Detrimental and Protective Action of Microglial Extracellular Vesicles on Myelin Lesions: Astrocyte Involvement in Remyelination Failure

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DETROIMINAL AND PROTECTIVE ACTION OF MICROGLIAL EXTRACELLULAR VESICLES ON MYELIN LESIONS: ASTROCYTE INVOLVEMENT IN REMYELINATION FAILURE

Marta Lombardi

Director of Studies: Dr. Claudia Verderio
External Supervisor: Dr. Roberto Furlan, Dr. Bobbi Fleiss
DETRIMENTAL AND PROTECTIVE ACTION OF MICROGLIAL EXTRACELLULAR VESICLES ON MYELIN LESIONS: ASTROCYTE INVOLVEMENT IN REMYELINATION FAILURE

Thesis submitted for the degree of Doctor of Philosophy (PhD)

Marta Lombardi

Clinical and Research Institute Humanitas
Open University PhD Program

Director of Studies: Dr. Claudia Verderio
External Supervisor: Dr. Roberto Furlan, Dr. Bobbi Fleiss

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LIST OF ABBREVIATIONS

AEA: Endocannabinoid N-arachidonoyl ethanolamine
APCs: Antigen-presenting cells
ArgI: Arginase 1
aSMase: Acid-sphingomyelinase
BBB: Blood–brain barrier
BDNF: Brain-derived neurotrophic factor
BB: Blood–brain barrier
BDNF: Brain–derived neurotrophic factor
BPM: Bone morphogenic proteins
CB1: type-1 cannabinoid receptors
CC: Corpus callosum
CD31+ EEVs: Endothelial CD31 positive EVs
CIS: Clinical isolated syndrome
CNPase: 2′,3′-cyclic nucleotide-3′-phosphodiesterase
CNS: Central nervous system
CNTF: Ciliary neurotrophic factor
CR3: Complement receptor 3
Cryo-EM: Cryo-electron microscopy
CSF: Cerebrospinal fluid
Cx30: Connexins 30
Cx32: Connexins 32
Cx43: Connexins 43
Cx47: Connexins 47
DMTs: Disease-modifying treatments
dpl: Days post lesion
DRG: Dorsal root ganglion
DTI: Diffusion tensor imaging
EAE: Experimental autoimmune encephalomyelitis
EBV: Epstein-Barr virus
EM: Electron microscopy
ER: Endoplasmic reticulum
ET-1: Endothelin-1
ETN: Etanercept
EVs: Extracellular vesicles
FACS: Fluorescence-activated cell sorting
FGF: Fibroblast growth factor
ICC: Immunofluorescence
d-EVs: EVs from inflammatory microglia
IFN-γ: Interferon-gamma
IGF-1: Insulin-like growth factor 1
IGF-2: Insulin-like growth factor 2
IHC: Immunohistochemistry
IL4-EVs: EVs derived from IL4-treated microglia
IL4-MG: IL4-treated microglia
i-MG: inflammatory microglia
iNOS: inducible NO synthase
LIF: Leukemia inhibitory factor-like protein
LIFR: Leukemia inhibitory factor receptor
LINGO-1: Leucine-rich repeat and Immunoglobulin-like-domain-containing
LPC: Lysolecithin
LPS: Lipopolysaccharide
MACS: Magnetic-activated cell sorting
MAG: Myelin-associated glycoprotein
MBP: Myelin basic protein
MG: Microglia
MHCII: Major histocompatibility complex-II
MMPs: Metalloproteinases
MOG: Myelin-oligodendrocyte glycoprotein
MRI: Magnetic resonance imaging
MS: Multiple sclerosis

MSC-EVs: EVs derived from MSC-treated microglia
MSC-MG: MSC-conditioned microglia
MSCs: Mesenchymal stem cells
MTR: Magnetization transfer ratio
MV: Microvesicles
NAWM: Normal-appearing white matter
NF: Neurofilament
NGF: Nerve growth factor
NGS: Next Generation Sequencing
NO: Nitric oxide
NS-MG: Non stimulated microglia
NT3: Neurotrophin-3
NTA: Nanosight Tracking Analysis
Olig1: oligodendrocyte transcription factor 1
Olig2: oligodendrocyte transcription factor 2
OLs: Oligodendrocytes
omGP: Oligodendrocyte myelin glycoprotein
OPCs: Oligodendrocyte precursor cells
OSP: Oligodendrocyte surface protein
PCA-LDA: Principal Component Analysis and Linear Discriminant Analysis
PDGF: Platelet-derived growth factor
PEVs: platelet derived EVs
PLP: Myelin proteolipid protein
PPMS: Primary progressive form of multiple sclerosis
PS: Phosphatidylserine
ROS: Reactive oxygen species
RRMS: Relapsing-remitting form of multiple sclerosis
RS: Raman spectroscopy
RUNX1: Runt-related transcription factor
S1P: Sphingosine 1 phosphate
Sema 3A: Semaphorin 3A
Sema 3F: semaphorin 3F
S-ene: FTY720 Vinylphosphonate
SGZ: Subgranular zone
SIRP-α: Signal regulatory protein-alpha
South 10: Sex determining region Y-box 10
South2: Sex determining region Y-box 2
SPMS: Secondary progressive form of multiple sclerosis
SVZ: Subventricular zone
T3: Thyroid hormone
TGF-β: Transforming growth factor β
Th2: Type 2 T helper
tmTNF: Transmembrane TNF
TNF: Tumor necrosis factor
TNFR-1: Tumor necrosis factor receptor 1
TNFR-2: Tumor necrosis factor receptor 2
Treg: Regulatory T cells
TREM2: Triggering receptor expressed on myeloid cells 2
UVB: Ultraviolet B light
VEGF: Vascular endothelial growth factor
WB: Western-blot
ABSTRACT (max 300 words)
Microglia (MG) are the immune cells of the brain. They have an enormous plasticity in response to brain injury, acquiring different activated phenotypes depending on the stimuli they receive. During multiple sclerosis, MG participate to myelin injury and repair by influencing the function of oligodendrocyte precursor cells (OPCs), the brain cells that differentiate into myelin-forming cells. However, the mode(s) of action of MG in promoting or inhibiting brain repair is still largely unknown. Here, we explored the action of Extracellular Vesicles (EVs) produced in vitro by either pro-inflammatory or pro-regenerative MG on OPCs at myelin lesion induced by lysolecithin injection in the mouse corpus callosum. Immunolabelling for myelin proteins and electron microscopy showed that EVs released by pro-inflammatory MG (i-EVs) impaired remyelination, whereas EVs produced by MG co-cultured with immunosuppressive mesenchymal stem cells (MSC-EVs) promoted OPC recruitment and differentiation at the lesion site. The molecular mechanisms underlying the detrimental/beneficial action of MG EVs were dissected using primary OPC cultures, a powerful system with easy access for EVs. i-EVs promoted OPC differentiation in monoculture, while they caused a clear block of OPC maturation when OPCs were co-cultured with astrocytes, implicating these cells in remyelination failure. Moreover, biochemical fractionation of EV components revealed that astrocyte may be converted in oligotoxic cells by i-EV cargo whereas the lipid components of EVs promoted OPC maturation and migration as indicated by immunohistochemical and qPCR analyses. While the lipid species that enhance OPC maturation remains undefined, we demonstrated a central role for EV-associated S1P in stimulating OPC migration, a fundamental process in remyelination. Collectively, these results uncover a role for microglial EVs as key players in myelin repair, given their ability to influence OPC and astrocyte functions at myelin lesion, and support the study of EV content and function for developing novel approaches for remyelination.

KEYWORDS: Microglia/ Extracellular Vesicles/ Mesenchymal stem cells/ Myelin lesion/ S1P/ Astrocytes
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CHAPTER I

INTRODUCTION
1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) characterized by focal lesions of inflammation, axonal loss, gliosis and demyelination, which affects both the white and the grey matter (Frischer et al., 2009; Lassmann, Bruck, & Lucchinetti, 2007).

Demyelination is the process resulting from destruction of myelin, a lipid-rich membrane produced by oligodendrocytes (OLs), a type of glial cells which enwraps around the axons of neurons allowing rapid propagation of electrical impulses across CNS and axon integrity (Nave & Werner, 2014; Olsen & Akirav, 2015).

Although MS etiology remains unknown, the disease results from a combination of genetic susceptibility (e.g. MHC) and environmental factors, such as vitamin D or ultraviolet B light (UVB) exposure, Epstein-Barr virus (EBV) infection, obesity and smoking (Ascherio, 2013). Among environmental factors a recent evidence shows that the gut microbiota also contribute to MS pathogenesis (J. Chen et al., 2016).

The course of MS is highly variable. Disease heterogeneity is not limited to the symptoms but also involves neuro-radiologic and histologic appearances of white and grey matter lesions, and response to therapy (Lucchinetti et al., 2000).

85% of MS patients initially present flare-up of symptoms (fatigue, numbness, blurred vision, tingling, walking difficulties) followed by periods of remission when symptoms improve or disappear, namely, the classic relapsing-remitting form of multiple sclerosis (RRMS). In RRMS, brain lesions are called “classical active lesions” and are characterized by profound lymphocytic infiltration, with a predominance of T-cells rather than B-cells and plasma cells, and macrophages and astrocytes activation (Dobson & Giovannoni, 2019; Filippi et al., 2012). Most of RR patients advance to a secondary progressive disease (SPMS), while 15% of MS patients develop primary progressive disease (PPMS), where the illness is progressive from the onset (Cunniffe & Coles, 2019). Both SPMS and PPMS are characterized by gradual neurological deterioration and symptoms such as muscle stiffness, cognitive deficits and difficulties with urination and defecation (Lassmann & Bradl, 2017; Tanaka & Yoshida, 2014). The lesions are circumscribed and hypocellular, and devoid of active demyelination. They present an inactive lesion core surrounded by a narrow rim of activated microglia and macrophages, high levels of B-cells and plasma cells (Frischer et al., 2009), fibrillary gliosis, loss of axons and OLs (Filippi et al., 2012).
Fig. 1.1 Multiple Sclerosis types. Magnetic resonance imaging (MRI) activity (vertical arrows) indicates an inflammatory process as measured on brain MRI by gadolinium enhancement or new T2 hyperintense brain lesions. MRI activity increases during clinical relapses (spikes in clinical disability). Reduction of brain volume and increase in disease burden (total volume of lesions), both measured on MRI, are signs of the permanent brain damage in the secondary progressive phase of MS. Adapted from (Fox & Cohen, 2001).

Three theories have been proposed to explain how MS progresses: i) the brain damage is caused by inflammatory process and the microenvironment favors activation of inflammatory cells; ii) MS starts as inflammatory disease but then neurodegeneration mediates disease progression; iii) MS is mainly a neurodegenerative disease and inflammation occurs later, contributing to progressive phase of disease (Correale, Gaitan, Ysraelit, & Fiol, 2017).

However, studies in this field support the idea that the interaction between multiple components of the immune system and elements of the CNS is at the basis of MS development. Indeed, it has been shown that T-cells in the periphery become activated by a viral or another infectious antigen, presented by dendritic cells, which shows molecular similarity with some CNS antigen (Sospedra & Martin, 2005), probably myelin antigen (Mallucci, Peruzzotti-Jametti, Bernstock, & Pluchino, 2015). Activated T-cells can differentiate into Th1 (IFN-γ producing cells) or Th17 cells (IL-17, IL-22, IL-21) which produce inflammatory products besides cytokines, able to damage myelin and axons. After crossing the blood–brain barrier (BBB), reactive T-cells are reactivated upon interaction with CNS antigen-presenting cells, namely microglial cells, the immune cells resident in brain parenchyma. Specifically, activated CD8⁺ T cells, a class of T-cells known to have cytotoxic functions, contribute to axon damage through secretion of cytokines as well as granzyme and perforin (J. M. Fletcher, Lalor, Sweeney, Tubridy, & Mills, 2010).
Together with T-cells, B-cells and plasma cells can also enter the CNS, where contribute to neurodegeneration by producing antibodies, which can bind the antigens and activate the complement cascade. This inflammatory cascade leads to myelin lesion, especially after repeated injury, and consequentially the axons degenerate because they are deprived of the trophic support provided by myelin (Piaton, Williams, Seilhean, & Lubetzki, 2009).

The innate immune system, consisting of microglia and macrophages, play also an important role in the initiation and progression of MS. They produce factors that attract further inflammatory cells to the CNS amplifying the inflammatory process (Barnett, Henderson, & Prineas, 2006). In particular, microglial cells, when activated, contribute to the development of the disease trough secretion of pro-inflammatory cytokines, chemokines, free radicals and high level of glutamate (Thompson, Baranzini, Geurts, Hemmer, & Ciccarelli, 2018).

Other important players in MS lesions are astrocytes, which also contribute to T-cell recruitment, activation and differentiation through pro-inflammatory cytokines and chemokines production (Xie & Yang, 2015). Astrocytes also increase the permeability of BBB by secreting metalloproteinases (MMPs) and directly exert detrimental action on OLs through the release of the cytokine tumor necrosis factor (TNF) (A. Williams, Piaton, & Lubetzki, 2007).

**Fig.1.2 Immune responses in multiple sclerosis.** T- and B-cell responses are primed in the periphery by antigens that are released from the CNS or by cross-reactive foreign antigens. Dendritic cells present antigens and activate T-cell
response. After clonal expansion, B cells and T-cells infiltrate the CNS. B cells re-encounter their specific antigen, mature to plasma cells and release large amounts of immunoglobulin-γ antibodies. Clonally expanded CD8+ and CD4+ T-cells invade the brain and encounter their specific peptide ligand, presented by glial or neuronal cells, and damage neurons and oligodendrocytes by releasing inflammatory cytokines. Astrocytes release inflammatory cytokines, metalloproteases and chemokines, inducing damage of BBB, axons and oligodendrocytes.

Given that MS is considered an autoimmune CNS disease, several immunosuppressive or immunomodulatory therapies are currently available to treat the disease. These disease-modifying treatments (DMTs) reduce the incidence and severity of lesions by blocking T-cell infiltration into the CNS, B-cells activity or altering the functionality of immune system (Baecher-Allan, Kaskow, & Weiner, 2018; Rahamanzadeh, Weber, Bruck, Navardi, & Sahraian, 2018). However, their efficacy in reducing the development of brain atrophy and in decelerating the progressively increasing disability of MS-affected patients is strongly limited (Cunniffe & Coles, 2019; Ineichen et al., 2017). Therefore, one well-acknowledged approach to prevent disease progression would be via a boost of myelin repair.

Currently, the study of the intrinsic and extrinsic regulatory pathways involved in remyelination has allowed the identification of possible targets for therapeutic manipulation. For example, the human monoclonal antibody Opicinimab has been developed to inhibit Lingo-1, a negative regulator of myelination, and its utility has been already tested in patients with acute optic neuritis (Cadavid et al., 2017). Other compounds such as the antihistamine/anticholinergic benzatropine, the corticosteroid clobetasol and the anti-fungal miconazole have been demonstrated to exert remyelinating effect in both experimental autoimmune encephalomyelitis (EAE) and cuprizone mice models (Deshmukh et al., 2013). In addition, a cluster of compounds with an anti-muscarinic effect has been shown to promote the formation of myelin sheath by OLs (Mei et al., 2014). Among them, Clemastine was already translated into a clinical trial (Green et al., 2017).

All these therapeutic approaches aim to stimulate the differentiation of oligodendrocyte precursors cells (OPCs), which is still considered a rate-limiting step in the remyelination process. However, clinical studies revealed that a combination of therapies might be necessary to repair MS lesions. Moreover, there is the possibility that these drugs are effective in the preclinical studies but have not beneficial effect in humans (R. J. M. Franklin & Ffrench-Constant, 2017; Hooijmans et al., 2019).
1.1.1 Biomarkers in multiple sclerosis

In addition to possible MS therapies, considerable interest has raised over the last six decades in finding biomarkers of MS that can improve disease diagnosis, predict disease progression and improve clinical outcomes.

Currently, there are two biomarkers in clinical use in MS diagnosis and treatment: Oligoclonal bands in the cerebrospinal fluid (CSF) and white matter lesions on Magnetic resonance imaging (MRI). Oligoclonal bands and high level of IgG were the first biomarkers found in the CSF collected from patients with MS. Particularly, oligoclonal bands have proven to be predictive of conversion from clinically isolated syndrome (CIS) to MS (Ciccarelli & Toosy, 2015).

Together with oligoclonal bands, MS subjects can be identified by the presence in the brain of gadolinium-enhancing lesions detectable by MRI. These lesions are indicative of active inflammation and lesion burden, and their number and size are predictive of both onset and severity of relapses (Bruck et al., 1997; Hemond & Bakshi, 2018). However, gadolinium-enhancing lesions do not correlate with some MS symptoms such as cognitive decline in RRMS patients (Rocca et al., 2015), that strongly impact the patients’ quality of life.

By contrast, there is a positive correlation between gray matter atrophy and cognitive dysfunction in MS, suggesting that gray matter atrophy, rather than white matter lesion load, may be a useful biomarker for prediction of clinical severity (Geurts, Calabrese, Fisher, & Rudick, 2012).

In concomitance of these biomarkers, further studies have identified new potential biomarkers in MS. One of the most reliable marker in MS is CNS neurofilaments (Nfl), consisting of neurofilament heavy and light chains, which are released proportionally to the degree of axonal damage (Gaetani et al., 2019; Kuhle et al., 2013). Increased levels of Nfl have been found in both CSF and blood of RRMS and progressive MS patients compared to healthy subjects (Cai & Huang, 2018). In RRMS patients, Nfl levels in CSF increase at all disease stages, but fluctuate depending on the clinical course and the appearance of active lesions (Bielekova & McDermott, 2015). Quantification of Nfl at beginning of MS is thus important for predicting disease severity and progression to secondary progressive MS (Gaetani et al., 2019). In addition, treatment of MS patients with Fingolomod, Natalizumab, and Rituximab decrease CSF Nfl levels, suggesting a clinical amelioration of patients (Housley, Pitt, & Hafler, 2015).

Other markers of neuronal/glial cell damage or immune system activation include Tau, NCAM, NGF (associated to neuronal damage), GFAP, CNTF, S100B (indicative of astrocyte damage and astrogliosis), CXCL13 (a B cell chemoattractant) and ferritin, which have been shown to be upregulated in the CSF of MS patients compared to controls (Avsar et al., 2012; Petzold et al., 2002). These markers are not unique to MS but are frequently expressed in other CNS inflammatory diseases.
(Verbeek, De Jong, & Kremer, 2003). However, they are useful for understanding the susceptibility and progression mechanisms of the disease.

A large body of studies has focused on small non-coding RNAs (miRNAs) as possible MS biomarkers. miRNAs can be isolated from peripheral blood mononuclear cells (PBMC), purified immune cell subsets, serum or CSF of MS patients.

Specifically, miR-18b and miR-599 have been found increased in PBMC isolated from MS patients during relapses compared to both healthy individuals and patients in remission (Otaegui et al., 2009). By contrast, miR-193a is upregulated only in patients in remission phase (Otaegui et al., 2009). Keller and colleagues demonstrated an increased expression of miR-422a and miR-223 in whole blood samples of RRMS patients compared to controls. In detail, miR-422a is decreased in plasma of MS patients, while miR-223 is reduced in the serum of RRMS and PPMS patients (Siegel, Mackenzie, Chaplin, Jablonski, & Griffiths, 2012). Like miR-223, miR-15b and miR-23a are reduced in serum of these patients (Fenoglio et al., 2013), and have proven to be potential biomarkers for disease progression. Moreover, Du and colleagues have found that miR-326 levels are high in peripheral blood leukocytes derived from RRMS patients and correlate with disease activity in both MS and EAE model (Du et al., 2009). By contrast, a report by Cox et al. showed that miR-17 and miR-20a are significantly decreased in peripheral blood cells from RRMS, SPMS and PPMS subtypes and are able to modulate T cell activation genes (Cox et al., 2010). Other miRNAs able to influence T cell functions are miR-128 and miR-340, which increase in naïve CD4+ T cells and memory CD4+ T cells from MS patients, respectively (Guerau-de-Arellano et al., 2011).

Despite evidence that miRNAs can be measured in many body fluids suggests clinical feasibility of these molecules as potential biomarkers for MS (Raphael, Webb, Stuve, Haskins, & Forsthuber, 2015), the specificity of these biomarkers requires further validation.

MS is an autoimmune neuro-inflammatory disease, thus myelin-reactive T-cells and their inflammatory profiles may represent additional disease biomarkers. Indeed, myelin-reactive CD4+ and CD8+ cells have been found increased in the peripheral blood from MS patients as compared to healthy controls (Raddassi et al., 2011). Interestingly, these cells are characterized by high expression of IFN-γ and GM-CSF genes, and IL-17 production (Y. Cao et al., 2015). However, it is not yet clear whether analyzing myelin-reactive T-cells and their phenotypic and transcriptional profile may be useful for MS diagnosis and prognosis.

Further research has focused on understanding how the microbiome may be influencing MS susceptibility and progression. It is known that the gut microbiome participates in immune-regulatory pathways that can both contribute to, and protect individuals from, disease (Lynch & Pedersen, 2016).
The first studies in this research field demonstrated that antibiotic-treated and germ-free mice develop attenuated EAE with reduced levels of Th1 and Th17 cells and increased number of T-reg cells (Berer et al., 2011). Accordingly, the infection of germ-free mice with segmented filamentous bacteria (SFB) restores the sensitivity to EAE (Y. K. Lee, Menezes, Umesaki, & Mazmanian, 2011). Studies in humans found that the bacterial species Methanobrevibacter and Akkermansia are increased and the bacteria Butyricimonas are decreased in patients with MS relative to control subjects (Jangi et al., 2016). Moreover, it has been demonstrated that i) the levels of intestinal Th17 cells correlate with disease activity in MS (Cosorich et al., 2017); ii) gut bacteria from MS individuals exacerbate EAE when transplanted to germ-free mice (Baecher-Allan et al., 2018); iii) gut microbiota from unaffected twins promote EAE and reduce IL-10 production in recipient mice (Berer et al., 2017). However, the predictive value of microbiome in MS is still unknown.

In this scenario, extracellular vesicles (EVs), membrane structures released from different cell types, also arouse interest (Scolding et al., 1989; Verderio et al., 2012). EVs are involved in both MS pathogenesis and progression: they act as immunomodulator agents in the disruption of the BBB and spread inflammation in CNS tissue, but, on the other hand, they also contribute in brain repair. Evidence supporting that EVs can be used as biomarkers of MS are described in the Chapter 1, paragraph 1.6.1. Of note, the results regarding this aspect are sometimes contradictory, depending on the type of EV (MVs or exosomes), their cellular origin, the methods used and the analyses performed.

1.2 Animal Model of Myelin Loss

Several animal models have been developed to study the mechanisms underlying demyelination and remyelination. Among them, experimental autoimmune encephalomyelitis (EAE) (Gold, Linington, & Lassmann, 2006), virus-induced demyelination/inflammation (Mecha, Carrillo-Salinas, Mestre, Feliu, & Guaza, 2013) as well as toxin-induced demyelination models with cuprizone (Kipp, Clarner, Dang, Copray, & Beyer, 2009), lysolecithin, ethidium bromide, and complement/anti-galactocerebroside antibodies (Woodruff & Franklin, 1999) are the most commonly used. However, all these experimental models have strengths and limitations (Hooijmans et al., 2019).

EAE is a model of CNS demyelination that shows the major pathologic characteristics of MS. In mice, EAE can be induced by injection of myelin-derived antigen (MOG35-55) in combination with an adjuvant capable of inducing an inflammatory response. Immunization leads to activation and expansion of peripheral antigen-specific T-cells, which enter the CNS and bind the specific myelin antigens causing secretion of pro-inflammatory cytokines and damage of the BBB.
Microscopic examination of EAE brains revealed lesions similar to those observed in MS patients (Denic et al., 2011) with an evident destruction of myelin and the presence of immunoglobulins in both the brain and the cerebrospinal fluid. However, in this model demyelination and remyelination processes are less extensive and more acute than in humans, and are difficult to dissect because they proceed simultaneously (Osorio-Querejeta, Saenz-Cuesta, Munoz-Culla, & Otaegui, 2017; Tanaka & Yoshida, 2014).

Given that epidemiological data support a possible involvement of viruses in MS pathogenesis, models of virus-induced demyelination have been established, including demyelination induced by the Theiler’s murine encephalomyelitis virus. Models of viral infection have two advantages: i) the disease is chronic-progressive in susceptible mice; and ii) pathological anomalies are limited to the CNS (Sarchielli, Trequattrini, Usai, Murasecco, & Gallai, 1993; Sibley, Bamford, & Clark, 1985). However, they are not extensively used due to the long incubation periods between infection and the pathology onset and high mortality rates among infected animals (Osorio-Querejeta et al., 2017).

The most used toxins to model MS are bis-cyclohexanone-oxaldihydrazone (cuprizone) and lysophosphatidylcholine (lysolecithin), which are both able to induce the death of oligodendrocytes (OLs).

Cuprizone is a copper chelator that inhibits copper-dependent mitochondrial enzymes causing mitochondrial dysfunction in OLs and toxicity (Matsushima & Morell, 2001). Dietary administration of cuprizone to young and adult mice induces demyelination in different brain regions, especially in the corpus callosum that predominantly contain white matter tracts. This model present microglia and astrocyte activation similar to MS, but the BBB remains intact differently from MS and EAE model (Matsushima & Morell, 2001). Despite this mouse model is reproducible and simple, it should be noted that the age, sex and animal species can influence the experimental results (Kipp et al., 2009).

Lysolecithin (LPC) is a demyelinating chemical that is administered through a stereotactic injection into white matter area in the CNS (Rawji & Yong, 2013). LPC injection alters membrane composition, specifically of OLs, inducing a focal myelin lesion that spontaneously recovers over time in mice. Demyelination is not immune-mediated and is evident even in immune-deficient mice. In the acute phase immediately following the lysolecithin injection, microglia and macrophage infiltrate the lesion with minimal T-cell involvement (Imai et al., 2008), reactive astrogliosis occurs, axonal homeostasis is altered, and oligodendrocyte progenitors (OPCs) proliferate and migrate to the lesion site (Keough, Jensen, & Yong, 2015). However, chronic inflammation in lesions is minimal and complete remyelination occurs in 5–6 weeks. If young animals are used, lysolecithin lesions show rapid repair. Conversely, repair in older animals is much slower (Shields, Gilson, Blakemore, & Franklin, 1999).
This model is highly reproducible but it is advantageous because demyelination is rapid and remyelination has prolonged course (Tanaka & Yoshida, 2014). Because a single model does not accurately mimic all pathological and clinical features of human MS, a combination of \textit{in vitro} and \textit{in vivo} model systems should be used to investigate the mechanisms of myelin repair and the causes of remyelination failure occurred in MS.

