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REVIEW

Microglia-derived extracellular vesicles in homeostasis and demyelination/remyelination processes

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Abstract

Microglia (MG) play a crucial role as the predominant myeloid cells in the central nervous system and are commonly activated in multiple sclerosis. They perform essential functions under normal conditions, such as actively surveying the surrounding parenchyma, facilitating synaptic remodeling, engulfing dead cells and debris, and protecting the brain against infectious pathogens and harmful self-proteins. Extracellular vesicles (EVs) are diverse structures enclosed by a lipid bilayer that originate from intracellular endocytic trafficking or the plasma membrane. They are released by cells into the extracellular space and can be found in various bodily fluids. EVs have recently emerged as a communication mechanism between cells, enabling the transfer of functional proteins, lipids, different RNA species, and even fragments of DNA from donor cells. MG act as both source and recipient of EVs. Consequently, MG-derived EVs are involved in regulating synapse development and maintaining homeostasis. These EVs also directly influence astrocytes, significantly increasing the release of inflammatory cytokines like IL-1β, IL-6, and TNF-α, resulting in a robust inflammatory response. Furthermore, EVs derived from inflammatory MG have been found to inhibit remyelination, whereas Evs produced by pro-regenerative MG effectively promote myelin repair. This review aims to provide an overview of the current understanding of MG-derived Evs, their impact on neighboring cells, and the cellular microenvironment in normal conditions and pathological states, specifically focusing on demyelination and remyelination processes.

KEYWORDS
demyelination, extracellular vesicles, microglia, oligodendrocytes, remyelination

Abbreviations: ApoE, apolipoprotein E; AST, astrocytes; ATP, adenosine triphosphate; BBB, Blood–brain-barrier; BDNF, brain-derived neurotrophic factor; CDKL, cyclin-dependent kinase-like 5; CNS, central nervous system; CPZ, Cuprizone; CSF-R1, colony-stimulating factor receptor-1; EAE, encephalomyelitis autoimmune experimental; EVs, Extracellular vesicles; FGF, fibroblast growth factor; GDNF, glial cell-derived growth factor; GFAP, glial fibrillary acidic protein; Iba-1, binding adaptor molecule 1; IGF, insulin growth factor; IL, Interleukin; iNOS, Inducible nitric oxide synthase; KLF4, Krüppel-like factor 4; LPL, lipoprotein lipase; Mfge8, Milk fat globule-EGF factor 8 protein; MG, microglia; MHC, major histocompatibility complex; miRNA, micro RNA; MMP, Matrix metalloproteinase; MS, multiple sclerosis; MSC, mesenchymal stem cells; NGF, nerve growth factor; NO, Nitric oxide; NT-3, Neurotrophin-3; OLG, oligodendrocytes; Olig3, Oligodendrocyte Transcription Factor 3; OPC, oligodendrocyte precursor cells; PAD12, peptidylarginine deiminase 12; PKC, protein kinase C; scRNA, small conditional RNA; SPP1, Secreted Phosphoprotein 1; TGF, transforming growth factor; Tmem119, transmembrane protein 119; TNF, tumor necrosis factor; TNFR2, tumor necrosis factor receptor 2.

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1 | INTRODUCTION

Microglia (MG) are the most abundant resident myeloid cells in the central nervous system (CNS), representing 5%–20% of CNS cells and 0.2% of total retinal cells. Unlike oligodendrocytes (OLG) and astrocytes (AST), which originate in the neuroectoderm, MG have their origin in the mesoderm. MG are produced through hematopoiesis in the yolk sac during embryogenesis (Ginhoux et al., 2010) and can self-renew throughout life, their number remaining stable in the brain through the coupled processes of apoptosis and mitosis (Askew et al., 2017; Askew & Gómez-Nicola, 2017; Li & Barres, 2017; Li, Cheng, et al., 2019; Li, Liu, et al., 2019; Li, Tong, et al., 2019; Salter & Stevens, 2017; Sierra et al., 2019). Studies using the Microfetti mouse, which randomly expresses one of four possible fluorescent proteins and thus enables the monitoring of MG dynamics and fate, have shown that MG are long-lived and their population is renewed at random locations three times during the 2-year half-life of a mouse. These observations suggest that the microglial network in the healthy adult mouse brain is largely stable and that microglial self-renewal is likely a stochastic process. In humans, MG live for decades and their turnover is thus slow, which suggests that MG may have a memory of past events imprinted on their epigenome (Askew et al., 2017; Sierra et al., 2019; Tay et al., 2017). It has been further shown that depleted MG are replaced by new MG derived from residual MG in adults (Huang, Ge, et al., 2018; Huang, Xu, et al., 2018; Wolf et al., 2017).

Being in contact with different cell types and detecting multiple extracellular signals, MG fulfill critical functions in physiological conditions; more specifically, MG (i) actively survey surrounding parenchyma via dynamic movement of processes, (ii) maintain synaptic remodeling, phagocytosis of dead cells or cell debris, and myelin homeostasis, and (iii) protect the brain against infectious pathogens and injurious self-proteins (Aires et al., 2021; Hickman et al., 2018). MG can integrate into, translate, and respond to different stimuli, rapidly changing their state to maintain homeostasis (Salter & Stevens, 2017). During development, MG are chiefly responsible for remodeling synapses, rearranging neural circuits, and engulfing apoptotic neurons (Marín-Teva et al., 2004). In adult life, MG closely interact with neurons, the movement of MG processes depends on neuronal activity (Szepesi et al., 2018). The maintenance of homeostasis by MG partly relies on signals derived from neuronal and AST-derived factors. Healthy neurons secrete membrane-bound signals such as CD200 (Hatherley & Barclay, 2004) and fractalkine (CX3CL1; Harrison et al., 1998). In addition, neurons and AST release immune-related soluble factors that bind to cognate receptors on MG and promote specific MG phenotypes. These factors comprise neurotrophins (e.g., NT-3, BDNF, and NGF), neurotransmitters (e.g., glutamate), and anti-inflammatory cytokines (e.g., TGF-β; Biber et al., 2007; Kerschensteiner et al., 2009).

Notably, a reduction in fractalkine signaling observed in neuroinflammatory settings results in MG activation and pro-inflammatory activity (Pawelec et al., 2020). AST can influence several functions of the MG. They can modulate phagocytosis by releasing CXCL10 and regulate macrophage colony-stimulating factor expression, which modulates MG activation. Moreover, AST release advanced glycation end-products, whose receptors are expressed by MG and induce NFκB activation and pro-inflammatory cytokine release. Additionally, iron transport regulation by AST may influence MG function, as a decrease in iron reduces the microglial secretion of pro-inflammatory cytokines TNF-α and IL-1β (Molina-Gonzalez & Miron, 2019). Conversely, a regulatory negative feedback loop has been recently identified, driven by MG-AST interactions and mediated by amphiregulin and IL-33 receptor signaling. Amphiregulin produced by MG limits NF-κB-driven AST pro-inflammatory responses which promote CNS pathology in experimental autoimmune encephalomyelitis (EAE) and, potentially, multiple sclerosis (MS) (Wheeler et al., 2023).

Extracellular vesicles (EVs) are heterogeneous lipid bilayer-enclosed structures originated from the intracellular endocytic trafficking pathway or from the plasma membrane which are released by cells into the extracellular space and are present in all body fluids (György et al., 2011; Harding et al., 1983). EVs were initially thought to be a disposal mechanism to discard unwanted materials from cells. However, later studies showed several EVs biological functions in both physiological and pathological conditions (D'Anca et al., 2021). EVs are one of the most recently discovered communication mechanisms between surrounding and distant cells, facilitating the transfer of functional proteins, lipids, multiple RNA species, and even DNA fragments from donor cells (Skog et al., 2008). EVs recognize and interact with specific target cells (Lösche et al., 2004). They can influence the behavior of these target cells through various mechanisms, including: (i) acting through signaling complexes mediated by surface-expressed ligands (Ratajczak, Wysoczynski, et al., 2006), (ii) receptor-mediated binding to the surface of target cells (Quah et al., 2008), (iii) delivering functional proteins (Sarkar et al., 2009), or (iv) transferring genetic information via mRNA, microRNA, or transcription factors (Ratajczak, Miekus, et al., 2006). However, EVs can also directly interact with the surrounding extracellular matrix (ECM; Patel et al., 2023) and actively degrade it with their surface-associated enzymes (Sung et al., 2015), thus playing an integral role in ECM evolution in both physiological and pathological conditions.

In this context, this review provides an overview of the current knowledge of the role of MG-derived EVs and their impact on neighboring cells and the cellular microenvironment in homeostasis and pathological conditions, with a particular emphasis on demyelination and remyelination processes.

