells after 24 h of incubation in the free wound area after excision (Figure 11C). After the application of irisin (Figure 11D), as shown in Figure 3, there was no significant increase in BV2 cell motility, with a free wound surface which was almost comparable to the control condition after 24 h (Figure 11F). This indicates that the migratory capacity of these cells was significantly lower than in LPS-stimulated cells. Figure 3F demonstrates that, in cells treated with both LPS and irisin, irisin reversed the effect of LPS, leading to a significant reduction in cell migration compared to BV2 cells treated with LPS alone. Thus, in both migration assays, irisin worked as a regulator of migratory capacity by reducing the LPS-induced increase in BV2 cell motility.

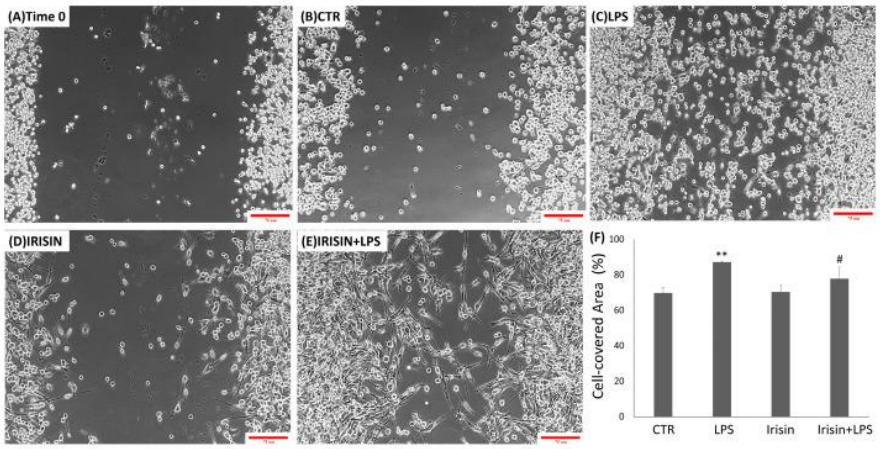

Figure 11. The analysis of the migratory capacity of microglia after the administration of irisin in the presence or absence of LPS. A wound was generated in a sub-confluent layer of BV2 cells, and the resulting space was captured at the wound site 0 and 24 h after the treatment: (A) BV2 cells at time 0, (B) 24 h after the cut in the control condition, (C) treated with 1 µg/mL LPS, (D) with 5 nM irisin, and (E) with 5 nM irisin in the presence of 1 µg/mL LPS. The images are representative of an experiment with three independent replicates. The percentage of the wound gap was analyzed using the ImageJ software and subsequently plotted and statistically analyzed as the percentage of wound closure compared to the 0 time condition (F). The values are presented as means ± standard deviations. Bar: 75 µm (20× objective). ** $p < 0.01$ compared to the control. # $p < 0.05$ compared to the LPS condition.

4.1.4 NLRP3 Inflammasome Activation Decreased in BV2 Cells Treated with Irisin

The Western blot assay and semi-quantitative analysis showed that NLRP3 expression was significantly reduced in BV2 cells treated with irisin compared to cells treated with LPS. Similarly, the expression of Pycard significantly decreased with the use of irisin. Moreover, we could see a clear reduction in the expression of these two pro-inflammatory factors in BV2 cells treated with both irisin and LPS with respect to cells treated with LPS alone. Taken together, these

data demonstrate that the NLRP3 inflammasome is reduced in microglial cells under irisin conditions (Figure 12).

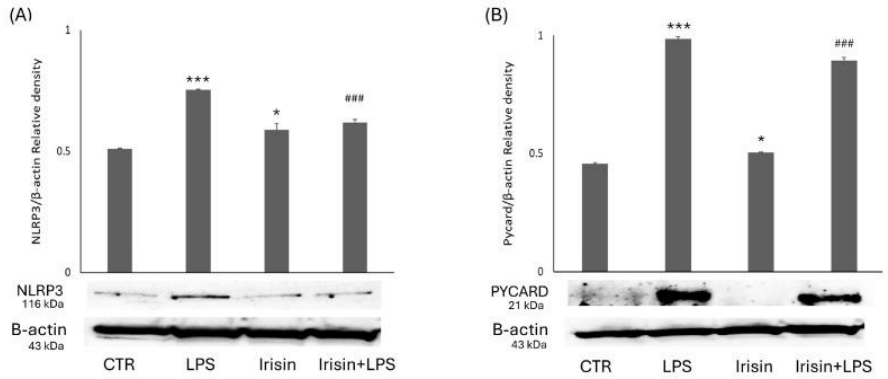

Figure 12. Evaluation of NLRP3 and Pycard expression following the administration of irisin, with or without LPS. Western blotting detection and densitometric analysis of the expression of the pro-inflammatory NLRP3 (A) and Pycard (B) in control cells (CTR), BV2 cells treated with irisin, BV2 cells treated with LPS, and BV2 cells treated with irisin + LPS. The protein expression values are expressed in arbitrary units after normalization against β-actin. The data are presented as means ± SDs (* $p < 0.05$ vs. CTR; *** $p < 0.001$ compared to the control; and ### $p < 0.001$ compared to the condition of LPS-stimulated microglia).

4.1.5 Irisin's Effects on Pro-Inflammatory Marker Expression

We further investigated the role of irisin in inhibiting LPS-induced inflammation in the microglia. Irisin treatment downregulated NLRP3-induced inflammasome activation, as reflected by a reduction in the protein expression levels of IL1β (Figure 13A) and caspase-1 (Figure 13B) in cells pretreated with irisin and LPS compared to those treated with LPS alone over 24 h. We then examined the expression of CD14 (Figure 13C), a glycosylphosphatidylinositol-anchored monocyte antigen, which, together with TLR4, represents an early event in the activation of the

neuroinflammatory signaling pathway. Upon binding to LPS, CD14 associates with the extracellular domain of TLR4. Again, the protein expression level of CD14 was upregulated in response to LPS exposure and was reduced by irisin pretreatment in LPS-treated cells.

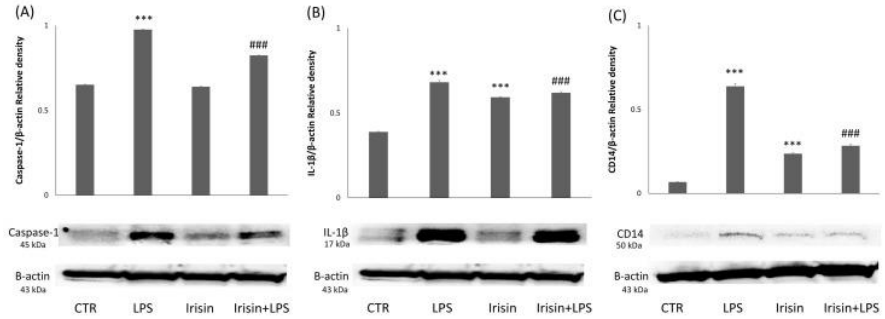

Figure 13. Evaluation of caspase-1, IL1β, and CD-14 expression following the administration of irisin, with or without LPS. Western blotting detection and densitometric analysis of the expression of pro-inflammatory caspase-1 (**A**), IL1β (**B**), and CD-14 (**C**) in control cells (CTR), BV2 cells treated with irisin, BV2 cells treated with LPS, and BV2 cells treated with irisin + LPS (*** $p < 0.001$ compared to the control condition; and ### $p < 0.001$ compared to the condition of LPS-stimulated cells).

4.1.6 Irisin's Effects on Anti-Inflammatory Marker Expression

To further investigate the effect of irisin on the phenotypic transition of BV2 cells, Arg-1 was assessed as a marker of M2 microglial polarization. The results demonstrated that irisin alone significantly increased Arg-1 levels compared to the control. In contrast, LPS treatment reduced Arginase1 (Arg-1) expression relative to the control. Notably, co-treatment with irisin and LPS

produced the opposite effect, resulting in elevated Arg-1 expression in BV2 cells (Figure 14).