1.3 Oligodendrocytes and (re)myelination

Remyelination is a spontaneous regenerative process in which new myelin sheaths are generated on demyelinated axons to restore an efficient electric conduction (R. J. Franklin & Ffrench-Constant, 2008).

In the CNS (re)myelination is mainly supported by oligodendrocytes (OLs). OLs account for 5-8% of all brain cells and derive from differentiation of adult oligodendrocyte progenitors (OPCs), which constitute the largest population of dividing neural cells (Dawson, Polito, Levine, & Reynolds, 2003). OPCs, also called NG2 cells, are self-renewing, highly proliferative, motile and bipolar cells, which express high level of gangliosides identified by A2B5 antibody, the receptor of PDGF alpha (PDGFαR) and the NG2 proteoglycan (Zuchero & Barres, 2013). These cells are normally present in a quiescence state, characterized by a low rate of proliferation, and are distinguished into two main populations: early postnatal-derived OPCs, that are homogenously distributed within the CNS (Nishiyama, Lin, Giese, Heldin, & Stallcup, 1996), and new OPCs that are continuously originated from the endogenous neural precursor cells (eNPCs) in the subgranular zone of the dentate gyrus (SGZ) in the hippocampus and the subventricular zone of the lateral ventricles (V-SVZ) (Menn et al., 2006). Under normal conditions, these adult V-SVZ progenitors generate more neurons than OLs, but after demyelination, V-SVZ-derived progenitor cells preferentially differentiate towards OLs (Maki, Liang, Miyamoto, Lo, & Arai, 2013).

In response to demyelination, both parenchymal OPCs and eNPCs get promptly activated and differentiate into OLs, thus representing a potential reservoir for myelin repair (Domingues, Portugal, Socodato, & Relvas, 2016). OLs derived from parenchymal OPCs are only able to migrate short distances, just populating damaged areas in the proximity of their progenitor cells, whereas eNPCs can migrate long distances into the corpus callosum, striatum, and fimbria fornix, where they continue to divide or differentiate into mature myelinating and non-myelinating OLs (Kaneko, Kako, & Sawamoto, 2013; Menn et al., 2006).

More recently, Serwanski and colleagues showed that after acute demyelination in the corpus callosum local OPCs rapidly expand and differentiate into remyelinating OLs within the first two weeks. By contrast, eNPCs in the SVZ contribute to myelin repair with a temporal delay of two weeks.
with an increased number of SVZ-derived OPCs at myelin lesion four weeks after demyelination (Serwanski, Rasmussen, Brunquell, Perkins, & Nishiyama, 2018). However, the specific contribution of parenchymal OPCs or eNPCs during remyelination and whether one population of cells is more important than the other still remain largely unknown. (Maki, 2013 #268)

Numerous studies on animal models of MS provided evidence that remyelination requires several steps: i) generation of new OPCs; ii) OPC migration to the demyelinating area iii) OPC differentiation into myelinating OLs. More in details, following myelin damage, factors released by microglia and astrocytes activate OPCs that shift from a quiescent to a regenerative phenotype. This process is accompanied by upregulation of specific genes such as oligodendrocyte transcription factor 1 and 2 (Olig1, Olig2), Sex determining region Y-box 2 (Sox2) and 10 (Sox10) (Zuchero & Barres, 2013), Myt1, Homeobox protein Nkx-2.2 (Nkx2.2) and Nkx2.6 (Dulamea, 2017a), which probably interact with promoters of myelin genes (Miron, Kuhlmann, & Antel, 2011; Sohn et al., 2006).

Among chemo-attractant factors released from microglia and astrocytes, there are platelet derived growth factor (PDGF) and semaphorin 3F (Sema 3F), (Dulamea, 2017a), and molecules of extracellular matrix (tenascin-C, fibronectin, merosin) which, in association to metalloproteases, allow OPCs to push their way through the extracellular environment and extend their processes in order to contact the axons (Baumann & Pham-Dinh, 2001).

Once activated, OPCs migrate to the lesion site. The migration of OPCs is important for brain development and is regulated by different pathways. Some of them, including the Notch1/jagged1 signalling pathway, prevent a premature differentiation of OPCs, allowing the myelin production where it needs. Accordingly, OPCs derived from Notch1 knockout mice differentiate faster than wild-type OPCs (Y. Zhang et al., 2009).

At the lesioned area, the recruited OPCs proliferate and then differentiate into remyelinating oligodendrocytes, by progressively increasing their morphological complexity and expressing specific markers of distinct maturation steps (R. J. Franklin & Ffrench-Constant, 2008).

The differentiation of OPCs is promoted by a few mediators such as insulin-like growth factor 1 (IGF-1), ciliary neurotrophic factor (CNTF) and thyroid hormone (T3) (M. Zhang, Ma, Qin, & Yao, 2016), which are commonly use in vitro to culture OLs. CNTF and IGF-1 are also able to promote myelination in vivo, in demyelination animal models, and to protect mature OLs from a pathological insult (Mason, Ye, Suzuki, D'Ercole, & Matsushima, 2000; J. Zhang et al., 2011).

In particular, CNTF is one of the most effective neurotrophic factor in promoting OPC differentiation and survival (Q. Cao et al., 2010). Indeed, CNTF reduces EAE severity by decreasing the OL
apoptosis and enhancing OPC proliferation and differentiation (Butzkueven et al., 2002; Linker et al., 2002). Accordingly, CNTF-deficient mice display exacerbated EAE disease and decreased number of proliferating OPCs (Linker et al., 2002). However, administration of CNTF is not sufficient to promote remyelination in mice with demyelinating lesions induced by ethidium bromide injection (Talbott et al., 2007), although transplantation of adult OPCs expressing CNTF has been reported to promote myelin repair and functional recovery following traumatic injury to the spinal cord (Q. Cao et al., 2010).

IGF-1 is another neuroprotective trophic factor for cells of the oligodendrocyte lineage. It can protect mature OLs from apoptotic death in vivo after a demyelinating insult. Transgenic mice that continuously express IGF-1 (IGF-1 tg) after cuprizone intoxication display lower number of apoptotic mature OLs at myelin lesion compared to wild-type mice. An early recovery from demyelination occurs in these mice thanks to the ability of IGF-1 to retain the mature oligodendrocyte population. IGF-1 has been reported to be protective also in EAE mouse model. IGF-1 injection into EAE animals reduces the numbers and area of demyelinating lesions and increases the number of axons containing regenerating myelin segments (Yao, Liu, Hudson, & Webster, 1995). A rapid clinical and pathological recovery has been found in treated mice, however the clinical amelioration is transient and occurs during the acute and not chronic phase of disease (Cannella, Pitt, Capello, & Raine, 2000).

In addition to CNTF and IGF-1, significant evidence implicates the neurotrophin brain-derived neurotrophic factor (BDNF) as a key pro-myelinating molecule in vivo. BDNF potentiates normal CNS myelination in development and enhances recovery after myelin injury (J. L. Fletcher, Murray, & Xiao, 2018). Accordingly, BDNF knock-out mice exhibit decreased expression of the key myelin proteins myelin basic protein (MBP) and proteolipid protein (PLP) in the hippocampus and cortex (Cellerino, Carroll, Thoenen, & Barde, 1997).

Despite the success of CNTF, IGF-1 and BDNF in preclinical studies of in vivo demyelination, the trophic factors were ineffective in the clinical trials (J. Zhang et al., 2011). Failure of clinical trials was likely due to the inability of the neurotrophins to access to the CNS parenchyma through the BBB. Thus, understanding of the cellular and molecular mechanisms by which these mediators exerts their functions could provide novel therapeutic strategies to promote OPC differentiation.

Once differentiate, OPCs lose in part their capacity to migrate and proliferate.

Mature OLs must establish the contact with the injured axons before generating myelin. Myelin is not only a lipid-rich membrane but contains about 30% of proteins (Jackman, Ishii, & Bansal, 2009). Among them, myelin-associated glycoprotein (MAG) is known to maintain the contact between myelin and axons (Poltorak et al., 1987), whereas myelin proteolipid protein (PLP) and myelin basic
Protein (MBP) are known to aid in stabilizing the myelin sheath (Schwob, Clark, Agrawal, & Agrawal, 1985).

During repair, several layers of myelin are wrapped around the axon until remyelination is complete. An important role is exerted by a network of microtubules which support the extension of the OL membranes along axons and reduce the space between OL cell body and myelin sheath favoring the synthesis of membrane proteins at the remyelination site rather than within the OL cell body (Simons & Trajkovic, 2006).

The final product of the remyelination process is a compacted layer of myelin that is thinner and shorter than that produced during developmental myelination (Blakemore, 1974), but, despite its smaller dimensions, it appears sufficient to ensure full functional recovery of the axon (Crawford, Chambers, & Franklin, 2013).

Finally, in addition to (re)myelination, OLs provide trophic factors and lactate as energy source to axons (Funfschilling et al., 2012), contributing to normal brain function. Indeed, experimental evidence showed that inhibition of OL maturation prevents mice from learning of new motor skills, suggesting an important role of mature OLs in neuronal plasticity (McKenzie et al., 2014). Moreover, depletion of NG2+ immature cells in the prefrontal cortex of adult mice impairs excitatory neurotransmission in the pyramidal neurons and glutamate uptake by astrocytes, indicating a role of OPCs in regulating CNS homeostasis (Birey et al., 2015).
1.3.1 Oligodendrocytes and Remyelination Failure in MS

In MS, the remyelination is mostly restricted to the peripheral areas of lesions, starts early during the formation of lesions, and occurs in lesions with active demyelination. However, observations from a magnetization transfer ratio (MTR) study supported the hypothesis that entirely demyelinated lesions are the result of multiple episodes of demyelination and incomplete remyelination (Brown, Narayanan, Banwell, Arnold, & Canadian Pediatric Demyelinating Disease, 2014).

Possible cause of remyelination failure in MS may be an altered balance between the dysfunction and then the loss of OLs, and the reduced formation of new OLs from OPCs, likely due to a paucity of pro-regenerative factors or excess of inhibitory mediators (R. J. M. Franklin & Ffrench-Constant, 2017).

Research on *in vitro* models and animal models of MS revealed that myelin damage and OL loss in MS are caused by cytotoxic T lymphocytes and specific auto-antibodies (Fields, 2005; Lassmann, 2014). Accordingly, other studies identified in serum and cerebrospinal fluid (CSF) of MS patients various antibodies against proteins expressed in OLs that may be targets for autoimmune response such as MBP, PLP, MAG, MOG, transaldolase, oligodendrocyte surface protein (OSP), oligodendrocyte myelin glycoprotein (omGP), NOGO, NG2 and glycolipids (Dulamea, 2017b; Hecker et al., 2016).

*In vitro*, these antibodies are able to block the migration of OPCs, the synthesis of myelin and may lead to lysis of OLs, suggesting that such antibodies may reflect remyelination failure in MS patients. Together with OL damage, one of the causes of remyelination failure in MS may be the impaired migration of OPCs to the lesioned area.
Several studies have identified factors that negatively modulate OPC migration during remyelination. In particular, at chronic active lesions two chemoreppelent factors, semaphorin 3A (Sema 3A) and netrin-1, are highly expressed (Dulamea, 2017a; Piaton et al., 2011). In line with this observation, Boyd and colleagues showed an inverse correlation between the number of OPCs and the expression of the inhibitory signal Sema3A (Boyd, Zhang, & Williams, 2013). With respect to netrin-1, despite the protein and its fragments may play a positive role in the mature CNS by limiting cell migration, axon growth, and sprouting, in pathological circumstances it inhibits the capacity of OPCs to access and repair demyelinated plaques (Bin et al., 2013). Thus, the development of strategies to block Sema3A and netrin-1 function holds promise for future treatment of demyelinating diseases, including MS.

Another cause of remyelination failure may be the low differentiation rate of OPCs. As mentioned previously, the differentiation of OPCs is regulated by a complex of factors, which play a role during both brain development and remyelination in MS. Olig2 has a crucial role in OL determination and is predominantly detected in maturing OLs at active lesion borders rather than at silent and shadow plaques (Wegener et al., 2015). By contrast, Leucine-rich repeat and Immunoglobulin-like-domain-containing Nogo receptor-interacting protein 1 (LINGO-1) is considered an inhibitory regulator of OL differentiation and axonal myelination. Knockout mice for LINGO-1 exhibited enhanced myelin sheath formation and recovery from EAE (Mi et al., 2007). In addition, the treatment with LINGO-1 antagonists resulted in increasing OPC differentiation and myelination in both EAE and in LPC-mediated demyelination model (M. Zhang et al., 2016). On this basis, researchers have recently developed BIIB033 (Biogen), a LINGO-1 monoclonal antibody suitable for use in humans (Bothwell, 2017).

An additional factor that inhibit remyelination is Endothelin-1 (ET-1), a protein released by reactive astrocytes that has been found in demyelinating sites (Hammond et al., 2014a). ET-1 regulates the expression in astrocytes of Jagged1, a ligand that directly binds to Notch receptor on OPCs, inhibiting OL differentiation (Chamberlain, Nanescu, Psachoulia, & Huang, 2016). Moreover, endocrine receptors are considered as potential remyelination targets. Estrogen and progesterone are able to reduce the immune responses and improve OL and astrocyte function (Kipp, Amor, Krauth, & Beyer, 2012). Several studies showed that low levels of estrogens exacerbate MS in women (Triantafyllou et al., 2016), and low level of progesterone and testosterone metabolites are detectable in the cerebrospinal fluid of MS patients (Caruso et al., 2014).
Myelin components, in particular cholesterol, the major lipid of myelin sheath, also play an important role in the remyelination process. Knockout mice for a squalene synthase gene, an enzyme necessary for cholesterol synthesis, exhibit an irregular myelin sheath compared to wild type mice (Saher et al., 2005), suggesting the contribution of this lipid component not only in myelination but also in myelin repair (Olsen & Akirav, 2015).

To better understand why remyelination fails in MS, it should be considered other two crucial contributory processes: the immune system and the age.

The involvement of the immune system in the remyelination process is well documented and a large body of evidence shows an important role of both microglia/macrophages and astrocyte in the control of OL function. These glial cells can exert either protective or deleterious effects on OLs by synthesizing and releasing different cytokines, trophic factors, extracellular matrix components and neurotransmitter-like molecules. The functional role of microglia/macrophages and astrocyte in MS and their impact on OPC activity will be discussed in the following paragraphs.

Regarding the contribution of age to remyelination failure, it is known that the remyelination process become less efficient with time, and this is related to deficit in OPC differentiation. Despite the high number of reactive OPCs at myelin lesions, only some of them are able to successfully complete their maturation in aged mice (Woodruff, Fruttiger, Richardson, & Franklin, 2004). Experimental evidence showed that the age-associated reduction of remyelination efficacy is related to changes in the

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**Fig.1.5 Factors modulating remyelination.** OPC: oligodendrocyte progenitor cell; OL: oligodendrocyte; Sema3A: semaphorin 3A; LINGO-1: Leucin-rich repeat and immunoglobulin-like-domain-containing nogo receptor-interacting protein 1; ET-1: endothelin-1; PDGF: platelet derived growth factor; Sema3F: semaphorin 3F; Olig2: helix-loop-helix transcription factor Olig2; IGF-1: insulin-like growth factor 1; CNTF: ciliary neurotrophic factor. Modified from (Dulamea, 2017a).
response of macrophage and other inflammatory mediators to demyelination (Zhao, Li, & Franklin, 2006). In old mice, reactive macrophages and microglia lose their capacity to release pro-differentiating factors and to phagocyte myelin debris, which are known to limit OPC differentiation. This inefficient process may be reverted in experiments of heterochronic parabiosis, where circulation of aged mice is coupled with that of young mice (Ruckh et al., 2012). By considering intrinsic age-related changes in OPCs, Kuhlmann and colleagues demonstrated that aged OPCs are less responsive to pro-differentiating factors, contributing to their reduced remyelination capacity at chronic MS lesions (Kuhlmann et al., 2008).

Moreover, RNA sequencing from young and old mice indicated an important role of mTOR pathway in age-related changes of OPC function (Cunniffe & Coles, 2019). mTOR is a downstream mediator of PI3K/Akt signalling and is activated by growth factors such as insulin or IGF-I or neuregulin (Wood et al., 2013). Neumann and colleagues showed that the manipulation of mTOR pathway in old rats with caloric restriction or with the AMPK-agonist metformin reduces the inefficiency of OPCs to differentiate and restores myelination (Neumann et al., 2019).

Several in vitro and in vivo studies demonstrated the importance of mTOR signaling pathway in CNS myelination. Transgenic mice that express constitutively Akt, an mTOR activator, in mature OLs show a significant hyper-myelination throughout the CNS and increased myelin protein expression and mTOR activity (Flores et al., 2008). Accordingly, treatment with the mTOR inhibitor rapamycin induces CNS hypo-myelination in wild type mice (Dai, Bercury, & Macklin, 2014) and inhibits OPC differentiation in cell culture (Gaesser & Fyffe-Maricich, 2016). PI3K/Akt/mTOR pathway also plays a crucial role during remyelination by ensuring the adequate generation of OPCs and the appropriate differentiation of OLs. In LPC-induced model of focal demyelination, the reduction of Akt levels by deletion of the regulator Cdk5 from OLs induces an impaired OPC differentiation and delays remyelination (Luo, Burke, Kantor, Miller, & Yang, 2014). By contrast, the stimulation of the estrogen receptor β in EAE mice increases Akt activity, improves remyelination and reduces functional disability (S. Kumar et al., 2013).

Additional reports suggested that mTOR and its associated complexes are regulators of myelination at the transcriptional and translational level. MBP is the only myelin protein known to be specifically affected by mTOR signaling loss. Interestingly, mTOR inactivation significantly decreases MBP protein expression with no changes in MBP mRNA levels, indicating that mTOR controls the trafficking and/or local translation of MBP (Bercury et al., 2014). On the other side, mTOR regulates the expression of the transcription factors SREB1 and SREB2, which in turn control genes encoding enzymes required for myelin synthesis, thus favoring myelination. Specifically, SREB1 and SREB2
control the genes mediating lipid synthesis FA synthase (FASN), stearoyl-CoA desaturase-1 (SCD1), HMG-CoA reductase (HMGCR) and isopentenyl-diphosphate delta isomerase 1(IDI1).

Of note, there are alternative signaling pathways to mTOR, such as Wnt/β-catenin and ERK/MAPK, which may be able to compensate effectively for the absence of mTOR and to ensure appropriate OL development and myelination.
1.4 Microglia

Microglia are the tissue-resident macrophages of the central nervous system (CNS). They constitute 5-10% of the total glial cells and are broadly distributed throughout the brain and the spinal cord (Ginhoux, Lim, Hoeffel, Low, & Huber, 2013). Unlike monocytes, microglia (MG) develop early in embryogenesis from precursors cells in the embryonic yolk sac that migrate to the CNS at approximately embryonic day 8.5 (Wieghofer & Prinz, 2016). This process is regulated by transcriptional factors such as PU.1 and Runt-related transcription factor (RUNX1), which influence MG differentiation into CD11b+ cells. PU.1 is a transcription factor expressed in hematopoietic cells and in resting and activated MG in both rodents and humans. RUNX1 is another regulator of microglia development; its progressive loss of expression at postnatal day 10 is associated with the transformation of MG from amoeboid to ramified morphology (Nayak, Roth, & McGavern, 2014).

Microglia are currently considered as a cell population distinct from macrophages. They are self-renewing, long-lived, and cannot be replaced by bone marrow-derived macrophages (Kabba et al., 2018).

Under homeostatic conditions, MG exist in a “resting state” characterized by ramified morphology, small cell soma and highly motile long cellular processes, through which the cells continuously survey the surrounding environment (Gonzalez, Elgueta, Montoya, & Pacheco, 2014). Resting MG play many functions, including synaptic organization, control of neuronal excitability, myelin homeostasis, removal of debris by phagocytosis and trophic support (Asai et al., 2015).

In healthy brain, MG are characterized by a homeostatic function and express high level of microRNA-124, IL-10 and extracellular matrix related genes, important for cell survival and quiescent state maintenance (Orihuela, McPherson, & Harry, 2016; Varnum & Ikezu, 2012), and low level of CD46, major histocompatibility complex-II (MHCII) and CD11b. In addition, these cells secrete neurotrophic factors such as IGF-1 and nerve growth factor (NGF), brain–derived neurotrophic factor (BDNF) and transforming growth factor β (TGF-β) (Saijo & Glass, 2011).

This state of MG is favored by several factors i.e. neurotrophins, anti-inflammatory cytokines and prostaglandins released by neurons, astrocytes and also microglia. Among signalling pathways involved in neuron-MG activation/inhibition there are neuron-derived fractalkine (CX3CL1) that binds to CX3CR1 and MG cell receptors CD172, CD200R and CD45, which interact with neuronal cell-surface proteins CD47, CD200 and CD22, respectively (Loane & Kumar, 2016; Saijo & Glass, 2011).

In response to infection, trauma or ischemia, MG become rapidly activated, acquire an ameboid-phagocytic morphology and migrate to the site of injury (Kabba et al., 2018). Activated MG secrete
a broad spectrum of both pro-inflammatory and anti-inflammatory cytokines, other molecular mediators, reactive oxygen species and nitrogen species, prostanoids, complement components and excitatory amino acids (Kabba et al., 2018), induce phagocytosis and direct cytotoxicity (Lampron, Pimentel-Coelho, & Rivest, 2013). Depending on the nature and duration of stimuli, MG can acquire a variety of different phenotypes, specializing their function and ‘personality’ towards the most effective one (Ransohoff & Perry, 2009).

Similar to peripheral macrophages, MG has long been classified into two major phenotypes, defined as “classical activation”, also termed M1 phenotype, or “alternative activation”, M2 phenotype. However, the M1/M2 phenotype classification has been proposed based on experiments where MG were exposed to specific stimuli in vitro, a condition that does not reflect the complexity of in vivo environment (Ransohoff, 2016). Indeed, recent studies showed a much complex activation pattern of MG in vivo. Single cell genome sequencing indicated that microglial cell can dynamically transit from pro-inflammatory to anti-inflammatory function, depending on the environmental context (Chu et al., 2018; Hammond et al., 2019; A. Kumar, Alvarez-Croda, Stoica, Faden, & Loane, 2016) and can display a mixed transitional MG phenotype, called Mtran, which co-express M1 and M2 markers in vivo (A. Kumar et al., 2016).

Despite this limitation, the M1/M2 paradigm is still useful to define the function that microglia polarized towards extreme inflammatory or pro-regenerative states may exerts, at least in principle, in various pathological contexts.
**Fig.1.6 Microglia functions in the CNS.** Microglia play many functions: axonal tract development and myelin homeostasis (A), programmed cell death and removal of debris by phagocytosis (B), synaptic remodelling by pruning and control of neuronal excitability (C), and response to signals of injury, leading to release of pro-inflammatory or anti-inflammatory cytokines (D). Modified from (Sominsky, De Luca, & Spencer, 2018).

1.4.1 Microglia M1/M2 phenotypes

M1 microglia (M1-MG) are pro-inflammatory cells, which can be induced by *in vitro* stimulation with lipopolysaccharide (LPS) or interferon-γ (IFN-γ) and cellular or bacterial debris (Kabba et al., 2018). M1-MG are characterized by larger branching body and by high expression level of MHCII, scavenger receptors and CD40, the antigen presentation receptors which could fuel the pro-inflammatory responses against bacterial, viral or fungal pathogens (J. Wang et al., 2019).

To neutralize the insult, M1-MG release several pro-inflammatory cytokines, such as IL-1β, IL-12, IL-6, IFN-γ, IL-23, TNF; chemokines, including CCL4, CCL5, CCL8, CXCL2, CXCL4, and CXCL9; and express high levels of inducible nitric oxide synthase (iNOS), an enzyme catalyzing the production of nitric oxide (NO). In an oxidative environment NO reacts with superoxide leading to peroxynitrite formation and cell toxicity (Pacher, Beckman, & Liaudet, 2007).

In contrast, pro-regenerative microglia, also called M2 microglia (M2-MG), contribute to tissue repair and inflammation resolution. The cells exhibit smaller cell bodies and a branched structure (Ransohoff & Perry, 2009), and highly express markers of “alternative activation” such as arginase 1 (ArgI), chitinase-like Ym1, mannose receptor CD206, receptors CD14, CD163, CD204 and chemokine ligands CCL17, CCL22 and CCL24 (Sasaki, 2017). Specifically, ArgI is an enzyme that metabolizes arginine to urea and ornithine. The arginase pathway limits arginine availability for NO synthesis and ornithine itself can activate the pathways of polyamine and proline syntheses, which are important for cellular proliferation and tissue repair (Lisi et al., 2017). By contrast, CD206 is a C-type lectin that functions in endocytosis and phagocytosis, and plays an important role in immune homeostasis by scavenging unwanted mannose glycoproteins (Zhou et al., 2017).

The beneficial phenotype of MG can be induced *in vitro* by a variety of anti-inflammatory molecules, including IL-4, IL-13, IL-3, IL-21, IL-33, CCL2 and CXCL4.

Differently from M1 cells, M2-MG do not have cytotoxic properties; they contribute to immunoregulation by repressing inflammation, remodelling tissue, promoting angiogenesis and clearing parasites (Banerjee et al., 2013). These functions depend on the ability of MG to secrete anti-inflammatory cytokines (IL-10, IL-13 and TGF-β), neurotrophic factors (IGF-1, BDNF, VEGF, EGF) and IL-1 receptor antagonist, and to block inducible NO synthase (iNOS). Moreover, their elongated
shape and high levels of F-actin compared to M1 cells favor phagocytic activity and phagosome formation (Xia, Zhang, Gao, Wang, & Chen, 2015).

**Fig.1.7 Microglia M1/M2 phenotypes.** The morphology and the phenotype associated with different functional states of microglia are shown. During neuroinflammation, microglia assume ameboid morphology and acquire classical M1 or alternative M2 phenotype according to the nature of local milieu.

### 1.4.2 Functional Role of Microglia in MS

Microglia (MG), together with macrophages, are considered key players in the development of MS and its animal model EAE. They represent the most abundant immune cells in active MS lesions and in normal-appearing white matter (NAWM) of MS patients (Singh et al., 2013).