1.1 | MG phenotypes

MG are highly heterogeneous cells characterized by a dual function. Like peripheral macrophages, MG play important roles in both homeostatic and inflammatory conditions (Epelman et al., 2014). They express surface markers typically present on many other tissue macrophages and/or monocytes, including colony-stimulating factor receptor 1 (CSF-1R), integrin CD11b, glycoproteins CD68 and F4/80,

ionized calcium-binding adapter molecule 1 (Iba-1), proto-oncogene tyrosine-protein kinase MER (MerTK), and common leukocyte antigen CD45, the latter showing lower expression than in circulating monocytes/macrophages (Amici et al., 2017; Tay et al., 2017). By contrast, unlike most other tissue macrophages, adult MG constitutively express high levels of fractalkine receptor CX3CR1. At the same time, specific markers distinguish MG from other myeloid cells. In physiological conditions, Tmem119 is specifically expressed in homeostatic human and murine MG, but not in other brain-resident cells or infiltrating macrophages (Bennett et al., 2016; Ruan et al., 2020; Satoh et al., 2016). However, more recent studies have shown that Tmem119 immunoreactivity decreases in reactive MG under pathological conditions to levels comparable to those of blood-borne macrophages, which blurs discrimination between these myeloid populations after brain injury (Mercurio et al., 2022). In addition, conventional immunohistochemistry and double-labeled immunofluorescence studies have shown that CD163 specifically reveals perivascular macrophages in the normal human CNS. However, in MS lesions, CD163 staining is observed in foamy macrophages and MG, together with an increase in the number of perivascular macrophages stained. In contrast, mannose receptor expression is restricted to perivascular macrophages in both normal and inflamed brain tissue (Fabriek et al., 2005). Table 1 shows markers specific to MG in their different stages, as well as markers specific to peripheral macrophages.

MG had been long believed to be quiet, resting cells in steady-state conditions; however, due to advances in immunological and molecular techniques that allow for the in-depth study of cell populations, MG are now known to have extremely mobile processes and protrusions which are in constant movement to survey the environment (Figure 1). Moreover, ongoing research has shown that MG do not only change morphology (for example, branched, primed, reactive or amoeboid) but also present different phenotypes in both homeostatic and pathological conditions (Torres-Platas et al., 2014). In the initial paradigm, MG were classified into two opposite types: classical (M1) or alternative (M2). This classification was eventually found to oversimplify the real complexity of MG (Ransohoff, 2016), and a continuum of intermediate phenotypes was established between M1 and M2 along which MG can transit (Colonna & Butovsky, 2017). M1 MG release pro-inflammatory mediators, inducing inflammation and neurotoxicity. In particular, M1 MG produce CCL2, IL-12, IL-1β, IL-6, IL-18, IL-23, and TNF-α, reactive oxygen species (ROS), and inducible and nitric oxide synthase (iNOS). The release of these cytokines into the surrounding tissues creates a feed-forward loop, activating neighboring MG and promoting further inflammation. Furthermore, MG express the main histocompatibility complex type II (MHC-II), Fc receptors, integrins, co-stimulatory molecules, and matrix metallopeptidase (MMP)-12. In contrast, M2 MG are proposed as anti-inflammatory, healing cells, releasing anti-inflammatory cytokines such as TGF-β and IL-10 and secreting growth and neurotrophic factors such as FGF, IGF-1, CSF-1, NGF, BDNF, GDNF, arginase 1, and the pro-survival factor progranulin. CD206 has been considered a specific marker for this population (Miron et al., 2013).

It is worth noting that marker expression varies according to the regions in which MG are studied. Additionally, a critical issue to consider is the disparity in microglial markers expressed in rodents as compared to humans (Martinez & Gordon, 2014). This disparity often hinders the translation of primary study findings to human disease (Jubb et al., 2016). Moreover, microglial subsets in the developmental brain differ considerably from those in the adult brain, especially during aging (Réu et al., 2017).

Given that MG perform pleiotropic functions in the CNS, studies first focused on potentially specialized subsets of MG. Early analyses such as immunohistochemistry, in situ hybridization, and flow cytometry were unable to probe and monitor the MG landscape in different conditions. However, the recent introduction of single-cell (scRNA-seq) and single-cell mass spectroscopy technologies has made it possible to profile single cells with high-throughput datasets, which may allow the identification of new markers, pathways, and microglial states with critical roles in homeostasis and disease (Ajami et al., 2018; Masuda et al., 2019; Morris et al., 2020; Trapnell, 2015). In conditions of homeostasis, MG subtypes differ in gene expression profiles, stages of development, and CNS regions populated, which hints at local MG specificity. Grabert et al. (2016) demonstrated that MG exhibit unique transcriptional profiles dependent on the region, and these profiles are associated with aging in mice. The influence of aging on this diversity also implies a foundation for regional disparities in susceptibility to age-related neurodegenerative processes involving neuro-inflammatory mechanisms. More recently and using multiplex mass cytometry of post-mortem samples, Böttcher et al. (2019) determined the expression levels of 57 postmortem human MG-specific markers from up to five different brain regions. These signatures were different from those of peripheral myeloid cells but comparable to those obtained from fresh human MG. In subsequent work, Masuda et al. (2019) used single-cell analysis of homeostatic CNS tissues in mice and revealed specific time- and region-dependent MG subtypes. MG development and survival critically rely on class III tyrosine kinase CSF-1R. Recently, studies by our groupusing animal cuprizone (CPZ)-induced demyelination have shown that pharmacological inhibition of CSF-1R generates different responses to microglial depletion in terms of myelin protection and axonal degeneration across the cortex, striatum, corpus callosum, hippocampus, and cerebellum, which may be associated with the regional heterogeneity of MG (Wies Mancini et al., 2019, 2022).

Moreover, a growing body of evidence demonstrates that demyelinating and neurodegenerative diseases evoke context-dependent MG subtypes with distinct molecular hallmarks and cellular kinetics. Analysis of disease-specific signatures in MG has revealed that subpopulations emerge in demyelination and remyelination processes, which indicates that different MG phenotypes may perform different functions (Flowers et al., 2017; Grabert et al., 2016; Hammond et al., 2019; Masuda et al., 2019; Rodríguez-Gómez et al., 2020). In EAE, purinergic receptor P2X4 is expressed by MG at the recovery peak, suppressing pro-inflammatory genes such as Nos2, which codes for *iNOS* and *Tnf* (Lloyd & Miron, 2019; Yu et al., 2015; Zabala et al., 2018). Indeed, P2X4 blockade worsens EAE severity, impairing

TABLE 1 Cell-specific markers of MG, macrophages, and vesicles derived from these cells.

(a) Selective markers for MG, macrophages, perivascular macrophages, choroid plexus macrophages, and meningeal macrophages

Cells	Specific markers	Reference
MG		
Ramified or steady state	IBA1$^+$, P2RY12$^+$, TMEM119$^+$, CD74$^+$, Cx3cr1^{hi}, CD11b$^+$, CD115$^+$, CD64$^+$, MerTK$^+$, F4/80$^+$, FCRLS$^+$, Siglec-H$^+$, CD206$^-$ CD45^{low}, CD163$^-$ CD11b$^+$, MHCII$^+$, Ly6C$^-$, Sall1$^+$, HexB$^+$	Ginhoux et al. (2010) Kierdorf et al. (2013) Greter et al. (2015) Jurga et al. (2020) Lier et al. (2021) Kenkhuis et al. (2022)
Pro-inflammatory, M1-like	IBA1$^+$, CD74$^+$, CD68$^+$, ferritin, CD45$^+$, CD11b$^+$, F4/80$^+$, Cx3cr1 ^{hi}, CD11c^{int}, MHCII^{int}, CD14$^+$, CD16$^+$, CD32$^+$, CD40$^+$, CD86$^+$, TLR2$^+$, TRL4$^+$, CD36+, iNOS, Cox-2, Trem-1$^+$	Greter et al. (2015) Jurga et al. (2020) Lier et al. (2021) Wu et al. (2022)
Anti-inflammatory, M2-like	IBA1$^+$, CD74$^+$, CD68$^+$, CD301$^+$, MHCII^{low}, ferritin, CD163$^+$, CD206$^+$, CD204$^+$, arginase, TGM2, NR1C3, Trem-2$^+$	Abellanas et al. (2019) Lier et al. (2021) Wu et al. (2022)
Amoeboid	IBA1$^+$, CD74$^+$, CD68$^+$, MHCII$^+$	Lier et al. (2021)
Dystrophic	IBA1$^+$, CD74$^+$, TMEM119$^+$, ferritin$^+$	Lier et al. (2021)
Macrophages		Lier et al. (2021)
Steady-state	CD44$^+$, CD45^{hi}, CD169$^+$, CD206^{hi}	Jurga et al. (2020)
Pro-inflammatory, M1-like	CD80$^+$, CD86$^+$, TRL-2$^+$, TLR-4$^+$, iNOS$^+$, MHC-II$^+$, CD16$^+$, CD32$^+$	Yao et al. (2019) Lyu et al. (2020)
Anti-inflammatory, M2-like	CD163$^+$, CD206$^+$, CD209$^+$, FIZZ1$^+$, Ym1/2$^+$, Arg-1$^+$, CD14$^+$, CD204$^+$, CCL17$^+$, CCL22$^+$, CCD24$^+$	Yao et al. (2019) Chu et al. (2018)
M2a	IL-1R$^+$, CD206$^+$, Arg-1$^+$, FIZZ1$^+$, Ym1/2$^+$	Yao et al. (2019)
M2b	IL-10R$^+$, IL-12R$^+$, CD86$^+$, IL-6R$^+$	Yao et al. (2019)
M2c	Arg-1$^+$, TRL-8$^+$, TLR-1$^+$, CD206$^+$, CD163$^+$	Yao et al. (2019)
M2d	IL-10R$^+$, IL-12R$^+$	Yao et al. (2019)
Perivascular macrophages	CD206$^+$, CD163$^+$, CD45^{hi}, CD11b$^+$, MHCII^{hi}, Ly6C^{low}, F480$^+$, Cx3cr1^{low}, Iba1^{low}, LYVE1$^+$	Zeisel et al. (2015) Faraco et al. (2016) Goldmann et al. (2016)
Choroid plexus macrophages	CD206$^+$, CD163$^+$, CD45^{hi}, CD11b$^+$, MHCII^{hi}, Ly6C^{low}, F480$^+$, Cx3cr1^{low}, Iba1^{low}	Chinnery et al. (2010) Goldmann et al. (2016)
Meningeal macrophages	CD206$^+$, CD163$^+$, CD45^{hi}, CD11b$^+$, MHCII^{hi}, Ly6C^{low}, F480$^+$, Cx3cr1^{low}, Iba1^{low}, LYVE1$^+$	Chinnery et al. (2010) Goldmann et al. (2016)

