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MicroRNAs expression in serum exosomes from Multiple Sclerosis (MS) patients and the role of exosomes derived from MS patients and Mesenchymal Stem Cells to modulate the inflammatory profile of microglia

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INTRODUCTION

Chapter 1

Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) characterized by the formation of inflammatory plaques disseminated in the white matter of the brain and spinal cord with consequent demyelination, loss of oligodendrocytes and axonal damage. The etiology is still unknown, and the clinical evolution of the disease is variable.

MS patients manifest variable symptoms which include blurred vision, loss of balance, poor coordination, slurred speech, tremors, numbness, extreme tiredness, memory and concentration problems, painful muscle spasms, sleep disturbances, ataxia and serious conditions such as paralysis and blindness (Mulakayala et al., 2013). The accumulation of disability is quantified in clinical practice with the Expanded Disability Status Scale (EDSS) (Fisniku et al., 2008). The EDSS is an ordinal scale ranging from 0 (normal neurologic examination) to 10 (death owing to MS).

In 1996 by the U.S. National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis, four MS phenotypes have been proposed: relapsing remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS), and progressive relapsing MS (PRMS). In 2013, the Committee (sponsored by NMSS and the European Committee for Treatment and Research in MS) reviewed the proposed nomenclature by eliminating the PRMS and introducing two new disease courses: radiologically isolated syndrome (RIS) and clinically isolated syndrome (CIS) (Lublin et al. 2014).

1.1 - Radiologically isolated syndrome (RIS)

The term RIS identifies patients with magnetic resonance imaging (MRI) abnormalities highly suggestive of demyelination in the absence of clinical signs or symptoms. Although RIS is not considered a distinct MS phenotype (Lublin et al. 2014), those patients are at increased risk of developing clinically definitive MS (CDMS) in the future.

1.2 - Clinically isolated syndrome (CIS)

CIS is an established disease course characterized by a first clinical event highly suggestive of demyelinating CNS disease that is isolated in time or not preceded by any neurologic event. It usually causes optic neuritis, a brainstem syndrome, or an incomplete transverse myelitis (Miller et al., 2012; Miller et al., 2005).

Like RIS, certain demographic and imaging characteristics influence the risk of CDMS conversion. The presence and number of T2 white matter lesions and abnormal cerebrospinal fluid (CSF) profile, defined as an elevated IgG index or the presence of oligoclonal bands (OCBs), are the two predictors most used in clinical practice.

1.3 - Relapsing remitting MS (RRMS)

The most common MS phenotype, found in about 85% of MS patients, RRMS, is characterized by alternating periods of neurological dysfunction (relapses) and periods of relative clinical stability free of new neurological symptoms (remissions).

1.4 - Primary progressive MS (PPMS)

PPMS affects about 10%–20% of patients who will develop this disease phenotype, characterized by the lack of initial RR phase and ongoing progression from the disease onset (Compston and Coles 2008; Ransohoff et al. 2015). Pathology in PPMS is complex and includes neurodegeneration occurring along with mild-to-moderate inflammation.

1.5 - Secondary progressive MS (SPMS)

It is the form of disease into which most patients with RRSM convert, characterized by a progressive and irreversible course similar to that of PPSM. The pathology in SPMS involves some degree of persistent inflammation combined with neurodegeneration caused by mitochondrial dysfunction and resultant axonal damage.