Given the difficulty in distinguishing monocytes and MG, both morphologically and functionally, the complex roles of MG versus monocytes in the inflammatory/degenerative processes in MS have not been fully clarified yet. However, recent comparative transcriptome studies identified new molecular markers, including Siglec-H, Sal-1, olfactomedin-like3, TMEM119 and P2Y12 to distinguish MG from infiltrating macrophages, which can be of help to understand MG-specific functions in different phases of MS (Bennett et al., 2016; Butovsky et al., 2014; Han, Harris, & Zhang, 2017).

Microglial functions relevant to the disease include antigen presentation, phagocytosis, synaptic pruning, and secretion of pro- and anti-inflammatory mediators (Q. Li & Barres, 2018).

The involvement of MG in MS development and progression is supported by the presence of clusters of activated MG in the white matter of MS patients, in particular in proximity of “pre-active lesions”, which are characterized by an absence of leucocyte infiltration and demyelination but may eventually develop into active demyelinating MS lesions (Singh et al., 2013).
In contrast to healthy controls, the number of P2Y12+ homeostatic MG has been found significantly reduced in the white matter of MS patients, whereas TMEM119+ activated MG have been found increased in density in active MS lesions, suggesting that MG activation contribute to the initial and early stages of plaque formation and tissue damage (Zrzavy et al., 2017). Moreover, interference with MG activation attenuates disease severity and demyelination, as indicated by experiments of MG depletion performed using bone marrow chimeric mice (Ponomarev, Shriver, Maresz, & Dittel, 2005) or herpes simplex virus thymidine kinase (HSVTK) suicide gene (Heppner et al., 2005) or MG-specific deletion of TGF-β-activated kinase 1 in CX3CR1-CreERT2 mouse model (Goldmann et al., 2013).

Activated MG within the damaged brain area may play both detrimental and beneficial roles, participating both to myelin injury and repair (Domingues et al., 2016). Earlier studies reported that the majority of MG in early active and chronic MS lesions show a pro-inflammatory phenotype and that their activity correlates with the axonal and oligodendrocyte pathology (Baufeld, O'Loughlin, Calcagno, Madore, & Butovsky, 2018). These reactive microglial cells express the surface markers CD68, MHC I and II, P22phox, and CD86, as well as molecules involved in phagocytosis, antigen presentation, T-cell stimulation, oxidative injury and iron metabolism (Zrzavy et al., 2017).

In both EAE and cuprizone-induced demyelination models, MG show high expression level of MHC and costimulatory molecules CD80, CD86 and CD40, suggesting a role of MG in antigen presentation and local stimulation of myelin-autoreactive T-cells (Brendecke & Prinz, 2015). Consistent with this, time-course studies in EAE mice showed that the appearance of inflammatory T-cells in the CNS correlates with the activation of CD11b+ MG (Murphy, Lalor, Lynch, & Mills, 2010). Conversely, other studies revealed that the activation of CNS-specific T cells occurs in the periphery before their entry in the CNS, and thus MG contribute to antigen spreading in the CNS and not to direct T-cells stimulation (Engelhardt, Vajkoczy, & Weller, 2017).

Another major function described for MG is the coordination of local tissue inflammation through secretion of pro-inflammatory cytokines (IL-6, IL-1β, IL-18, IL-12, IL-23, TNF) (Merson, Binder, & Kilpatrick, 2010) and chemokines (CCL2, CCL3, CCL4, CCL5, CCL7, CCL12, CCL-22) (Jiang, Jiang, & Zhang, 2014), which are normally detected at low level in the healthy CNS (J. Wang et al., 2019). However, whether these inflammatory agents have a MG-specific role in the disease is not clear yet, because also peripheral immune cells secrete the same inflammatory mediators at the lesion sites.

Although MG are involved in synaptic plasticity, this function is destroyed under pathological conditions, causing synaptic loss in MS and thus cognitive impairment (Di Filippo, Portaccio,
Indeed, activated MG may produce reactive oxygen species (ROS), reactive nitrogen species, large amounts of TNF and glutamate, which are known to induce demyelination and neurodegeneration. Accordingly, the inhibition of the ROS-producing enzyme NADPH oxidase attenuates MOG-induced EAE and reduces the pro-inflammatory response of MG to MOG in vitro (Domingues et al., 2016).

Importantly, the negative impact of MG on remyelination may also be mediated by astrocytes, recognized players in MS immunopathology (Brambilla, 2019; Colombo & Farina, 2016). Indeed, through secretion of three inflammatory mediators (IL-1α, TNF and C1q), activated MG are able to convert astrocytes to harmful cells, which cause dysfunction in both OLs and neurons (Liddelow et al., 2017). However, whether harmful astrocytes contribute to remyelination failure in MS is still unclear.

On the other hand, MG also exert beneficial functions in MS, through phagocytosis of myelin debris and dead cells, and secretion of neuroprotective and anti-inflammatory mediators (Miron et al., 2013; Olah et al., 2012; Voss et al., 2012).

The capability of MG to phagocyte myelin debris, which is important for the remyelination process, is associated to the expression of several receptors, including CX3CR1, RXR-γ, triggering receptor expressed on myeloid cells 2 (TREM2), complement receptor 3 (CR3) and signal regulatory protein (SIRP)-α, as well as IFN-β and transmembrane TNF (tmTNF). For instance, blocking TREM2 using a monoclonal antibody increase demyelination in EAE mouse model (Piccio et al., 2007). Ablation of microglial TNFR2 leads to earlier disease onset and increases myelin damage and leucocyte infiltration, thus indicating that pro-inflammatory cytokine tmTNF has neuroprotective function in the disease (H. Gao et al., 2017).

In addition, MG secrete anti-inflammatory molecules, including IL-4, IL-10, IL-13 and TGF-β, which together induce the differentiation of protective type 2 T helper (Th2) and regulatory T (Treg) cells (Voet, Prinz, & van Loo, 2019). In particular, IL-4 enhance oligodendrogenesis (Butovsky et al., 2006) while activin-A, a member of TGF-β superfamily, promote OL differentiation during remyelination (Miron et al., 2013).

Other evidence indicates that MG favor remyelination through P2X4R signalling. Indeed, P2X4R signalling blockade exacerbate clinical signs in EAE model, induce MG activation towards pro-inflammatory phenotype and inhibit myelin phagocytosis (Zabala et al., 2018).

Support to protective microglial functions comes from a recent study showing that MG-specific deletion of A20, a crucial regulator of MG activation, exacerbate EAE as a result of hyperactivation of the NLRP3 inflamasome and an enhanced secretion of IL1-β and IL-18 (Voet et al., 2018).
In conclusion, it is now clear that microglia play crucial roles in healthy, inflamed, injured, and recovering CNS given their dual nature. However, additional research is necessary to design therapeutic approaches able to block the pro-inflammatory action of microglia during MS disease and to boost their anti-inflammatory functions.

1.4.3 Microglia interaction with Oligodendrocytes

A large body of evidence supported that the cross-talk between oligodendrocytes (OLs) and microglia (MG) is important for OL development, proliferation, migration and differentiation. However, this cross-talk is very complex and contradictory because MG exert both protective and harmful functions on myelin-forming cells. In turn, OLs can control microglial activity through secretion of chemokines, cytokines, chaperokines and nucleotides (Kabba et al., 2018; Peferoen, Kipp, van der Valk, van Noort, & Amor, 2014). The contribution of MG in supporting oligodendrogenesis has been demonstrated in vivo using a knockout mice for CSF-1R, a tyrosine kinase receptor required for MG survival. Although MG depletion is not complete in CSF-1R knockout animals, the brain architecture in the periventricular area is destroyed and the number of OLs in the cerebral cortex is reduced by 50% in transgenic mice (Erblich, Zhu, Etgen, Dobrenis, & Pollard, 2011). In addition, microglial cells have been observed near dying OLs after spinal cord injury, where they may promote OL survival via insulin-like growth factor 2 (IGF-2) (Miller et al., 2007).

However, the activation state of MG may differently influence OPC behaviour and the remyelination process.

In three different focal demyelination models (lysolecithin in corpus callosum or spinal cord and ethidium bromide in caudal cerebellar peduncles), pro-inflammatory MG (identified by expression of iNOS, TNF and CD16/32) have been observed in the early phase after myelin lesion, during the proliferation phase of OPCs. Depletion of M1 microglia (M1-MG) using gadolinium chloride III has been reported to induce a reduction of OPC proliferation (Miron, 2017), the first important step in the remyelination process. This suggests that MG characterized by a typical inflammatory phenotype may exert a pro-regenerative function. By contrast, several other studies showed that M1-MG inhibit both in vitro and in vivo OPC proliferation and induce OPC apoptosis through secretion of pro-inflammatory cytokines, including IL(1,2,3), interferons (α,β and γ), TNF, ROS and lymphotoxin (LT), which are detectable in demyelinating MS lesions (He, Chen, Qian, Chen, & Buzby, 2010; Peferoen et al., 2014). Specifically, microglial TNF, by binding TNF-R1 on OLs and activating the mitogen-activated protein kinase signalling pathway induces OL death. This finding has been demonstrated in transgenic mice over-expressing TNF that spontaneously develop chronic
demyelinating disease (Peferoen et al., 2014). Like TNF, LT directly induces OL death by increasing the intracellular concentration of ceramide in OLs (Plo et al., 1999). Unlike LT, IL1-β does not induce apoptosis directly, but its systemic and intracerebral injection in neonatal mice delays remyelination during disease (Deng, Lu, Sivakumar, Ling, & Kaur, 2008). IFN-γ increases *in vitro* the expression of caspases and Fas in OLs, making them more susceptible to cell death (Lin et al., 2006), and inhibits remyelination in the cuprizone-treated mice by inducing stress of the endoplasmic reticulum (ER). IFN-γ also activates iNOS, an enzyme involved in production of nitric oxide (NO) that is highly toxic to OLs (J. Li, Baud, Vartanian, Volpe, & Rosenberg, 2005). Besides the apoptotic effects, MG contribute to OL damage releasing glutamate. Since OPCs express diverse subtypes of glutamate receptors, they are very vulnerable to calcium-mediated excitotoxicity induced by glutamate (Matute & Ransom, 2012).

On the other hand, several studies indicate that pro-regenerative microglia (M2-MG) play beneficial role on OPC functions. A transition in microglia activation to a beneficial phenotype (identified by expression of ArgI, CD206 and IGF-1) has been observed during the OPC differentiation phase of remyelination process. Indeed, depletion of M2-MG using mannose-coated clodronate liposomes impairs OPC differentiation (Miron, 2017). Importantly, studies of MS tissues revealed that beneficial MG is predominant in post-mortem lesions which have been actively remyelinating (acute active lesions, rim of chronic lesions), and not in those characterized by impaired remyelination (chronic inactive lesions), suggesting a correlation between beneficial MG and remyelination efficiency in human disease (Miron, 2017).

*In vitro* experiments demonstrated that M2-MG favor OL survival by limiting their apoptosis and promote OPC maturation into myelinating OLs by the release several pro-differentiating factors (Nicholas, Stevens, Wing, & Compston, 2002). For instance, IL4-treated MG enhance differentiation of cultured OPCs into mature OLs via IGF-1 (Butovsky et al., 2014), while conditioned media derived from TGF-β stimulated MG promote OPC chemotaxis (Lalive et al., 2005). Other studies showed that conditioned media derived from MG treated with IL-3 or IL-10 significantly enhance OL differentiation via activin A (Miron et al., 2013), and myelination in OL-neuron cocultures through release of PDGF-AA, IGF-1 and VEGF (Pang et al., 2013). *In vivo* studies further elucidated the mechanisms through which M2 microglia impact OPCs, myelination and remyelination. In the developing subventricular zone, MG support OL development via production of TNF, IL1-β, IL-6 and IFN-γ (Shigemoto-Mogami, Hoshikawa, Goldman, Sekino, & Sato, 2014), while promote myelination via iron release (X. Zhang, Surguladze, Slagle-Webb, Cozzi, & Connor, 2006).

Furthermore, gene expression analysis of MG isolated from the corpus callosum in the cuprizone mouse model identified TNF, FGF-2 and Osteopontin-M as principal regulators of OPC proliferation
(Selvaraju et al., 2004), whereas IGF-1 and galectin-3 were identified as inducers of OPC differentiation (Hoyos et al., 2014; Voss et al., 2012).

**Fig.1.8 Oligodendrocyte-microglia cross-talk.** Microglia play a dual role on oligodendrocyte functions. Such production of chemokines, cytokines, and growth factors may induce mild damage to death of oligodendrocytes (A) or, conversely, promote neuroprotection and repair including proliferation or recruitment or differentiation of oligodendrocyte progenitors (B). Microglia also interact with oligodendrocytes via production of extracellular vesicles. Modified from (Peferoen et al., 2014).

In conclusion, there is increasing evidence that microglia play a critical role in regulating oligodendrocyte function during (re)myelination. However, how the interplay between microglia and oligodendrocytes is dysregulated in different CNS disorders, including MS, is still not completely understood. Reprogramming microglia towards beneficial functions may represent a therapeutic strategy to promote myelin repair and to dampen inflammation, especially in progressive form of MS.

**1.4.4 Microglia versus Macrophages in MS**

In several neurological diseases including multiple sclerosis (MS), infiltration of immune cells from the periphery into the CNS is largely observed and mainly consists in an accumulation of macrophages within the brain.
Macrophages are important components of innate immune system. Differently than microglia (MG), these cells originate from either embryonic progenitor cells and hematopoietic stem cells in the bone marrow, which differentiate into blood monocytes that circulate in the peripheral vasculature and infiltrate all tissues such as the liver, lungs, and non-parenchymal areas of the CNS, including the meninges, choroid plexus and perivascular space (Rawji & Yong, 2013). Under physiological conditions, few bone marrow derived macrophages are present in the brain. However, upon brain injury, these cells infiltrate the CNS and accumulate at the lesion site, contributing to both damage and tissue repair (Aguzzi, Barres, & Bennett, 2013).

Because macrophages and MG share many surface markers (F4/80+/Cd11b+ phenotype) and have many similar functions including inflammatory response, phagocytosis, homeostasis and tissue remodeling, it is difficult to distinguish these two cell types both morphologically and functionally (Chu et al., 2018). Several tools have been developed to distinguish the two cell types such as bone marrow chimeras, obtained by transplantation of labeled bone marrow cells into irradiated mice, and parabiosis, which allows a shared blood circulation between two mice. However, transcriptomic gene assays and computation biology, along with proteomic and epigenomic analysis, have contributed to better define the biomarker profile of these cell type.

Indeed, studies showed that during the early phase of immune response infiltrating macrophages show high expression of CD45, CCR1, CCR2 and CCR5 but low level of TGF-β expression, while MG express low level of the molecular markers CD45, CCR1 and CCR5 and high level of TGF-β (Chu et al., 2018). In addition, transmembrane protein 119 (Tmem119) has been showed to be specifically expressed in microglia not in macrophages (Bennett et al., 2016).

A central role of macrophages in MS pathogenesis and progression has been demonstrated in EAE mouse model. In the early stages of EAE, macrophages enter into CNS as a result of a destroyed BBB, which is in turn caused by the increased expression of chemokine receptors. Specifically, chemokine receptor CCR2 and its ligand CCL2 are involved in regulating macrophages infiltration and tissue damage in EAE (Baufeld et al., 2018). Indeed, lack of CCR2, or the deletion or inhibition of CCL2, impairs monocyte-derived macrophage recruitment into the CNS of EAE mice (Dogán, Elhoy, & Karpus, 2008) and reduces disease severity (Hsieh et al., 2014). Accordingly, other studies reported that EAE progression correlates with macrophage infiltration in mice (Ajami, Bennett, Krieger, McNagny, & Rossi, 2011) and the disease is attenuated when macrophages are depleted (Tran, Hoekstra, van Rooijen, Dijkstra, & Owens, 1998).

During the acute phase of the disease, macrophages, together with MG, acquire a pro-inflammatory phenotype (M1) and release a large amount of inflammatory cytokines leading to tissue damage, demyelination and neuronal loss in the CNS (Almolda, Gonzalez, & Castellano, 2011). Indeed, the
administration to EAE mice of ganciclovir, a drug that reduces MG/macrophage density, suppresses the development of EAE suggesting a detrimental role of MG/macrophages activation in the disease (Heppner et al., 2005). In addition, Nikic et al. showed that the reactive oxygen and nitrogen species released from macrophages induce mitochondrial pathology and axonal degeneration (Nikic et al., 2011).

However, in the later stage of EAE, MG/macrophages acquire the beneficial phenotype M2 and release a variety of anti-inflammatory cytokines, which are implicated in tissue repair and inflammation resolution. In this regard, recent study provides evidence that the transfer of M2-polarized monocytes into a blood vessel or M2 microglia into a ventricle in EAE animals promotes oligodendrocyte differentiation and ameliorates EAE (McMurran, Jones, Fitzgerald, & Franklin, 2016). In addition, estrogen treatment can protect animals from EAE by promoting M2 polarization of macrophages/MG, which is associated to regulatory B-cells activation (Benedek et al., 2017).

Based on these findings, it is hypothesized that the dynamic balance between M1/M2 phenotypes is crucial for disease development and that the transition from the pro-inflammatory to the anti-inflammatory phenotype can lead to restoration of homeostasis and improve functional outcomes (Alvarez et al., 2016; Butovsky et al., 2006; Mikita et al., 2011).

Several factors have been found to modulate macrophage/MG polarization such as lysosomal adaptor protein Lamtor1 deficiency, amino-acid starvation, inhibition of v-ATPase and mTOR pathway, which are able to increase M1 activation and suppress M2 polarization (T. Kimura et al., 2016). By contrast, new compounds such as resveratrol, valproic acid, tuftsin, and spermidine have been developed for MS treatment by enhancing M2 MG/macrophage or inhibiting M1 MG/macrophage (Chu et al., 2018; Hou et al., 2012; Yang et al., 2016). Recently, also miRNA have been included as potential therapeutics able to suppress local MG/macrophage activation. For instance, miR-124 is one of the most expressed miRNA in the brain. In EAE, the reduction of miRNA-124 activity correlates with an increased activation of MG/macrophages, and administration of exogenous miRNA in these mice induces the M2 phenotype in MG/macrophages by suppressing the disease (Ponomarev, Veremeyko, & Weiner, 2013).

In conclusion, it is important to keep in mind that because MG and macrophage responses could be both neuroprotective and neurotoxic depending on the stage and progression of the disease, the modulation of the immune pathways is very tricky and timing of therapeutic intervention is pivotal.
1.5 Astrocytes

Astrocytes constitute approximately 30% of glial cells in the CNS, where they occupy a unique territory delimited by non-overlapping processes (Sofroniew & Vinters, 2010; Verkhratsky & Nedergaard, 2018).

The heterogeneity of astrocytes is very complex and depends on their diverse morphology, antigenic phenotypes, anatomical location and functions (Domingues et al., 2016).

Through their highly ramified processes, one single astrocyte connects with several neuronal terminals forming the so-called tripartite synapse, made by the pre- and the post-synaptic terminals and the astrocyte process (Ventura & Harris, 1999). Within this structure, astrocytes regulate synaptogenesis, synaptic transmission and plasticity by releasing several bioactive molecules, i.e. glutamate, D-serine, ATP (Araque et al., 2014), uptaking glutamate (Oliet, Piet, & Poulain, 2001) and buffering extracellular K⁺ (Higashi et al., 2001). Astrocyte also make contact by gap junctions with the cerebral vasculature and the parenchymal basal lamina, contributing to the maintenance of the blood-brain barrier (BBB). Indeed, as a BBB component, astrocytes regulate the movement of small molecules such as water, ions and soluble factors, and limit the entry of peripheral immune cells into the CNS (Keaney & Campbell, 2015). Being located at the interface between blood and neurons, astrocytes provide lactate to neurons, the main energy substrate in neurons, and use glucose derived from the circulation to form glycogen, the storage form of glucose, and to sustain neuronal activity. In addition, astrocytes regulate lipid metabolism by supplying cholesterol to neurons and OLs, which is important for membrane homeostasis and myelin synthesis (Camargo et al., 2017; Verkhratsky & Nedergaard, 2018).

Importantly, astrocytes also contribute to immune-modulation. Upon stimulation, astrocytes produce and release cytokines (e.g., TNF, IL1-β, IL-6), chemokines (e.g., CCL2, CCL20 CXCL1), neurotrophic factors (e.g., NGF, BDNF, VEGF) and oxidants (NO). They express MHCII molecules and certain co-stimulatory molecules, at least in vitro (Filippello et al., 2016), being recognized as weak antigen-presenting cells (APCs), that moderately activate CD4⁺ and CD8⁺ T-cells (Brambilla, 2019).

Like MG, astrocytes respond to CNS alterations with a complex process of activation consisting of induction of cell proliferation, morphological modifications and new function acquisition. This phenomenon is called “astrogliosis” and influences either positively or negatively disease pathogenesis and progression.

Recently, two distinct phenotypes of reactive astrocytes, A1 and A2, have been described in neuro-inflammatory conditions and following ischemic injury, respectively.
A1 astrocytes are induced by classically activated inflammatory microglia and are toxic for neurons and OLs by inhibiting OPC proliferation and differentiation. Their number is significantly higher in MS active lesions compared to chronic active and inactive. On the contrary, A2 astrocytes release neurotrophic factors, play protective and anti-inflammatory functions and are predominant during remyelination (Liddelow et al., 2017).

These two types of reactive astrocytes can be identified according to their individual genetic expressions. A1 astrocytes upregulate genes involved in the complement (C3, Serping1), antigen presentation (H2-D1, H2-T23, Psmb8), cytokine pathway (Gbp2), cell adhesion (Fbln5, Amigo2), and apoptosis (Srgn). Among them, C3, Serping1 and Amigo2 are the most characteristic of A1 harmful phenotype. In detail, C3 (Complement 3) and Serping1 (Serpin peptidase inhibitor, clade G, member 1) play a key role in the complement pathway activation and contribute to innate immunity. Their upregulation in A1 astrocytes may reflect ongoing inflammation in the brain (Gorelik, Sapir, Woodruff, & Reiner, 2017). Amigo2 (adhesion molecule with IG-like domain 2) is a cell adhesion molecule widely expressed in the CNS implicated in the formation and functioning of tripartite synapses (Hillen, Burbach, & Hol, 2018).

By contrast, A2 astrocytes upregulate genes associated to protein synthesis (tgm1), immunomodulation (Ptx3, Sphk1, Cd14), intracellular trafficking and cell migration (S100a10), and cell proliferation (Ptgs2, Tm4sf1, Emp1) (Clarke et al., 2018; Liddelow et al., 2017).

Among them, PTX3, Cd14 and Tm4sf1 are some of markers significantly upregulated in A2 astrocytes and not expressed in A1 astrocytes. PTX3 (Pentraxin 3), an acute-phase protein released during inflammation, aids phagocytic clearance of pathogens and apoptotic cells by astrocytes and immune cells (Ummenthum et al., 2016) and regulates macrophage activity in tissue injury (Shiraki et al., 2016). In addition, this protein supports BBB integrity and promotes astrocytic scar formation by increasing the expression of tight junctions proteins such as ZO-1 and Claudin-5 (Shindo et al., 2016).

A2 astrocyte-related gene Cd14 (cluster of differentiation 14) is a GPI (glycosyl phosphatidylinositol-anchored cell membrane) protein that facilitates cellular responses to low doses of lipopolysaccharide (LPS). Although LPS is the main ligand, CD14 also recognizes other pathogen-associated molecular patterns (Ranoa, Kelley, & Tapping, 2013). Differently, Tm4sf1 (transmembrane 4 L Six Family Member 1) is a small plasma membrane glycoprotein that regulates cell proliferation (Wei et al., 2018) and may also promote cell migration by increasing the formation of filopodia (Neal et al., 2018).

Of note, Haindl and colleagues showed that A1 and A2 astrocytic phenotypes change their distribution and functions in EAE mouse model during lesion evolution. During early remyelination,
A1 astrocytes are detectable in a granular pattern spreading over the lesion and are able to attract microglia and T cells. On the contrary, during later remyelination, the A1 astrocytes form a ring surrounding the lesion, which might acts as a barrier, protecting adjacent healthy tissue. At this chronic disease phase, A2 astrocytes are predominant in the lesion and support tissue repair by inhibiting inflammation and secreting neurotrophic factors (Haindl, Kock, Zeitelhofer-Adzemovic, Fazekas, & Hochmeister, 2019).

Hence, similar to MG, the astrocyte response appears to be dependent on the physiological context (type of injury, location in the CNS) and on the microenvironment. Similar to M1/M2 microglia polarization A1 and A2 dichotomy is likely an oversimplification of multiple functional states that astrocytes sequentially acquire during different phases of disease, also explaining the co-existence of mixed A1 and A2 features in vivo (Pekny et al., 2016).

**Fig.1.9 Role of astrocytes under physiological or pathological condition (a) Functions of the astrocytes in physiological conditions, which are in favor of the homeostasis of the brain. (b) Reactive astrogliosis, which has a double function highly discussed, one for cell death and one for pro-neuroprotection depending on the microenvironment. Modified from (Becerra-Calixto & Cardona-Gomez, 2017).**
1.5.1 Role of Astrocytes in (re)Myelination

Astrocytes play detrimental as well as beneficial roles in MS. The dual role of reactive astrocytes during the phases of MS development and progression has been investigated by using ablation strategies, such as administration of ganciclovir to GFAP-HSV-TK mice.

Depletion of astrocytes prior to onset and at the acute phase of EAE exacerbates the disease and is associated with increased infiltration of immune cells in CNS (Brambilla, 2019). This is probably caused by the loss of astrocyte barrier function, which prevents uncontrolled immune cell entry into the brain and widespread damage of the surrounding parenchyma (Toft-Hansen, Fuchtbauer, & Owens, 2011). Conversely, astrocytes become harmful with the progression of disease pathogenesis. Astrocytic depletion during the chronic phase of the disease (at 30 days post demyelination) ameliorates EAE clinical outcome and is associated with reduced leucocyte infiltration into the CNS (Colombo & Farina, 2016). Accordingly, several studies showed that astrocytes act as antigen presenting cells and upregulate the T-cell co-stimulatory molecules B7-1 and B7-2 (Ulivieri et al., 2019) promoting encephalitogenic T-cell activation and thus contributing to neuro-inflammation. Importantly, astrocytes are themselves targets of infiltrating autoreactive T-cells. Indeed, via contact-independent mechanisms, infiltrating Th1 and Th17 cells induce the release of inflammatory mediators and repress the expression of anti-inflammatory cytokines in astrocytes (Prajeeth et al., 2017).