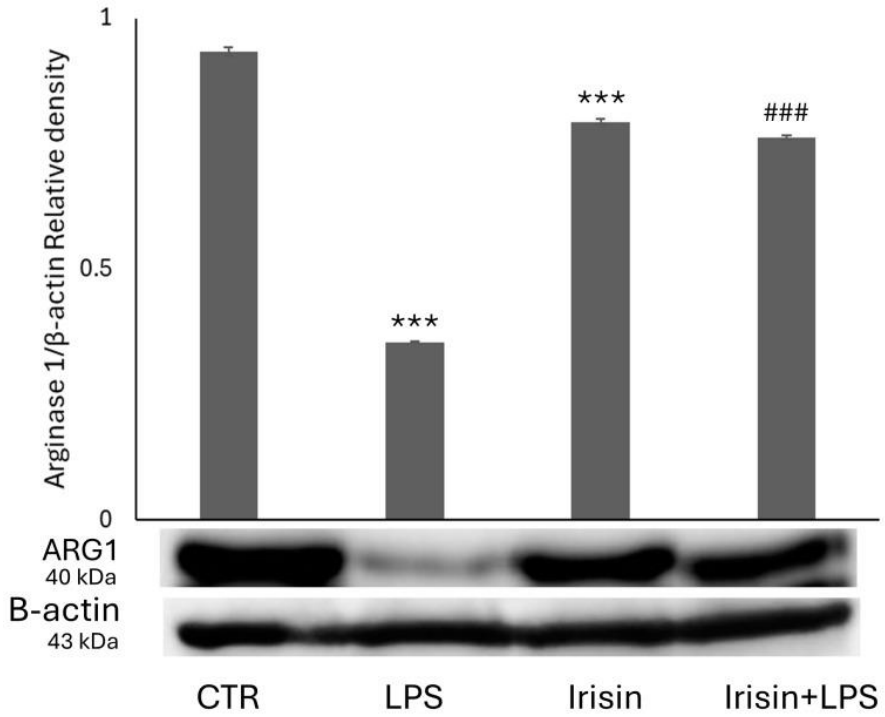

Figure 14. Evaluation of Arginase 1 expression following the administration of irisin, with or without LPS. Western blotting detection and densitometric analysis of the expression of the anti-inflammatory agent in control cells (CTR), BV2 cells treated with LPS, BV2 cells treated with irisin, and BV2 cells treated with irisin + LPS (*** $p < 0.001$ compared to the control condition; and ### $p < 0.001$ compared to the condition of LPS-stimulated cells).

4.2 Chrysin-Loaded Extracellular Vesicles

Chrysin-Loaded Extracellular Vesicles Attenuate LPS-Induced Neuroinflammation in BV2 Microglial Cells In Vitro: A Novel Neuroprotective Strategy.

Filannino F.M., Soleti R., Ruggiero M., de Stefano M.I., Panaro M.A., Lofrumento D.D., Trotta T., Maffione A.B., Benameur T., Cianciulli A., Calvello R., Zoila F., Porro C.. Molecules. 2025 Jul 25;30(15):3131. doi:10.3390/molecules30153131. PMID: 40807306; PMCID: PMC12348394. Figure 15.

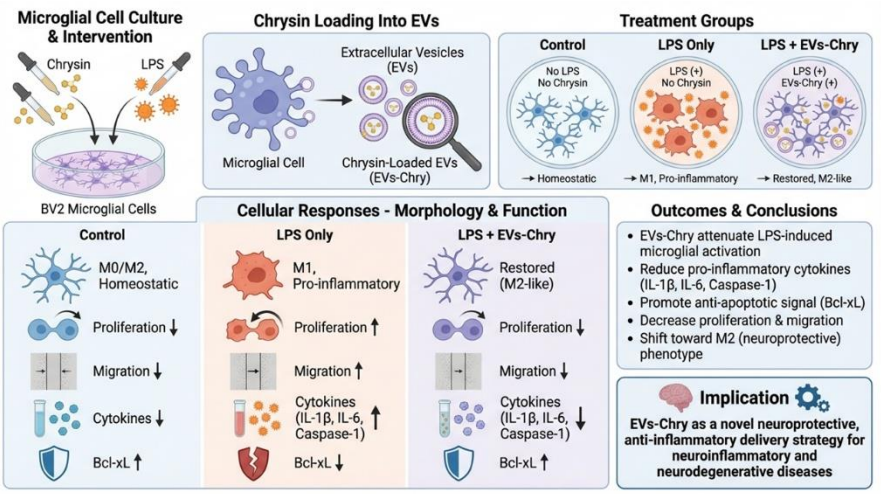

Figure 15. Chrysin-Loaded Extracellular Vesicles

4.2.1 Chrysin Cytotoxicity and Microglial Proliferation Effects

To evaluate the cytotoxicity of chrysin (Figure 16A) and its effects on BV2 microglial cell viability, the CyQUANT® Cell

Proliferation Assay was performed. BV2 cells were treated with chrysin at concentrations ranging from 5 to 50 μM for 24 h. Our results showed that chrysin was non-cytotoxic at all tested concentrations. As shown in Figure 16A, treatment with chrysin at 5 μM, 10 μM and 20 μM significantly enhanced BV2 cell proliferation compared to untreated control cells. Based on these findings, the CyQUANT® assay was repeated using 5 and 20 μM chrysin, with or without 1 μg/mL LPS, following a 24 h incubation (Figure 16B). Pre-treatment with chrysin in combination with LPS for 24 h significantly altered the viability of BV-2 microglial cells compared to the proliferative effects observed with LPS stimulation alone.

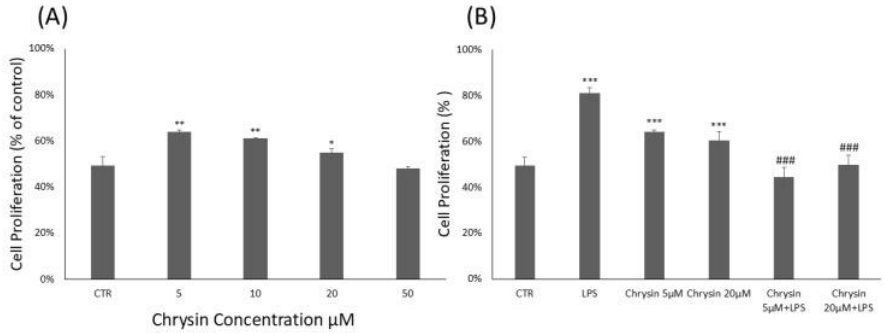

Figure 16. Chrysin effects on BV2 microglial cells proliferation. (**A**). Chrysin concentration-dependent effects on BV2 microglial cells. The results are representative of three independent experiments. (**B**) Effects of Chrysin at various concentrations on BV2 microglial cells proliferation in the absence or presence of LPS. Data are expressed as percentage of control values. Data are expressed as mean ± standard deviation (S.D.). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control group; ### $p < 0.001$ vs. LPS-treated group.

4.2.2 Isolation, Characterization and Cytotoxic Effect of Chrysin-Loaded EVs from Microglial Cells

EVs were isolated from BV2 microglial cell lines under three conditions: untreated cells (control) and stimulated with chrysin at concentrations of 5 µM and 20 µM. After 24 h of treatment, EVs were isolated from the culture medium through a series of centrifugations, as described below. The isolated EVs, designated as EVs-CTR (from unstimulated cells; (Figure 17A), EVs-Chry5µM (from cells treated with 5 µM chrysin; (Figure 17B), and EVs-Chry20µM (from cells treated with 20 µM chrysin; (Figure 17C) were initially analysed for particle diameter distribution using the ZetaView nanoparticle tracking system.

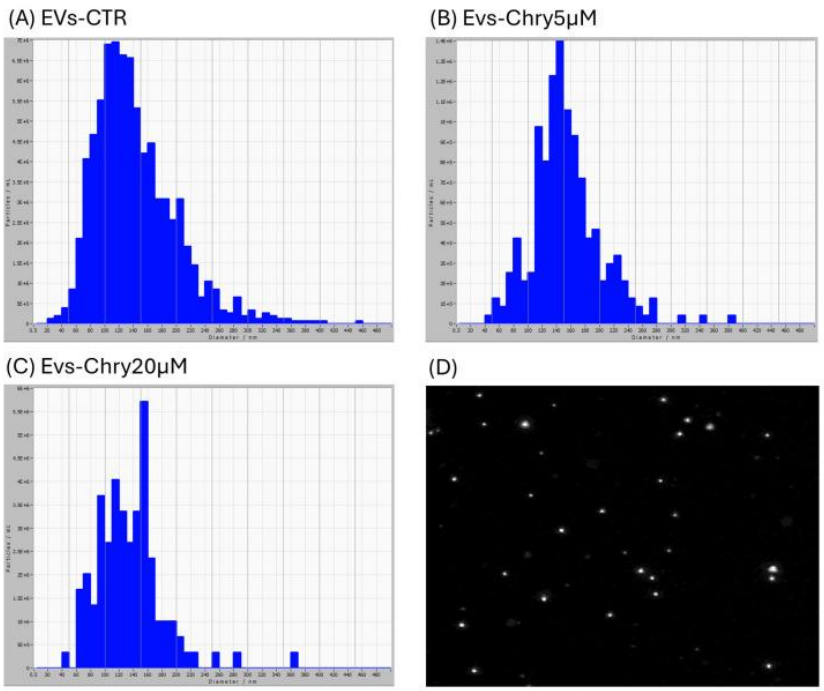

Figure 17. Characterization of EVs obtained from BV2 cell supernatant. EV samples examined with ZetaView are predominantly composed of vesicles with diameters ranging from 20 to 420 nm. In the EVs-CTR group (**A**), most vesicles show a diameter between 100 and 150 nm. The EVs-Chry5μM sample (**B**) shows a high number of vesicles with diameters ranging from 110 to 200 nm. The EVs-Chry20μM sample (**C**) presents slightly larger vesicles, with a diameter mainly ranging from 150 to 160 nm. Image of EVs obtained with ZetaView (**D**).