(b) Selective markers for MG- and macrophage-derived EVs

Cells	Specific EV markers	Reference
MG	TMEM119$^+$ GFP-transgenic rats Iba-1$^+$/PKH67$^+$ TMEM119$^+$/CD14$^+$ CD11b$^+$	Visconte et al. (2023) Zhang et al. (2023) Lombardi et al. (2019) Xin et al. (2020) Roseborough et al. (2023) Cohn et al. (2021)
Macrophages	CD14$^+$ PKH67 staining kit	Albrecht et al. (2023) Chu et al. (2023) Zhang et al. (2023)

the removal of myelin debris. In addition, the expression of homeobox transcription factor protein MSX3 is associated with a pro-remyelination microglial activation state (Yu et al., 2015), which increases the expression of *Mrc1* (encoding CD206) and *Igf1* and reduces the expression of *Nos2* (encoding iNOS) and *Tnf* (Yu et al., 2015). Furthermore, cells isolated from the brains of patients with histologically confirmed early active MS express increased levels of *APOE*, the transcription factor *MAFB*, SPP1, PAD12, and LPL, whereas the expression levels of *TMEM119* and the purinergic receptor *P2RY12* are significantly lower or null. Notably, MG gene profiles enriched in the brain of MS patients are similar to those observed in murine demyelination models in demyelination and remyelination (Masuda et al., 2019).

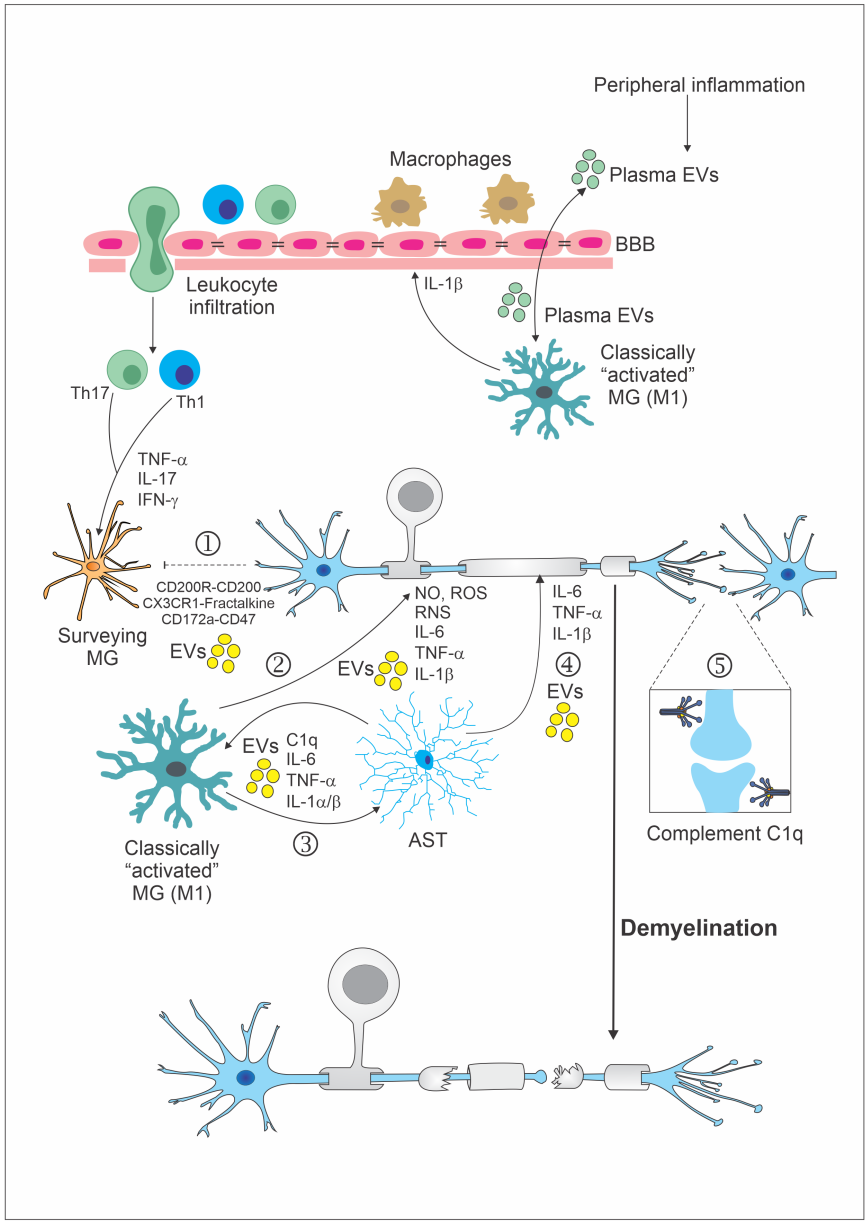

FIGURE 1 In a surveillance state, MG monitor the brain parenchyma and detect danger signals. This state is maintained through interactions with neurons, such as CD200-CD200R, CD47-CD172a, and fractalkine-CX3CR1 interactions. As a consequence of neuroinflammation mediated, for example, by cytokines released by Th1 and Th17 cells, these interactions are lost, and resident MG change phenotypes to an "activated" state. In addition, EVs induced by peripheral inflammation can cross the BBB and activate M1 MG (1). The expansion and activation of activated MG are associated with the production of EVs capable of releasing pro-inflammatory cytokines and toxic metabolites such as nitric oxide (NO), reactive oxygen species (ROS), and nitric oxide species (RNS). EVs can also transport glutaminase, which increases pro-inflammatory miRNAs and reduces anti-inflammatory miRNAs. Furthermore, EVs transfer the inflammasome adapter ASC, increasing the activation of NLRP3 and the production of IL-1β (2). EVs from activated MG also release IL-1α, TNF, and the C1q component of complement, promoting the transformation of AST into harmful A1 cells. In turn, A1 AST can re-stimulate MG, promoting a retro-stimulation circuit (3). In addition, the release of MG-derived EVs containing ATP stimulates the purinergic receptor P2X7, inducing the release of pro-inflammatory cytokines IL-1, IL-6, and TNF-α by AST (4). Through their cargos, MG-derived EVs exert multiple actions on synapses, including detrimental effects on dendritic spines, the stimulation of Glu release, the down-regulation of GABAergic transmission, and the silencing of pre-synaptic neuroligin-1 and post-synaptic synaptotagmin-1, among other effects, thus promoting synaptic dysfunction (5). For further details on these effects, see Table 3. ASC, apoptosis-associated speck-like protein containing a CARD; C1q, complement component C1q; EVs, extracellular vesicles; GABA, Gamma-aminobutyric acid; Glu, glutamic acid; IL, interleukin; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; TNF, tumor necrosis factor.

2 | EXTRACELLULAR VESICLES

EVs play an important role in cellular processes such as signal transduction (Gangoda et al., 2015), antigen presentation (Mittelbrunn et al., 2011), and immune responses (Greening et al., 2015). The contents transported by EVs and their function depend on age (Alibhai et al., 2020; Lazo et al., 2021; Pusic & Kraig, 2014), cell type (Nazarenko et al., 2010; Prada et al., 2018; Zhang, Zou, et al., 2021; Zhang, Wei, et al., 2021), and status of cell activation (Bhargava et al., 2019; Lombardi et al., 2019). EVs can survive in circulation, keep the cargo safe from the immune system, and even cross anatomical barriers like the blood–brain barrier (BBB) in a bidirectional manner between the bloodstream and brain parenchyma (Basso & Bonetto, 2016; Rajendran et al., 2014; Ridder et al., 2014). This communication is of vital importance, as neurons have a high metabolism and the BBB controls iron and molecule traffic. This makes EVs important mediators, as they might play a major role in health and disease, interfering with neuroinflammatory (Selmaj et al., 2017) and neurodegenerative processes (Quek et al., 2017). Biochemical fractionation has revealed that both EVs cargo and surface lipids can have either harmful or protective effects. For example, AST may be converted into harmful cells by inflammatory EVs cargo, whereas EV surface lipid components released by MG promote oligodendroglial progenitor cell (OPC) migration and/or differentiation, which links EV lipids to myelin repair (Lombardi et al., 2019). In the CNS, recent evidence suggests that the protein content of EVs might reflect the phenotype of the tissue of origin (Yates et al., 2022).