The detrimental action of astrocytes in the secondary disease phase is also associated to formation of the glial scar around the core of the demyelinated plaque, a physical barrier that prevents both OPCs and axons from entering demyelinated plaques, thereby inhibiting remyelination (Tanaka & Yoshida, 2014). Consistent with this, in vitro studies showed that reactive astrocytes inhibit myelination of dorsal root ganglion (DRG) axons by mature OLs (Domingues et al., 2016) through the release of several factors such as PDGF and FGF2, tenascin C, bone morphogenic proteins (BPM) 2/4 and hyaluronan. Among them, PDGF and FGF2 are both potent mitogens for OPCs and inhibit premature OL differentiation, as observed in focal demyelinating lesions induced by lysolecithin or cuprizone (Tanaka & Yoshida, 2014). On the contrary, hyaluronan is a glycosaminoglycan that binds CD44, a receptor expressed by OPCs, and blocks OPC differentiation both in cultures and in the lysolecithin mouse model. This factor, indeed, accumulated in chronic MS or EAE demyelinated lesions (Back et al., 2005).

Astrocyte-derived endothelin-1 (ET-1) is another negative regulator of OPC differentiation and remyelination because it promotes Jagged1 expression and the Notch pathway activation in OPCs (Hammond et al., 2014a).
Of note, the cytokine TNF has been found highly upregulated in astrocytes present in MS active lesions, both acute and chronic. TNF has been attributed both detrimental and beneficial roles in the CNS. In particular the soluble form of TNF (solTNF), differently from the transmembrane form (tmTNF), is responsible for pro-inflammatory and pro-apoptotic processes via activation of TNFR1 (Brambilla et al., 2011), and it damages OLs in MS by initiating the process of necroptosis (Pasparakis & Vandenabeele, 2015).

On the other hand, other studies showed protective effects of astrocyte on remyelination by suppressing T-cell and supporting OL functions. Indeed, astrocytes promote surface upregulation of inhibitory molecules on T cells, including the inhibitory receptor CTLA-4 and the ectonucleotidases CD39 and CD73, promoting an immunosuppressive program in T-cells (Filipello et al., 2016). Additionally, reactive astrocytes at the edge of active MS lesions express chemoattractant molecules for OPCs, including CXCL8, CXCL1 and CXCL10, which induce migration of OPCs to the lesion site (Omari, John, Sealfon, & Raine, 2005). Accordingly, Franklin and colleagues showed that in vivo transplantation of astrocytes enhance OL remyelination and increase the thickness of myelin sheaths (R. J. Franklin, Crang, & Blakemore, 1991).

In cuprizone-induced demyelination, TNFR2 activation by tmTNF in astrocytes has been shown to induce autocrine expression of CXCL12 that promotes OPC proliferation and differentiation. Together with tmTNF, other soluble factors have been implicated in enhancing myelination by astrocytes, such as IL-10, leukemia inhibitory factor-like protein (LIF), neuregulin-1, gamma-secretase, CNTF, IGF-1, osteopontin and neurotrophin-3 (NT3). Specifically, CNTF has been identified within reactive astrocytes in and around spinal cord remyelinating lesions and regulates FGF-2 production by astrocytes during early remyelination, suggesting that it is an important survival factor for OLs (Albrecht, Enterline, Cromer, & Levison, 2007). Furthermore, cocultures of astrocytes and OLs revealed that the physical contact between these cells promotes the maturation of OLs and myelination where axons are present (Iacobas & Iacobas, 2010; Sorensen, Moffat, Thomson, & Barnett, 2008). Given that astrocytes express connexins 43 (Cx43) and Cx30 while OLs express Cx32 and Cx47, these cells can establish heterotypic gap junctions composed of Cx47-Cx43 or, to a lesser extent, Cx32-Cx30 (Orthmann-Murphy, Abrams, & Scherer, 2008). Gap-junction elimination delays myelination in mice and favors disease progression in EAE animals, revealing a role of connexins in myelin maintenance (Markoullis et al., 2014). Through connexins, astrocytes also support OLs metabolically; they transfer cytosolic contents to OLs and buffer the concentration of K+ accumulating in the white matter during neurotransmission (Nagy & Rash, 2000).
Overall, these studies suggest that the functions of OPCs and the (re)myelination process depend on the surrounding microenvironment, which differently influences the activation state of astrocytes. A key factor controlling the impact of astrocytes on oligodendrocytes is represented by the distance of astrocytes from the lesion site. It has been hypothesized that distal astrocytes contribute to regeneration, while astrocytes in proximity of the lesion are harmful and impair remyelination (Nash, Ioannidou, & Barnett, 2011).

![Fig.1.10 Dual role of astrocytes in multiple sclerosis (MS). Astrocytes interact with neurons by secreting neurotoxic factors. Microglia/macrophage recruitment and T lymphocyte activation are induced by astrocytes as well. Furthermore, astrocytes are able to inhibit oligodendrocyte precursor cell (OPC) recruitment and differentiation (left side of the scheme). On the other hand, astrocyte release neurotrophic factors for neurons, suppress T-cell and immune cells activation and promote both OPC migration and maturation (right side of the scheme). Modified from (Allnoch, Baumgartner, & Hansmann, 2019).](image-url)
1.6 Extracellular Vesicles

Intercellular communication is a key process for the functioning and regulation of all biological processes. In addition to direct cell-to-cell communication and release of soluble molecules, extracellular vesicles recently emerged a fundamental mechanism of intercellular communication. Extracellular vesicles (EVs) are circular membrane structures released by all cell types (e.g. fibroblast, epithelial, hematopoietic, immune, tumor and stem cells, neurons, microglia, astrocytes, oligodendrocytes and neural progenitors) in both physiological and pathological conditions (Colombo, Borgiani, Verderio, & Furlan, 2012; Turola, Furlan, Bianco, Matteoli, & Verderio, 2012). Two main types of EVs have been identified based on size, content and mechanism of formation (Raposo & Stoorvogel, 2013): exosomes and ectosomes. Exosomes originate from multivesicular endosomal cell compartment and represent a homogeneous population in size (from 30 to 150 nm in diameter), enriched in specific components such as tetraspanning proteins, CD63, CD19 and Alix (Barreca, Aliotta, & Geraci, 2017). On the contrary, ectosomes, also called microvesicles (MVs) or shed vesicles, are larger vesicles (from 0.3 to 1 µm in diameter), which bud directly from the plasma membrane and are released into the extracellular space upon cell activation (Cocucci, Racchetti, & Meldolesi, 2009). Due to their heterogeneous composition, no universal markers have been yet identified to define MVs. Given the difficulties in partitioning EVs into microvesicles (MVs) and exosomes without cross-contamination, EVs are now preferentially classified in small (< 100-200 nm in size) and medium-large EVs (> 200 nm) and on the basis of other physical characteristics (density, biochemical composition) (Thery et al., 2018). Furthermore, another type of membrane vesicles are apoptotic bodies, that are larger than exosomes and membrane vesicles (1-5 µm in size) and are formed exclusively during cell apoptosis (Gyorgy et al., 2011). Currently, the generic term EV is used to refer to all secreted vesicles (Gould & Raposo, 2013).

Upon release, EVs may either remain in the extracellular space adjacent to the site of origin, or move by diffusion and enter into biological fluids, such as blood, urine, milk, CSF and synovial fluid, controlling biological processes also at significant distance from their site of origin. EVs contain a variety of cell surface receptors, intracellular signalling proteins and genetic materials (DNA, RNA, long non-coding RNA, miRNA) derived from the originating cells. Despite their cargo commonly reflects the identity of the cells from which they were released from, EVs may also be enriched in some molecules not present in donor cells (C. C. Li et al., 2013).

Importantly, EVs are able to specifically interact with target cells and can transfer their luminal cargo to recipient cells, thereby strongly influencing their phenotype. Several types of interactions between EVs and target cells have been described. EV can fuse with the plasma membrane of recipient cells or be taken up by endocytosis. Alternatively, EVs can undergo rupture and release their luminal active
components, modulating the activity of target cells by protein secretion (Deregibus et al., 2007; Valadi et al., 2007).

**Fig.1.11 Extracellular vesicles (EVs).** EVs are circular membrane structures released by all cell types. Two distinct type of vesicles have been described: Exosomes, which are generated inside multivesicular bodies (MVBs) and are released upon fusion of MVBs with plasma membrane, and Ectosomes, also known as shed vesicles, which bud directly at the cell surface. Both vesicle population contain a variety of cell surface receptors, intracellular signalling proteins and genetic materials derived from donor cells and, by transferring these components to target cells, EVs strongly influence the phenotype of the recipient cells. Image taken from (Garikipati, Shoja-Taheri, Davis, & Kishore, 2018).

Recent studies showed that EVs are involved in both physiological and pathophysiological processes controlling coagulation, cell proliferation and differentiation, inflammation, tumorigenesis and stem cell biology (Kim et al., 2005; Muralidharan-Chari, Clancy, Sedgwick, & D'Souza-Schorey, 2010; Robbins & Morelli, 2014).

In the brain, EVs are released by neurons and glial cells, and are involved in fundamental physiological processes, such as neuronal development (Marzesco et al., 2005), synaptic activity (Antonucci et al., 2012; Gabrielli et al., 2015), nerve regeneration (Lai & Breakefield, 2012) and myelin formation (Greening, Xu, Gopal, Rai, & Simpson, 2017; A. D. Pusic & Kraig, 2014; K. M. Pusic, Pusic, & Kraig, 2016; van Niel, D'Angelo, & Raposo, 2018).
In particular, EVs contribute to the crucial role of glial cells in the maintenance of CNS homeostasis. Specifically, EVs released by OLs possess myelin proteins and RNAs specific for the promotion of myelination not only during development but also during brain damage (Fruhbeis et al., 2013). Studies also showed that OLs-derived EVs are released close to the axonal surface in response to glutamate, where they are able to increase neuronal firing rate and influence the expression of neuronal genes associated to synaptic plasticity, such as VEGF and BDNF (Frohlich et al., 2014). Similarly, MG-derived EVs, produced in response to the danger signal ATP, increase the presynaptic release of glutamate by stimulating neuronal sphingolipid metabolism (Antonucci et al., 2012; Riganti et al., 2016). In addition, MG-derived EVs carry the active endocannabinoid N-arachidonoyl-ethanolamine (AEA), which inhibits inhibitory transmission via stimulation of type-1 cannabinoid receptors (CB1) and, target inhibitory neurons. MG EVs may also sustain synaptic activity by providing neurons with glycolytic enzymes and substrates involved in energy metabolism (Drago et al., 2017; Potolicchio et al., 2005). Thus, glial EVs influence both excitatory and inhibitory neurotransmission, providing a regulatory feedback on presynaptic activity (Holm, Kaiser, & Schwab, 2018).

1.6.1 EVs as biomarkers in Multiple sclerosis

Due to their universal presence in biofluids, including plasma and CSF (J. Lee et al., 2016), EVs have been recognized as potential biomarkers to monitor MS activity and response to treatments. Compared to the MS biomarkers described in Chapter 1, paragraph 1.1, EVs are easy to process at a low cost and hence their use could be extended to large study populations. In addition, EVs can provide information on inaccessible neural cells, such as neurons, astrocytes, oligodendrocytes and microglia, along with ependymal and leptomeningeal cells when searching the CSF, and brain endothelial cells when searching the blood. EV content can thus contribute to understand the pathogenesis of neurological diseases (Croese & Furlan, 2018).

Several independent studies reported increased levels of EVs in CSF and serum in MS patients compared to healthy donors and many authors have investigated the role of different types of EVs in MS pathogenesis and resolution (Carandini et al., 2015).

The team of my laboratory showed that the production of vesicles secreted by immune cells (MG and peripheral macrophages) dramatically increases in the CSF isolated from MS patients and EAE mice as compared to matched-age controls, especially in the acute phase of the disease. The number of EVs increases at disease onset and during relapses while decreases in the remission phase of disease, providing a strong correlation between CSF EV levels and demyelinating lesions in MS patients (Verderio et al., 2012). Accordingly, EAE ameliorates in transgenic mice impaired in EV production.
(acid-sphingomyelinase –aSMase- deficient mice, lacking the enzyme essential for EV shedding) (Verderio et al., 2012). Furthermore, as mentioned above, microglial MVs enhance transmission in excitatory neurons (Antonucci et al., 2012) and inhibit inhibitory transmission, thus possibly contributing to excitotoxicity phenomena that occur in MS (Centonze et al., 2009). Because CSF collection by lumbar puncture is an invasive procedure, most of the studies focused on a possible correlation between EV level in the peripheral blood and MS activity. Increased levels of EVs released by different components of the blood such as monocytes, leukocytes and platelets have been found in MS patients compared to healthy donors (Blonda, Amoruso, Martino, & Avolio, 2018). Minagar and colleagues demonstrated a correlation between the level of endothelial CD31 positive EVs (CD31+ EEVs) in the plasma and MS progression, suggesting that these vesicles may reflect endothelium inflammation and disruption of BBB integrity (Minagar et al., 2001; Minagar et al., 2003; Minagar, Maghzi, McGee, & Alexander, 2012). Along EEVs, platelet derived EVs (PEVs) were reported to be significantly increased in all MS clinical forms and to enhance endothelial permeability and leukocyte infiltration (Marcos-Ramiro et al., 2014). Other studies also highlighted the contribution of EVs released by the endothelial cells of the BBB, platelets or leukocytes to MS activity, in both inflammatory progression and lesion repair (Saenz-Cuesta et al., 2014). For instance, endothelial-derived EVs were shown to activate CD4+ and CD8+ T cells, which are involved in the autoimmune reaction of MS (Wheway, Latham, Combes, & Grau, 2014). These vesicles contain several metalloproteinases, which are able to induce BBB destruction (Lacroix et al., 2012), the cleavage and release of tmTNF and its receptors (TNFR-1 and TNFR-2) (Canault et al., 2007) and the migration of monocytes and lymphocytes into the brain, contributing to demyelination (Jy et al., 2004). Together with endothelial-derived MVs, platelet-derived MVs from MS patients are involved in BBB destruction given their capability to increase the permeability of endothelial layers in vitro by activation of P-selectin-integrin signalling (Marcos-Ramiro et al., 2014). Very recently, by the use of nanoplasmonic approach to quantify EVs it has been confirmed that the number of total plasmatic EVs significantly increase in clinical isolated syndrome (CIS) and MS patients compared to healthy controls (Mallardi, Nuzziello, Liguori, Avolio, & Palazzo, 2018). In recent years, large interest has been raised towards the content of EVs and its possible alterations in the disease. Galatzka and colleagues found that serum exosomes contain myelin proteins, including MOG, and that the content of MOG fragments strongly correlates with disease activity (Galazka, Mycko, Selmaj, Raine, & Selmaj, 2018). By contrast, serum exosomes isolated from pregnant mice were shown to directly support the maturation of oligodendrocyte precursors and facilitate their migration to active lesions in the CNS (J. L. Williams et al., 2013). This beneficial effect of serum exosomes has been related to two factors expressed in pregnancy-derived exosomes: leukemia
inhibitory factor receptor (LIFR) and ceruloplasmin. LIFR is a neurotropic factor of the IL-6 family able to completely rescue OL loss during EAE and favor myelin repair (Marriott et al., 2008). Ceruloplasmin is another protein that promotes OPC maturation by catalyzing the oxidation of ferrous iron, which is taken by oligodendrocytes and converted into ferric iron, essential for remyelination (Todorich, Pasquini, Garcia, Paez, & Connor, 2009).

Using Next Generation Sequencing (NGS), four circulating miRNA (hsa-miR-122-5p, hsa-miR-196b-5p, hsa-miR-301a-3p, and hsa-miR-532-5p) have been identified in serum exosomes, whose expression is reduced during relapses in RRMS patients compared to healthy donors (Selmaj, Cichalewska, et al., 2017). However, their role in MS is still unknown.

In addition, Ebrahimkhani et al. have identified other exosomal miRNAs, which are differentially expressed in the serum of both RRSM and progressive MS patients (miR-15b-5p, miR-23a-3p, miR-223-3p, miR-374a-5p, miR-30b-5p, miR-433-3p, miR-485-3p, miR-342-3p, miR-432-5p), suggesting that miRNAs represent a useful tool to discriminate the diverse disease stages and to monitor disease activity (Ebrahimkhani et al., 2017).

During EAE, the miRNA cargo of serum-derived exosomes, including miRNA-124, has been shown to control the activation of immune cells. Peripheral administration of miRNA-124 in EAE mice led to systemic deactivation of macrophages, reduced activation of myelin-specific T cells and caused marked suppression of disease activity. In addition, the intranasal administration of these exosomes induced an increase in myelination in the rat brains, an effect which might be mediated by miRNA-219. Accordingly, dendritic cell-derived exosomes were shown to contain high level of miRNA-219, which directly favors oligodendrocyte progenitor growth, increases the amounts of myelin, decreases oxidative stress, and promotes repair processes in demyelinated lesions (A. D. Pusic & Kraig, 2014).

Thus, during EAE exosomes not only suppress inflammation but may also promote myelin repair. EVs are also recognized as potential biomarkers of therapeutic efficacy in MS. Initially it was reported a reduction of endothelial MVs upon IFN-β1b administration (Sheremata et al., 2006), and a decrease of total plasma microparticles after treatment with IFN-β1a (Lowery-Nordberg et al., 2011). On the contrary, another study reported higher levels of lymphocyte-derived exosomes in the blood of patients under IFN-β and Natalizumab treatment, probably due to the block of lymphocytes transmigration through the BBB induced by the drugs (Saenz-Cuesta et al., 2014).

Verderio et al. demonstrated that EAE mice treated with Fingolimod displayed a reduction in CSF levels of myeloid MVs, indicating that Fingolimod might prevent MV shedding from reactive MG and inhibit the spreading of inflammation throughout the brain (Verderio et al., 2012).
Very recently, other MS treatments such as IFN-β1a and Teriflunomide have been reported to reduce the production of monocyte-derived MVs and their IL1β cargo in relation to the duration of the therapy (Blonda, Amoruso, Grasso, Di Francescantonio, & Avolio, 2017). Globally, these findings indicate that EVs and their cargo might influence the course of the demyelinating disease. However, it still remains tricky to understand the information carried by EVs. Available methodologies to measure and analyse EVs in biological fluids have some limitations such as the capability to separate the different types of EVs, to optimize cargo analysis and to identify disease-specificity of EV content.
CHAPTER II
AIM OF STUDY
Neuroinflammation plays a central role in multiple sclerosis (MS) by damaging myelin and causing axonal injury. Brain resident microglia (MG) are among the main effector cells of the inflammatory response associated to MS (Mallucci et al., 2015). However, besides detrimental functions, MG are believed to also have a beneficial role in MS, supporting remyelination and tissue repair (Miron et al., 2013). The ultimate fate of MS lesions seems to be regulated by the acquisition of distinct effector functions and polarization states by MG. However, the mode(s) of action of MG in fostering or inhibiting CNS repair are far from being elucidated.

A panel of markers of pro-regenerative or detrimental activation states of MG have been recently defined thanks to proteomics and transcriptomic approaches (Chiu et al., 2013). However, classifying the phenotypes of MG in vivo is still challenging, due to the intrinsic plastic properties of these cells and lack of antigenic markers able to distinguish MG from macrophages in the injured tissue. Murine models which allow selective manipulation of MG, i.e. inducible CX3CR1-creERT2 mice, have been recently developed (Goldmann et al., 2013). However, no methodologies are yet available to selectively deplete MG without causing massive increase in cytokines and astrogliosis (Bruttger et al., 2015). Hence, despite the important role of MG in MS, we still lack a full understanding of how and through which mechanism the cell types are involved in MS pathogenesis. This knowledge could be useful in identifying factors that contribute to the development of MS and other demyelinating disorders, and in designing effective therapeutic strategies to treat or prevent these diseases.

In past years the host lab has extensively characterized extracellular vesicles (EVs) released by MG and studied the role in neuro-inflammation. EVs are membrane vesicles of endosomal (exosomes) or plasma membrane (ectosomes/microvesicles) origin, which are released by most cell types. In the brain, EVs are produced by neurons and by glial cells (Raposo & Stoorvogel, 2013). By exposing cell-type specific adhesion receptors, EVs can interact with specific cells and deliver complex "signals", including proteins, lipids and RNA between cells (Prada et al., 2018). Several lines of evidence suggest a role for MG-derived EVs in MS and other inflammatory diseases. Among these are studies from the host laboratory showing that: i) EV production from myeloid cells increases at disease onset and during relapses in mice affected by EAE, reflecting disease activity; ii) mice impaired in EV secretion are highly resistant to EAE; iii) EV production from myeloid cells is more abundant in relapsing patients than patients at remission, and in CIS subjects as compared to sex and age-matched healthy controls; iv) the amount of myeloid EVs in the cerebrospinal fluid of MS patients correlates with the number of active lesions, reflecting disease severity; v) EVs derived from reactive cultured MG propagate an inflammatory reaction to neighbouring glial cells (Verderio et al., 2012); vi) MG-derived EVs cause an excitation/inhibition unbalance, likely participating in excitotoxicity phenomena associated to brain inflammation (Antonucci et al., 2012; Gabrielli et al.,
vii) Chronic exposure to MG-derived EVs induce a pathological loss of excitatory synapses (Prada et al., 2018).

On these bases, my PhD project aimed to study EV-dependent crosstalk between MG and oligodendrocyte precursors cells (OPCs), the glial cell type able to generate myelin-forming cells, under physiological condition or in response to myelin lesion.

Specifically, I pursued the following aims:

**AIM 1) To explore the action of EVs produced *in vitro* by inflammatory or pro-regenerative microglia in the remyelination process (Chapter IV, paragraph 4.3, 4.4).**

We investigated whether EVs released from inflammatory or pro-regenerative MG may promote OPC recruitment and remyelination in the lysolecithin (LPC) mouse model of focal demyelination.

In this model, a single injection of LPC into the mouse corpus callosum causes a myelin lesion that spontaneously recovers over time, offering a robust approach to study remyelination (Miron et al., 2013).

By immunofluorescence and electron microscopy (EM) analysis we examined the action of EVs produced by differently activated MG on the early response to demyelination as well on remyelination in both young (2.5-4 months old) and aged mice (8.5-12 months old), where remyelination process occurs more slowly (Shields et al., 1999). Saline-injected mice were used as control.

Data from this objective advanced our understanding of the mechanism by which inflammatory or pro-regenerative MG influence the inflammatory damage and the remyelination process in a model of focal demyelination, and suggested novel strategies to modulate the phenotype of MG in patients.

**AIM 2) To explore the effects of inflammatory or pro-regenerative MG-derived EVs on *in vitro* OPC migration, proliferation and myelination (Chapter IV, paragraph 4.5, 4.6).**

To investigate the molecular mechanisms through which MG-derived EVs exert harmful and protective action on remyelination, we took advantage of OPCs in primary culture, a powerful system that allows to dissect the effects directly mediated by EVs.

Specifically, we analyzed the impact of microglial EVs on OPC proliferation, migration, differentiation and myelin deposition.

These experiments enabled me to define the direct effects of EVs in MG-oligodendrocyte crosstalk and to compare the *in vitro* EV action to that induced in *in vivo*, in the LPC mouse model of focal myelin lesion.
AIM 3) To define the molecular mechanisms by which microglial EVs induce responses in OPCs (Chapter IV, paragraph 4.5, 4.6).

Understanding the molecular mechanisms by which EVs influence OPC behaviors by identifying the signals, which repress or promote OPC differentiation and myelination, will help finding new ways to modulate endogenous neuro-regenerative responses after demyelination. We thus carried out biochemical fractionation of EVs to investigate *in vitro* whether the effects of MG-derived EVs on OPC maturation are mediated by factor/s present at the surface or in the lumen of these organelles, and mainly depend on protein or lipid EV components. Furthermore, we tested the hypothesis that astrocytes may mediate the detrimental response of EVs released from inflammatory microglia, as suggested by the experiments carried out to explore AIM 1 and 2.

The results of these experiments increased our understanding of how microglia interact with astrocyte and myelin-forming cells and provided a possible mechanism responsible for remyelination failure in MS.
IMPORTANT NOTES
This thesis cites the following article, of which I am the first author:

**DETRIMENTAL AND PROTECTIVE ACTION OF MICROGLIAL EXTRACELLULAR VESICLES ON MYELIN LESIONS: ASTROCYTE INVOLVEMENT IN REMYELINATION FAILURE**

Marta Lombardi¹-², Roberta Parolisi³, Federica Scaroni², Elisabetta Bonfanti⁴, Alice Gualerzi⁵, Martina Gabrielli², Nicole Kerlero de Rosbo⁶, Antonio Uccelli⁶-⁷, Paola Giussani⁸, Paola Viani⁸, Cecilia Garlanda⁹, Maria P. Abbracchio⁴, Linda Chaabane¹⁰, Annalisa Buffo³, Marta Fumagalli⁴, Claudia Verderio²*

¹IRCCS Humanitas, via Manzoni 56, 20089 Rozzano, Italy.
²CNR Institute of Neuroscience, via Vanvitelli 32, 20129 Milan, Italy.
³Department of Neuroscience Rita Levi-Montalcini and Neuroscience Institute Cavaleri Ottolenghi, University of Turin, Regione Gonzole 10, 10043 Orbassano, Italy.
⁴Department of Excellence: Department of Pharmacological and Biomolecular Sciences (DiSFeB), University of Milan, via Balzaretti 9, 20133 Milan, Italy.
⁵IRCCS Fondazione Don Carlo Gnocchi, via Capecelatro 66, 20148 Milan, Italy.
⁶Department of Neurology, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health (DINOGMI), University of Genoa, Largo Paolo Daneo 3, 16132 Genoa, Italy.
⁷IRCCS Ospedale Policlinico San Martino, Largo Rosanna Benzi 10, 16132 Genoa, Italy.
⁸Department of Biotechnology and Translational Medicine, University of Milan, 20090 Segrate, Italy.
⁹Humanitas University, via Manzoni 56, 20089 Rozzano, Italy.
¹⁰Institute of Experimental Neurology (INSPE), San Raffaele Scientific Institute, Via Olgettina Milano 58, 20132 Milan, Italy.
CHAPTER III

MATERIAL & METHODS
3.1 Lysolecithin-induced myelin lesions

Demyelinating lesions were induced in the corpus callosum (CC) of adult (2.5-4 months old) and aged (8-12 months old) male C57BL/6 mice by stereotaxic unilateral injection of 1 μl of 2% lysolecithin the mouse corpus callosum (CC, coordinates: 1.0 mm lateral, 1.0 mm rostral to Bregma and 1.5 mm deep). Three days post lesion (dpl), 2x10^8 EVs, produced by 1.5x10^6 microglia and dissolved in 150 μl of sterile saline, were delivered to the mice with mini-pumps (Alzet osmotic pumps 1007D) at the same coordinates over 4 days at 1.5 μl/h delivery rate. To limit EV degradation, minipumps were filled with freshly isolated EVs and implanted within a few hours. Controls were obtained with delivery of saline solution. To examine cell proliferation, we used the thymidine analogue 5-bromo-2-deoxyuridine (BrdU), which is incorporated into DNA during S-phase of the cell cycle and remains in the DNA even when the cell has exited the active phases of the cells cycle. Mice were i.p. injected with BrdU (100 mg/kg body weight; 2 pulses 2h apart) one day from the start of EVs delivery. A separate group of mice received a single injection of 6.6x10^7 EVs, released from 0.5x10^6 microglia and dissolved in 1 μl of sterile saline, at the site of lesion at 7dpl. Controls were obtained by injecting saline. Mice were transcardially perfused with 4% paraformaldehyde in phosphate buffer (PB 0.1M, pH 7.4) at 4 days after mini-pump implantation or at 3 days after acute injection and brains were post-fixed overnight and cryoprotected in 30% sucrose in 0.12 M PB. Surgery and perfusion were carried out under deep general anaesthesia (ketamine, 100 mg/kg, Ketavet, Bayern, Leverkusen, Germany; xylazine, 5 mg/kg, Rompun, Bayer, Leverkusen, Germany). All animal procedures were performed in accordance with the European directive (2010/63/EU) and the Italian Law for Care and Use of Experimental Animals (DL116/92; DL26/2014), and the studies were authorized by the Italian Ministry of Health (Authorization: 1112-2016PR) and the Bioethical Committee of the University of Turin.