Subsequently, EV concentrations were quantified using the BCA protein assay. These EVs were then applied to BV2 cells for an additional 24 h incubation to assess their biological activity.

Therefore, the CyQUANT® assay was repeated after 24 h, with the addition of either EVs-Chry at 5 μM or at 20 μM, in the presence or absence of LPS. Pretreatment with EVs-Chry at 5 μM in combination with LPS had a significant effect on the viability of BV2 microglial cells, resulting in the inhibition of LPS-induced proliferation (Figure 18A). Based on these results, EVs-Chry 5μM were selected for all subsequent analyses and are hereafter referred to as EVs-Chry. The absorbance at 348 nm (the λmax of chrysin) of the lysates from the EVs-CTR and EVs-Chry samples was analysed. Figure 18B shows higher absorbance only in EVs-Chry. Further characterization of the vesicle preparation using the ZetaView system provided detailed information about the extracellular vesicles, including particle concentration and size distribution of the EV samples. The vesicle samples were predominantly composed of vesicles ranging from 20 to 420 nm in diameter. In the EVs-CTR group, the majority of vesicles measured between 100 and 150 nm. The EVs-Chry5μM sample contained a high number of vesicles with diameters ranging from 110 to 200 nm. However, the EVs-Chry20μM sample displays slightly larger vesicles, with a diameter primarily

ranging from 150 to 160 nm. Based on these size distributions, we concluded that EVs were successfully isolated from all samples, with a predominance of microvesicles and a relative reduction in exosome content.

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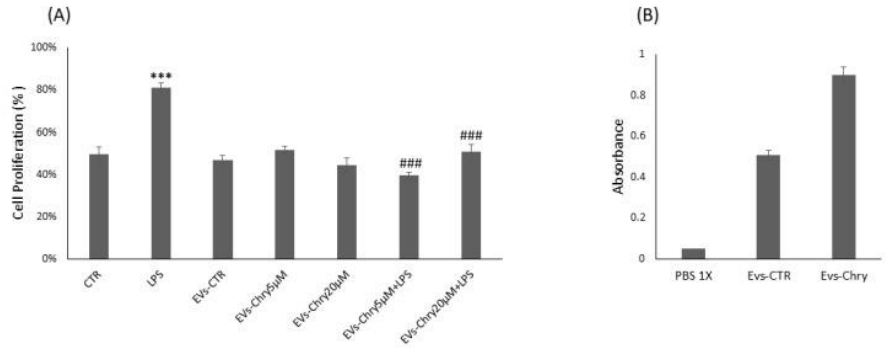

Figure 18. Characterization Effects of EVs-Chry5 µM and EV-Chry20 µM, at a concentration of 10 µg/mL, on the viability of BV2 microglial cells in the absence or presence of LPS (**A**). Cell proliferation was assessed using the CyQUANT® assay. Absorbance of EVs at 348 nm (**B**). All results are representative of three independent experiments. Data are expressed as mean ± standard deviation (SD). *** $p < 0.001$ vs. control. ### $p < 0.001$ vs. LPS group.

4.2.3 BV-2 Cells Activated with LPS Show Anti-Inflammatory M2 State After EVs-Chry Stimulation

The morphological changes observed in microglial cells are characterized by the progressive development of processes and cell body alterations in response to various stimuli. Therefore, the initial analysis focused on evaluating the morphological changes in BV2 microglial cells. Untreated control BV2 cells and BV2 cells treated with only EVs-CTR (Figure 19A), exhibited a quiescent microglial

morphology, characterized by a small central body and numerous elongated processes. In contrast, BV2 cells stimulated with LPS only (Figure 19B) displayed an activated amoeboid shape, marked by an enlarged cell body and retracted processes, typical morphological characteristics of the M1 pro-inflammatory phenotype. As shown in Figure 19E, the treatment with EVs-Chry alone led to a ramified morphology characterized by a small central soma, closely resembling the resting state observed in control cells in both size and shape. Cells treated with both EVs-Chry and LPS (Figure 19F) exhibited a marked reduction in the amoeboid morphology induced by LPS, suggesting that EVs-Chry exert anti-inflammatory effects and promote a shift toward the anti-inflammatory M2 phenotype. The quantitative analysis of cellular areas (Figure 20) confirmed these observations visible under the optical microscope: LPS-treated cells showed a significant increase in cell size compared to controls. Conversely, cells treated with both EVs-Chry and LPS demonstrated a significant reduction in cell area, indicating a reversal of the LPS-induced morphological changes.

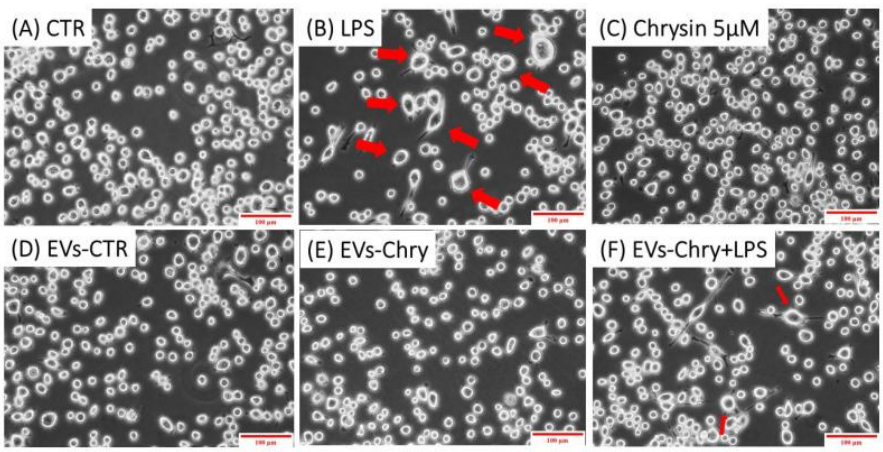

Figure 19. Effects of EV-chrysin on BV2 cell morphology over a 24-h period. Red arrows in the images highlight cells exhibiting morphological features characteristic of the M1 pro-inflammatory phenotype, including an enlarged cell body and retracted cellular processes. Control BV2 cells (**A**) and cells treated with EVs-CTR (**D**) show a quiescent microglial morphology. BV2 cells stimulated with LPS alone (**B**) show an amoeboid phenotype characteristic of the pro-inflammatory state, characterized by a small central body and numerous elongated processes. Treatment with EVs-Chry (**E**) and Chrysin (**C**) alone led to a ramified morphology characterized by a small central soma, very similar to the resting state observed in control cells both in size and shape. Cells treated with both EVs-Chry and LPS (**F**) showed a marked reduction in LPS-induced amoeboid morphology. Scale bar represents 100 µm (10× objective). Arrows in the images highlight cells exhibiting morphological changes. Cell area (µm^2) was quantified using ImageJ software (version 1.8.0) with 64-bit Java 8 support.