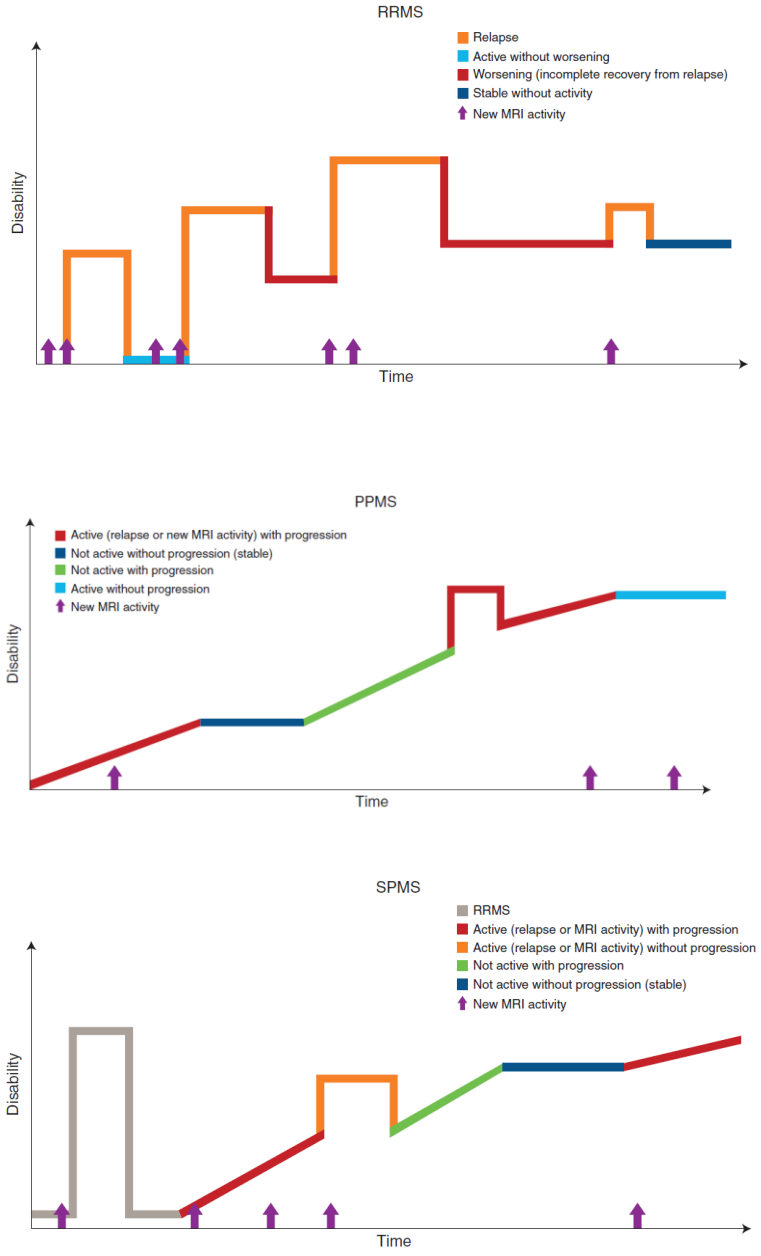


Figure A. Disease courses of Multiple Sclerosis (MS). RRMS, Relapsing remitting multiple sclerosis; PPMS, primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; MRI, magnetic resonance imaging (from the National Multiple Sclerosis Society).

1.6 - Epidemiology

MS mainly affects young people, between 20 and 40 years of age, with a female predominance (Noseworthy et al., 2000; Compston and Coles 2008) The global median prevalence of MS is 33 per 100,000 people, with the highest prevalence in North America and Europe and the lowest in Asia and sub-Saharan Africa countries (Belbasis et al., 2015). In Italy, the hypothesized prevalence is 70 per 100,000 people; the region with the highest prevalence is Sardinia due to genetic isolation (Rosati, 2001; Marrosu et al., 2002).