3.2 Electron microscopy of myelin lesions

Conventional electron microscopy was carried out as in (Bertero et al., 2018). LPC-lesioned old mice injected with saline, i-EVs or MSC-EVs were perfused transcardially with PB followed by 2% paraformaldehyde and 2.5% glutaraldehyde in PB. The brain was removed and post-fixed overnight at 4°C in the same fixative. Vibratome sagittal sections (250 μm thick) were cut, and post-fixed with 1% osmium tetroxide for 1h at 4°C, then stained with uranyl acetate replacement stain (Electron Microscopy Sciences, USA). After dehydration in ethanol, samples were cleared in propylene oxide and embedded in Araldite (Fluka, Saint Louis, USA). Semithin sections (1 μm thick) were obtained at the ultramicrotome (Ultracut UCT, Leica, Wetzlar, Germany), stained with 1% toluidine blue and 2% borate in distilled water and then observed under a light microscope for precise lesion location.
Ultrathin sections (70-100 nm) were examined under a transmission electron microscope (JEOL, JEM-1010, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany) for computerized acquisition of the images. Profiles of microglia, astrocytes, oligodendrocytes and myelinated axons were identified according to well established criteria (Quigley, 2017). Microglia were distinguished by their specific ultrastructural features including frequent stretches of endoplasmic reticulum and a condensed, electron-dense cytoplasm (Bisht, Sharma, Lacoste, & Tremblay, 2016; Tremblay, Lowery, & Majewska, 2010). In the core of the lesion, electron micrographs were taken at 25K magnification and ImageJ software (https://imagej.nih.gov/ij/) was used to measure the proportion of myelinated axons (on at least 150 axons/animal) and perform the measurements needed to obtain G-ratios, the ratios of the axonal diameter (d white, Fig.4.4k) to the outer fiber diameter including the myelin sheath (D yellow, Fig.4.4k), of at least 50 myelinated axons/animal. Quantifications were performed on three mice (8-12 months old) per experimental condition.

### 3.3 Immunohistochemistry

Brains were processed according to standard immunohistochemical procedures (Boda et al., 2015). They were cut into 30-µm-thick coronal sections, collected in PBS and stained with the antibodies reported in the table I. OPCs were labelled by anti-NG2 antibodies while more mature cells were detected by anti-CC1 serum. Anti-Olig2 and anti-Sox10 stained the whole oligodendrocyte lineage. Anti-MBP antibodies labelled myelin. To follow cell proliferation, we used anti-BrdU. DAPI or Hoechst33258 were used to label nuclei. Anti-GFAP, anti-PTX3 and anti-C3 were used to label astrocytes, Iba-1 to reveal microglia. Sections were incubated overnight with primary antibodies at 4°C in PBS with 1.5% donkey serum, 2% bovine serum albumin and 0.5% Triton X-100. Sections were then exposed for 2 h at room temperature to secondary anti-rabbit, anti-chicken antibodies conjugated with Alexa Fluor 488 or anti-rabbit antibodies conjugated with Alexa Fluor 546 (1:500; Invitrogen, Waltham, MA USA), anti-mouse, anti-rabbit antibodies conjugated with Cy3 (1:500; Jackson ImmunoResearch, West Grove, PA, USA), anti-goat antibodies conjugated with Alexa Fluor 649 (1:500; Invitrogen) or anti-mouse antibodies conjugated with Cy5 (1:500; Jackson ImmunoResearch). Double staining with rabbit anti-GFAP and rabbit anti-PTX3/anti-C3 was performed using the High Sensitivity Tyramide-Rhodamine Signal Amplification kit (Perkin-Elmer, Monza, Italy) following the manufacturer’s instructions. Following counterstaining with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Co., St. Louis, MO, United States) or Hoechst 33258 (Life Technologies, Monza, Italy) to label nuclei, slides were coverslipped with Mowiol (Millipore, Burlington, MA, USA) or with a fluorescent mounting medium from Dako (Milan, Italy).
All images were collected using a Nikon Eclipse 90i confocal microscope (Nikon, Melville, NY), or using a Zeiss LSM 800 confocal microscope (Carl Zeiss S.p.A, Milano, Italy). Adobe Photoshop 6.0 (Adobe Systems) was used to adjust image contrast and assemble the final plates. Measurements were derived from at least three sections per mice. Three to five animals were analyzed per each time point or experimental condition. Image analysis was performed with the Image J software to quantify the proportion of NG2 or MBP-stained pixels throughout the entire lesioned area in each section (Kalakh & Mouihate, 2017; Kucharova & Stallcup, 2017; Miron et al., 2013). Density of oligodendroglial, proliferative cells and PTX3- and C3-positive astrocytes in the lesion was calculated as number of cells/mm², using the ImageJ software.

Table I: List of primary antibodies used for immunohistochemistry (IHC), immunofluorescence (ICC) and western-blot (WB) analyses.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Supplier</th>
<th>IHC dilution</th>
<th>ICC dilution</th>
<th>WB dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-NG2</td>
<td>Rabbit</td>
<td>Millipore (Burlington, MA, USA)</td>
<td>1:200</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>anti-CC1</td>
<td>Mouse</td>
<td>Millipore (Burlington, MA, USA)</td>
<td>1:1500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CNPase</td>
<td>Rabbit</td>
<td>SantaCruz (Dallas, Texas)</td>
<td></td>
<td>1:250</td>
<td></td>
</tr>
<tr>
<td>anti-Olig2</td>
<td>Rabbit</td>
<td>Millipore (Burlington, MA, USA)</td>
<td>1:500</td>
<td>1:300</td>
<td></td>
</tr>
<tr>
<td>anti-SOX-10</td>
<td>Goat</td>
<td>SantaCruz (Dallas, Texas)</td>
<td>1:200</td>
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<td></td>
</tr>
<tr>
<td>anti-MBP</td>
<td>Rat</td>
<td>AbD Serotec (Oxford, UK)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>anti-MBP</td>
<td>Rat</td>
<td>Covance (Princeton, NJ)</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-MBP</td>
<td>Rat</td>
<td>Millipore (Burlington, MA, USA)</td>
<td></td>
<td>1:200</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-BrdU</td>
<td>Rat</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-pentraxin3</td>
<td>Rabbit</td>
<td>home-made generated as previously described, (Bonavita et al., 2015)</td>
<td>1:250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-C3</td>
<td>Rabbit</td>
<td>Dako (Milan, Italy)</td>
<td>1:250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>Rabbit</td>
<td>Dako (Milan, Italy)</td>
<td>1:30000</td>
<td>with amplification kit</td>
<td></td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>Mouse</td>
<td>Sigma-Aldrich (Milan, Italy)</td>
<td>1:100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Neurofilaments (SMI32)</td>
<td>mouse</td>
<td>Cell Signalling (Beverly, MA)</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4 Magnetic Resonance Imaging (MRI)

In vivo MRI was performed using a 7 Tesla scanner (Bruker-Biospin) equipped with a radiofrequency coil for mouse. Mice were anesthetized with an intramuscular injection of Ketamine (100 mg/kg; Ketavet, Bayern, Leverkusen, Germany) mixed with xylazine (5 mg/kg; Rompun, Bayer, Leverkusen, Germany). High Resolution coronal and sagittal images, T2 weighted (TR/TE=3000/48ms, slice thickness=0.7 mm, pixel=78x80 µm) were acquired to localize lesions in the corpus callosum.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-neurofilaments</td>
<td>Mouse</td>
<td>Cell Signalling (Beverly, MA)</td>
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<tr>
<td>Anti-α-tub</td>
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<td>Sigma-Aldrich</td>
<td>1:2000</td>
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<tr>
<td>Anti-GSTpi</td>
<td>Rabbit</td>
<td>MBL International Corporation (Sunnyvale, CA)</td>
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</tr>
<tr>
<td>Anti-GPR17</td>
<td>Rabbit</td>
<td>home-made generated as previously described, Ciana 2006</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-GPR17</td>
<td>Rabbit</td>
<td>Cayman Chemical (Michigan, USA)</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>Chicken</td>
<td>AVES Labs</td>
<td>1:700 1:1400</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>Rabbit</td>
<td>Life Technologies (Monza, Italy)</td>
<td>1:3000</td>
</tr>
<tr>
<td>Anti-Iba1</td>
<td>Rabbit</td>
<td>Wako Chemicals (Richmond, VA)</td>
<td>1:1000</td>
</tr>
<tr>
<td>4′,6-diamidino-2-phenylindole DAPI</td>
<td></td>
<td>Fluka (Buche, Switzerland)</td>
<td>1:1000</td>
</tr>
<tr>
<td>DAPI</td>
<td></td>
<td>Molecular Probes (Life Technologies, Monza, Italy)</td>
<td>1:20.00</td>
</tr>
<tr>
<td>Hoechst 33258</td>
<td></td>
<td>Life Technologies (Monza, Italy)</td>
<td>1:50000 1:10000</td>
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<tr>
<td>Anti-Alix</td>
<td>Rabbit</td>
<td>Covalab (Villeurbanne, France)</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-Flotilin 2</td>
<td>Mouse</td>
<td>BD transduction (San Jose, CA)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-GAPDH</td>
<td>Rabbit</td>
<td>Synaptic Systems (Gottingen Germany)</td>
<td>1:1000</td>
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<tr>
<td>Anti-GS28</td>
<td>Mouse</td>
<td>BD transduction (San Jose, CA)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Diffusion Tensor Imaging (DTI: 30 directions, b=1000 sec/mm²) was also used to measure water molecule diffusivity across white matter fibers to further characterize CC lesions.

3.5 Primary cultures

Mixed glial cell cultures, containing both astrocytes and microglia, were established from rat Sprague–Dawley pups (P2) (Charles River, Lecco, Italy) and maintained for 10 days in the presence of South American fetal bovine serum (Life Technologies, Monza, Italy) that optimizes microglia expansion or fetal bovine serum (EuroClone, Milan, Italy) which favors OPC proliferation.

**Microglia.** Microglia were harvested by orbital shaking for 40 min at 1,300 r.p.m. and re-plated on poly-L-ornithine-coated tissue culture dishes (50 μg/mL, Sigma-Aldrich, Milan, Italy). To minimize the activation, pure microglia (> 98%, (Prada et al., 2018)) were kept for 24 hrs in low-serum (1%) medium. Cells were then stimulated with a cocktail of Th1 cytokines, i.e. 20 ng/ml IL-1β (Peprotech, Milan, Italy), 20 ng/ml TNF (Peprotech, Milan, Italy) and 25 ng/mL IFN-γ (Sigma Aldrich, Milan, Italy), or with 20 ng/ml IL-4 for 48 hrs (R&D, Milan, Italy). In addition, microglia were co-cultured in transwell with MSCs at a microglia-to-MSCs ratio of 1:1 for 48 hrs in the presence of Th1 cytokines. MSCs were plated on the filter of the upper chamber and microglia in the lower chamber of the transwell. At the end of treatment, MSCs were removed, microglia were washed and stimulated with ATP to increase EV production (Prada et al., 2018). To obtain GFP-labelled EVs, microglia were established from GFP-transgenic rats expressing GFP under the chicken beta actin promoter (Okabe, Ikawa, Kominami, Nakanishi, & Nishimune, 1997). Indeed, GFP is included in EVs released by GFP-positive microglia (Suppl. Fig.5.3h).

**OPCs.** After microglia removal, OPCs growing on top of astrocyte monolayer were isolated by shaking cells on an orbital shaker at 200 rpm for 3h and incubated on an uncoated Petri dish for 1hr to further eliminate microglia. Pure OPCs (> 95% (Fumagalli et al., 2011)) were seeded onto poly-D,L-ornithine-coated glass coverslips or plates (50 μg/mL, Sigma-Aldrich, Milan, Italy) in Neurobasal (Life Technologies, Monza, Italy) supplemented with 2% B27 (Life Technologies, Monza, Italy), 2 mM L-glutamine (EuroClone, Milan, Italy), 10 ng/mL human platelet-derived growth factor BB (Sigma-Aldrich, Milan, Italy), and 10 ng/mL human basic fibroblast growth factor (Space Import Export, Milan, Italy), to promote proliferation (proliferating medium). After 3 days, cells were either detached with accutase (Millipore, Burlington, MA, USA) and used for migration assay, or switched to a Neurobasal medium lacking growth factors and supplemented with triiodothyronine T3 (10 ng/ml, Sigma Aldrich, Milan, Italy) to allow differentiation (differentiating medium).
Dorsal root ganglion (DRG)-OPC co-cultures. DRG-OPC co-cultures were prepared according to a previously described protocol (Fumagalli et al., 2015). Briefly, DRG from E14.5 mouse embryos were plucked off from spinal cord, put in culture (1 DRG/coverslip) in Neurobasal supplemented with B27 in the presence of nerve growth factor (NGF) (100 ng/ml; Harlan, Milan, Italy) and cycled with fluorodeoxyuridine (10 μM; Sigma Aldrich, Milan, Italy) to eliminate all non-neuronal cells. After 20 days, when neurites were well extended radially from DRG explants, 35x10^3 OPCs were added to each DRG in culture and kept in Minimum essential media MEM (Life Technologies Monza, Italy) supplemented with glucose (4 g/L; Sigma Aldrich, Milan, Italy), 10% FBS and 2 mM L-glutamine (EuroClone, Milan, Italy). Myelination was induced the following day by the addition of recombinant chimeric tyrosine kinase receptor TrkA Fc (1 μg/ml; Sigma Aldrich, Milan, Italy) to the culture medium.

Astrocyte-OPC co-cultures. Purified astrocytes were isolated from P2 rat whole brains by magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-GLAST (ACSA 1) MicroBeads according to the manufacturer's instructions. After 1 week, OPCs were plated on top of astrocytes at an astrocyte-to-OPC ratio of 1:1. In a set of experiments astrocytes were isolated from P2 mouse whole brains and cultured alone for qPCR analysis of specific markers for A1 and A2 astrocytes.

MSCs. MSCs were prepared and expanded as described previously (Zappia et al., 2005). Briefly, marrow cells were flushed out from tibias and femurs of 6-8-week-old C57BL/6J mice and cultured in plastic plates as adherent cells using murine Mesencult as medium (Stem Cell Technologies, Vancouver, BC, Canada). Medium was refreshed every 3 days until cells reached 80% confluence. Following treatment with 0.05% trypsin solution containing 0.02% EDTA (Euroclone, Milan, Italy), the cells were plated in 75 cm^2 flask at the density of 4x10^5 cells. Mature MSCs, obtained after four to five passages in culture, were defined by the expression of CD9, Sca-1, CD73, and CD44 and the lack of the hematopoietic markers CD45, CD34, and CD11b on their surface, and their immunosuppressive activity was verified in T-cell proliferation assays (Zappia et al., 2005).

3.6 RNA isolation and qRT-PCR
Total RNA was isolated from rat primary microglia using Direct-zol™ RNA MiniPrep (Zymo Research, Irvine CA, USA) following the manufacturer’s protocol. cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City CA, USA) and Random Hexamers as primer. The resulting cDNAs were amplified using TaqMan® Gene Expression Assay (Applied Biosystems, Foster City CA, USA) using QuantStudio™5 (ThermoFisher Scientific, Waltham MA, USA) real-time PCR system. The mRNA expression was normalized to the
label of Rpl13 (Ribosomal Protein L13) mRNA. Data obtained were quantified using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Q-PCR for A1 and A2 markers was performed on murine astrocytes with the Luna Universal Probe qPCR Master Mix (M3004S, New England Biolabs) using the StepOne™ Plus Real-Time PCR System (Life Technologies, Monza, Italy). The expression of selected genes was normalized to the expression of the housekeeping gene β-actin. The list of primers used can be found in Table II.

Table II: List of gene expression assays for qPCR.

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<tr>
<th>Gene Symbol</th>
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<tr>
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<td>Complement C1q</td>
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<td>Nitric Oxide Synthase 2</td>
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<tr>
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<td>Ribosomal Protein L13</td>
<td>Rn00821946_g1</td>
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<tr>
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<td>Suppressor Of Cytokine Signalling 3</td>
<td>Rn00585674_s1</td>
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3.7 EV isolation and quantification

EVs were isolated from microglia exposed to Th1 cytokine cocktail (i-EVs), IL-4 (IL4-EVs) or MSC (MSC-EVs). To isolate EVs, polarized microglia were stimulated with 1 mM ATP for 30 min in KRH (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO, 2 mM CaCl2, 6 mM D-glucose, and 25 mM HEPES/NaOH, pH 7.4). The culture supernatant was collected and released EVs were pelleted at 100k g after pre-clearing from cells and debris at 300 g, as previously described (Gabrielli et al., 2015). In some experiments, an ectosome-enriched fraction was pelleted at 10k g. EV pellets were resuspended and used immediately after isolation. The number and dimension of EVs were
assessed using NanoSight NS300 (NanoSight, UK) configured with a 488 nm laser and SCMOS camera. Videos were collected and analyzed using the NTA-software (version 2.3), with the minimal expected particle size, minimum track length, and blue setting, all set to automatic. Camera shutter speed was fixed at 15 ms and camera gain was set to 300. Room temperature was ranging from 25°C to 28°C. EV pellets were re-suspended in 800 μl of 0.1 μm-filtered sterile KRH and four recordings of 30 seconds were performed for each sample.

EVs were destroyed by hypo-osmotic stress and re-pelleted to remove their luminal cargo as previously described (Gabrielli et al., 2015). For biochemical fractionation of EVs, total lipids were extracted with 2:1 (by volume) of chloroform and methanol. The lipid fraction was evaporated under a nitrogen stream, dried for 1h at 50°C and resuspended in PBS at 40°C in order to obtain multilamellar vesicles. Small unilamellar vesicles were obtained by sonicating multilamellar vesicles, following the procedure of (Barenholz et al., 1977).

3.8 Quantification of sphingolipid content in EVs

[3H] Sphingosine ([3H]Sph) was stocked in absolute ethanol. For the quantification of sphingolipid content in EVs, 1x10^6 microglia were pulsed with [3H]Sph (0.3 μCi/ml) for 24hrs followed by a 48hrs chase in order to obtain a [3H]GM3/[3H]SM ratio corresponding to that of endogenous compounds (data not shown), an index for a steady-state labelling of cell sphingolipids (Viani et al., 2003). At the end of the chase, microglia were stimulated with ATP, EVs were pelleted and the cells were washed twice with PBS at 4°C and harvested. Total lipids were extracted from both EVs and cells and processed as previously described (Riboni, Viani, Bassi, Giussani, & Tettamanti, 2000; Riboni, Viani, & Tettamanti, 2000). The organic phase and the aqueous phase were analyzed by high performance thin layer chromatography (HPTLC) using chloroform/methanol/water (55:20:3 by volume) and butanol/acetic acid/water (3:1:1 by volume) as the solvent systems.

3.9 Raman Spectroscopy

The Raman analysis was performed following a previously described protocol (Gualerzi et al., 2017). Briefly, freshly isolated EVs were laid on a calcium fluoride slide and allowed to air dry. All of the measurements were performed with a Raman microspectroscope (LabRAM Aramis, Horiba Jobin Yvon S.A.S, Lille, France) equipped with a laser line operating at 532 nm and a Peltier-cooled CCD detector. Acquisitions were performed with 50X objective (NA 0.75, Olympus, Tokyo, Japan), 1800 grooves/mm diffraction grating, 400 μm entrance slit, and confocal mode (600 μm pinhole) in the spectral ranges 600–1800 cm\(^{-1}\) and 2600–3200 cm\(^{-1}\). Accumulation times were 2x30s per spectrum. Silicon reference sample was used for the instrument calibration. At least 30 independent replicates
of the Raman spectra were obtained for every EV type. After acquisition, polynomial baseline correction and unit vector normalization were performed before the multivariate statistical analysis.

3.10 OPC migration assay
Migration of OPCs was performed in Boyden chambers (8 μm pore size filter; Constar, Corning, NY, USA) as previously described (Bonfanti et al., 2017). Briefly, the chamber was nested inside the well of 24-well plates and 5x10⁴ OPCs were seeded in the top of each insert with 200 μl of neurobasal medium. The bottom well was filled with 600 μl of medium containing EVs released from 1x10⁵ microglia (~4x10⁸ EVs/ml). The chemotactic agent sphingosine-1 phosphate (S1P) (100 nM) was used as positive control. After 16h, non-migrated cells were removed from the top compartment with a cotton swab, whereas cells that had migrated to the lower side of the filter were fixed with 4% paraformaldehyde and stained with Hoechst33258 (Life Technologies, Monza, Italy). Images were acquired at 20X magnification under an inverted fluorescence microscope (200M; Zeiss, Oberkochen, Germany) and cells were counted using ImageJ cell counter plugin in 45 random fields per well. All conditions were run in triplicate. Data are expressed as a percentage of basal migration, that is the migration of OPCs without chemoattractant.

3.11 EV delivery by optical manipulation
An IR laser beam (1064 nm, CW) for trapping was coupled into the optical path of an inverted microscope (Axiovert 200M, Zeiss, Oberkochen, Germany) through the right port of the microscope. The trapping beam was directed to the microscope lens (Zeiss 63X, NA 1.4) by the corresponding port mirror (100%) and the tube lens. Optical trapping and manipulation of EVs was performed following the approach previously described (Prada et al., 2016). Immediately before recording, EVs were added in the temperature-controlled recording chamber, where OPCs plated on glass coverslips were maintained in 400 μl of Neurobasal medium with B27. As soon as an EV appeared in the recording field, it was trapped and positioned on a selected OPC by moving the cell stage horizontally and the microscope lens axially. After about 30s from initial contact, the laser was switched off to prove EV-OPC interaction, as previously described (Prada et al., 2016). During the experiments, OPCs were live-imaged with a spinning disk confocal microscope (UltraVIEW acquisition system, Perkin Elmer Waltham, MA, United States) using a digital camera (High Sensitivity USB 3.0 CMOS Camera 1280X1024 Global Shutter Monochrome Sensor, Thorlabs, Newton, NJ, United States) at a frame rate of 2 Hz.
3.12 OPC proliferation assay
One day after plating on glass coverslips, OPCs were co-exposed to EVs derived from twice as many microglia (~2x10^7 EVs/500 µl) and to the thymidine analog EdU (Click-iT® EdU Assay, Life Technologies, Monza, Italy) for 24hrs in proliferating medium. Cells were fixed with 4% paraformaldehyde and stained for EdU following the manufacturer’s instructions. Coverslips were then incubated with DAPI (1:20000, Molecular Probes, Life Technologies) to reveal total nuclei and mounted with a fluorescent mounting medium (Dako, Milan, Italy). 40-50 fields per coverslip were imaged at 20X magnification under an inverted fluorescence microscope (200M Zeiss, Oberkochen, Germany) connected to a PC computer equipped with the Axiovision software (Zeiss). OPC proliferation was assessed by quantifying EdU-DAPI double positive nuclei in at least three coverslips/condition, using ImageJ cell counter plugin. Cells with nuclei larger than 15 microns, belonging to astrocytes occasionally present in the cultures, were excluded from the analysis.

3.13 OPC differentiation assay
OPCs were kept for 3 days in proliferating medium, shifted to differentiating medium for 24hrs and then exposed to EVs for 48hrs (2x10^7 EVs/500 µl). Cells were fixed and labelled with anti-G Protein-Coupled Receptor 17 (GPR17) and anti-MBP (table I) in Goat Serum Dilution Buffer (GSDB; 450 mM NaCl, 20 mM sodium phosphate buffer, pH 7.4, 15% goat serum, 0.3% Triton X-100), followed by secondary antibodies conjugated to Alexa Fluor 555 or Alexa Fluor 488 (1:200; Molecular Probes, Life Technologies). Differentiation towards mature oligodendrocytes was determined by counting MBP+ cells over total DAPI+ cells in 35-45 fields per coverslip with ImageJ software. GPR17 staining was used to reveal immature oligodendrocytes, the most abundant oligodendrocyte population after 3 day in differentiating medium.

3.14 OPC myelination assay
OPC-DRG co-cultures were kept in 1 µg/ml TrkA-Fc for 5 days and then exposed to EVs for 11 days (fresh EVs, 2x10^7 EVs/500 µl, were added at day in vitro (DIV5, DIV8 and DIV12). Cells were fixed at DIV16 with paraformaldehyde and labelled with anti-MBP and anti-high-molecular-weight neurofilaments (NF) antibodies (SMI31 and SMI32 in table I) in GSDB, followed by secondary antibodies conjugated to Alexa Fluor 555 or Alexa Fluor 488 (1:600; Molecular Probes, Life Technologies). Nuclei were labelled with DAPI. Coverslips were mounted with a fluorescent mounting medium (Dako, Milan, Italy). For the co-culture analysis, stacks of images of MBP- and SMI31- and SMI32-positive cells were taken under confocal microscope at 40X magnification (at 6 fields/coverslip) and the ZEISS LSM Image Browser was utilized to automate quantification of the
myelination index. Images in the stack were merged at each level and pixels overlapping in the red and green fields above a predefined threshold intensity value were highlighted in white. The amount of myelin per axon (myelination index) was calculated as the ratio between the white pixel and the green pixel areas.