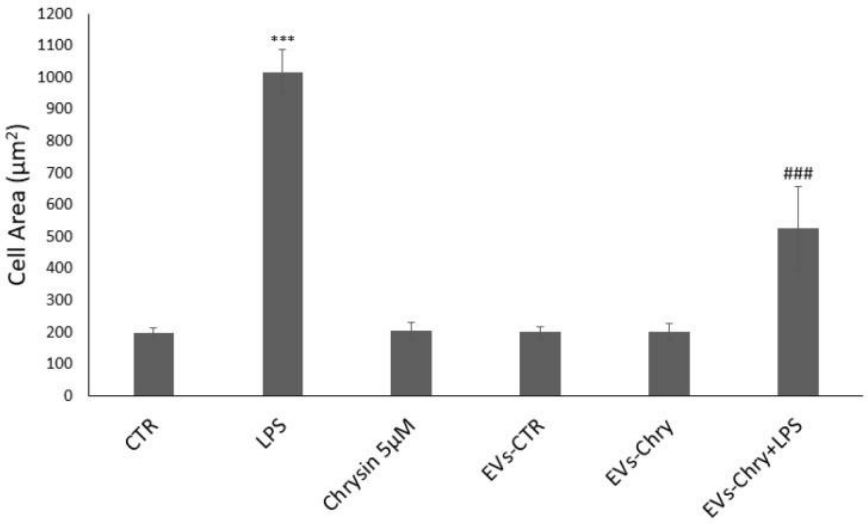

Figure 20. Quantitative analysis of cell areas. LPS-treated cells have a significant increase in cell size compared to controls. In contrast, cells treated with EVs-Chry and LPS showed a significant reduction in cell area, indicating a reversal of LPS-induced morphological alterations. Values are reported as mean ± standard deviation. A highly statistically significant difference was observed between the control and LPS-treated groups (*** $p < 0.001$), as well as between the EVs-Chry + LPS group and LPS-only groups (### $p < 0.001$).

4.2.4 EVs-Chry Attenuate LPS-Induced Migration of BV2 Microglial Cells

To evaluate the effects of EVs-Chry on BV2 cells migration, a wound healing assay was performed. BV2 cells were scratched and treated with LPS, Chrysin (5 μM), EVs-CTR, EVs-Chry, or a combination of EVs-Chry and LPS. After a 24 h incubation period, BV2 cells treated with LPS alone (Figure 21C) exhibited a significantly accelerated rate of wound repair compared to the untreated cells (CTR). In contrast, treatment of BV2 with EVs-Chry (Figure 21F) resulted in wound opening comparable to that observed in CTR and EVs-CTR treated cells. Finally, co-treatment with EVs-Chry and LPS (Figure 21G) significantly reduced wound repair capacity compared to treatment with LPS alone, indicating an inhibitory effect on LPS-induced microglial migration. These findings are quantified in Figure 22, where analysis of the wound area confirms the statistical significance of both LPS and EVs-Chry+LPS treatments. Collectively, these results demonstrate that EVs-Chry attenuates BV2 microglial migration and may counteract the pro-migratory effects induced by LPS.

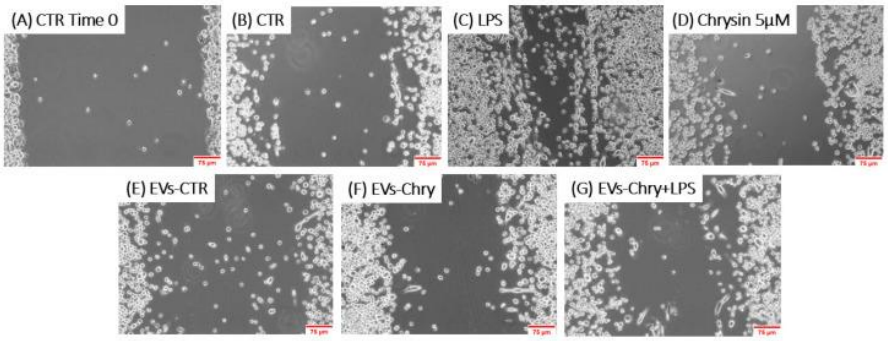

Figure 21. The analysis of the migratory capacity of microglia after the administration of EVs-Chry in the presence or absence of LPS. A wound was introduced into a sub-confluent monolayer of BV2 cells, and images of the wound area were captured at 0 and 24 h post-treatment: (**A**) BV2 cells at time 0, (**B**) 24 h under control conditions, (**C**) treated with LPS, (**D**) with Chrysin at 5 µM, (**E**) with EVs-CTR, (**F**) with EVs-Chry, and (**G**) cotreated EVs-Chry + LPS. The images shown are representative of one of three independent experiments. Scale bar: 75 µm (20× objective).

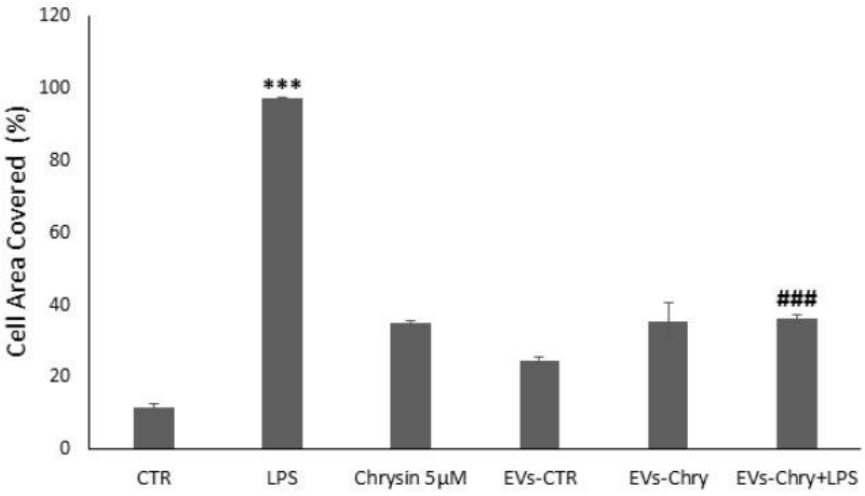

Figure 22. Wound closure was quantified using ImageJ software and subsequently plotted as the percentage of gap closure relative to the 0 h condition. Wound closure was quantified using ImageJ software, and results were plotted as the percentage of gap closure relative to the 0 h condition. Data are expressed as

mean ± standard deviation. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. LPS-treated group.

4.2.5 Suppression Effect of EVs-Chry on Expression of Pro-Inflammatory Molecules in BV2 Cells

In neurodegenerative diseases, inflammation plays a fundamental role in promoting both cell proliferation and migration. Indeed, prolonged inflammation can increase the levels of pro-inflammatory cytokines and proteases in the CNS, which can induce neuronal damage and cell death. In order to assess the anti-inflammatory potential of EVs-Chry, we evaluated its efficacy in suppressing the expression of pro-inflammatory cytokines. BV2 microglial cells were treated for 24 h with one of the following conditions: LPS alone, 5 μM chrysin, EVs-CTR, EVs-Chry alone, or a combination of EVs-Chry and LPS. As expected, LPS treatment significantly increased the expression of pro-inflammatory cytokines, including IL-1β and IL-6. However, EVs-Chry treatment reduced the expression levels of IL-1β and IL-6.

Furthermore, co-treatment with EVs-Chry and LPS significantly reduced the levels of these cytokines compared to LPS treatment alone (Figure 23). In addition, western blot analysis revealed a significant decrease in the protein expression level of caspase-1 following EVs-Chry treatment (Figure 24). Caspase-1 is associated with the inflammasome pathway and is upregulated in response to LPS stimulation, where it facilitates the release of IL-1β. These results suggest that EVs-Chry can drive microglial activation and promote the transition from pro-inflammatory state to neuroprotective phenotype by suppressing the expression of pro-

inflammatory cytokines and associated inflammasome signaling components.

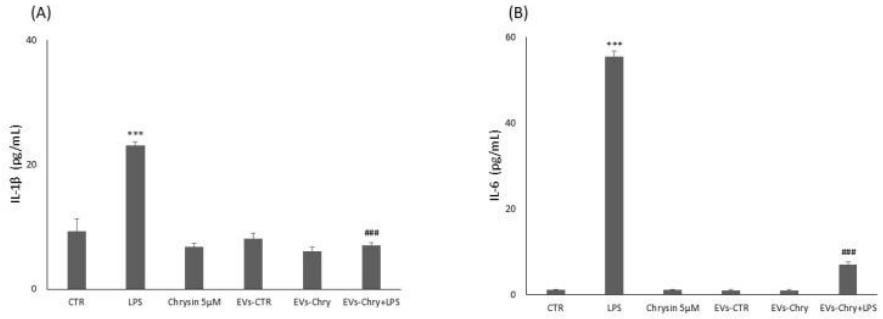

Figure 23. Modulatory effect of EVs-Chry on proinflammatory markers. Evaluation of IL-6 and IL1β expression is performed with ELISA following administration of EVs-Chry, with or without LPS. Data relating to cytokine concentrations of IL-6 (**A**) and IL-1β (**B**) show how EVs-Chry reduced the expression levels of IL-1β and IL-6. In particular, concomitant treatment with EVs-Chry and LPS significantly reduced the levels of these cytokines compared to treatment with LPS alone. Data are expressed as means (pg/mL) ± standard deviations (SD). (*** $p < 0.001$ compared to the control condition; and ### $p < 0.001$ compared to the condition of cells stimulated with LPS).