1.7 - Etiology

The ultimate cause of MS is unknown, and a multifactorial etiology is accepted. Thus, genetic and environmental factors have been identified that influence the risk of disease onset. Genetic studies revealed highly polygenic inheritance, indicating the HLA (Human Leukocyte Antigen) region as the main locus associated with the disease (Olerup and Hillert, 1991). In particular, the HLA-DR1501 and HLA-DQ060 alleles, which encode T-cell restriction elements, are associated with a 2-4-fold increased risk of developing MS in the white population (Olerup and Hillert, 1991). The existence of 350 potential susceptibility genes was also estimated in addition to the MHC complex (Major Histocompatibility Complex) of which 50 loci with confirmed association with MS: among these are included the genes involved in the metabolism of vitamin D (Australia and New Zealand Multiple Sclerosis Genetics Consortium, 2009; Wang et al., 2011; Sawcer et al., 2011) and polymorphic variants of the genes for IL2Rα, IL7R and CD58 (Hafler et al., 2007). Environmental risk factors are mainly infectious agents including Epstein-Barr virus (Cullen et al., 2012), measles virus, endemic mumps, rubella, herpes virus (HHV-6) and MSRV retrovirus. Risk factors are also vitamin D deficiency and cigarette smoking (Cullen et al., 2012).

1.8 - Pathogenesis: activation of autoimmunity

MS pathogenesis implies the entry into the CNS of autoreactive T lymphocytes, specific for myelin antigens, with activation of the immune system and consequent myelin sheath and oligodendrocytes

destruction, axonal damage and neuronal loss. The first event is represented by the activation in the periphery of the autoreactive T lymphocytes specific for myelin antigens and their entry into the CNS. It is still unclear how T cells expressing receptors (T cell receptors, TCRs) specific for myelin antigens can be activated in the peripheral immune compartment since their specific antigen is sequestered in the intrathecal compartment and, therefore, not presented to peripheral T cells (Mulakayala et al., 2013). However, different mechanisms of activation of autoreactive T lymphocytes have been hypothesized. The main one is represented by molecular mimicry: there is the possibility that self-reactive T cells are activated in the peripheral immune compartment by cross-reactivity with non-self-antigens, of viral or bacterial origin (Korn, 2008). Some lymphocytes may also express a double TCR: a specific TCR for a viral antigen, which determines the activation of the clone, and a specific TCR for the myelin antigen, responsible for the subsequent autoimmune reaction. The hypothesis of the existence, on the β chain of the TCR, of a superantigenic binding site shared by different lymphocyte clones, would provide a further explanation for the simultaneous activation of numerous T lymphocytes, specific for different antigens, including autoantigens. Finally, self-reactive T lymphocytes could be activated by antigen-independent mechanisms such as: the high concentration of pro-inflammatory cytokines, the change in the expression of the autoantigen and the breakdown of the blood-brain barrier (BBB) with consequent loss of antigenic sequestration. TCRs of autoreactive clones can recognize different epitopes of both surface proteins and secreted by CNS cells. Possible candidates are myelin antigens expressed by oligodendrocytes, neuronal antigens and antigens of infectious agents epidemiologically associated with MS (Levin et al., 2005; Soldan et al., 1997). The most represented antigens are shown in **table 1**.

PROTEIN (%)	MOLECULAR WEIGHT (kDa)	LOCALIZATION
MBP (Myelin Basic Protein) (30%)	18.5	cytoplasm of oligodendrocytes and myelin membrane
PLP (Proteolipid protein) (50%)	30	myelin membrane

MOG (*Myelin Oligodendrocyte Glycoprotein*) (≤0.1%)	54	myelin membrane, on the surface of the oligodendrocytes
MAG (*Myelin-associated glycoprotein*) (1%)	100	myelin membrane, periassonal space
CNPase (2', 3'-cyclic nucleotide 3'-phosphodiesterase) (4%)	46	cytoplasm of oligodendrocytes and myelin membrane
αβ CRYSTALLIN	23	intracellular in oligodendrocytes and astrocytes
S100β	21	Non-myelin antigen: cytosolic protein, which binds Ca2 + in astrocytes

Table 1. Potential autoantigens in MS.