Tetraspanins such as CD9, CD63, CD81, CD82, and CD15 are highly enriched in EVs (Andreu & Yáñez-Mó, 2014). Differential protein expression has been observed in EVs released by various cell types (Dozio & Sanchez, 2017; Xu et al., 2016). It should be noted, however, that while tetraspanins have been proposed as markers for some EVs, it is unclear whether all EVs contain tetraspanins. On the other hand, separate EVs populations isolated from the blood of patients with specific clinical pictures have been reported to express the same miRNA sequences (Nuzziello et al., 2017; Ramirez et al., 2018). These small, single-stranded, non-coding RNA molecules can post-transcriptionally regulate gene expression. Therefore, improvements in EVs isolation and characterization may raise awareness of additional common markers for and properties of different EVs subtypes. These differences may prove significant for EVs to serve as disease biomarkers, as they are highly stable, reach sufficient concentrations in biological fluids, and carry molecules that reflect their parental cells. However, a panel approach of various biomarkers, along with large-scale multicenter longitudinal approaches, will be needed to fully evaluate the utility of EVs as biomarkers in diagnosing and monitoring recovery following disease or injury onset (Kawata et al., 2018).

2.1 | EV biogenesis, composition, and cargo

The characterization of EVs is key to their analysis and application in experimental models (Théry et al., 2018). EVs comprise three main vesicle subtypes: exosomes (40–100 nm in diameter), microvesicles or ectosomes (>100 nm in diameter), and apoptotic bodies (1–5 μm in diameter) (Beyer & Pisetsky, 2010; Cocucci et al., 2009; Mathivanan et al., 2010; Théry et al., 2009). Although the last decade has seen a marked increase in the number of scientific publications describing physiological and pathological functions of EVs the criteria for EV classification into subclasses is still matter of debate. Therefore, the International Society for Extracellular Vesicles has endorsed "extracellular vesicle" as the generic term for particles naturally which are released by cells, are delimited by a lipid bilayer, and cannot replicate, that is, do not contain a functional nucleus (Théry et al., 2018). This heterogeneity is a consequence of the variety and functional states of releasing cells, as well as of the different cell death mechanisms.

Almost all cells can produce and release EVs which can both exert their action locally or migrate long distances through biological fluids to host cells, a recently discovered mode of intercellular communication for both short and longer-range signaling events (Simons & Raposo, 2009). EVs are taken up by recipient cells via receptor-ligand interaction (Hoshino et al., 2015), directly by fusion with the plasma membrane (Mathieu et al., 2019; Montecalvo et al., 2012), or endocytosis (Chivet et al., 2014; Mulcahy et al., 2014). EVs carry a rich cargo of DNA, RNA, enzymes, proteins, lipids, and metabolites (de Hornung et al., 2020; Doyle & Wang, 2019; Dutta et al., 2015; Théry et al., 2002, 2018; Thompson et al., 2016). In addition, circulatory EVs can carry coding and non-coding RNAs such as microRNAs (miRNAs), long-noncoding RNAs (ln cRNAs), small-interfering RNAs (siRNAs), and circular RNAs (circRNAs), captured by EVs in a differential, controlled manner with further active influence upon recipient cells. Some of these non-coding RNAs can regulate gene transcription at the post-transcriptional level by cleaving or blocking mRNAs for further translation (Zimta et al., 2020). Therefore, they can regulate processes such as development, cell fate, apoptosis, metabolisms, and responses to physiological and environmental changes (Ambros & Ruvkun, 2018).

2.2 | EV sources

There are multiple sources of EVs in the CNS, as they are secreted by both neuronal and glial cells. Therefore, circulating EVs in the bloodstream and other peripheral biofluids constitute a non-invasive and rapid alternative to studying brain-related disorders. The characterization of EVs isolated from human and mice serum and plasma has demonstrated similarities in vesicle size, shape, concentration, and presence of exosomal markers, which suggests that serum and plasma are equally helpful for EV isolation (Mattera et al., 2020; Soares Martins et al., 2018). EVs obtained from other biofluids, such as cerebrospinal fluid and saliva, have also been used for diagnostic purposes (Cao et al., 2019; Yoo et al., 2018). Although obtaining saliva from patients is less invasive than obtaining blood as a source of biomarkers in neurological diseases, saliva has been less widely studied than plasma or serum (Cao et al., 2019; Han et al., 2018). By contrast, biomarkers measured in CNS-derived serum or plasma

EVs representing CNS diseases are similar to those of cerebrospinal fluid, which indicates that the same confidence level can be achieved using a less invasive procedure. Another advantage of blood-derived EVs as compared to those of cerebrospinal fluid is that they allow marker comparison across different cell populations. MG represent a challenge, however, as they share surface markers with macrophages and peripheral monocytes and lack a specific EVs marker (de Hornung et al., 2020).

Much like saliva, tears may be a valuable non-invasive source of biomarkers thanks to the smaller protein dynamic range of these biological fluids with respect to serum and plasma (Ruhen & Meehan, 2019). Actually, tear EVs represent an attractive tool for disease diagnosis and monitoring using proteomics analysis. Indeed, the presence of EVs of both neuronal and microglial origin has been demonstrated in tears (Pieragostino et al., 2019).

3 | METHODS FOR ISOLATION OF EXTRACELLULAR VESICLES, THEIR LIMITATIONS, AND MOLECULAR MARKERS

The methodology used to obtain EVs essentially depends on the source. Théry et al. (2006) published a basic differential centrifugation protocol which for many years was the most widely used method to obtain EVs from culture media and body fluids. However, several studies have reported that ultracentrifugation at 100000×*g* may cause aggregation or morphological changes which could cause artifacts and lead to erroneous conclusions on EV composition or phenotype (Issman et al., 2013; Linares et al., 2015). This method allows EVs to be separated by their size and density but not by their subcellular origin. To isolate EVs from tissue samples, such as mouse brain tissue, this basic protocol is followed by a sucrose density gradient purification step (Muraoka et al., 2020; Perez-Gonzalez et al., 2012; Vella et al., 2017) or commercial gradients such as Optiprep Iodixanol (Crescitelli et al., 2021; Hurwitz et al., 2019). Alternative methods for obtaining EVs are size exclusion chromatography or ultrafiltration (Benedikter et al., 2017).

EVs can be detected through their specific markers using Western blot assays, immunohistochemistry, and flow cytometry. They can also be observed through transmission electron microscopy (Alberro et al., 2019; Santiago et al., 2023; Soares Martins et al., 2018; Vinuesa et al., 2019; Wang et al., 2020; Wei et al., 2021) and scanning microscopy (Barranco et al., 2019; Du et al., 2021; Mattera et al., 2020; Sung et al., 2019), while their size distribution and number can be determined by Dynamic Light Scattering (DLS; Mattera et al., 2020) and nanoparticle tracking analysis (NTA) (Alberro et al., 2019; Almansa et al., 2022; Santiago et al., 2023; Soares Martins et al., 2018; Zhu et al., 2020). Classic markers used for EV detection by Western blot and immunohistochemistry include flotilin-1, tetraspanins CD9, CD63, and CD81, ALIX, HSP70 and TSG101 (Casella et al., 2018; Lombardi et al., 2019; Mattera et al., 2020; Muraoka et al., 2021; Santiago et al., 2023; Soares Martins et al., 2018; Wang et al., 2020; Wei et al., 2021; Zhu et al., 2020). For detection by flow cytometry, different authors have reported the use of anti-CD63 FITC, anti-CD81 APC, and anti-CD9PE antibodies (Alberro et al., 2019; Wang et al., 2020).

4 | PHYSIOLOGICAL FUNCTIONS, CELL–CELL COMMUNICATION, AND EV-MEDIATED CROSSTALK

The coordination and orchestration of cellular events in multicellular systems depend on cell–cell communication. In the CNS, cells communicate via gap junctions, cell adhesion, and EVs, which are loaded with proteins, transcription factors, and nucleic acids, and carry carbohydrates and lipids in their membranes (Caruso Bavisotto et al., 2019; Zhang & Yang, 2018). EVs serve as vehicles for this communication in the brain by transferring molecules from diverse origins. In the CNS, each cell type is capable of secreting and taking up EVs, which gives them a key role in both health and during disease. EVs also possess versatile biological activity and can modulate different target cells. Physiological processes include the maintenance of myelination, synaptic plasticity, neuronal trophic support, and antigen presentation, among others. EVs have also been found to promote pathogenesis in neurological diseases such as Alzheimer's disease, Parkinson's disease, and prion diseases, by carrying misfolded proteins or their coding material between neurons (Brenna et al., 2020; Hill, 2019; Takeuchi, 2021; You et al., 2022). Successful intercellular communication entails the maintenance of EVs integrity, EVs contact with target cells, EVs internalization, and the activation of signaling cascades or the release of EVs content into the extracellular space (Antonucci et al., 2012; Budnik et al., 2016; Domingues et al., 2020; Goetzl et al., 2016; Krämer-Albers et al., 2007; Lee et al., 2012; Thompson et al., 2016).