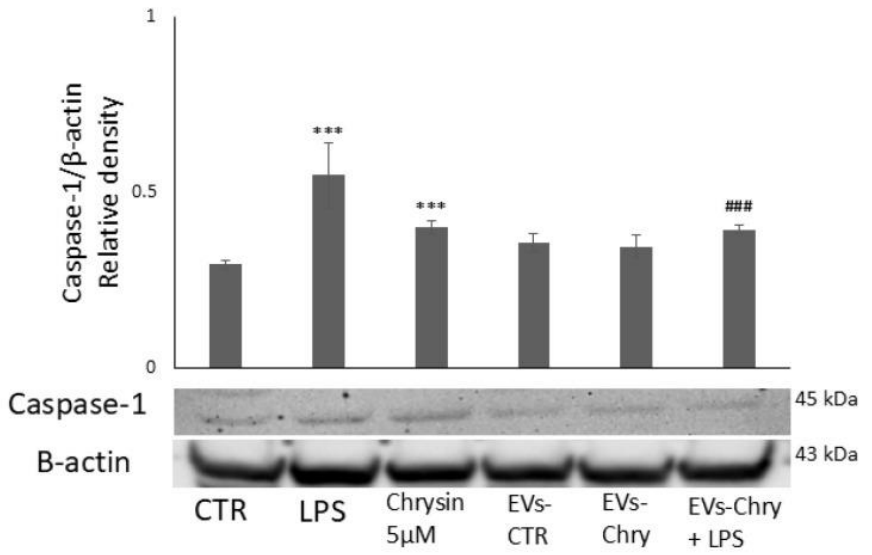

Figure 24. Modulatory effect of EVs-Chry on caspase-1. EVs-Chry modulate the expression of caspase-1, which is associated with the inflammasome pathway and appears increased in response to LPS stimulation, where it facilitates the release of IL-1β. Detection of caspase-1 expression by Western blotting and densitometric analysis. Data are expressed as mean ± standard deviation (SD). *** $p < 0.001$ vs. control. ### $p < 0.001$ vs. LPS group.

4.2.6 EVs-Chry Stimulate the Increase in the Anti-Apoptotic Protein Bcl-xL in BV-2 Cells

To evaluate the impact of EVs-Chry on the regulation of the synthesis of mitochondria-associated apoptotic proteins, we analysed the expression levels of the anti-apoptotic protein Bcl-xL (Figure 25). Treatment with LPS alone led to a significant reduction in Bcl-xL expression, consistent with the pro-apoptotic environment induced by inflammatory stimuli. In contrast, treatment with EVs-Chry alone resulted in a marked upregulation of Bcl-xL expression compared to CTR, indicating a potential protective effect. In the course of our research, we examined the impact of a double EVs-Chry treatment in the presence of LPS. Furthermore, co-treatment with EVs-Chry in the presence of LPS effectively restored Bcl-xL expression to levels significantly higher than those observed with LPS treatment alone. These results suggest that EVs-Chry prevent LPS-induced downregulation of anti-apoptotic signaling and exert a protective effect against inflammation-induced cellular apoptosis. This highlights the potential of EVs-Chry not only as an anti-inflammatory agent but also as a modulator of apoptosis through mitochondrial pathways.

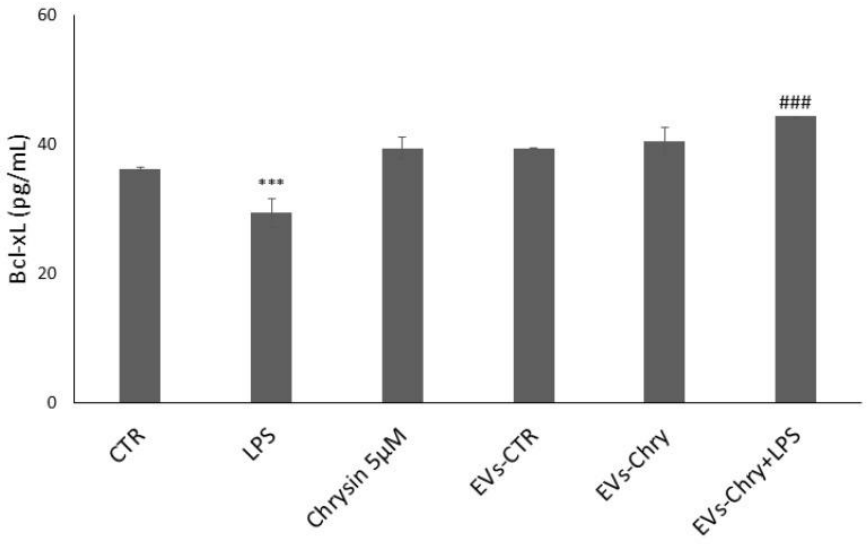

Figure 25. EVs-Chry prevents the downregulation of LPS-induced anti-apoptotic signaling and exerts a protective effect against inflammation-induced cell apoptosis. LPS treatment alone led to a significant reduction in Bcl-xL expression. In contrast, EVs-Chry treatment alone led to a marked upregulation of Bcl-xL expression compared to CTR, indicating a potential protective effect. Co-treatment with EVs-Chry in the presence of LPS effectively restored Bcl-xL expression. Assessment of anti-apoptotic Bcl-xL protein by ELISA. Data are expressed as means (pg/mL) ± standard deviations (SD). *** $p < 0.001$ compared to control condition. ### $p < 0.001$ compared to LPS-stimulated microglia condition. *** $p < 0.001$ compared to CTR.

5. DISCUSSION

The global demographic transition toward an aging population represents one of the most significant challenges facing modern healthcare systems. As life expectancy continues to increase worldwide, with more than 2 billion individuals expected to be older than 60 by 2050, the gap between total life expectancy and healthy life expectancy has widened substantially [154]. This discrepancy reflects the reality that individuals are living several years with functional limitations, chronic diseases, and age-related decline. The aging process is characterized by multidimensional changes affecting tissues and organs, leading to decreased physiological integrity over time. These changes can be categorized into primary hallmarks, including genomic instability, epigenetic alterations, telomere attrition, and loss of proteostasis; antagonistic hallmarks representing compensatory responses to primary damage; and integrative hallmarks such as stem cell exhaustion and impaired intercellular communication [155]. Biological aging, which encompasses a wide range of physical, physiological, and cognitive functions influenced by molecular and cellular processes, provides a more accurate prediction of health outcomes than chronological age alone. The age gap between biological and chronological age serves as a complementary indicator of aging, revealing insights into individual health risks and susceptibility to age-related diseases. My research trajectory began with an investigation into the neuroprotective potential of physical activity, with a particular focus on yoga as a mind-body intervention.

NDs represent an escalating global health challenge, with AD and PD constituting the most prevalent forms affecting millions of individuals worldwide. As the global population continues to age, the incidence of these debilitating conditions is projected to increase dramatically, with dementia cases expected to reach 115.4 million by 2050 [156]. The pathophysiological mechanisms underlying NDs are complex and multifactorial, involving chronic neuroinflammation, oxidative stress, mitochondrial dysfunction, protein misfolding and aggregation, and progressive neuronal loss in specific brain regions. Current pharmacological interventions remain largely symptomatic, offering temporary relief without addressing the underlying neurodegenerative processes or halting disease progression [157]. Consequently, there is an urgent need for complementary therapeutic strategies that can modulate multiple pathogenic pathways simultaneously, promote neuroplasticity, and enhance overall brain health. Physical activity and exercise emerge as cornerstones in the pursuit of optimal aging, with research consistently highlighting their profound impact on various dimensions of wellbeing in later life. Regular physical activity can help mitigate the decline in muscle mass and strength, combatting the effects of sarcopenia and promoting better mobility and independence in older adults [158]. Additionally, exercise, particularly weight-bearing and resistance training, can contribute to maintaining bone density and reducing the risk of fractures and osteoporosis. Furthermore, physical activity has positively impacted cognitive function, including improved memory, attention, and executive function, which can significantly contribute to maintaining independence and quality of life as individuals age. In terms of chronic disease prevention, participating in regular exercise

has been associated with a reduced risk of conditions such as heart disease, diabetes, and certain types of cancer [158]. Through its influence on weight management, blood pressure regulation, and insulin sensitivity, physical activity can help mitigate the risk factors for these diseases, thereby promoting overall health and wellbeing as individuals age [158]. Moreover, staying physically active also contributes to emotional and psychological wellbeing, promoting a sense of purpose, satisfaction, and fulfilment in later years, supporting the concepts of active, healthy, and successful aging. Physical activity and exercise have emerged as powerful interventions capable of modulating the aging process and potentially slowing neurodegenerative disease progression. Regular exercise influences multiple biological systems simultaneously, providing benefits that extend from cardiovascular and metabolic health to cognitive function and emotional well-being [159].