1.9 - Pathogenesis: role of the immune system cells

Upon their activation, autoantigen specific CD4 + T cells cross the BBB and enter the CNS. The presence of metalloproteases (MMP) and gelatinase, together with the action of cytokines, interleukin (IL)- 17 and IL-22, causes an impairment of the BBB, making it more permeable to the passage of further autoreactive leukocytes in the brain parenchyma. CD4 + T lymphocytes localize in the perivascular spaces of the CNS and are reactivated here by the antigens presented on the dendritic and microglial cells - which up-regulate the expression of MHCII molecules - and by pro-inflammatory cytokines released locally. CD4 + T lymphocytes recruit macrophages which, by releasing pro-inflammatory cytokines (IL-1, IL-6, tumor necrosis factor (TNF) α), nitric oxide and MMP, cause tissue damage especially in oligodendrocytes and neurons. Activated perivascular macrophages, in turn, by expressing MHC class II molecules, process and re-present antigen to T cells. Dendritic cells recruited into inflammatory CNS lesions capture and process antigen, migrate to cervical lymphnodes through the cerebro-spinal fluid (CSF) and, mainly localizing in the follicular area, activate the B cell response (De Vos et al., 2002; Hatterer et al., 2006). CD8 + T lymphocytes invade the tissue parenchyma, localizing mainly in the central area and at the edges of the lesion (Hemmer et al., 2006). In addition

to secreting pro-inflammatory molecules, they exert a direct cytolytic effect on neurons and oligodendrocytes. T helper lymphocytes (T CD4 +), both Th1, Th2 and Th17, show a critical role in onset and especially in the progression of the disease. Following the activation of TCR, in the presence of co-stimulatory molecules, naïve T cells, depending on the cytokine context, can differentiate into different cell types, with specific effector functions (**figure B**).

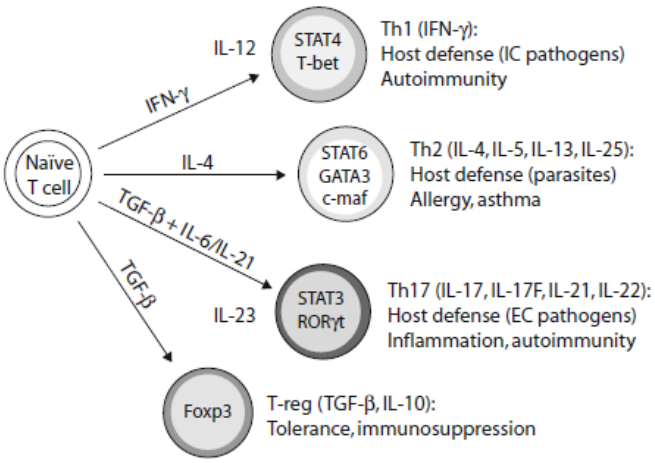


Figure B. Differentiation of naïve T lymphocytes (Korn, 2008).

Th1 lymphocytes produce large quantities of pro-inflammatory cytokines, such as interferon (IFN)- γ and IL-12 and their differentiation is determined by IL-12 and IFN-γ; IL-4 determines the differentiation of Th2 that produce the anti-inflammatory cytokines IL-4 and IL-13; Th17 produce IL-17 and IL-26 and their differentiation is supported by IL-21 and IL-23 (Korn, 2008; Esendagli et al., 2013). IFN-γ produced by autoreactive Th1 during MS exerts pro-inflammatory effects on various cell types such as:

- brain endothelial cells, by inducing adhesion molecules and chemokines expression and tight junctions destruction;
- microglia and macrophages, by inducing MHC class I and II molecules expression, cytokines, nitric oxide and protease production, antigenic presentation and phagocytosis;
- astrocytes, by inducing antigenic presentation and cytokines production;
- oligodendrocytes, by inhibiting proliferation and differentiation.