4.1 | Specific physiological functions of MG-derived EVs

MG are both sources and recipients of EVs. EVs released by MG mirror the dynamic nature of their donor cells, exhibiting important and versatile functions in the CNS. In basal conditions, MG play key roles in modulating neuronal activity by pruning excessive or dysfunctional synapses, distributing supportive growth factors to active neurons and AST, and regulating synaptic function (Wang et al., 2011). While MG indeed modulate neuronal activity, accumulating evidence shows that neurons inform MG of their status and are thus capable of controlling microglial activation and motility (Kierdorf & Prinz, 2019). Studies have recently demonstrated that MG-derived EVs can regulate synapse development and homeostasis through their miRNA content (e.g., miRNA146a-5p) and the transport of bioactive lipids exerting their function on neuronal activation (Prada et al., 2018). Furthermore, MG-derived EVs play a role in excitatory neurotransmission, as their release stimulates the neuronal production of ceramide and sphingosine

both in vitro and in vivo (Augusto-Oliveira et al., 2019). EVs released by MG also have a direct effect on AST. MG stimulation with ATP through the activation of the P2X7 receptor massively increases EV release and modifies their proteomics and their effect on AST, as evidenced by a significant increase in the release of IL-1β, IL-6, and TNF-α, which induces a robust inflammatory response (Bianco et al., 2009; Drago et al., 2017). On the other hand, MG exposed to ATP release EVs containing proteins promoting neurite outgrowth and synaptogenesis—that is, thrombospondin 1 and 4 (Arber & Caroni, 1995; Eroglu et al., 2009) together with proteins that negatively regulate neuron apoptosis. Moreover, MG-derived EVs contain lactate, which serves as an energy source for neurons, isoform-1 of lactate DH, required for lactate synthesis, and lactate transporter MCT-1 (Potolicchio et al., 2005). These findings suggest that MG-derived EVs may have a direct, protective action toward neurons.

The prevention of myelin damage and the regeneration of oligodendrocytes capable of remyelination represent challenges to new therapies for functional brain recovery. Using an MG depletion model, Raffaele et al. (2020) showed that the intracranial injection of regenerative MG-derived EVs restores early MG/macrophage protective functions, rescues dystrophic, senescence-like traits in resident immune cells, and leads to oligodendroglial progenitor cell differentiation and functional recovery. Supporting these findings, the number of MG-derived EVs is significantly larger in EAE mice at the peak of the disease, which reflects disease course and severity. Likewise, the number of MG-derived EVs is also higher in patients with active MS as compared to patients with stable disease or healthy controls (Verderio, Muzio, et al., 2012).

Heterogenous microglial EV composition and function reflect MG multiplicity and a variety of activation phenotypes. Microglial EV release is induced by environmental stimuli, such as ATP (Asai et al., 2015; Drago et al., 2017; Lombardi et al., 2021; Takeuchi et al., 2015), pro-inflammatory cytokines (Casella et al., 2018; Prada et al., 2018), IL-4 (Casella et al., 2018; Lombardi et al., 2019; Prada et al., 2018; Raffaele et al., 2021), and lipopolysaccharide (Yang et al., 2018). In addition, EV production and content in MG and macrophages in vivo are affected by pathological conditions such as MS, Alzheimer's disease, and traumatic brain injury (Agosta et al., 2014; Dalla Costa et al., 2021; Gelibter et al., 2021; Joshi et al., 2014; Kumar et al., 2017; Liu et al., 2017; Verderio, Cagnoli, et al., 2012; Verderio, Muzio, et al., 2012). On the basis of this evidence, microglial EVs in body fluids emerge as a valuable parameter for diagnosing and monitoring disease progression and treatment efficacy.

4.2 | MG regulation by EVs from different cell populations

MG can act as target cells for EVs secreted by OLG, AST, and neurons, and are thus part of a complex cell–cell communication system. OLG-secreted EVs are transferred to MG by macropinocytosis and then functionally degraded by lysosomal trafficking (Fitzner et al., 2011; Frühbeis et al., 2020). In healthy conditions, this process does not affect cytokine expression, which indicates that MG are specialized in the elimination of excessive OLG membrane (Fitzner et al., 2011), a clearance mechanism for myelin maintenance in homeostatic conditions (Domingues et al., 2016). Several reports have shown that this action occurs through the CX3CR1 or IFN-β pathways (Delpech et al., 2019; Fröhlich et al., 2014; Kocur et al., 2015). Extracellular release, rather than lysosomal processing in OLG, might constitute an advantage for cells with poor degradation capacity. OLG have, in fact, a high capacity for membrane synthesis (Pfeiffer et al., 1993) but probably a low capacity for excess membrane degradation. Therefore, membrane synthesis and degradation tasks can be divided among different cell types (Fitzner et al., 2011). If permanently ongoing, however, this process may trigger an immune response by MG, regulated by the levels of MHC Class II expression. The role of EVs derived from OLG (OLG-EVs) in regulating chronic inflammation must be better understood. In this context, Van den Broek et al. (2022) demonstrate that OL-EVs are internalized by activated MG and play a pivotal role in maintaining cellular homeostasis during chronic inflammation. They achieve this by enhancing the formation of autophagic vesicles and reducing oxidative stress-induced apoptotic cell death.

EV secretion by neurons is enhanced by potassium-induced depolarization and facilitates microglial neurite shedding (Bahrini et al., 2015). In a neuronal context, in particular, EVs play a key role in the regulation of MG phenotypes in neurological diseases. Indeed, EVs derived from motor neurons of the NSC-34 lineage transfer inflammation by miR-124 to their neighboring cells substantially affecting the MG phenotype, reducing its phagocytic capacity, and inducing a pro-inflammatory phenotype characterized by an increase in the release of IL-1β, TNF-α, NO, and iNOS, and in the expression of MMP-2, MMP-9, and MHC-II (Pinto et al., 2017). Similarly, EVs derived from glioblastoma modulate human MG by inducing the expression of membrane type 1 MMP through EV-secreted miR-451 and miR-21 (de Vrij et al., 2015). In the same line, in vivo studies have demonstrated that MG capture glioma-derived EVs, which hints at a mechanism by which tumor cells communicate over long distances to evade the host's immune response (van der Vos et al., 2016). Table 2 summarizes MG regulation by EVs from different cell populations.

Intravenous treatment with EVs derived from mesenchymal stem cells (MSC) from adipose tissue in Theiler's murine encephalitis might mediate protection and/or recovery. This treatment modulates neuroinflammation by reducing GFAP and Iba-1 staining in the brain and increasing myelin protein expression. Furthermore, changes in MG morphology in the spinal cord suggest that EVs also modulate the MG activation state. These findings indicate that EVs can modulate neuroinflammation and increase myelin protein synthesis. The immunomodulatory, neuroprotective, and neurodegenerative properties of MSC make them promising candidates with therapeutic potential for different diseases, including MS (Laso-García et al., 2018).

TABLE 2 MG regulation by EVs secreted by different cell types in homeostasis and disease.

Cells	Effects	Mediators	Reference
Neurons	Facilitate MG neurite shedding	Potassium-induced depolarization	Bahrini et al., (2015)
Motor neurons	Affect the MG phenotype, reducing phagocytic capacity and inducing a pro-inflammatory phenotype	Transfer inflammation by miR-124	Pinto et al. (2017)
Glioblastoma	Induce the expression of membrane type 1 MMP	miR-451 and miR-21	de Vrij et al. (2015)
Oligodendrocytes	Degradation of OLG membrane	Macropinocytosis	Fitzner et al. (2011)
	Control chronic inflammation, increasing the formation of autophagic vesicles	HSPB8 conveying LC3B II and BAG 3. LC3B is involved in the formation of autophagosomes, playing a role in mitophagy and preventing excess ROS production	Van den Brock et al. (2022)
Astrocytes	Attenuate MG-mediated neuroinflammation	miR-873a-5p	Long et al. (2020)

Abbreviations: BAG 3, Bcl2-associated athanogene 3; HSPB8, heat-shock protein beta-8; LC3B II, microtubule-associated protein 1A/1B-light chain 3; MG, microglia; miR, micro-RNA; MMP, matrix metalloproteinases; OLG, oligodendrocytes; ROS, reactive oxygen species.