Also, Yoga, an ancient mind-body practice integrating physical postures (asanas), breathing exercises (pranayama), and meditation (dhyana), has emerged as a promising non-pharmacological intervention with demonstrated benefits in improving motor function, cognitive performance, mood, and quality of life in individuals with NDs [160]. The therapeutic potential of yoga in neurodegenerative conditions extends beyond its well-documented effects on physical fitness and psychological well-being. At the molecular level, yoga practice appears to modulate several key biological pathways implicated in neurodegeneration, including the enhancement of neurotrophic factors such as BDNF, reduction of pro-inflammatory cytokines such as IL-6, TNFα, mitigation of oxidative stress markers, and potential preservation of gray matter volume in

critical brain regions such as the hippocampus [161]. I delved deeper into the literature examining among the various exercise-induced myokines and signaling molecules, irisin emerged as a particularly compelling candidate worthy of further investigation.

Irisin, a novel myokine cleaved from the membrane protein FNDC5, is secreted by skeletal muscle during exercise and appears to cross the blood-brain barrier to exert beneficial effects on brain function and structure [162]. The discovery of irisin's neurobiological actions has opened new avenues for understanding how exercise-based interventions, including yoga, may confer protection against neurodegenerative processes. The potential mechanisms by which yoga practice stimulates irisin secretion are multifaceted [162]. Unlike conventional aerobic or resistance exercise that primarily focuses on cardiovascular fitness or muscle strengthening, yoga combines isometric and dynamic muscle contractions with controlled breathing patterns and parasympathetic nervous system activation. This integrated approach may create optimal conditions for irisin production through several pathways. First, the sustained muscle engagement required to maintain yoga postures can activate the PGC-1α/FNDC5/irisin pathway in skeletal muscle, particularly when poses are held for extended periods or when more physically demanding styles such as power yoga or Hatha yoga are practiced [163]. Second, the emphasis on diaphragmatic breathing and pranayama techniques in yoga may enhance oxygen delivery to tissues and improve metabolic efficiency, potentially augmenting exercise-induced irisin secretion. Third, the stress-reduction effects of yoga, mediated through decreased cortisol levels and enhanced parasympathetic tone, may create a hormonal milieu conducive to irisin production and

action. The reduction in chronic stress is particularly important, as elevated glucocorticoids can suppress muscle protein synthesis and may interfere with the exercise-induced upregulation of myokines including irisin [164]. The importance of irisin in the context of aging and neurodegenerative disease stems from its multifaceted biological activities. Irisin crosses the blood-brain barrier and directly influences brain metabolism and function. In the central nervous system, irisin promotes expression of BDNF and other neurotrophic factors, supporting neuronal survival, synaptic plasticity, and neurogenesis in the hippocampus, regions critical for memory formation and cognitive function that are particularly vulnerable in AD [165]. Moreover, irisin demonstrates potent anti-inflammatory properties, in fact capable of modulating microglial activation and reducing production of pro-inflammatory mediators [166]. Irisin influences also metabolic health through multiple pathways, promoting browning of white adipose tissue, enhancing glucose uptake and insulin sensitivity, and improving mitochondrial function. These metabolic effects are particularly relevant for older adults at risk for metabolic syndrome and type 2 diabetes, conditions that significantly increase vulnerability to cognitive decline and dementia. Irisin demonstrates antioxidant properties, enhancing cellular defences against oxidative stress and protecting neurons from ROS-mediated damage [166]. This antioxidant action complements its anti-inflammatory effects, as oxidative stress and inflammation are intimately linked in neurodegenerative pathology. Irisin may influence protein homeostasis and clearance of pathological protein aggregates, potentially affecting the accumulation of amyloid-β and tau in AD or alpha-synuclein in PD, though this mechanism requires further

investigation. The elevation of irisin through exercise represents a physiological mechanism by which physical activity translates into neuroprotective benefits, bridging the gap between peripheral muscle contraction and central nervous system health [167].

Understanding the role of irisin provides insight into the molecular mechanisms by which exercise promotes healthy aging and may contribute to the development of exercise prescriptions optimized for neuroprotection. Moreover, the potential for pharmacological irisin mimetics or therapies targeting the FNDC5/irisin pathway represents a promising avenue for individuals unable to engage in sufficient physical activity due to disability or advanced disease [168]. The integration of exercise-induced myokine signaling, including irisin, into our understanding of neuroprotection exemplifies the systemic nature of exercise benefits and highlights the importance of maintaining muscle mass and physical activity throughout the lifespan as a strategy for brain health and resilience against neurodegenerative disease.

The study of irisin effects on microglia fits into this context. What captured my attention was a critical gap in the existing research: while irisin's neuroprotective properties had been documented in various contexts, its specific effects on microglial inflammatory responses remained largely unexplored. The convergence of my initial interest in yoga-based interventions with the identification of this uncharted territory in irisin research led me to formulate my hypothesis: that irisin may exert anti-inflammatory effects directly on microglial cells, potentially representing a key mechanistic link between physical activity and neuroprotection.

Microglia, the resident immune cells of the CNS, play a crucial role in maintaining neuronal homeostasis and responding to pathological insults. In their resting state, these cells actively scan the microenvironment with ramified processes, contributing to tissue homeostasis and providing neurotrophic substances while regulating neurotransmitters and hormones. However, upon activation by various stimuli including foreign pathogens, abnormal protein aggregates, and apoptotic cells, microglia undergo dramatic morphological and functional transformations that can significantly impact neurological health [169].

The activation of microglia can be induced by multiple stimuli, with LPS serving as a prominent trigger. LPS, a heat-stable glycolipid component of the outer membrane of Gram-negative bacteria, induces robust inflammatory responses by binding to LPS-binding protein and the glycosylphosphatidylinositol-anchored protein CD14, which associates with TLR4 [38]. This interaction activates pro-inflammatory signaling cascades, leading to the production and release of TNFα, IL-6, and NO, ultimately resulting in neuronal cell death. Microglial activation traditionally follows a polarization paradigm, wherein cells adopt either a pro-inflammatory M1 phenotype or an anti-inflammatory M2 phenotype, depending on the nature of the lesion and activating stimulus. The M1 phenotype is characterized by the secretion of high levels of pro-inflammatory factors including IL-1β, IL-6, and TNFα, along with increased production of NO and ROS [170]. While these responses serve as the first line of defence against pathogens, chronic microglial activation and the resulting excessive levels of inflammatory mediators can cause substantial neuronal damage, as confirmed in NDs such as AD,

PD, and ALS. Morphologically, the transition between microglial phenotypes is striking. When adopting the pro-inflammatory M1 phenotype, microglia retract their branching processes and migrate toward areas of tissue damage, displaying an amoeboid morphology with enlarged, thickened cell bodies and reduced cellular processes. This morphological switch is accompanied by enhanced migratory capacity, mediated by increased release of inflammatory mediators such as TNF-α, monocyte chemotactic protein-1, and ROS. In contrast, the alternative M2 activation phenotype is characterized by cells retaining branching processes with reduced cytoplasm and diminished migratory capacity, similar to resting microglia. An imbalance in microglial polarization favouring the M1 phenotype is widely recognized as a contributing factor in neurodegeneration pathogenesis, making the modulation of microglial activation toward a more balanced or M2-dominant state a critical therapeutic objective [171].

Irisin represents a particularly intriguing therapeutic candidate, as it links physical exercise to neuroprotection. Regular exercise is associated with reduced risk of dementia and neurodegenerative diseases, effects mediated through multiple mechanisms including reduction of chronic oxidative stress, enhancement of mitochondrial biogenesis, positive regulation of autophagy, and stimulation of key neurotransmitters and trophic factors such as dopamine, glial-derived neurotrophic factor, insulin-like growth factor-1, BDNF, and fibroblast growth factor-2. Irisin exerts its effects by binding to αV class integrins, specifically αV/β5 integrin in microglial cells, thereby activating downstream signaling pathways [172].

We have studied for the first time the effects of irisin on microglial cell in the order to demonstrate the potential of irisin to decrease neuroinflammation. Using BV2 microglial cells we have demonstrated that irisin, even at low physiological concentrations as minimal as 5 nM, effectively modulates microglial inflammation without affecting cell viability. When administered alongside LPS, irisin induces a profound shift in microglial phenotype, reversing LPS-induced inflammation and promoting a transition from the pro-inflammatory amoeboid M1 morphology to a resting phenotype. This morphological change is accompanied by significant reduction in CD14 expression, a marker of microglial activation that mediates monocytic cell activation and triggers production of multiple inflammatory mediators. Furthermore, irisin treatment increases Arg-1 protein expression, a marker of M2 polarization, thereby promoting microglial polarization toward the anti-inflammatory phenotype.