3.15 Western blot analysis
OPCs were lysed with a buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM HEPES, 2 mM EDTA pH 7.4. A modified version of the Laemmli buffer (20 mM Tris pH 6.8, 2 mM EDTA, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue) was then added to a final 1X concentration and proteins were separated by electrophoresis, blotted on nitrocellulose membrane filters and probed using the using the primary antibodies reported in the table I and the HRP-conjugated secondary antibodies (goat anti-rat 1:1000, goat anti-rabbit 1:4000 and goat anti-mouse 1:2000; Sigma Aldrich, Milan, Italy). Photographic development was by chemiluminescence (ECL, GE Healthcare) according to the manufacturer’s instructions. Densitometric analysis of the protein bands was performed with ImageJ software. Band intensities were measured as integrated density volumes (IDV) and expressed as percentage of control lane values.

3.16 ELISA
The concentration of IL-1α or C1q or TNF in microglial cells or EVs were quantified using a solid-phase sandwich ELISA (enzyme-linked immunosorbent assay) kit following the manufacturer’s protocol (Rat IL-1α ELISA kit, Invitrogen Waltham, MA USA, BMS627; Rat TNF ELISA kit, Invitrogen Waltham, MA USA, BMS622, Rat C1q ELISA kit, Novus Biologicals Centennial, CO, USA, NBP2-74988). IL-1α or C1q or TNF content in the cells was determined after cell lysis with RIPA Buffer (Sigma Aldrich, Milan, Italy) whereas inside EVs after detergent permeabilization with 0.6% Triton X-100 (Sigma Aldrich, Milan, Italy) in presence of protease inhibitors (1:1000, Sigma Aldrich, Milan, Italy). Sample absorbance was measured with a spectrophotometric system (1420 Multilabel Counter Victor 2; Wallac) at 450 nm at 10 Hz. The amount of IL-1α, C1q or TNF in EVs was estimated on the basis of a standard curve in the presence 0.6% Triton X-100.

3.17 Cryo-electron microscopy
Cryo-EM allows imaging of samples without the addition of any heavy metals or fixatives, which might cause artefacts, with the drawback of yielding a lower contrast. The sample is frozen so rapidly that the water vitrifies forming no ordered crystals, and the native structure of the sample is preserved.
(Dubochet et al., 1988; Orlov et al., 2017). Freshly prepared vesicles resuspended in saline were plunge frozen in liquid propane using a Vitrobot Mark IV (ThermoFisher Scientific, Oregon, USA).

3.18 Drugs and reagents
S-FTY720-Vinylphosphonate (kind gift from Prof. Robert Bittman) was dissolved in fatty acid free bovine serum albumin (1 mM in PBS). S1P (Enzo Biochem. Inc, Farmingdale, NY) was dissolved in fatty acid free bovine serum albumin (4 mg/ml in distilled water). Stock solutions were diluted in fresh Krebs-Ringer solution.

3.19 Statistical analysis
All data are presented as mean ± SEM from the indicated number of experiments “N”. Statistical analysis was performed using SigmaStat 3.5 software (SigmaStat software, San Jose, California). After testing data for normal distribution, the appropriate statistical test was used and the overall p-value indicated in the figure legends. Group differences were considered significant when P was <0.05, indicated by an asterisk; those at P was <0.01 are indicated by double asterisks; those at P<0.001 are indicated by triple asterisks. For the Raman spectra, Principal Component Analysis and Linear Discriminant Analysis (PCA-LDA) were performed by means of Origin2018 (OriginLab, Northampton, MA, USA). To test the sensitivity, specificity and accuracy of the classification model to distinguish the EV phenotype by the overall biochemical composition, leave-one-out cross-validation was used.

3.20 Author Contribution
During my PhD period, I focused on improving my technical and communication skills and on increasing my knowledge in Neuro-immunology research. I mostly performed in vitro experiments described in this thesis by using an array of different experimental approaches, including glia cell culture preparation, immunocytochemistry, qPCR, WB and ELISA analysis. In addition, I carried out specific methodologies for vesicle isolation and characterization (ultracentrifugation, Nanosight Tracking Analysis, and EV preparation for Raman spectroscopy and Cryo-EM) as well as imaging of EV-oligodendrocyte interaction by using optical tweezers.

The characterization of the morphology and composition of microglial EVs by Cryo-EM and Raman spectroscopy were performed by Dr Paolo Swuec (University of Milan, Italy) and Dr Alice Gualerzi (IRCCS Fondazione Don Carlo Gnocchi) respectively.
In collaboration with Dr. Marta Fumagalli and Dr. Elisabetta Bonfanti (University of Milan, Italy), I established primary cultures of oligodendrocyte precursor cells for proliferation, migration and differentiation assays. Dr. Marta Fumagalli and Dr. Elisabetta Bonfanti analyzed myelin deposition in OPC-DRG co-cultures.

Thanks to the collaboration with Dr Annalisa Buffo e Dr Roberta Parolisi (University of Turin), I acquired knowledge of the basic and standard procedures for mouse surgery (such as mouse anesthesia, injection with lysolecithin or EVs or saline into the corpus callosum of mouse, implantation of Alzet minipumps in mouse cerebral cortex, brain tissue collection) and slice immunostaining. Roberta and I performed in vivo experiments and immunohistochemistry analysis. Dr Roberta Parolisi performed electron microscopy analysis of myelin lesions induced by LPC injection in mice, whereas Dr. Linda Chabaane (San Raffaele Scientific Institute, Milan) carried out MRI analysis.

Dr Paola Giussani (University of Milan, Italy) performed quantification of sphingolipid content in cells and EVs as well as lipid extraction of EVs.

During my PhD, I also improved my ability in experimental design, critical thinking and data interpretation, thanks to my Director of Studies Dr Claudia Verderio and her scientific background and experience.
CHAPTER IV

RESULTS
4.1 Budding of Extracellular vesicles (EVs) at the surface of microglia infiltrating the myelin lesion

Highly relevant for my PhD project a switch of microglia (MG) from inflammatory (M1) to pro-regenerative (M2) phenotype has been recently reported to promote remyelination in an animal model of demyelination induced by local injection of lysolecithin (LPC) (Miron et al., 2013). In this model, a single injection of the detergent LPC (2%) into the corpus callosum (CC) of mice produces a demyelinating lesion within 2–3 days, that spontaneously recovers thanks to recruitment, proliferation and differentiation of oligodendrocyte precursors cells (OPCs) to the lesion site (Oláh et al., 2012). Importantly, in this model infiltrating and activated macrophage/MG at the site of injury play a crucial role in the remyelination by influencing OPC functions (Miron et al., 2013). However, the biological activity of the EVs released by MG has not been tested in vivo yet.

In our study, LPC-induced demyelination model offers the distinct advantage of having spatial and temporal control over demyelination, so it is suitable for studying the complex mechanisms of remyelination. By inducing a demyelinating lesion at a specific site within the CNS, this model also allows us to deliver MG derived-EVs at the site of lesion and to analyse their specific effects on myelin repair.

More in detail, first objective of my research activity was to test the hypothesis that EVs released from both inflammatory and pro-regenerative MG may influence OPC recruitment and remyelination in the LPC mouse model of focal demyelination. This idea was suggested by high-resolution electron microscopy (EM) analysis of the lesion site. EM analysis showed that ten days after LPC injection (dpl) MG infiltrated the lesion site (Fig.4.1a) along with astrocytes and oligodendrocytes (Fig.4.1b). Of note, infiltrating microglial cells were very similar to “dark” microglia, which are characterized by condensed, electron-dense cytoplasm and nucleoplasm, as recently described by the Tremblay’s group (Bisht et al., 2016; El Hajj et al., 2019; St-Pierre, Bordeleau, & Tremblay, 2019).

Despite the narrow conformation of the extracellular space, consisting of a spider web of small gaps filled with interstitial fluid, commonly constrains EV visualization, we observed a dark microglia, infiltrating the lesion, with several blebs at the cell surface and surrounded by membrane vesicles in the pericellular space (Fig.4.1c, arrows), suggesting intense EV production. In the isolated MG, (Fig 4.1c) dark EVs are clearly detectable at distance from the cell membrane thanks to massive cell shrinkage and/or the fortuitous expansion of the pericellular space during sample processing.

This observation prompted us to examine the action of EVs in the crosstalk between MG and OPCs at myelin lesion. However, we still lack selective pharmacological or genetic tools to inhibit EV production from MG in vivo and explore the effects of endogenous EVs. Consolidated evidence indicated that inhibitors of neutral and acid sphingomyelinases decrease EV production in many cell
types, including MG. However, these blockers may interfere per se with the remyelination process as they alter the sphingolipid pathway (Verderio, Gabrielli, & Giussani, 2018). Accordingly, we used exogenous EVs produced in vitro from MG with different activation states as a tool to explore the action of microglial EVs. In addition, the use of LPC animal model in our experiments allowed us to know the location of demyelinating lesions, facilitating MG-derived EV administration at the lesion site.

![Fig.4.1](image-url)

**Fig.4.1 Microglia infiltrating the myelin lesion release EVs.** (a-c) EM images of the CC showing dark cells resembling microglia (a), oligodendrocytes (b) and astrocytes (b) infiltrating demyelinated lesion at 10dpl. High magnification inserts in (c) show examples of EVs budding from the surface of dark microglia (Scale bars, 2 μm).

### 4.2 Characterization of EVs released by pro-inflammatory and pro-regenerative microglia

We established rat primary MG and maintained the cells in resting conditions (NS) or in the presence of polarizing agents for 48hrs, i.e. Th1 cytokines for inducing inflammatory phenotype or IL-4 for polarizing microglia towards a pro-regenerative phenotype. Since immunomodulatory mesenchymal stem cells (MSCs) drive a neuroprotective phenotype in MG (Giunti et al., 2012; Zanier et al., 2014), MG were also co-cultured with MSCs in a transwell system.
in the presence of Th1 cytokines for 48hrs (Fig. 4.2a). Cells were exposed to the inflammatory cytokines because previous evidence indicates that MSCs increase their immunosuppressive and pro-regenerating abilities when treated *in vitro* with pro-inflammatory cytokines such as TNF and IL1-β (Carrero et al., 2012 589; Fan et al., 2012 590). More specifically, it has been described that MSCs induce pro-regenerative functions in MG through the release of chemokine fractalkine/CX3CL1, which is driven by inflammatory signals (Giunti et al., 2012; Zanier et al., 2014).

We verified the phenotypic profile of MG measuring by qPCR analysis the expression of 7 genes (IL-1α, C1q, TNF, IL-1β, iNOS, Arg1, Socs-3) that helps with the discrimination between the opposite (beneficial or detrimental) polarization states. In inflammatory MG (i-MG) pro-inflammatory genes IL-1α, C1q, TNF, IL-1β and iNOS were upregulated, while in IL-4 treated MG (IL-4 MG) only the expression of the anti-inflammatory gene Arg1 was increased. MSCs-conditioned MG (MSC-MG) presented a mixed phenotype characterized by the upregulation of pro-regenerative marker Arg1 but also pro-inflammatory markers IL-1α, C1q, IL-1β, TNF and iNOS (Fig. 4.2b). Despite it is difficult to define the microglia function by analysing only a few polarization markers, we speculated that the upregulation of these pro-inflammatory markers may not be unequivocally associated with a harmful MG function.

We also found that MSC-MG selectively upregulated a few genes, including Suppressor of cytokine signaling 3 (Socs-3), which negatively regulates cytokine pathways and Choline Phosphotransferase 1 (Chpt1), an enzyme involved in the synthesis of phosphatidylcholine (Fig. 4.2b), indicating the acquisition of a distinct phenotype.

After stimulation of MG cultures with ATP for 30 min, a condition that promotes EV secretion, medium-large vesicles (ectosomes) and small vesicles (exosomes) were isolated from the cell supernatants through ultracentrifugation at 100,000g, after removal of cells and debris, following a previously established protocol widely used in the lab (Gabrielli et al., 2015). Specifically, during the past years we have characterized the morphology and purity of EVs isolated by differential centrifugation by a combination of different techniques: i) negative staining electron microscopy, ii) fluorescence microscopy of EVs incubated with NBD C6-HPC to stain vesicle membrane, or with FITC-annexin V to reveal phosphatidylserine (PS) expressed on EV surface, iii) flow cytometry analysis (FACS) of annexin V binding to vesicles, iv) Western blotting analysis for EV specific markers (CD63, cannabinoid receptor CB1 and Alix for exosomes, and Tsg101, Flotilin for ectosomes) (Bianco et al., 2009).

To further characterize the morphology, structure, size of EVs and their purity, EVs isolated from non-stimulated MG (NS-MG) were analyzed by Cryo-electron microscopy (cryo-EM).
Cryo-EM is a powerful technique that enables the characterization of EV collected from different body fluids revealing structural details. MG derived-EVs were highly heterogeneous in shape and electron density. Most EVs were made by a single bilayer (88%) (Fig.4.2c, c'', c'''), had a round shape (Fig.4.2c-c'') and were very heterogeneous in size (mean diameter=81,14 ± 9,11nm) (Fig.4.2d). In addition, about 10% of EVs were multilamellar, containing two or more vesicles in their lumen. An example is shown in Fig.4.2c’, which shows a round vesicle enclosed in a larger one round in shape (Fig.4.2c’). This morphological variability of EVs suggests the existence of different subpopulations, which can possess different functions and composition.

For instance, 15% of the EVs show surface protrusions, electron-dense spikes protruding from the membrane, or part of the membrane (Fig.4.2e’’), suggesting the presence of transmembrane or membrane-associated proteins which may facilitate membrane fusion or cell-to-cell interaction (Zeev-Ben-Mordehai, Vasishtan, Siebert, & Grunewald, 2014). Cryo-EM also confirmed no contamination by apoptotic bodies or intracellular organelles derived from broken cells. For all the experiments, the concentration and size distribution of EVs released from MG polarized towards pro-inflammatory or pro-regenerative phenotype were checked by NanoSight Tracking Analysis (NTA). NTA showed no significant changes in total EV production from reactive MG compared to NS MG (Fig.4.2d).

Conversely, EVs derived from polarized microglia presented differences in molecular composition as showed by Raman spectroscopy (RS). RS is a sensitive optical technique, rapid and label-free, already used for bulk biochemical characterisation of EV preparations. Compared to measurements on particle counts, which may give a quick first overview of EV size and concentration, Raman assess both EV purity and composition. It provides information on the chemical content of EVs from cell culture supernatants and biofluids, and was previously proven to be able to highlight differences in the EV content related to changes in the activation state or in the type of donor cells (Gualerzi et al., 2019; Gualerzi et al., 2017). Indeed, Gualerzi and colleagues demonstrated that the Raman spectra of the EVs allows distinguishing the vesicles of the three cytotypes, such as bone marrow and adipose tissue-derived MSCs and human dermal fibroblast EVs with an accuracy of 93.7%. In addition, specific Raman fingerprint also represents the isolation procedure used for EVs and provides insights into the biochemical characteristics of EVs from different sources, in particular into the contribution of sphingomyelin, gangliosides and phosphatidylcholine to the Raman spectra themselves (Gualerzi et al., 2019; Gualerzi et al., 2017).
The multivariate statistical analysis (PCA-LDA) of the Raman spectra (Fig.4.2e) showed that major molecular differences were revealed between EVs released by pro-regenerative cells (IL4-EVs and MSC-EVs) and inflammatory microglia (i-EVs) compared to NS-EVs, whereas similarities were found between IL-4-EVs and MSC-EVs (Fig.4.2e). Despite such similarities, significant differences among IL-4-EVs and MSC-EVs spectra were highlighted by linear discrimination analysis, suggesting that the two types of EVs may indeed exert different action. Importantly, the main differences between different EV types were detected in the peaks and bands related to lipid components (mainly 2700–3200 cm\(^{-1}\)) (Fig.4.2f), with main peaks attributable to cholesterol and cholesterol ester (700; 1127; 1440 cm\(^{-1}\)) and in peaks corresponding to the CH2 deformations (around 1300 cm\(^{-1}\)) and CH, CH2, and CH3 bonds (in the spectral range 2600–3200 cm\(^{-1}\)) (Harris & Baffy, 2017). The classification model PCA-LDA demonstrated that RS can distinguish the microglia phenotypes of the EV source with an overall accuracy of 91.7% and an error rate of 18.24% after cross-validation. The error rate was reduced to 10.16% when analysing spectra from broken EVs, busted by hypoosmotic shock (Gabrielli et al., 2015) and re-pelleted to remove soluble components supporting the major contribution of membrane components in the spectral differences between the EV subtypes (Suppl. Figure 5.1).
Fig. 4.2 Production and characterization of EVs released by microglia with different activation states. (a) Scheme of microglial polarization in vitro. (b) Gene expression of inflammatory markers (IL1α, C1q, TNF, IL-1β and iNOS), pro-regenerative markers (Arg1 and Socs-3) and the metabolic gene Chpt1 in unstimulated microglia (NS-MG), IL-4-treated microglia (IL-4-MG) or microglia stimulated with inflammatory cytokines in the absence (i-MG) or in the presence of MSCs (MSC-MG) (number of independent experiments (n)=7-11/group). (c) Representative cryo-EM images of the heterogeneous population of microglial EVs in the 100,000g pellet. Example of single (c), double (c'), coated (c'') vesicle and large tubule (c'''). (d) Size profile of EVs pelleted from 1×10⁶ microglia, re-suspended in 100μl of 0.1 μm-filtered PBS and analysed using NTA (top). Histograms show production of EVs from NS or polarized microglia during 30 min stimulation with 1mM ATP (bottom) (n=4). (e) Mean Raman spectra obtained using 532 nm laser line from EVs of NS or polarized microglia. All spectra were baseline corrected, aligned and normalised before averaging. (f) Multivariate statistical analysis performed on the Raman spectra (n ≥ 30 per sample). The scatter plot represents the values obtained for the Canonical Variable 1 and Canonical Variable 2 after LDA. In the classification model, spectra from EVs were grouped based on the cell of origin to test RS ability to discriminate the molecular composition of EVs from different microglial phenotypes. The first 10 PC scores calculated by means of PCA were used for the LDA. Each dot represents a single spectrum.
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4.3 Action of MG-derived EVs on the oligodendroglial response to focal demyelinating lesions

We first explored the action of EVs secreted in vitro from inflammatory microglia (i-EVs) and pro-regenerative cells (IL4-EVs and MSC-EVs) on remyelination process in the LPC mouse model of focal demyelination.

EVs were administered to mice with different protocols, either chronic delivery or a single injection, and at different time points after the lesion, either at 3 or at 7 days post lesion (dpl), times that correspond to key steps in the remyelination process: OPC recruitment into the lesion by proliferation and migration, and initiation of remyelination by the differentiation of these OPCs into myelin-forming cells (Keough et al., 2015; Miron et al., 2013).

In a first set of experiments, mice were injected 3 dpl with EVs produced by 1.5 million cells (~2-3x10^8) at the lesion site via pre-filled Alzet mini-pumps for 4 days (at 2x10^6 EVs/h) to explore the effect of chronic delivery of MG-derived EVs on the early response to demyelination. Saline-injected mice were perfused as control, given that also NS-EVs may have biological activity.

NTA analysis revealed that EVs were still present in the mini-pumps 3 days after re-suspension, albeit the concentration was reduced by ~30% (EV concentration ± SEM= 2.80x10^8±0.19 at day 0; 2.60x10^8±0.13 at day 1; 1.90x10^8±0.08 at day 3).

After treatment, mice received BrdU (100 mg/Kg) twice (2h apart) at 4 dpl and were sacrificed 7 dpl in order to analyze EV effects on OPC proliferation at the site of lesion (Fig.4.3a).

Although quantification of BrdU positive cells showed that all types of EVs significantly increased cell proliferation in the lesion area compared to control mice (Fig.4.3b), MG-derived EVs did not influence the proliferation of OPCs, as indicated by quantification of cells double positive for BrdU and the oligodendroglial specific marker Olig2 (Fig.4.3c). These results indicate that other brain cells such as Iba1-positive microglia/macrophages and S100β positive astrocytes (data not shown) proliferated at the lesion site upon EV treatment.

However, staining for the transcription factor Sox10 showed that both IL4-EVs and MSC-EVs enhanced the density of oligodendroglial cells in the lesioned area, suggesting that pro-regenerative EVs promoted the migration of OPCs to the lesion site (Fig.4.3d,e).

Importantly, we also observed that pro-regenerative EVs enhanced the differentiation of OPCs into mature oligodendrocytes (OLs), as indicated by the analysis of immunoreactivity for the myelin protein MBP at the lesion sites, which showed an increase in the fraction of MBP positive area (Fig.4.3f,g). Accordingly, we observed a reduction of immature cells positive for NG2 (NG2+/Sox10+ cells) (Fig.4.3e, red y axis) and an increase in density of OLs undergoing differentiation (NG2−Sox10+ cell density±SEM: saline, 721.3±88.64; i-EVs, 562.0±40.31; IL4-EVs, 755.1±82.13; MSC-EVs,
1080±98.97; One Way ANOVA main effect of treatment p=0.0114 with Holm-Sidak's multiple comparisons test) after MSC-EVs infusion. No change in NG2 positive-area after pro-regenerative EVs injection (Fig.4.3i) suggest a balance between repopulation/migration of OPCs and their differentiation at the lesion site. We speculate that both IL4- and MSC-EVs contribute not only in OPC differentiation but also in maintaining the progenitor pool at demyelinating lesion.

By contrast, infusion of i-EVs inhibited OPC maturation as showed by the significant decrease in MBP positive fraction area (Fig.4.3f,g) with a parallel increase in NG2 staining (Fig.4.3h,i) and in the percentage of NG2+/Sox10+ early progenitors compared to saline-injected mice (Fig.4.3e, red y axis).

Collectively, these data indicate that, while inflammatory microglia inhibit OPC differentiation and remyelination through secretion of EVs, microglia with beneficial phenotype, especially after MSC conditioning, release EVs which promote OPC recruitment and maturation at the demyelinating lesion.

To specifically assess the impact of EVs on OPC differentiation and verify whether the dose of injected EVs may differently influence OPC functions, we administered a smaller amount of i-EVs or MSCs-EVs (7x10⁷ EVs, corresponding to about one third of EVs delivered over 4 days via the mini-pumps) to LPC-treated mice by a single injection at 7 dpl, during the phase of OPC maturation (Miron et al., 2013). The action of EVs on the remyelination process was analyzed at 10 dpl in both young (2.5-4 months old) and aged mice (8.5-12 months old) (Fig.4.4a), where remyelination process occurs more slowly (Shields et al., 1999).

This time point was selected for the analysis of EV impact on OPC differentiation on the basis of preliminary MRI experiments. Indeed, MRI analysis revealed that myelin lesion extended in the entire CC at 5-6 dpl, before EV treatment, as indicated by hyper-intense signal on T2-weighted MR images, whereas at 10 dpl the lesion in the CC fully recovered given that the signal returned hypointense almost similar to healthy controls (Suppl Fig.5.2a). Accordingly, Diffusion Tensor Imaging (DTI) showed that diffusivity perpendicular to white matter fibers considerably increased at 5dpl to then returned to normal values at 10 dpl, confirming complete myelin repair (Suppl Fig.5.2b).

To check whether EVs were able to reach the lesion site we injected EVs produced by GFP labelled microglia, established from GFP-transgenic rats. Twenty minutes after EV injection, we found GFP+ EVs at the lesion site, evidenced by double staining of the lesion site for DAPI (Suppl Fig.5.3a-d’). Some of them appeared inside Iba1+ microglia/macrophages (Suppl Fig.5.3e-e’’’), S100β+ astrocytes (Suppl Fig.5.3f-f’’) or NG2+ OPCs (Suppl Fig.5.3g-g’’’) as shown by the acquisition of confocal images and Imaris 3D reconstruction. After 60 min from injection, GFP+ EVs were no longer visible at the lesion site (not shown).
Importantly, despite EVs were administered in a small amount and in a single pulse, both i-EVs and MSC-EVs resulted to exert a strong effect on OPC differentiation. Specifically, i-EVs induced a clear block of OPC maturation in both young and old mice, as indicated by the significant decrease in MBP+ area at the lesion site compared to saline-injected animals (Fig.4.4c,d; Fig.4.5a,b). No differences in NG2 staining (Fig.4.4 e,f) and in the percentage of NG2+ early progenitors (Fig.4.4b) were observed in the lesioned area. This suggests that although i-EVs limit OPC maturation, the rate of OPC repopulation/regeneration is preserved.

Treatment of MG with MSCs completely counteracted the detrimental action of i-EVs. In old mice MSCs-EVs not only rescued but even enhanced MBP staining compared to controls (Fig.4.4c,d), indicating that they favored remyelination. The increase in MBP immunoreactivity was associated to a parallel reduction in NG2 staining at the lesion site (Fig.4.4e,f), consistent with differentiation of NG2+ OPCs into MBP+ mature oligodendrocytes. This result was confirmed by the increase in the density of OLs positive for CC1, a myelinating OL marker that stains the cell body (Bin, Harris, & Kennedy, 2016) (Fig.4.4g,h), and by a reduction in the percentage of NG2+/Sox10+ early progenitors at lesion site (Fig.4.4b).