4.3 | Regulation of other cells by MG-derived EVs

The main effects of MG-derived EVs on other CNS cells are summarized in Table 3. It has been well established that, when activated, MG can increase EV secretion. These vesicles activate signaling pathways mediated by contact or by the delivery of genetic material, which has a key role in the functions and molecular processes of target cells. The molecular composition and function of microglial EVs reflect the activation state of donor cells (Drago et al., 2017). Indeed, whereas EVs derived from inflammatory MG have been shown to block remyelination, EVs produced by pro-regenerative MG efficiently promote myelin repair. AST and neighboring MG are the main target cells of MG-derived EVs. Notably, EVs have differential effects on neurotoxicity depending on the source of MG activation (Beneventano et al., 2017; Horn & MacLean, 2021). EVs produced by MG co-cultured with immunosuppressive MSC promote OPCs recruitment and myelin repair. On the other hand, exposure of OPCs in the presence of AST to EVs derived from pro-inflammatory MG produces a blockage in progenitor maturation, implying a failure in the remyelination process. By contrast, lipid components (particularly sphingosine 1 phosphate) on the surface of EVs promote OPCs migration and/or differentiation, linking EV lipids to myelin repair. Thus, microglial EVs emerge as multimodal signaling mediators capable of influencing both OPCs and AST around myelin lesions (Lombardi et al., 2019).

Primary MG incubated with saturated fatty acid palmitate acquire a pro-inflammatory profile. The EVs isolated from these MG induce an immature dendritic spine phenotype in hippocampal neurons, which suggests MG-neuron communication (Vinuesa et al., 2019). In a physiological setting, MG-derived EVs regulate synaptic transmission by different mechanisms, including a dose-dependent increase in the spontaneous release of glutamate and miniature excitatory postsynaptic currents in cultured neurons. Likewise, in vivo experiments have demonstrated that the injection of MG-derived EVs increases excitatory synaptic transmission through the enhancement of ceramide and sphingosine production (Antonucci et al., 2012). On the other hand, MG-derived EVs can deliver N-arachidonoyl ethanolamine, suppressing the spontaneous inhibition of presynaptic transmission in GABAergic neurons via the stimulation of type 1 cannabinoid receptors (Gabrielli et al., 2015; Paolicelli et al., 2019). However, in inflammatory brain conditions, MG respond by releasing EVs into the cerebrospinal fluid of MS patients (Verderio, Cagnoli, et al., 2012; Verderio, Muzio, et al., 2012) and can propagate inflammatory responses across distant brain regions, contributing to disease pathogenesis (Takeuchi et al., 2015). Depending on the stimulus, EVs can differentially influence synapses, with extracellular ATP being an important stimulant for the release of EVs in MG. In addition, EVs derived from inflammatory MG are enriched in miRNAs capable of regulating pre- and post-synaptic proteins. Among them, miR-146a-5p controls the expression of presynaptic protein synaptotagmin 1 and post-synaptic neuroligin 1, a protein important in dendritic spine formation and synaptic stability (Prada et al., 2018). In addition, MG-derived miR-146a-5p can suppress neurogenesis by directly repressing neurogenic factors KLF4 and CDKL5 (Fan et al., 2022).

MG-derived EVs can also have protective effects on neurons (Figure 2). Indeed, miRNA analysis of EVs from MG activated after brain injury have shown a significant increase in the expression of miR-124-3p and miR-9-5p, which promotes anti-inflammatory M2 polarization in MG and inhibits inflammation in injured neurons (Huang, Ge, et al., 2018; Huang, Xu, et al., 2018) miR-124-3 is a well-known neuron-enriched miRNA, and ex vivo isolated MG show a decrease in miR-124-3p after some weeks in culture. Therefore, adult MG can only express miR-124-3 in the CNS microenvironment, which suggests that miR-124-3 expression in MG depends on paracrine action with, or transfer from, neurons (Ponomarev et al., 2011; Veremeyko et al., 2019). Likewise, M2 MG induced by IL-4 can foster an anti-inflammatory phenotype. EVs derived from these polarized MG acquire the ability to protect neurons from apoptosis, a

mechanism mediated by miR-124 which blocks the expression of protease 14. Furthermore, EVs derived from M2 MG may inhibit AST proliferation and glial scar formation (Casella et al., 2018).

MG infiltrating demyelinated lesions secrete a large number of EVs, which suggests that EVs play an essential role in the communication between MG and OLG. In mice with demyelinated lesions caused by lysolecithin injection in the corpus callosum, the infusion of pro-regenerative MG polarized with IL-4 promotes OPC migration and differentiation, improving remyelination. Conversely, in the same setting, EVs released by pro-inflammatory MG inhibit remyelination (Lombardi et al., 2019). These effects are produced mainly on GPR17-expressing progenitors, a group of cells considered sensors of local damage to the myelin sheath (Lecca et al., 2020). Interestingly, further analysis has revealed that the blockage of remyelination by pro-inflammatory MG-derived EVs depends on AST transformation into deleterious cells rather than a direct effect on OLG (Lombardi et al., 2019). Broken EVs retain the capacity to promote OPC maturation, which suggests the primary involvement of surface molecules, particularly lipid components such as sphingosine-1 phosphate –whose inhibition abolishes the chemoatracttant effect of MG-derived EVs on OPCs and transmembrane TNF, which drives OPC migration and differentiation through the activation of oligodendroglial TNFR2 (Lombardi et al., 2019; Madsen et al., 2016). However, the pro-differentiating effect of EVs derived from M2 cells can also be mediated by their miRNA cargo. M2-EV treatment after experimental ischemia can promote OPC survival and differentiation and functional recovery via EV miR-23a-5p, miR-221-3p, miR129-5p, and miR-155-5p. In particular, M2-EV miRNA-23a-5pmay promote OPC differentiation by inhibiting Olig3 expression. Supporting this notion, the knockdown of miR-23a-5p in M2-EVs abolishes the effects on OPC differentiation and oligodendrogenesis (Li et al., 2022). In addition, M2-EVs can protect neurons against apoptosis and inhibit the proliferation of AST and glia scar formation through miR-124. These findings suggest that the beneficial effects of MG-derived EVs on OPC differentiation and remyelination are mediated by a combination of molecules rather than a single one. Overall, EVs derived from MG can exert both detrimental and protective effects on neighboring or distant cells depending on their contents and their surface structure, which are partly determined by their surrounding milieu and, consequently, their polarization state.

5 | EVS AS THERAPEUTIC TOOLS TARGETING MG

EVs regulate different signaling pathways in the CNS and, as mentioned above, can cross the BBB either by paracellular or transcellular transportation. For transcellular transportation, EVs need to be previously internalized by brain microvascular endothelial cells. In addition, EVs can increase BBB permeability by dysregulating tight junction proteins.

The selection of cell sources for the production of EVs as therapeutic tools is critical. In this regard, two primary sources have been investigated: (i) MSC-derived EVs isolated from unmodified cells, and (ii) MSC-derived EVs isolated from modified cells whose composition has been modified to expand their targeting and therapeutic capacity. Pre-isolated EVs load drugs or genetic material into the lumen or display targeting ligands. Thus, targeted EVs as drug carriers may be a promising therapeutic strategy to reach the compartmentalized CNS immune system, which drives neurodegeneration and demyelination failure (Hickman et al., 2018).

EVs can be designed to carry specific cargos to be released to a particular target cell. In this context, the murine MG cell line Bv2 has been recently engineered to release EVs with surface marker lactadherin (Mfge8) to target phagocytes and containing the specific cargo IL-4. After inoculation into the cisterna magna of EAE animals, these EVs are internalized by myeloid cells in the meningeal compartment and choroid plexus, reducing neuroinflammation and clinical signals (Casella et al., 2018). Likewise, EVs loaded with PKC agonists provide marked benefits in EAE animals and CPZ-fed mice by acting on MG, favoring the transition of myeloid cells from a pro-inflammatory to an anti-inflammatory phenotype (Kornberg et al., 2018; Wu et al., 2022). Similarly, EVs derived from bone marrow MSC (BMSC) administered intravenously may affect the MG polarization, attenuating both demyelination and inflammation and restoring neurological function in EAE rats. Pro-inflammatory MG treated with BMSC-derived EVs undergo a decrease in TNF-α secretion and an increase in TGF-β and IL-10 release. These in vivo results indicate a shift of MG from a pro-inflammatory to an anti-inflammatory phenotype, which suggests the protective effects of treatment. Part of these effects are mediated by the release of EV miR-467f and miR-466q, which can modify the pro-inflammatory phenotype of activated MG, downregulating *Tnf* and *Il1b* expression by modulating p38MAPK signaling pathway (Giunti et al., 2021). These findings suggest that MSC-derived EVs could reduce neuroinflammation in the CNS through specific immunomodulatory miRNAs acting on MG (Giunti et al., 2021; Zhang et al., 2022). In addition, treatment is followed by a decrease in the expression of CD68, a marker of M1 MG, and an increase in CD206, an indicator of M2 MG, in both brain and spinal cord (Li, Cheng, et al., 2019; Li, Liu, et al., 2019; Li, Tong, et al., 2019).