Beyond its effects on microglial morphology and polarization markers, irisin demonstrates remarkable inhibitory activity on the NLRP3 inflammasome pathway, a critical mediator of neuroinflammation. The NLRP3 inflammasome has garnered substantial attention for its role in CNS diseases, including ALS, AD and PD. These multiprotein complexes, expressed primarily in myeloid cells, are essential for activating the protease caspase-1 and the downstream secretion of pro-inflammatory cytokines IL-1β and IL-18. In AD, amyloid beta peptides activate the inflammasome, while in PD, protein aggregates such as Lewy bodies induce inflammasome-dependent IL-1β secretion. Irisin significantly reduces the expression of NLRP3 inflammasome components including NLRP3, Pycard, and caspase-1 in response to LPS stimulation,

thereby attenuating inflammation and subsequent cell death. This inhibition extends to IL-1β expression, which irisin suppresses by downregulating NF-κB, demonstrating its capacity to inhibit the canonical NLRP3 inflammasome pathway and consequent cytokine release.

Neuroinflammation represents a fundamental pathophysiological process implicated in virtually all neurodegenerative disorders, ranging from AD and PD to MS and TBI. This complex inflammatory response within the CNS, primarily mediated by resident immune cells such as microglia and astrocytes, serves as a double-edged sword in brain homeostasis [173]. While acute neuroinflammatory responses are essential for pathogen clearance, debris removal, and tissue repair, chronic or excessive activation of these inflammatory cascades can precipitate devastating neuronal damage, synaptic dysfunction, and progressive neurodegeneration. The pathogenesis of neurodegenerative diseases is intricately linked to the sustained activation of glial cells, particularly microglia, which function as the primary mediators of inflammation in the brain environment. In this context of complex neuroinflammatory pathophysiology, bioactive natural substances have garnered tremendous scientific interest as promising therapeutic agents capable of modulating inflammatory responses while exhibiting favorable safety profiles compared to conventional pharmaceutical interventions [174]. These naturally occurring compounds, derived from diverse botanical and nutritional sources, possess pleiotropic mechanisms of action that enable them to simultaneously target multiple pathological pathways implicated in neuroinflammation and neurodegeneration. The therapeutic potential

of bioactive natural substances stems from their ability to exert anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective effects through interference with key molecular targets and signaling cascades [175].

In my research activity I have investigated the anti-inflammatory and neuroprotective actions of these bioactive natural substances which are multifaceted and involve intricate molecular interactions at various levels of cellular signaling. I also participated in several other projects carried out in the laboratory, in which the effects of Curcumin, α-tocopherol, and Lactoferrin were investigated.

Curcumin's therapeutic efficacy in neuroinflammation is mediated through direct inhibition of inflammasome assembly, particularly preventing the oligomerization of NLRP3 with ASC and subsequent caspase-1 activation, thereby blocking the maturation and secretion of IL-1β and IL-18 [134]. Additionally, curcumin activates the Nrf2 pathway, a master regulator of antioxidant responses, leading to enhanced expression of cytoprotective enzymes including thioredoxin reductase, heme oxygenase-1, and superoxide dismutase. This dual anti-inflammatory and antioxidant action positions curcumin as a particularly valuable therapeutic candidate for conditions characterized by oxidative stress and inflammation, such as AD, and various pulmonary disorders including acute lung injury and COVID-19-associated respiratory complications [176]. The compound's ability to suppress TLR4/MyD88/NF-κB signaling pathways, inhibit microglial pyroptosis, modulate macrophage polarization, and reduce the production of pro-inflammatory mediators has been demonstrated across numerous *in vitro* and *in vivo*

experimental models, with emerging clinical evidence supporting its safety and efficacy in humans [177].

Similarly, α-tocopherol exerts its neuroprotective effects through mechanisms extending beyond classical antioxidant activity, actively modulating microglial functional responses and inflammatory signaling cascades [178]. The vitamin's capacity to preserve the branched morphology typical of quiescent microglia, reduce cellular migration toward sites of injury, suppress TLR4 expression, downregulate CD40 activation, inhibit PI3K/AKT pathway signaling, and decrease TNF-α production while enhancing IL-10 secretion demonstrates its multifaceted anti-inflammatory potential. These actions collectively contribute to maintaining CNS homeostasis and preventing the transition from acute, beneficial inflammatory responses to chronic, detrimental neuroinflammation [135]. Lactoferrin similarly exhibits remarkable anti-inflammatory properties in neural contexts, demonstrated by its ability to reduce TLR4, GFAP, and IL-6 expression while upregulating IL-10 and NRF2 in activated astrocytes. Beyond inflammation modulation, lactoferrin possesses unique neurogenic properties, promoting the reprogramming of reactive astrocytes into proliferative neuroblasts through SOX2 overexpression, ultimately facilitating their conversion into neuronal lineages [179]. This dual capacity to simultaneously suppress neuroinflammation and enhance neurogenesis positions lactoferrin as an exceptionally promising therapeutic candidate for neurodegenerative disorders characterized by both inflammatory pathology and neuronal loss.

The clinical translation of bioactive natural substances faces certain challenges, particularly regarding bioavailability, stability, and

tissue penetration, which have prompted innovative delivery strategies including nanoencapsulation, liposomal formulations, and chemical modifications to enhance therapeutic efficacy. Despite these challenges, accumulating evidence from preclinical studies and emerging clinical trials supports the therapeutic potential of these compounds in preventing or ameliorating neuroinflammatory conditions. The favorable safety profiles of naturally occurring substances, combined with their multi-target mechanisms of action, position them as attractive candidates for long-term preventive strategies and adjunctive therapeutic approaches in neurodegenerative disease management. As our understanding of neuroinflammatory mechanisms continues to evolve, and with increasing recognition of the limitations and adverse effects associated with conventional anti-inflammatory pharmaceuticals, bioactive natural substances represent a paradigm shift toward safer, more holistic approaches to neuroprotection, offering hope for millions of individuals affected by devastating neurological disorders worldwide. This foundation in natural bioactive substances naturally led to the second phase of my research, which cantered on exploiting chrysin's anti-inflammatory activity. The biggest challenge I faced with chrysin was its poor bioavailability. The compound gets rapidly metabolized in the intestine, liver, and various target cells, undergoing conjugation, biotransformation, and conversion to glucuronides and sulfur derivatives. I realized I needed a way to protect chrysin and get it across the blood-brain barrier, which is when I came up with the idea of using EVs derived from microglia, exploiting their natural targeting capabilities to deliver the compound where it was needed.

Alongside my work on irisin, I dove into studying EVs in the central nervous system. I became fascinated by how these structures act as sophisticated messengers, carrying complex molecular cargo between different brain cells. EVs have emerged as critical mediators of intercellular communication within the CNS, serving as sophisticated biological carriers that transport complex molecular cargo between diverse neural cell populations. Within the CNS microenvironment, EVs cross the blood-brain barrier bidirectionally, enabling communication between peripheral organs and the brain parenchyma, and can be detected in cerebrospinal fluid and blood, where they serve as potential biomarkers for neurological disorders [180]. The therapeutic potential of EVs as natural drug delivery vehicles has gained considerable attention due to their intrinsic properties, including low immunogenicity, excellent biocompatibility, ability to penetrate the BBB, and capacity for targeted gene delivery, making them superior to conventional synthetic nanocarriers for CNS-directed therapeutics. In the context of neuroinflammation and neurodegenerative diseases, EVs play a particularly complex and often paradoxical role, as they can transport both neuroprotective and neurotoxic substances depending on the activation state of their parental cells [181].

In the first study I participated in, together with the anatomy laboratory team, we demonstrated the ability of extracellular vesicles to activate microglia and influence their functional status [181]. Microglia cells release EVs with dramatically different molecular compositions depending on whether they are in a resting or activated state. Under physiological conditions, microglia-derived EVs

contribute to synaptic homeostasis by regulating sphingolipid metabolism, promoting synaptic stability, and modulating neuronal excitability through the transfer of bioactive lipids and endocannabinoids.