In young mice, MSC-EVs was less effective. They significantly enhanced MBP immunoreactivity (Fig.4.5a,b), decreased NG2 staining (Fig.4.5c,d) and enhanced the density of CC1+ differentiated oligodendrocytes (Fig.4.5e,f) compared to i-EVs, but failed to promote remyelination over control levels (Fig.4.5a,b and e,f). This finding is in line with a recent study demonstrating that remyelination is accelerated in young mice compared to old mice (Gingele et al., 2020 594). In young animals, the process of repair starts early and is associated to a rapid activation of microglia and early proliferation and differentiation of OPCs, resulting in effective remyelination of the CC (Pfeifenbring, Nessler, Wegner, Stadelmann, & Bruck, 2015). In addition, microglia and astrocytes from young animals are more efficient in myelin removal by phagocytosis which creates a remyelination-supportive environment (Gingele et al., 2020 594). Given the high speed of the remyelination process, dissecting the effects of EVs on remyelination in young mice is much complex.
Fig. 4.3 Action of EVs on early response to EVs. (a) Experimental design of EV delivery to LPC-treated mice during the phase of OPC recruitment. (b-c) Histograms show the density of total proliferating cells (b) (Number of animals (N)=3-5/group) and the percentage of BrdU+ proliferating OPCs (c) (N=3-5/group) in saline, i-EVs-, IL4-EVs- or MSC-EVs-injected lesions. (d) Representative images of Saline, i-EVs, IL4-EVs or MSC-EVs-injected lesions (area delimited by dotted line) immunostained against Sox10 (red) (Scale bars of images and insets, 50 μm). Low magnification inserts show Sox10/DAPI double staining to visualize nuclei. (e) Corresponding density of Sox10+ cells (histograms) (N=3-5/group) and percentage of immature (NG2+/Sox10+) oligodendrocytes (red line) (N=3-5/group). (f-h) Representative images of Saline, i-EVs, IL4-EVs or MSC-EVs-injected lesions (area delimited by dotted line) immunostained against MBP (green) and DAPI (blue) (f) or against NG2 (green), Sox10 (red) and DAPI (blue) (h) (Scale bars, 50 μm). (g, i) Histograms show the percentage of the lesioned area immunoreactive for MBP (g) (N=3-7/group) or NG2 (i) (N=3-4/group) in saline-injected mice and mice that received different types of EVs.
4.4 EM analysis of remyelination upon EV treatment

The action of MG-derived EVs on the remyelination process was also evaluated by EM analysis of the CC of LPC-injected old mice at 10 dpl. We found that EVs from differently activated MG strongly affected myelin ultrastructural features. Specifically, mice that received i-EVs presented a higher G ratio, a measurement of axon diameter in relation to myelin thickness (Rushton, 1951), (Fig.4.4k,n) and thinner myelin (Fig.4.4m), while mice injected with MSC-EVs displayed higher percentage of myelinated fibers (Fig.4.4i,j), lower G ratio (Fig.4.4k,n) and higher myelin sheath thickness (Fig.4.4m) compared to saline-injected mice. Thus, EM analysis confirmed that i-EVs inhibit remyelination whereas MSC-EVs promote myelin repair.
Altogether, these data indicate that EVs released from inflammatory microglia block the remyelination process while EVs released from beneficial microglia promote OPC recruitment and maturation at the demyelinating lesion.
Fig. 4.4 Action of EVs on myelin deposition in acute LPC-mediated focal demyelination. (a) Experimental protocol of EV delivery to LPC-treated mice during the phase of OPC differentiation. (b) Histograms show percentage of immature (Sox10+/NG2+) and differentiated cells (Sox10+/NG2-) oligodendrocytes in saline-injected mice and mice that received i-EVs or MSC-EVs (N=3-4/group). (c, e, g) Representative images of Saline, i-EVs or MSC-EVs-injected lesions (area delimited by dotted line), immunostained against MBP (green, c), NG2 (green, e), or CC1 (yellow, g) (Scale bars, 50 μm). (d, f, h) Histograms show the percentage of the lesioned area immunoreactive for MBP (d) (N=3-4/group), NG2 (f) (N=4/group) or the density of mature oligodendrocytes (CC1) (h) (N=3/group). (i) Representative electron micrographs of CC in saline-injected mice and mice that received i-EVs or MSC-EVs (Scale bars of images, 1 μm; original magnification 25000). (j) Histograms show the percentage of unmyelinated/myelinated fibers (N=3/group). (k) Myelin thickness can be quantified by the G-ratio, defined as the ratio between the inner (axon, d, white) and outer (overlying myelin, D, yellow) diameters of myelinated axons. Scale bar of image, 1.5 μm. (l) Histograms show quantifications of the G-ratio (N=3/group). Each dot represents an individual value. (n) Scatter plots of G-ratio against axon diameter. The G-ratio of each measured myelinated fiber is indicated by a single circle (n=160 saline, n=167 MSC-EVs, n=169 i-EVs). Correlation between axon diameter (x1) and g-ratio (y1) is expressed by the correlation coefficient (r) of the linear regression curve (saline: r=0.503, i-EVs: r=0.518; MSC-EVs: r=0.448).

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Fig. 4.5 Action of microglia-derived EVs on OPC differentiation at myelin lesion in young mice. (a, c, e) Representative images of saline, i-EVs and MSC-EVs-injected lesions (area delimited by dotted line), immunostained against MBP (green, a), NG2 (green, c), or CC1 (yellow, e) (Scale bars, 50 μm). Low magnification inserts show double labelling for DAPI. (b, d, f) Histograms show the percentage of the lesioned area immunoreactive for MBP (b), NG2 (d) or the density of CC1 positive cells (f) at 10dpl in saline-injected mice and mice injected with i-EVs or MSC-EVs. (Number of animals (N) = 3-5/group).

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*Holm-Sidak’s multiple comparisons test

(P value of main effect)
4.5 EVs attract OPCs: involvement of sphingosine-1-phosphate

To investigate the molecular mechanisms through which MG-derived EVs exert harmful and protective action on remyelination, we took advantage of OPCs in primary culture, a powerful system that allows to analyse the effects directly mediated by EVs. In detail, we explored the possibility that EVs released from pro-inflammatory or pro-regenerative MG (either IL4-treated MG or MSC-treated MG) can influence OPC proliferation and/or migration, fundamental steps in the remyelination process.

OPCs were exposed to the amount of EVs released by a double number of MG (2:1 ratio between donor MG and recipient OPCs) to mimic the in vivo situation, where OPCs are less common than MG. To further investigate how the phenotype of MG influences EV action, NS-EVs were included in most in vitro experiments.

The action of EVs on OPC proliferation was assessed in purified cultures of OPCs co-exposed to MG-derived EVs and to the proliferation marker EdU for 24hrs. In agreement with in vivo observations, we observed very mild effects of EVs on OPC proliferation. Indeed, quantification of OPCs positive for EdU showed that EVs produced by pro-inflammatory cells inhibited the proliferation of cultured OPCs, albeit with no statistical significance, while EVs released by pro-regenerative MG significantly increased OPC proliferation compared to i-EVs-treated, but not to NS-EVs or control OPCs. Of note, pre-conditioning with MSCs significantly attenuated the anti-proliferative action of i-EVs, suggesting that exposure to MSCs may counteract the negative influence of pro-inflammatory milieu on MG (Fig.4.6a,b).

By contrast, the impact of EVs on OPC migration was much stronger. Using a classical transwell-based migration assay, we found that all types of EVs significantly enhanced transit of OPCs through the transwell filter (Fig.4.6c), suggesting that also in vitro MG-derived EVs may promote OPC migration, the first key step in myelin repair.

Given that MG-derived EVs contain several components of the sphingolipid pathways (Bianco et al., 2009), we next explored whether sphingosine-1-phosphate (S1P), a known chemoattractant (A. Kimura et al., 2008), might mediate EV-dependent chemoattraction.

We pulse-labelled MG with [3H]sphingosine ([3H]sph) under conditions of metabolic equilibrium and measured the content of [3H]sphingolipids in EVs and parental MG. We found detectable levels of [3H]S1P along with more abundant sphingolipids (sphingomyelin, lactosylceramide and monosialodihexosylganglioside (GM3)) in EVs produced by NS microglia, although S1P was not enriched in EVs compared to donor cells (Table III).

Consistent with a role of vesicular S1P in OPC migration, when OPCs were exposed to pro-regenerative EVs in the presence S-FTY720-Vinylphosphonate (S-ene) (100 nM) (Valentine et al.,
2010), a pan antagonist of the S1P receptors, we found that EV-dependent OPC migration was completely prevented (Fig. 4.6d).

Collectively, these data demonstrated that microglial EVs are able to act as chemoattractant for OPCs and identified vesicular S1P as an attractive guidance cue for OPCs.

**Graph 1: Sphingolipid content in EVs and parental microglia.**
Fig. 4.6 EV impact on OPC migration, differentiation and myelination. (a-b) Fluorescence images of cultured OPCs incubated with EdU (red), fixed and stained for NG2 (green) and DAPI (blue) after 24hrs exposure to i-EVs or MSC-EVs (Scale bars, 50 µm). The histograms in (b) show the percentage of EdU+ OPCs in cultures exposed or not to different EV types. Data have been normalized to control (number of experiments (n) =5-8 /group). (c) Histograms show the percentage of OPCs migrated through the filter of the Boyden chamber in control conditions and following addition of different types of EVs. Data have been normalized to control (n=3). (d) Percentage of migrated OPCs in response to S1P or IL-4-EVs in the presence or in the absence of the S1P receptor antagonist S-FTY720-Vinylphosphonate (n=3). (e)
Representative images of OPCs maintained in control conditions or exposed to different types of EVs for 2 days, fixed and stained for MBP (red), GPR17 (green) and DAPI (blue) (Scale bars, 50 µm). (f) Corresponding quantification of MBP+ OPCs. Data have been normalized to control (n=5-8/group). (g) Western blot of control OPCs and IL4-EVs-treated OPCs for the indicated markers of OPC differentiation. Tubulin has been used as loading control. Relative quantification of the band density is shown on the right (n=3). (h) Representative images of OPC-DRG co-cultures maintained in control conditions or exposed to i-EVs, IL4-EVs or MSC-EVs for 11 days, fixed and stained for MBP (red) and neurofilament (NF, green) (Scale bars, 20 µm). (i) Myelination index (MBP staining/NF staining) under different experimental conditions (n=3).

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4.6 EVs derived from both inflammatory and pro-regenerative microglia directly promote OPC differentiation

In order to explore the activity of EVs on OPC differentiation, postnatal OPCs were exposed to MG-derived EVs for 48hrs. Differentiation of OPCs towards myelinating cells was determined by quantifying the proportion of cells showing positivity for the differentiation marker MBP. Unexpectedly, immunofluorescence analysis revealed that i-EVs significantly increased the percentage of MBP+ OLs similar to IL4-EVs and MSC-EVs, whereas NS-EVs did not affect OPC maturation (Fig.4.6e,f).
To verify whether EV effects were dose-dependent, we treated cultured OPCs with half or double concentration of IL4-EVs, derived from IL-4 treated microglia, a type of EV that efficiently promoted OPC differentiation. We found no significant changes in the fraction of MBP+ cells under these experimental conditions (normalized MBP+ OPC fraction ± SEM: 1.53±0.26 standard EV dose; 1.42±0.22 half dose; 1.27±0.36 double dose), suggesting that the action of EVs on OPCs is not influenced by small variations in their concentration.

The pro-differentiating action of pro-regenerative EVs was confirmed by Western blot analysis. Indeed, IL4-EVs induced an increase in the expression of MBP along with other markers of mature OLs, i.e. 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase) and glutathione S-transferase (GST-pi) while downregulating GPR17, a marker of immature OLs (Fig.4.6g).

The capability of microglial EVs to influence myelin deposition was analyzed by co-culturing OPCs with murine Dorsal Root Ganglia (DRG) neurons, a condition that stimulates myelination (Taveggia et al., 2008). Co-cultures were exposed to EVs released from MG with different activation states for 11 days (three treatments, every three/four days), then fixed and immunostained with axon- and myelin- specific markers, high-molecular-weight neurofilaments (NF) and MBP, respectively. Quantitative analysis of the linear MBP+ segments extending along NF+ axons indicated that EVs derived from IL4-treated MG or MSCs-conditioned MG, but not resting cells (not shown), promoted myelin deposition compared to vehicle treated cultures (Fig.4.6h,i). Consistent with the capacity of i-EVs to drive OPC differentiation, EVs derived from inflammatory MG also increased myelination, albeit at a lower extent compared to pro-regenerative EVs (Fig.4.6h,i).

Importantly, we found that EVs derived from MSC-conditioned MG displayed higher capacity to promote myelin deposition compared to EVs released by MSCs (Suppl. Fig.5.4a).

Globally, these results show that EVs derived from activated MG exhibit direct pro-differentiating action on cultured OPCs. Of note, i-EVs favour OPC differentiation in vitro while they cause a clear block of remyelination in vivo. This suggests that other brain cells may be involved and account for the detrimental action of i-EVs in vivo.

To identify the molecular components of EVs driving OPC differentiation, we investigated whether broken EVs, depleted of their luminal content, maintain pro-differentiating action on OPCs. IL-4-EVs or MSC-EVs were busted by hypo-osmotic shock (Gabrielli et al., 2015) and membrane fragments pelleted to remove soluble components and added to cultured OPCs. Quantification of the percentage of mature OLs showed that broken EVs retained the capability to promote OPC differentiation (Fig.4.8b). This finding points to surface EV component(s) (proteins or lipids) as the agents responsible for the pro-differentiating action of EVs.
To distinguish between protein and lipid surface component(s), we exposed cultured OPCs to the lipid fraction extracted from IL-4 EVs (Fig.4.8c) or i-EVs (not shown). We found that EV lipids were able to enhance OPC maturation even more efficiently than intact EVs (Fig.4.8c), indicating that the stimulatory agent(s) are EV lipids.

Previous studies demonstrated that S1P and its analogue FTY720-p promote OPC differentiation and myelin deposition (Cui, Fang, Kennedy, Almazan, & Antel, 2014; Miron et al., 2010), however, we ruled out the involvement of S1P in the pro-differentiating action of EVs. In fact, the capacity of EVs to accelerate OPC maturation was unchanged when we treated OPCs with EVs in the presence of the S1P receptor antagonist S-ene (Fig.4.8d).

Still the lipid agent(s) driving OPC differentiation remain(s) undefined. A specific approach, such as lipidomic analysis, is thus required to validate and extend our results and to define candidate lipids, expressed in i-EVs, IL4-EVs and MSC-EVs and less abundant or not present in NS-EVs, which may have pro-differentiating activity. An alternative approach to identify the lipid(s), which stimulate OPC differentiation, may be to separate main lipid classes from the total lipid extract and then further distinguishing between members in the “active” families by testing their capacity to promote OPC differentiation. Whatever the approach, the identification of the EV lipid(s) responsible for OPC differentiation will take a long time and more investigations.

4.7 Astrocytes transform the pro-differentiating action of i-EVs to inhibitory activity

Recent evidence from Ben Barres’s group indicated that microglia can convert astrocytes into a neurotoxic A1 phenotype, which inhibit OPC differentiation (Yun et al., 2018). To explore whether astrocytes were involved in the block of remyelination caused by i-EVs in the LPC-model of myelin lesion, we grew OPCs on top of astrocytes (astrocyte-to-OPC ratio of 1:1) and maintained the co-cultures in the presence or in the absence of i-EVs for 48 hrs. Immunofluorescence staining for MBP revealed that, in the absence of i-EVs, astrocytes promoted OPC differentiation, as indicated by the increase of the percentage of MBP/Olig-2 double positive cells in astrocyte-OPC co-cultures compared to OPCs alone (Fig.4.7a,b). However, when exposed to i-EVs, astrocytes strongly inhibited OPC maturation, reverting the pro-differentiating activity of i-EVs on OPCs (Fig.4.7a,b).

The capability of i-EVs to transform astrocyte into A1 harmful cells was assessed by analysing the expression of markers for A1 (serping-1 and Amigo 2) and A2 astrocytes (PTX3, CD14 and Tm4sf1) in primary cultures of astrocytes exposed or not to i-EVs. qPCR analysis showed that the A1 reactive markers serping-1 and Amigo 2 were upregulated in cultured astrocytes exposed to i-EVs (Fig.4.7c), while the expression of the A2 markers PTX3, CD14 and Tm4sf1 was unaltered (Fig.4.7d).
We next treated astrocytes with lipids extracted from i-EVs to investigate whether the lipid components or the protein cargo of i-EVs were responsible of the acquisition of harmful phenotype by astrocytes. We found that astrocytes exposed to the lipid extract of i-EVs significantly upregulated A2 markers (Fig.4.7d), and slightly increased A1 markers (Fig.4.7c), suggesting that EV lipids were not involved in harmful astrocyte transformation.

Harmful astrocyte conversion was also validated in LPC-injected mice that received i-EVs for 4 days (Fig.4.3a). Immunofluorescence analysis indicated that GFAP+ astrocytes exhibited decreased PTX3 labelling (Fig.4.7e,f) and enhanced immunoreactivity for the A1 marker C3a at the lesion site in mice injected with i-EVs, albeit the increase was not statistically significant (Fig.4.7e,f). By contrast, MSC-EVs did not induce changes in PTX3 and C3a labelling nor in A1 and A2 markers expression at the lesion sites (Fig.4.7c,f).

Given that IL-1α, C1q and TNF were involved in A1 astrocyte conversion and were previously detected in MG-derived EVs (Drago et al., 2017; Soni et al., 2019), we asked whether these cytokines could be involved in astrocyte activation by i-EVs. By using specific ELISA, we first measured IL-1α, C1q and TNF content in i-EVs isolated from 15x10^6 microglial cells. This analysis revealed that i-EVs contained ~90 pg of IL1-α, ~ 78 pg of C1q and ~ 613 pg of TNF-α, indicating that, among the three inflammatory mediators, TNF was more enriched in i-EVs.

Interestingly, only TNF was suppressed in parental microglia by MSC conditioning, as indicated by ELISA (Fig.4.7g) and qPCR (Fig.4.2b) analysis. We therefore measured the TNF content in i-EVs and MSC-EVs and found that i-EVs displayed about 3-fold higher levels of TNF compared to MSC-EVs (i-EVs TNF content ~ 613 pg; MSC-EVs TNF content ~168 pg). These results suggest that the capacity of MSC-EVs to promote myelin repair may occur, at least in part, via TNF suppression.

To directly test this hypothesis we exposed astrocytes to i-EVs pre-treated with Etanercept (ETN, 200 ng/ml), a TNF inhibitor. qPCR analysis showed that i-EVs treatment with ETN inhibited serping-1 and Amigo2 upregulation and induced PTX3 and CD14 expression in vitro, preventing the astrocyte shift towards harmful functions (Fig.4.7c,d). These findings were validated in vivo by co-injecting i-EVs and ETN at myelin lesion in LPC-injected mice, followed by co-staining of astrocytes for A1 marker C3/A2 marker PTX3 and GFAP.

Immunofluorescence analysis revealed that co-injection of i-EVs and ETN induced a significant decrease in the density of C3+ astrocytes at lesions compared to i-EVs-treated mice. However, we observed that the inhibitor caused itself a reduction of the density of PTX3+ astrocytes at myelin lesion, suggesting that ETN itself influence the astrocyte phenotype (Fig.4.7e,f).
Collectively, these data indicate that TNF is involved in the harmful astrocyte transformation induced by i-EVs, despite its pharmacological inactivation is not sufficient to prevent astrocyte conversion in vivo.
Astrocytes transform the pro-differentiating action of i-EVs to inhibitory activity. (a) Representative images of OPCs cultured alone or with astrocytes in the presence or in the absence of i-EVs immunostained for MBP (red), Olig2 (green) and GFAP (cyan) (Scale bars, 20 μm). (b) Corresponding quantifications of MBP+ OPCs (n=2). (c-d) Representative qPCR analysis of A1 (c) and A2 (d) marker expression in unstimulated astrocytes (control), astrocytes exposed to i-EVs, the lipid fraction of i-EVs (lipids), MSC-EVs (left panels). Right panels show A1 and A2 marker expression in astrocytes exposed to i-EVs in the presence/absence of the TNF-a inhibitor etanercept (ETN). Three replicates/condition have been normalized to control. (e) Representative images of saline, i-EVs, or MSC-EVs-injected lesions, immunostained against C3 or PTX3 (red) and Hoechst (blue) (Scale bars, 50 μm). High magnification inserts show astrocytes double stained for GFAP (green) and C3 or PTX3 (red). (f) Density of C3- and PTX3-positive astrocytes at saline, i-EVs-, i-EVs+ETN, or MSC-EVs-injected lesions (C3, number of sections=5-10/group); (PTX3, number of sections=5-10/group). (g) ELISA quantification of IL-1α, C1q, and TNF in 1x10⁶ inflammatory microglia (i-MG) and in MSC-treated microglia (MSC-MG) (n=3).
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4.8 EV-OPC interaction

There is evidence that astrocyte reaction to EVs requires EV-astrocyte contact and an intact protein/mRNA cargoes of the EVs (Drago et al., 2017). However, how EVs interact with OPCs is completely unknown. We explored whether EVs physically interact with OPCs by using optical tweezers combined to time lapse imaging. By this approach, we placed single EVs (quite large EV, which are above the resolution limit of light microscopy) in contact with cultured OPCs and monitored EV-OPC dynamics using time lapse microscopy (Fig. 4.8a). We observed that about 70% of NS-EVs adhered to the OPC surface (17 out of 24), suggesting that most EVs have the capacity to signal to OPCs.

Fig. 4.8 EVs efficiently interact with OPCs. (a) Schematic representation of EV delivery to OPCs by optical tweezers. EVs are first trapped above the OPCs by the IR laser tweezers (left), then the stage is moved in plane (XY) and the objective/trap is moved axially (Z) to set the EVs in contact with the OPCs (middle). The trapping laser is switched off to
check whether EV adheres to the oligodendrocyte membrane (right). (a') Sequence of phase-contrast images showing one example of EV driven to an OPC following the procedure described in a (Scale bar, 10 μm). (b-c) OPCs were maintained in control condition or exposed to intact EVs, broken EVs or the lipid extract of EVs for 2 days, fixed and stained for MBP. Histograms show the percentage of mature MBP+ oligodendrocytes in cultures exposed to broken EVs derived from IL-4 microglia (Brk IL4-EVs) or MSC-treated cells (Brk MSC-EVs) (b; n=3), intact EVs or native lipids (lipids) extracted from IL4-EVs (c; One Way ANOVA p=0.0001 with Holm-Sidak's multiple comparisons test). Data have been normalized to control. (d) Percentage of differentiated MBP+ oligodendrocytes in cultures exposed for 2 days to i-EVs alone or in combination with S-FTY720-Vinylphosphonate, FTY720-Vinylphosphonate alone or S1P. Data have been normalized to control (n=3).

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control vs. brk MSC-EVs ** |
| Fig.4.8c | MBP+DAPI+ cells | One Way ANOVA | P=0.0001 | Holm-Sidak's multiple comparisons test | control vs. intact IL4-EVs **  
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control vs. s-ene ns  
control vs. S1P ns |
CHAPTER V
SUPPLEMENTARY INFORMATION
**Suppl. Fig. 5.1 Raman spectra of broken EVs.** Mean Raman spectra obtained using 532 nm laser line from broken (Brk) EVs of NS or polarized microglia. All spectra were baseline corrected, aligned and normalized before averaging.

**Suppl. Fig. 5.2 MRI analysis of the corpus callosum (CC) of LPC-treated old mice at different time points.** At 5 dpl, lesions along the CC are clearly observed as hypersignal on T2 weighted images (arrows on a, coronal sections; and b, sagittal sections). At 10 dpl, CC signal returned to normal, almost similar to healthy controls. CC demyelination was also observed with the increase of water molecule diffusivity perpendicular to fibers at 5 dpl (c, left image) which was decreased to normal at 10 dpl (c: right image).
Suppl. Fig.5.3 GFP-labelled EVs at the lesion site. (a) Photomicrograph showing GFP+ EVs at the lesion site (dotted line) 20 minutes after injection. (b) Imaris 3D reconstruction of the field of view shown in a. (c-d) High-magnification of the insets in a. (e’-d’) Imaris 3D reconstruction of the field of view in c and d. (e-g) Co-staining of GFP-labelled EVs (green) with Iba1+ microglia (grey, in e), S100β+ (grey, in f) or NG2+ OPCs (grey, g). Inserts on the right are co-staining of GFP+ EVs and the lineage marker (top inset) or DAPI (bottom) to reveal the internalized EVs (red arrowheads). Yellow arrowheads indicate GFP+ EVs outside the cell or anchored to the membrane. Insert e’-g’ Imaris 3D reconstruction of correspondent confocal images. e”-e”’, f’ g”-g”’. High-magnification of Imaris 3D reconstruction. Rotations and zoom have been applied to better visualize the EVs and their relations with the cells. Scale bars: a 10µm and c-g 5µm. (h) Western blot analysis of MVs- and exosomes-enriched fractions produced from rat microglia expressing cytosolic GFP with the indicated antibodies. Note the presence of GFP in both types of EVs. MVs and exosomes purity is indicated by low immunoreactivity for the Golgi marker GS28. The first lane shows 5 µg of cell lysates.
Suppl. Fig.5.4 EVs produced by MSCs are less effective than EVs produced by MSC-treated microglia in promoting myelination in vitro. (a) Histograms show myelination index in OPC-DRG co-cultures exposed to EVs released by MSCs (MSC-derived EVs) or MSC-treated inflammatory microglia (microglial EVs) (number of experiments (n)= 3). (b) Histograms show production of EVs from 1x10^6 MSCs (MSC-derived EVs) or MSC-treated inflammatory microglia (Microglial EVs) during ATP stimulation for 30 min. (c) Size profile of EVs-enriched fraction, pelleted from 1x10^6 MSCs or MSCs-treated microglia. (MSC-derived MVs: Mean±SEM 289.45±158.55, Mode 192.25; Microglial EVs: Mean±SEM 330.53± 162.67, Mode 228.60; n=3).

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CHAPTER VI

DISCUSSION
An increasing number of studies has focused on developing new therapeutic strategies to limit, prevent or reverse the progression of demyelinating diseases such as Multiple Sclerosis (MS) (R. J. M. Franklin & Ffrench-Constant, 2017).

Most of the current therapeutic strategies aim to reduce the impact of the immune system or to enhance endogenous remyelination (Cunniffe & Coles, 2019), a process supported by oligodendrocytes (OLs), the myelin-forming cells of the brain, which are primarily affected in MS (Baumann & Pham-Dinh, 2001; Brambilla, 2019; Goldmann et al., 2013; Miron et al., 2013). However, we still lack a full understanding of the mechanisms underlying the remyelination process and the remyelination failure, which occurs in MS. This knowledge is necessary to identify possible targets or pathways that can be modulated in the cure for MS.

This thesis aimed at advancing current knowledge in MS pathogenesis by characterizing the cross-talk between microglial cells (MG), the immune cells of the brain, and OLs, with the ultimate goal of uncovering novel approaches for promoting myelin repair.

Our results indicate that both MG and astrocytes, the most abundant glial cell in the brain, are able to influence OL homoeostasis in the healthy and lesioned brain. Specifically, we show that reactive MG release extracellular vesicles (EVs) at myelin lesions, which mediate either detrimental or beneficial function on OLs, reflecting the detrimental or pro-regenerative phenotype of donor MG. Importantly, we unveil an unprecedented role for astrocytes in mediating the detrimental effects of EVs released from pro-inflammatory MG at myelin lesion. Finally, we define the molecular components of EVs involved in the promotion of OPCs migration and/or differentiation as well as in the conversion of astrocytes to detrimental oligotoxic cells.

6.1 EVs are key mediators of Microglia-Oligodendrocytes communication

Microglia (MG) play a crucial role in the remyelination process by influencing OL proliferation, differentiation, migration and survival under both physiologic and pathologic conditions. Depending on the microenvironment and phenotype of MG, their impact on OLs can be either beneficial or detrimental (Michell-Robinson et al., 2015). However, the mechanisms underlying MG action are largely unknown.