Despite these findings, obstacles remain for the clinical application of EV-based therapies, including lack of data on EVs trafficking and biodistribution. In addition, EV membrane structure and core content are highly influenced by the origin and physiologic state of cells. Therefore, thorough analysis should be conducted on EV structure, particularly their lipid composition, and the permeability status of the BBB (Jakubec et al., 2020).

5.1 | EVs as biomarkers for MS

To be applied in a therapeutic context with potential for success, MS biomarkers should (1) be cost-effective, (2) correlate with disease biology or pathogenesis, such as inflammatory activity,

TABLE 3 Effects of MG-derived EVs on CNS cells in homeostasis and disease.

Cells	Effects	Mediators	Reference
Neurons	Neurite outgrowth support and synaptogenesis	Neurotrophic factors nGDF, TGF-β, Thrombospondin 1 and 4	Lemaire et al. (2019) Raffo-Romero et al. (2019) Eroglu et al. (2009)
	Conveyance of energy substrates	Metabolic enzymes for anaerobic glycolysis and lactate production	Potolicchio et al. (2005)
	Detrimental effects on dendritic spines	miR-146-5p	Prada et al. (2018)
	Stimulation of Glu release; down-regulation of GABAergic transmission	Sphingosine and sphingosine-1 phosphate, ceramide Endocannabinoids (anandamide, *arachidonoyl- ethanolamine)*	Antonucci et al. (2012) Riganti et al. (2016) Gabrielli et al. (2015)
	Silencing of post-synaptic protein neuroligin-1 and pre-synaptic protein synaptotagmin-1	miR-146-5p	Prada et al. (2018) Jovičić et al. (2013)
	Decrease in synapse density	Fatty acid palmitate	Vinuesa et al. (2019)
	Decrease in neurogenesis	miR-146-5p acting on KLF-4 and CDKL5	Fan et al. (2022)
	Induction of oxidative stress	Glutaminase	Chen et al. (2020)
	Induction of neurodegeneration mediated by TLR-7	miR let-7b and HMGB	Coleman et al. (2017)
	Synaptic pruning	ROS and complement factor C1q	Lombardi et al. (2019)
	Packaging and propagation of misfolded proteins	EVs containing misfolded proteins transfer them to other neurons in a trans-synaptic manner and contribute to neurodegenerative diseases	Gabrielli et al. (2022)
	Protection from apoptosis after oxygen–glucose deprivation and protection from neurodegeneration	miR-124-3p and suppression of PDE4B, miR-711, miR-135a-5p	Ge et al. (2020) Zhang et al. (2020) Liu et al. (2021)
Astrocytes	Transformation of astrocytes into harmful A1 cells	IL-1a, C1q, and TNF	Liddelow et al. (2017)
	Increase in the release of IL-1β, IL-6, and TNF-α	ATP stimulation of receptor P2X7	Bianco et al. (2009)
	Attenuation of glial scar formation	miR-124 targeting STAT3	Li et al. (2021)
	Increase in the expression of glutamate transporters Glt1 and Glast, enhancing Glu uptake	miR-124	Huang, Ge, et al. (2018); Huang, Xu, et al. (2018)
Oligodendrocytes	Promotion of OPC migration and differentiation	IL-4 polarization	Lombardi et al. (2019)
	Inhibition of remyelination	Pro-inflammatory cytokines	Lombardi et al. (2019)
	Increase in the number of GPR17-expressing OPCs at lesion boundaries and enhancement in maturation	IL-4 polarization	Raffaele et al. (2021)
	Chemoattractant effect on OPCs	Sphingosine-1-phosphate	Lombardi et al. (2019)
	Impairment of oligodendrocyte maturation	Pro-inflammatory cytokines, indirect effect mediated primarily by astrocytes	Lombardi et al. (2019)
	Promotion of OPC survival and differentiation	Transmembrane TNF and purified lipid fraction of EVs (endocannabinoids and sterols), miR-23a-5p, miR-221-3p, miR-129-5p, and miR-155-5p. miR-23a-5p could promote OPC differentiation by inhibiting Olig3 expression	Madsen et al. (2016) Lombardi et al. (2019) Gualerzi et al. (2021) Li et al. (2022)

(Continues)

TABLE 3 (Continued)

Cells	Effects	Mediators	Reference
Endothelial cells	Reduction in intracellular oxidative stress levels Increase in survival and cell migration	Activation of keap1/Nrf2/HO-1 pathway	Peng et al. (2021)
	Increase in the viability, migration, and tube-formation capacity of endothelial cells exposed to oxygen–glucose deprivation	TGF-β1/Smad2/3 pathway	Zhang, Zou, et al. (2021); Zhang, Wei, et al. (2021)
	Pro-angiogenic properties	miR-26a	Tian et al. (2019)
Microglial cells	Modulation of protective activities such as cellular movement, cell death and survival, cellular growth and proliferation of other MG	Surface phagocytic receptor TREM2	Huang, Ge, et al. (2018); Huang, Xu, et al. (2018); Huang et al. (2022)
	Modulation of protective activities of MG upon inflammatory stress	Downregulation of FAS, TNFSF10 (TRAIL), CXCL8, caspase 8, IL-6, and IL-1β transcripts	Van den Broek et al. (2020)
	Increase in the activation of NLRP3 inflammasome and induction of cell-to-cell communication	Transfer of the inflammasome adaptor protein ASC, increase in IL-1β production	Sarkar et al. (2019)
	Increase in pro-regenerative MG (higher expression of CD206)	TGF-β1	Zhang, Zou, et al. (2021); Zhang, Wei, et al. (2021)
	Facilitation of EV-mediated autophagy	Complement molecule C3 and MYD88	Van den Broek et al. (2020)
	Increase in pro-inflammatory miR-130, miR-145a, miR-23b, miR-146a, and decrease in anti-inflammatory miR-124 and let-7b	Glutaminase C	Gao et al. (2019)
	Induction of neuropeptide catabolism	Transmembrane aminopeptidase CD13	Potolicchio et al. (2005)
	Increase in neuronal survival	FGF, NGF and BDNF, miR-124	Song et al. (2019) Ponomarev et al. (2011)
	Inhibition of MG autophagy by targeting PTEN/AKT/mTOR	*miR-19a-3p*	Zhou et al. (2019)
	Inhibition of inflammation by targeting the TLR/NFκB pathway	miR-146a, miR-125b, miR-182, miR-17-5p, miR-140-5p, miR-9, miR-let7, and miR-181c	Vaz et al. (2019) Wang et al. (2018) Guo et al. (2019) Yang et al. (2020) Yin et al. (2017)
	MG migration	miR-9	Yang et al. (2018)
	Suppression of neurodegeneration by the decreased expression of TNF-α, IL-1β, and IL-6, and inhibition of neuronal autophagy	miR-124-3p	Li, Cheng, et al. (2019); Li, Liu, et al. (2019); Li, Tong, et al. (2019)
	Regulation of MG polarization toward an M2 phenotype	mi-R-223	Lo Sicco et al. (2017)

Abbreviations: AKT, protein kinase B; ASC, apoptosis-associated speck-like protein containing a CARD; BDNF, brain-derived neurotrophic factor; CDKL5, cyclin-dependent kinase-like 5; CXCL, C-X-C motif chemokine ligand; FAS, Fas cell surface death receptor; FGF, fibroblast growth factor; GABA, Gamma-aminobutyric acid; Glast, glutamate–aspartate transporter; Glt-1, glutamate transporter-1; Glu, glutamic acid; GPR17, G protein-coupled receptor 17; HMGB, High mobility group box; HO-1, Heme oxygenase 1; IL, interleukin; keap1, Kelch-like-associated protein 1; KLF4, Krüppel-like factor 4; miRNA, micro RNA; mTOR, mammalian target of rapamycin; MYD88, myeloid differentiation factor 88; nGDF, nervous growth and differentiation factor; NGF, nerve growth factor; Nrf2, nuclear factor-erythroid factor 2-related factor 2; OPC, oligodendrocyte progenitor cells; PDE4B, Phosphodiesterase 4B; PTEN, phosphatase and tensin homolog; ROS, radical oxygen species; SMAD, suppressor of mothers against decapentaplegic; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; TNFR2, tumor necrosis factor receptor 2; TNFSF, tumor necrosis factor superfamily; TREM2, triggering receptor expressed on myeloid cells 2.

the degree of neurodegeneration, demyelination, or remyelination, (3) be easy to access, primarily when used sequentially, and (4) be easily measured using precise and robust tests (Mathur et al., 2021; Paul et al., 2019). In this context, EVs secreted from different types of neural cells such as neurons, astrocytes, microglia, and oligodendrocytes reflect parental cells through cell-specific receptors on their surface and with their cargo, becoming a reservoir of potential biomarkers (Ramirez et al., 2018), EVs could serve as valuable biomarkers for MS diagnosis and monitoring of disease progression. The origin of these EVs has