I studied how microglia are activated microglia are activated by inflammatory stimuli such as LPS, they undergo polarization toward a pro-inflammatory M1 phenotype and release EVs enriched with pro-inflammatory cargo, including TNF-α, IL-1β, IL-6, and pro-inflammatory microRNAs such as miR-155. These LPS-stimulated EVs (EVs-LPS) can effectively propagate neuroinflammatory signaling by activating recipient microglia through engagement of TLR4 and downstream signaling cascades involving NF-κB, p-AKT, and p-IKBα, thereby amplifying the inflammatory response and inducing morphological transformation from ramified to amoeboid phenotypes, increased cellular proliferation, enhanced migratory capacity toward sites of injury, and elevated expression of pro-inflammatory markers such as CD40. In this first study on EVs, we have demonstrated that using the murine BV2 microglial cell line, that EVs isolated from LPS-activated microglia can induce similar pro-inflammatory responses in resting microglia as direct LPS stimulation, including altered cell morphology characterized by enlarged cell bodies and loss of processes, increased migration in wound healing assays, upregulation of pro-inflammatory cytokine expression and release, and activation of inflammatory signaling pathways, while EVs derived from unstimulated control microglia fail to polarize recipient cells toward an inflammatory state. The molecular mechanisms underlying these effects involve the transfer of functional TLR4 receptors, pro-inflammatory proteins, and

regulatory microRNAs that can modulate gene expression in target cells, with studies showing that EVs-LPS can induce even stronger activation of certain inflammatory mediators compared to direct LPS treatment, suggesting a potent amplification mechanism for neuroinflammatory signals. Beyond microglia, other CNS cell types also contribute to inflammation-associated EV signaling networks.

EVs in neurodegenerative conditions also transport misfolded pathological proteins, including amyloid-beta and tau in AD, alpha-synuclein in PD, and SOD1 in ALS, thereby facilitating the prion-like spread of protein aggregates throughout neural networks [182].

The dual nature of EVs in CNS pathology presents both challenges and opportunities: while they can exacerbate disease progression by disseminating toxic and inflammatory molecules, they also represent potential therapeutic targets for intervention strategies aimed at modulating their biogenesis, cargo loading, or uptake by recipient cells. Engineered EVs loaded with anti-inflammatory compounds such as curcumin or Bryostatin-1, or those derived from alternatively activated M2 microglia enriched in neuroprotective microRNAs like miR-124 and miR-137, have shown promise in preclinical models by reducing infarct volumes, promoting neuronal survival, suppressing inflammatory cytokine expression, and enhancing functional recovery after CNS injury [183]. Understanding the precise molecular mechanisms governing EV-mediated transport of pro-inflammatory substances, including the signals that trigger their release, the pathways that determine cargo selection, and the receptors that mediate their recognition and uptake by target cells, will be essential for developing novel diagnostic biomarkers and therapeutic interventions for neuroinflammatory and

neurodegenerative disorders, potentially enabling the transformation of pathological EV signaling into neuroprotective pathways through targeted molecular engineering and pharmacological modulation of vesicle biology [184].

In my PhD this led me to explore chrysin-loaded extracellular vesicles as an innovative delivery system that harnesses EVs' natural properties. These vesicles function as signal carriers between cells within the CNS, playing essential roles in both physiological conditions such as development and synaptic neurotransmission, and in pathological neurodegenerative conditions.

The therapeutic potential of EVs stems from their intrinsic biological composition and functional properties. They naturally encapsulate diverse cargo including cellular proteins, DNA, and RNA, while offering multiple benefits related to biocompatibility, reduced immunogenicity, stability, favorable pharmacokinetics, and efficient cellular uptake mechanisms [185]. Critically, EVs can cross the blood-brain barrier and selectively target specific cell types, facilitating the transfer of both intravesicular cargo and membrane receptors to recipient cells. These properties provide EVs with competitive advantages over synthetic drug delivery systems, which often elicit toxic immune responses *in vivo* and accumulate predominantly in the liver [186].

During my second year of my PhD, I was able to begin an in-depth study of EVs as carrier of Chrysin at the "Immunité Innée et Cancer" laboratory at the CHU d'Angers. My studies with EVs-Chry derived from BV2 cells revealed significant neuroprotective effects against LPS-induced microglial activation. EVs-Chry effectively reverse LPS-induced cell proliferation and restore resting microglial

morphology while reducing migratory capacity. Treatment with EVs-Chry, particularly at concentrations of 5 μM and 20 μM, maintains cell viability at levels significantly lower than those observed in LPS-treated groups, more closely resembling control levels. Morphological analysis reveals that microglia treated with EVs-Chry and LPS are dynamically directed toward a branched, non-amoeboid morphology associated with enhanced neuroprotective effects and significant reduction in pro-inflammatory features.

I noticed that EVs-Chry's anti-inflammatory mechanisms parallel those of irisin in several ways. Combined treatment with EVs-Chry and LPS demonstrates reduced expression of pro-inflammatory cytokines IL-1β and IL-6, while simultaneously increasing Bcl-xL levels. The reduction in IL-6, a key proinflammatory cytokine implicated in neurological disorders including stroke and epilepsy, confirms the downregulating and protective effects of these vesicles. Moreover, EVs-Chry significantly reduce caspase-1 levels, an essential effector protease involved in inflammasome activation, even when induced by LPS stimulation. This reminded me of irisin's effects, suggesting these different approaches might work through convergent mechanisms. The antiapoptotic effects of EVs-Chry, mediated through upregulation of Bcl-xL expression, represent an additional therapeutic dimension not explicitly examined in irisin studies. The Bcl-2 family plays a central role in regulating mitochondrial-mediated apoptosis through modulation of cytochrome c release, which initiates caspase activation and programmed cell death. By enhancing Bcl-xL expression even in the presence of LPS, EVs-Chry demonstrate the capacity to influence Bcl-2 family effectors and regulate apoptotic responses, providing a dual anti-

inflammatory and antiapoptotic effect that enhances cellular survival pathways associated with neuroinflammation.

Importantly, control EVs without chrysin loading exhibited no comparable anti-inflammatory or antiapoptotic activity, underscoring the critical importance of vesicle cargo composition in determining functional properties. This underlined how critical the cargo vesicle is in determining their function and highlighted the specificity of the therapeutic effects we observed.

6. CONCLUSIONS

As the global population ages, with over 2 billion individuals expected to exceed 60 years by 2050, developing effective interventions to contrast neurodegenerative diseases has become increasingly imperative. In this evolving landscape, both these studies suggest promising therapeutic strategies for modulating inflammatory responses within neurodegenerative contexts. These approaches share several fundamental mechanisms, including the induction of M2 microglial polarization, the reduction of pro-inflammatory cytokine expression, the inhibition of the NLRP3 inflammasome pathway, and the restoration of resting microglial morphology. Complementing this investigation of irisin, that offers distinct advantages as a naturally occurring, exercise-induced myokine that functions effectively at physiological concentrations, EVs-Chry provide superior delivery characteristics, characterized by enhanced biocompatibility, targeted cellular uptake, and efficient BBB penetration. The innovative core of this research lies in recognizing extracellular vesicles as sophisticated biological delivery systems, nanocarriers that fundamentally redefine our understanding of intercellular communication and therapeutic intervention. Unlike traditional pharmacological agents that face significant obstacles in crossing the blood-brain barrier and achieving precise cellular targeting, extracellular vesicles function as nature's own transport vehicles, engineered to ferry bioactive cargo between cells with exquisite specificity. The chrysin-loaded extracellular vesicles investigated in this thesis exemplify this revolutionary concept: these nanoscale carriers encapsulate therapeutic compounds within a biocompatible membrane envelope, facilitating efficient

BBB penetration, protecting cargo from degradation, and ensuring targeted uptake by recipient cells through surface-displayed recognition molecules. The convergence of these findings suggests that modulating microglial activation represents a viable therapeutic avenue for the prevention and treatment of neurodegenerative conditions. Such an approach addresses both immediate clinical needs and the broader objective of promoting healthy aging aiming to extend not merely lifespan, but the health span and overall quality of life for older adults. Furthermore, the intersection of exercise physiology, extracellular vesicle biology, and neuroinflammation research suggests that multi-modal interventions, which combine lifestyle modifications with advanced biotechnological approaches, will likely yield the most comprehensive treatment paradigm. Nevertheless, future investigations must address several critical areas to fully realize this therapeutic potential. Detailed mechanistic elucidation is required, particularly regarding cell-specific EV uptake mechanisms and the specific roles of cargo components in therapeutic efficacy. Finally, the optimization of therapeutic protocols will necessitate the standardization of EV isolation and characterization according to MISEV (Minimal information for studies of extracellular vesicles) guidelines, the development of precise dosing regimens, and the continued engineering of EVs for enhanced targeting efficiency.

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