In the present study, by combining immunohistochemistry and electron microscopy (EM), we show that EVs secreted from MG exert different effects on OLs depending on the stage of MG activation. We demonstrate that EVs released by pro-regenerative MG enhance the recruitment and the differentiation of OPCs at LPC-induced myelin lesions, while EVs released by inflammatory MG (i-EVs) block remyelination. Our study also shows that i) dark, reactive MG infiltrate myelin lesion and
release EVs, as revealed by EM, and ii) EVs mediate MG functions on OLs, reflecting the phenotype of donor cells, upon administration of exogenous EVs to the lesion site.

By analyzing the localization of EVs produced by GFP-labelled MG, we show that EVs remain intact for a short time at the injection site. After 60 min from the injection, GFP\(^+\) EVs are not visible at the lesion site, likely due to lysosomal degradation (Casella et al., 2018) or cell fusion and cargo release (Prada et al., 2018). However, EVs are able to induce long lasting effects on OLs surrounding the lesion. Accordingly, a recent study showed that MG-derived EVs engineered for delivering the anti-inflammatory cytokine IL-4 induce a prolonged immune modulation in the MS experimental model EAE (Casella et al., 2018).

Although the EV field is expanding exponentially, we still lack selective pharmacological or genetic tools to inhibit EV production from MG \textit{in vivo} and explore the effects of endogenous EVs on remyelination. Consolidated evidence indicates that inhibitors of neutral and acid sphingomyelinases decrease EV production in many cell types, including MG. However, these blockers may interfere \textit{per se} with the remyelination process as they alter the sphingolipid pathway (Verderio et al., 2018). Therefore, we used exogenous EVs produced \textit{in vitro} from polarized microglia as a tool to explore the action of microglial EVs.

6.2 Astrocytes transform the pro-differentiating action of i-EVs to inhibitory activity

Several studies have already reported that activated astrocytes can promote demyelination and inhibit remyelination by exacerbating inflammation, modulating BBB integrity and secreting pro-inflammatory cytokines and chemokines (Ponath, Park, & Pitt, 2018). In our study, we demonstrate that astrocytes impair remyelination by mediating the detrimental action of inflammatory MG-derived EVs (i-EVs) on OPCs.

Indeed, \textit{in vitro} data show that i-EVs directly promote the maturation of pure cultured OPCs to mature OLs. However, when OPCs are co-cultured with astrocytes, i-EVs induce a clear block of OPC differentiation, thus mimicking the detrimental action of i-EVs that we found \textit{in vivo} in the LPC demyelination model.

We show that the reduction of OPC differentiation in OPC-astrocytes co-cultures is associated with the transformation of astrocytes into A1 harmful cells, as indicated by their upregulation of the specific A1 markers Serping 1 and Amigo 2, and unaltered expression for the A2 markers Pentraxin 3 (PTX3), CD14 and Tm4sf1 (Liddelow et al., 2017). This finding is in line with our previous study showing that i-EVs are able to induce an upregulation of inflammatory markers in cultured astrocytes (Drago et al., 2017; Verderio et al., 2012).
A1 astrocyte conversion also occurs in the LPC-induced demyelination model, as indicated by the increase in the density of astrocytes positive for the A1 marker Complement component 3a (C3a) and the reduced number of astrocytes positive for the A2 marker PTX3 at the lesion site. Several studies have already reported the detrimental action of A1 astrocytes on OPC differentiation (Clarke et al., 2018; Yun et al., 2018). Indeed, astrocytes ablation in the cuprizone model of demyelination increase the number of mature oligodendrocytes (Madadi et al., 2019), while reactive astrocytes present in both acute and chronic MS lesions impair remyelination and damage OLs (Molina-Gonzalez & Miron, 2019).

The finding that i-EVs induce A1 astrocyte transformation contributes in understanding of the mechanisms and causes of remyelination failure in MS. Our hypothesis is that EVs chronically produced by inflammatory MG impair OPC maturation by transforming astrocytes in A1 harmful cells, which neutralize the direct pro-myelinating action of inflammatory EVs.

In addition, studies in EAE model have shown that depletion of activated astrocytes at the chronic phase of the disease ameliorates EAE clinical score (Mayo et al., 2014) and that remyelination by transplanted OPCs is delayed in the demyelinated area in the presence of astrocytes as compared to astrocyte-free areas (Blakemore, Gilson, & Crang, 2003). More specifically, astrocytes are known to inhibit remyelination by releasing factors that block oligodendrocyte lineage cell responses, including the extracellular matrix component fibronectin (Ponath et al., 2018), CXCL10 (Nash, Thomson, et al., 2011) and endothelin-1 (ET-1) (Hammond et al., 2014b).

Although astrocyte activation has been investigated as potential biomarker for disease progression, the role of astrocytes at distinct stages of myelin damage and repair lack a full understanding. Thus, our findings, by showing that A1 astrocytes inhibit the pro-myelinating action of EVs released by microglial cells, contribute to increase current knowledge on the involvement of astrocytes in the demyelinating processes.

6.3 Inflammatory EV cargo convert astrocytes into harmful cells

The mechanisms underlying astrocyte activation towards A1 phenotype are also currently unknown. Despite the glycolipid lactosylceramide (LacCer) and sphingosine-1-phosphate (S1P) have been involved in astrocyte activation in EAE model (Choi et al., 2011; Mayo et al., 2014; Rothhammer et al., 2017), we rule out a role of EV lipids in astrocyte transformation. Indeed, our results show that lipids extracted from i-EVs do not induce conversion of cultured astrocytes into toxic cells. On the other hand, recent evidence demonstrated that inflammatory MG secrete three mediators IL-1α, TNF-α, and complement 1q (C1q), that are necessary and sufficient for astrocyte conversion
to harmful cells. These factors alter astrocyte gene expression (e.g. upregulation of A1 markers) and function so that astrocytes become less phagocytic, stop promoting synapse formation and start damaging neurons and OLs (Liddelow et al., 2017). In particular, the detrimental contribution of microglial was already TNF indicated by previous studies. By binding TNF-R1 on OLs, microglial TNF induces OL death. TNF overexpressing transgenic mice develop spontaneous demyelination that reverses with anti-TNF administration (Peferoen et al., 2014; Probert, 2015), whereas demyelination is delayed in TNF deficient mice (Korner et al., 1997).

Our results indicate that i-EVs carry IL-1α, TNF-α, and C1q, the three inducers of A1 phenotype in astrocytes, in line with previous studies from the lab showing that i-EVs propagate an inflammatory signal to cultured astrocytes and microglia (Drago et al., 2017; Verderio et al., 2012). Of the three molecules, only TNF is reduced in EVs released from pro-regenerative MG (MSC-EVs) that are not able to induce astrocyte activation. This suggests that the capacity of MSC-EVs to promote myelin repair may be attributed to selective TNF suppression.

To verify the involvement of EV-associated TNF in A1 astrocyte conversion, we inactivated TNF by using the pharmacological inhibitor Etanercept (ETN), a decoy receptor widely used in clinics to treat auto-inflammatory diseases. qPCR analysis for inflammatory A1 and anti-inflammatory A2 astrocytic markers shows that TNF inactivation prevents the upregulation of A1 marker Serping-1 and induces the expression of A2 marker PTX3, indicating that i-EVs fail to activate an inflammatory response in the presence of the inhibitor. Moreover, immunofluorescence analysis reveals a significant decrease in the density of A1 astrocytes positive for C3a in the presence of the inhibitor, although TNF inactivation does not prevent the decrease in the density of A2 astrocytes positive for PTX3 caused by i-EVs in vivo. Collectively, these data indicate that TNF is necessary for i-EVs dependent A1 astrocyte conversion. However, our findings do not exclude that molecules may be sorted in MSC-EVs, such as TGF-β or FGF (Arab et al., 2019), which may counteract the action of the inflammatory cargoes, inhibiting A1 astrocyte transformation.

Increased levels of the cytokine TNF was previously reported in the serum, cerebrospinal fluid of subjects with MS, correlating with severity and progression of disease (Lock, Oksenberg, & Steinman, 1999; van Oosten et al., 1998). In addition, the production of TNF is predominantly sustained by MG or infiltrating macrophages during progressive EAE and its blockade significantly ameliorates clinical symptoms and decreases mortality (Valentin-Torres et al., 2016). However, TNF has been shown to play both detrimental and beneficial roles in MS (Brambilla et al., 2011; Pasparakis & Vandenabeele, 2015). In particular, the membrane bound form of the cytokine can promote tissue repair by activating type 2 TNF receptor (TNFR2) in OLs. Accordingly, serious side effects have been observed in patients treated with TNF blockers, including immune suppression and
demyelination aggravation (Kemanetzoglou & Andreadou, 2017), clearly indicating that TNF inhibitors are not a useful treatment for MS. Nevertheless, the quantification of TNF content in MG-derived EVs collected from MS patients, especially during progressive phase of disease, may lead to the identification of novel biomarker for evaluating disease activity or monitoring the efficacy of therapy.

6.4 Key role of lipids in the pro-myelinating action of EVs

An interesting observation of our study is that the lipid component of MG-derived EVs is involved in the promotion of OPC migration and/or differentiation. Interestingly, our lipid measurements indicate that microglial EVs contain sphingosine-1-phosphate (S1P), a known chemoattractant agent (A. Kimura et al., 2008) that promotes OPC migration, a crucial step in the remyelination process. In addition, analysis of OPC migration indicates that the S1P receptor antagonist S-ene-FTY720 Vinlyphosphonate (S-ene) completely prevents EV-dependent OPC attraction, highlighting a key role for vesicular S1P as attractive guidance cues for OPCs.

In line with our findings, previous studies showed that S1P either favors or inhibits extension of oligodendrocyte processes depending on whether it activates the S1P receptor type 1 (S1P1) or type 5 (S1P5) (Groves, Kihara, & Chun, 2013), and that the interaction in OLs between S1P and signalling factors, such as neurotrophin-3 (NT-3) and platelet-derived growth factor (PDGF), has profound effects on OL development and myelination (Halmer, Walter, & Fassbender, 2014). Importantly the pan S1P antagonist S-ene-FTY720 Vinlyphosphonate used in our study is a molecule different from FTY720 or Fingolimod, an analog of sphingosine 1P used as drug to treat MS. The latter drug acts by reversibly retaining central memory T cells and naive T cells in lymph nodes, thereby reducing the recirculation of autoreactive lymphocytes to the CNS. Other effects of Fingolimod consist in reducing B cell activation, interfering with the production of eicosanoid inflammatory mediators in mast cells (Payne et al., 2007), or changes in the permeability of the blood brain barrier (BBB) (Foster et al., 2009). Due to its lipophilic nature, FTY720 also crosses the BBB, accumulates in the CNS and binds to all of the high-affinity S1P receptors except S1P2 expressed in brain cells (Brinkmann & Lynch, 2002). Therefore, FTY720 also exerts direct actions on neurons, microglia, astrocytes and oligodendrocytes. Specifically, the drug promotes OL proliferation and differentiation primarily via S1P3 signalling (Coelho, Saini, & Sato-Bigbee, 2010). No effects of FTY720 treatment on OPC migration have been described. However, our results suggest that OPC attraction at the lesion site may represent an additional mechanism underlying the beneficial action of FTY720 on myelin lesions in MS.
Besides S1P, other chemotactic molecules, such as Wnt3a (Hooper et al., 2012) and the endocannabinoid anandamide (Gabrielli et al., 2015), are present in MG-derived EVs thus supporting the important role of EVs in packaging and transferring chemotactic signals (Kriebel et al., 2018). Through EV fractionation, we also show that the direct pro-myelinating function of microglial EVs is mediated by their lipid content. However, we exclude a role of S1P in the pro-differentiating activity of EVs, as this was not abrogated in the presence of the S1P receptor antagonist. Additional investigations are required to identify the lipid components mediating the pro-differentiating activity of EVs. This will be of great help to design new therapeutics to foster myelin repair.

Notably, previous studies showed a role of miRNA cargo of EVs on remyelination. Indeed, exosomes isolated from the serum of rats exert pro-myelinating activity by transferring to OPCs miR-219, a miRNA that controls the expression of multiple genes involved in OPC differentiation (K. M. Pusic et al., 2016). Although miR-219 is contained in MG-derived EVs and upregulated in i-EVs (Prada et al., 2018), our study supports a major role of EV lipids rather than miRNA in pro-differentiating activity of microglial EVs. Indeed, our biochemical experiments demonstrate that broken EVs, depleted of luminal miRNAs, as well as lipids extracted from EVs are able to promote OPC maturation, prompting us to conclude that the RNA and/or protein cargoes of EVs do not play a major role in the pro-myelinating action of EVs.

### 6.5 The pro-myelinating effect of MSCs is largely mediated by microglia

Cell-based therapies represent a promising strategy for boosting myelin formation and repair (Scolding et al., 2017). Thanks to their homing properties and immunosuppressive functions, mesenchymal stem cells (MSCs) are the most attractive tool for treatment of demyelinating diseases. Several lines of evidence demonstrated that co-transplantation of syngeneic MSCs with allogeneic OPCs increases OPC migration and maturation (Cristofanilli et al., 2011).

In addition, transplantation of MSCs or neural precursor cells (NPCs) not only ameliorates EAE but also stimulates CNS repair. These pro-regenerative effects are due to the release of soluble factors (Freedman et al., 2010) and the capacity of MSCs to shift the local inflammatory microenvironment to a pro-regenerative one (Cusimano et al., 2012).

In our study, we show that MSCs induce a neuroprotective phenotype in MG and counteract the detrimental response of inflammatory MG. We also reveal that EVs released from MSC-conditioned MG (MSC-EVs) favour OPC differentiation and myelin deposition both *in vitro* and at myelin lesion. Interestingly, their pro-myelinating activity exceeds that of EVs released from IL4-treated MG (IL4-EVs) or directly produced by MSCs, suggesting that MSCs are more effective than the classical polarizing agent IL-4 in directing MG towards a beneficial phenotype. Our findings also suggest that
the well-documented beneficial effect of MSCs in experimental model of MS is largely mediated by their impact on MG.

The versatility and the beneficial properties of MSCs make them an attractive candidate for repair strategies. However, transplantation procedures and risk of tumor formation and tissue rejection in patients limit their therapeutic use. Defining which molecular components of MSC-EVs drive remyelination and limit astrocyte activation may be useful for developing alternative therapeutic approaches for MS. Further work remains to be done to define the minimal components of MSC-EVs required to drive efficient remyelination.

6.6 Therapeutic Potential of Extracellular Vesicles

The pathogenesis of several diseases has been shown to be linked to EVs, including cancer (Robbins & Morelli, 2014), neurodegenerative diseases (Basso & Bonetto, 2016) and demyelinating diseases (Selmaj, Mycko, Raine, & Selmaj, 2017; Verderio et al., 2012). On the other hand, EVs are also used as a treatment for modulating the course of disease. Indeed, EVs are able to cross biological barriers, carry a wide array of nucleic acids and mediate their functional delivery into recipient cells (Valadi et al., 2007). As such, EVs hold great potential as a drug delivery vehicle, especially for delivery of biotherapeutics, which may be loaded in- or onto EVs.

However, there are some hurdles to overcome in EV application in therapy, such as the choice and characterization an appropriate cell source for EV, the optimization of loading procedures and isolation methods and the evaluation of EV safety/toxicity.

Mesenchymal stem cells (MSCs) and dendritic cells (DCs) are widely used as source of EVs, at least in certain disease settings, owing to their immunomodulatory properties (Wiklander, Brennan, Lotvall, Breakefield, & El Andaloussi, 2019). Clinical trials and numerous preclinical studies indicated that the use of autologous cell EVs is safe and well tolerated in patients. However, xenogeneic EVs may be also tolerated in vivo. Accordingly, EVs derived from human MSCs are tolerated and functional in immune-competent mice (Gyorgy, Hung, Breakefield, & Leonard, 2015). Other cell sources such as endothelial progenitor cells for using in myocardial infarction (C. W. Chen et al., 2018) or amnion epithelial cells for treatment of lung fibrosis (Tan et al., 2018) are ongoing in testing.

In addition, EVs can be produced by cells engineered to stably express the therapeutic of interest in order to enrich the specific RNA or protein drug in EVs.

To use EVs as off-the-shelf therapies, stability and storage of EVs must be examined.
EVs are intrinsically stable in circulation, due to their negatively charged surface and their ability to avoid the immune system reaction (de Jong et al., 2019). By contrast, other studies showed that EVs are rapidly cleared following intravenous injection (Kooijmans et al., 2016; Lai et al., 2014). Given that the storage conditions can affect EV integrity and biophysical properties (Elsharkasy et al., 2020)), once purified and modified, EVs must be stored in specific container, dissolved in an appropriate storage buffer at controlled temperature. They are often kept at -80 °C in presence of cryoprotectants (CPAs) or trehalose, which decrease osmotic damage and increase EV stability and functionality (Witwer et al., 2013). A storage alternative is lyophilisation or freeze drying of EVs (Elsharkasy et al., 2020).

Another aspect that need to be addressed is the safety of EVs in the clinical use. Compared to virus-derived vehicles or cell therapies, EVs are relatively safe, non-replicative, not mutagenic, not antigenic and do not induce adverse effects or neoplasia formation in treated patients. The low toxicity of EVs has been largely demonstrated in clinical trials (Wiklander et al., 2019), as well as numerous preclinical studies (X. Gao et al., 2018; Kamerkar et al., 2017; Zhu et al., 2017). For instance, intrapleural injection of tumour cell derived EVs loaded with chemotherapeutics has shown minimal toxicity and more clinical benefits (Guo et al., 2019). No adverse effects have been found in individuals treated with bone marrow MSC EVs in bone regeneration, or with umbilical cord MSC EVs for treating retinal lesions and chronic kidney disease (Nassar et al., 2016; X. Zhang et al., 2018), or with autologous DC derived EVs for activating the natural killer cell immune response (Morse et al., 2005). However, it is still necessary to assess whether the clinical application for EVs is really safe and efficacy, given that several trials use therapeutic EVs produced from immortalized cell lines, which may carry oncogenic material.

Despite the hurdles and the technical difficulties, the research in this field is constantly increasing and the therapeutic potential of EVs in different diseases still remain intact.

6.6.1 Clinical application Extracellular Vesicles in MS

Several studies demonstrated the therapeutic potential of EVs in Multiple sclerosis. For example, exosomes isolated from pregnant mice serum or human periodontal ligament stem cells reduce EAE by inhibiting immune response and by promoting OPC recruitment into lesions (Rajan et al., 2016; J. L. Williams et al., 2013). In addition, the intranasal injection of glioblastoma-derived exosomes loaded with curcumin ameliorates EAE course (Zhuang et al., 2011), while that of exosomes enriched in miR-219 favor myelin regeneration in aged rats (A. D. Pusic & Kraig, 2014).
In this context, our study reinforces the therapeutic potential of EVs or EV mimetics in remyelination and MS therapy. The use of EVs as biopharmaceuticals for treating MS requires that EVs are able to cross the BBB, reach the CNS, target OPCs and no other cells, and have minimal side effects.

The intranasal route may represent an efficient tool for delivering EVs directly to the brain. This is supported by experiments conducted in a model of Parkinson’s disease (PD), in which macrophage-derived exosomes loaded with catalase are reported to accumulate in the brain and mediate neuroprotection (Haney et al., 2015).

Among different and efficient sources, established cell lines or cells isolated from patient or compatible donor are the best sources of EVs in MS therapy (Osorio-Querejeta, Alberro, Munoz-Culla, Mager, & Otaegui, 2018). Biofluids such as plasma or urine are also an alternative. However, EVs produced in vitro still remain more reproducible and easy to manage.

Interestingly, EV loading with exogenous cargoes before administration is not always necessary for therapeutic effects in the CNS, as recently demonstrated in a status epilepticus mouse model. Long and colleagues demonstrated that delivery of unmodified exosomes derived from human bone marrow MSCs reduce neuron loss and inflammation, supporting a replacement of MSCs-based therapies with EV-based therapies (Long et al., 2017).

Despite these promising results obtained by using non-modified EVs (A. D. Pusic & Kraig, 2014; A. D. Pusic, Pusic, Clayton, & Kraig, 2014), the modification of EV cargo by bioengineering techniques may further increase their therapeutic potential. For instance, EVs derived from cells overexpressing specific miRNA (miR-138, miR-219 and miR-338) have been shown to induce OPC differentiation and remyelination by miRNA delivery (de Faria et al., 2012; Dugas et al., 2010; H. Wang et al., 2017), while vesicles loaded with curcumin reduce inflammation and astrogliosis (Kalani et al., 2016).

Notably, EVs can be modified not only in their cargo but also in their surface. The expression of surface molecules targeting membrane receptors on target cells would increase specific uptake of EVs by recipient cells, while the enrichment in surface lipids playing beneficial functions, as suggested by our results, may improve their beneficial impact on OLs.

Which are the therapeutic implication of our study?

Currently, it is not possible to obtain microglia from MS patients and is still technically challenging to differentiate human induced pluripotent stem cells (iPSCs) into microglia and likely not ready for clinical trials in the next 5 years. Nevertheless, these data will be invaluable in linking improvements in iPSC culturing protocols align with the capacity to designs appropriate therapies with them. Moreover, deeper knowledge of MSC-EV composition may lead to the assembly of EV mimetics, containing selected luminal components (proteins and/or RNAs) of MSC-EVs in combination with
pro-differentiating lipid(s), which may be used as tool for boosting myelin repair. The low complexity and the low chance of off-target effects of EV biomimetics make them ideal tools to develop novel therapeutic approaches in chronic MS.

Despite the implication of EVs in remyelination is well demonstrated, the knowledge on their use in MS therapy is just beginning and needs more investigations.

6.7 Future Plans

Our study contributes to MS research by defining the role of microglial EVs in the cross-talk between microglia and astrocytes and oligodendrocytes, and adding an important piece of information towards the understanding of the mechanisms possibly responsible for remyelination failure in the disease.

The most relevant findings have been obtained by taking advantage of the lysolecithin mouse model of focal myelin lesion, which offers spatial and temporal control over de- and remyelination. Nevertheless, we aim to validate our results in experimental autoimmune encephalomyelitis (EAE) mouse model, given that this model shows major pathologic characteristics of human MS.

EAE model is an induced inflammatory disease following the induction of the immune response against CNS-specific antigens, thus leading to demyelination and neurodegeneration (Burrows et al., 2019). Microglia and astrocytes are present in both acute and chronic demyelinating lesions in EAE, contributing to both disease development and progression (Constantinescu, Farooqi, O'Brien, & Gran, 2011). Importantly, EAE is the only animal model that exhibits a numerical point severity scale (Osorio-Querejeta et al., 2017), similar to the expanded disability scale (EDSS) in MS which describes disease progression in humans.

We will assess the impact of EVs released by inflammatory (i-EVs) or pro-regenerative microglia (MSC-EVs) on remyelination in EAE mice. EVs will be administered in mice by single intrathecal injection the day of clinical onset, as well described in (Casella et al., 2018). The status of inflammation and myelination will be evaluated at 30 days post-injection. Inflammation will be studied by evaluating gliosis, inflammatory cell infiltration and expression of inflammation indicators including GFAP and Iba1, whereas remyelination will be studied by luxol fast blue staining and evaluating the expression of oligodendrocyte specific markers NG2 and MBP, along with specific oligodendrocyte transcription factors.

We expect that the results described in this thesis will be confirmed in EAE mice. However, any outcome will be equally informative and will increase our knowledge on the role of microglial EVs on MS clinical outcome.

Furthermore, our findings shed light on the involvement of astrocytes in the demyelination process in response to i-EVs, thus elucidating the mechanisms involved in impaired oligodendrocyte
differentiation and myelin deposition. However, the molecular components of i-EVs driving astrocytes towards harmful function are unknown.

Given that we have found that EV lipids are not able to induce A1 astrocyte conversion, we will focus on the three inflammatory cytokines IL-1α, TNF-α, and C1q present in the lumen of i-EVs and which are known to be essential for A1 conversion. We will test in vitro their specific contribution in astrocyte activation by using neutralizing antibodies (anti-C1q blocking Ab (Hong et al., 2016) or anti-TNF-a neutralizing Ab (Joshi et al., 2014) or IL1-a neutralizing Ab (Cohen et al., 2011)) or pharmacological inhibitors available for each cytokine (for TNF-a: infliximab (Crisafulli, Galuppo, & Cuzzocrea, 2009) or etanercept or Xpro (Clausen et al., 2014); for C1q: Peptide 2J (Roos et al., 2001); for IL1a: MABp1 (Reichert, 2017)). Briefly, EVs will be pretreated with cytokine specific Ab/drug and then incubated with cultured astrocytes in the presence of the Ab/drug before qPCR analysis for A1 and A2 markers. The treatment (Ab/drug) most effective in preventing A1 transformation will be validated in vivo, by injecting EVs pre-treated with the selected Ab/drug at myelin lesion in LPC-injected mice, followed by co-staining of astrocytes for C3/PTX3 and GFAP.

The effects driven by i-EVs will be compared to those induced by MSC-EVs, known to favor remyelination and limit astrocyte activation.

We expect that the inflammatory cargo of i-EVs is responsible for astrocyte activation. However, EVs contain multiple bioactive molecules including mRNAs and miRNAs, which may participate to harmful astrocyte conversion. These experiments will be fundamental to increase current knowledge on astrocyte-MG crosstalk at myelin lesion and to identify new therapeutic targets that can be modulated in MS.

Another aspect that we will investigate is the lipid composition of microglial EVs, given that EV lipids display a pro-differentiating activity on oligodendrocytes. To this aim, by using Raman spectroscopy (RS) analysis we will compare the EV spectra with those of the main lipids of the double membrane layer (cholesterol, phosphatidylcholine, sphingomyelin, ceramide) in order to identify and quantify the lipid species present in EVs.

We will expect to obtain an overall biochemical characterization of microglial EVs by RS and, by taking advantage of additional techniques such as lipidomics combined to analysis of OPC differentiation, to uncover the lipid species involved in EV dependent oligodendrocyte differentiation. These results may lead to the production of EV mimetics containing functional lipids able to promote oligodendrocyte differentiation and myelin repair in MS patients.

Importantly, it will be necessary to check that EVs or EV mimetics do not cause excessive OPC differentiation, because misplaced or excessive myelination could also affect brain functions (Almeida et al., 2018). Hyper-myelination, however, is an unlikely event requiring ample supply of
an energy substrate to the myelin forming cells in order to sustain the metabolic costs for myelin synthesis.

In conclusion, we believe that EVs constitute one of the more promising fields of investigation in myelin repair and we hope that our findings can set the stage for exciting developments in the near future.
REFERENCE LIST


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