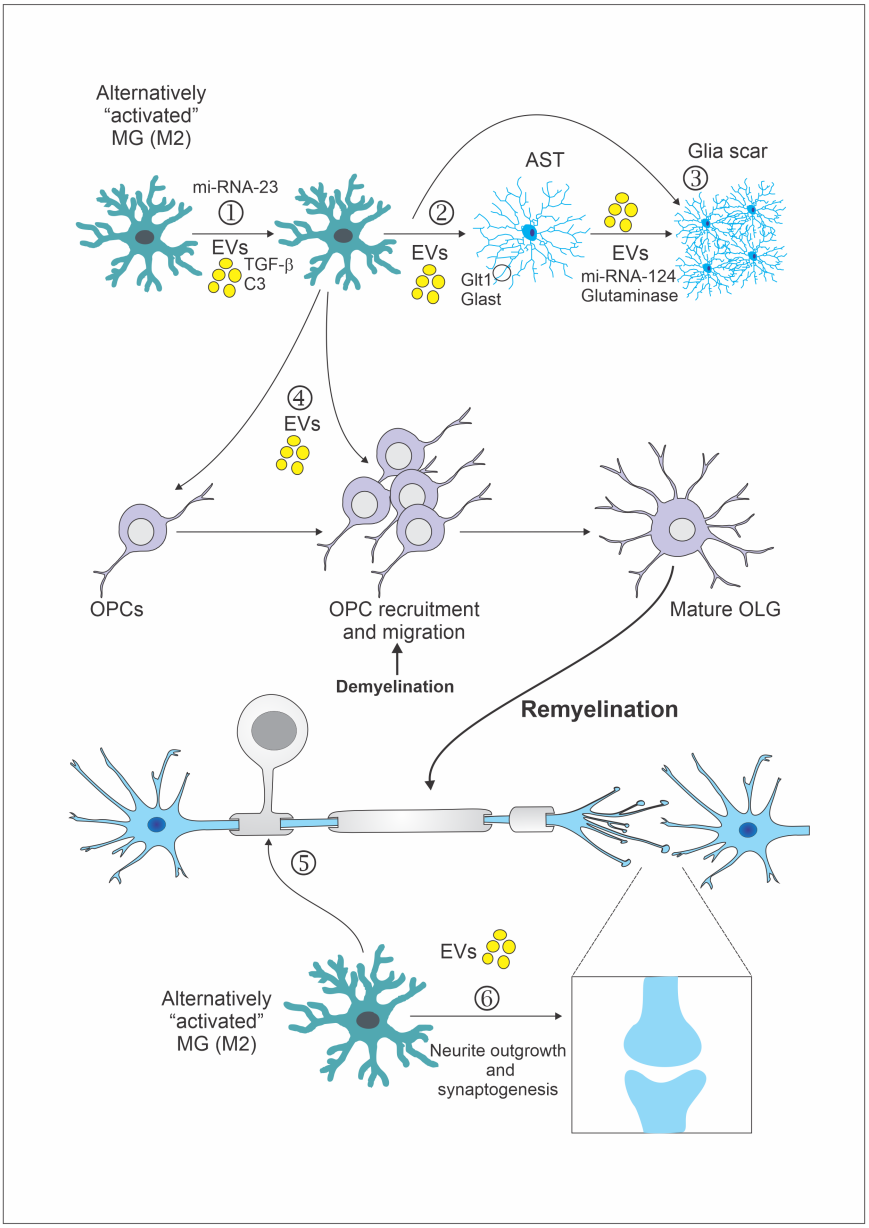

FIGURE 2 Pro-regenerative MG (M2) secrete TGF-β, which increases the expression of CD206 and complement molecules C3 and MYD88 in neighboring MG, facilitating autophagy (1). Furthermore, their cargos miR-124, MMPS, and transglutaminase-2 attenuate glial scar formation and increase the expression of Glu transporters, enhancing Glu uptake (2) and (3). MG-derived EVs also exert several effects on the recruitment, migration, and differentiation of oligodendroglial progenitors by releasing IL-4 and mi-RNA 23a 5p, activating TNFR2, and lipid-secreting fractions, including endocannabinoids and sterols (4). In addition, MG-derived EVs increase neuronal survival by secreting growth factors FGF, NGF, IGF-1 and BDNF, and miRNA-124, simultaneously transporting metabolic enzymes for anaerobic glycolysis and lactate production. Furthermore, several miRNAs released by MG-derived EVs inhibit the inflammatory process targeting the TLR/NFκB pathway (5). Some of these EVs also support neurite outgrowth and synaptogenesis through nGDNF, TGF-β, and thrombospondin 1 and 4 (6). For further details on these effects, see Table 1. BDNF, brain-derived neurotrophic factor; EVs, extracellular vesicles; FGF, fibroblast growth factor; Glast, glutamate transporter (EET1); Glt1, presynaptic glutamate transporter (EET2); Glu, glutamic acid; IGF, insulin growth factor; IL, interleukin; MMPS, metalloproteinases; MYD88, *myeloid differentiation primary response 88*; NFκB, nuclear factor Kappa B; nGDF, nervous growth/differentiation factor; NGF, nerve growth factor; OLG, oligodendrocytes, OPC, oligodendrocyte progenitor cells; TGF-β, transforming growth factor-β; TLR, toll-like receptor; TNFR, tumor necrosis factor receptor.

been verified through immunogold electron microscopy using CD11b/c antibodies, which has demonstrated an elevation in microglia/macrophage-derived EVs in cerebrospinal fluid during periods of inflammation. Additionally, IB4 has been employed as an additional marker for isolating microglial EVs from plasma, able to distinguish patients in the acute phase from the remission phase (Geraci et al., 2018; Verderio, Muzio, et al., 2012). Similarly, EVs originating from CNS endothelial cells could serve

as biomarkers indicative of BBB permeability and active MS (Mazzucco et al., 2022).

6 | CONCLUSIONS AND FUTURE PERSPECTIVES

Microglial activation is a common feature of MS. Although this activation can be harmful in some instances, the protective and regenerative functions of MG have also been documented (Figures 1 and 2). In particular, MG play a vital role in remyelination, a critical process for axonal health. Several cellular events involved in this process depend on cell–cell communication. EVs serve as communication vehicles in the brain, transferring different molecules in physiological and pathological conditions. EVs characterization remains challenging, as their populations are highly heterogeneous regarding content, size, and composition. Recent progress in high throughput transcriptome techniques has shown that EVs do not contain a random sampling of parent cell components but rather reflect parent cell state of activation and can be influenced by the pathological process of origin (Jeppesen et al., 2019). EVs can be found in body fluids of patients with different neurodegenerative diseases, which makes them attractive diagnostic tools and biomarkers.

Furthermore, EVs may have the appropriate capacity for loading and releasing molecules in controlled conditions. Due to their small size and lipophilic nature, EVs can cross the BBB and could therefore be used as nano-delivery vehicles for therapeutic molecules of choice. In this scenario, different approaches have been used for EVs to target MG-regulating diseases in animal models of demyelination. Despite EVs ability to cross the BBB, questions remain as to what the pathways are by which EVs can be taken up by the brain microvascular endothelial cells of the neurovascular unit to reach the CNS. Information is also still scarce about EV trafficking and biodistribution. Glucose-coated gold nanoparticles have been widely used to track EVs in the CNS and studied in vivo using computerized tomography (Betzer et al., 2017). Although intravenously administered EVs can reach the CNS, biodistribution studies have shown systemically administered EVs trapped in the liver, lungs, spleen, kidney, and gastrointestinal tract (Wiklander et al., 2018). Therefore, local administration routes that bypass the BBB may prove attractive for targeted CNS delivery. Due to non-invasiveness, the intranasal route is the most promising approach for preclinical and clinical applications (Mattera et al., 2023). EV composition also poses the problem of transferring undesirable immunogenic content derived from parent cells (Rufino-Ramos et al., 2017). Furthermore, the MG lines and mouse models that have been used to characterize EV cargos have limitations and fail to fully recapitulate the pathological hallmarks observed in humans. Currently, research on EV focuses on their ability to mediate cell–cell communication and their characteristics as biomarkers. Because EVs can be isolated from blood and other biological fluids, they are potentially non-invasive biomarkers for early diagnosis and prognosis. The profile of EVs has been referred to as a "liquid biopsy," as EVs may provide information about tissues without the need for an invasive approach (Poudineh et al., 2018). More research is needed to understand the mechanisms of bidirectional communication of EVs through the BBB and potential changes in cargo secretion. In addition, further studies should determine the type of cells to be used for EV production and the scale and methods of production which may yield high-purity EVs.

AUTHOR CONTRIBUTIONS

V. S. B. Wies Mancini: Writing – original draft. **V. S. Mattera:** Writing – review and editing. **J. M. Pasquini:** Conceptualization; writing – review and editing; supervision. **L. A. Pasquini:** Writing – original draft; writing – review and editing; conceptualization. **J. D. Correale:** Conceptualization; writing – original draft; writing – review and editing; supervision.

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CONFLICT OF INTEREST STATEMENT

Dr. Juana Pasquini is an editor for *Journal of Neurochemistry*. The other authors have no conflict of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in PubMed—NIH at https://pubmed.ncbi.nlm.nih.gov/.

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