Th1 cytokines levels are higher during relapses, whereas Th2 cytokines are prevalent in remission phase of the disease. The key mediators of neuroinflammation, tissue damage and MS progression are Th17 lymphocytes, localized in active brain lesions (Korn, 2008; Amedei et al., 2012; Esendagli et al., 2013; Imam et al., 2007). In MS patients, Th17 across the BBB more effectively than Th1 due to the greater expression of chemokine receptor CCR6 and adhesion molecules to the endothelium (Brucklacher-Waldert et al., 2009). In addition, they show high levels of basal activation, increased proliferative capacity and lower susceptibility to suppression by T regulatory cells (Tregs) compared to Th1 (Brucklacher-Waldert et al., 2009). In inflammatory lesions, Th17 activation is supported by IL-23 product by tissue antigen presenting cells (APCs). The activation of Th17 is followed by the consequent cytotoxic activity on neurons and by the production of IL-17, IL-26 (Kebir et al., 2007; Dong, 2008) and IL-22 (Amedei et al., 2012). The interaction between IL-17 and its receptors expressed on myeloid and epithelial cells determines protease, pro-inflammatory cytokines and chemokines up-regulation thus boosting the immune response (Gaffen, 2009; Akdis et al., 2011). IL-26, a member of the IL-10 family of immune-regulatory cytokines, seems to play a protective role although its specific function is not yet known (Akdis et al., 2011). B lymphocytes are also activated in the lymphnodes, in the presence of specific autoantigenic T-helper cells, and across the BBB locating itself in perivascular space and meninges. Differentiated into plasma cells, they release IgG immunoglobulins specific for myelin antigens (MBP and MOG), but also for DNA and neurofilament (Cross et al., 2001). These antibodies, binding to specific autoantigens expressed on oligodendrocytes and neurons, activate the complement classical pathway or induce antibody-mediated phagocytosis by macrophages, resulting in destruction of target cells. Furthermore, B lymphocytes amplify the inflammatory response through pro-inflammatory cytokines secretion (lymphotoxin, LT and TNF) and antigens presentation to auto-reactive T lymphocytes. B cells response is compartmentalized in CNS and characterized by constant antigenic stimulation. Indeed, in MS meninges lymphnode-like aggregates have been observed, consisting of B, T, dendritic and stromal cells. In these ectopic lymphoid organs, auto-reactive B lymphocytes undergo somatic hypermutation, clonal selection and differentiation into plasma cells,

typical reactions of the germinal center of lymphnode follicles (Uccelli et al., 2005). The auto-antibodies intrathecal synthesis is finally proved by the oligoclonal bands presence in MS patients CSF. Also, astrocytes, responsible of CNS homeostasis and interactions between the latter and the immune system, are involved in inflammation mechanisms and tissue damage in MS pathogenesis. They, being responsive to IFN-γ, up-regulate the expression of IL-17 and IFN-γ by T lymphocyte (Amedei et al., 2012), produce chemokines for T and B cells, macrophages and dendritic cells recruitment and secrete M-CSF (Macrophage Colony-Stimulating Factor) e GM- CSF (Granulocyte-macrophage colony-stimulating factor) for microglia activation. Microglia, following interaction between antigen and TCR of auto-reactive Th1 and co-stimulation mediated by CD40-CD40L and IFN-γ, releases IL-1 e TNFα which increase the adhesion molecules expression on endothelial cells and astrocytes cytokines production, thus amplifying inflammation. Microglia also performs myelin-toxic and neurotoxic functions following the production of reactive oxygen and reactive nitrogen species (ROS and RNS), proteolytic enzymes and excitotoxins. Inflammatory processes lead to myelin sheath destruction mediated by T cells, auto-antibodies and microglia/macrophages activation products. Following demyelination, oligodendrocytes and undifferentiated glial progenitors can proliferate and reconstitute the myelin sheath; even microglia IL-1 and TNFα, with astrocyte PDGF (Platelet-Derived Growth Factor) and NGF (Nerve Growth Factor), are involved in remyelination and neuroprotection. However, remyelination in MS brain lesions is often incomplete and reactive astrocytes form glial scars, thereby preventing the functional recovery of the damaged area. The described mechanisms are summarized in **figure C**. The loss of the myelin sheath, preventing the saltatory conduction of the action potential, slows down the nerve impulse conduction causing the neurological symptoms typical of clinical relapses. But axonal damage and the consequent neurodegeneration determine permanent functional damage in MS patients.

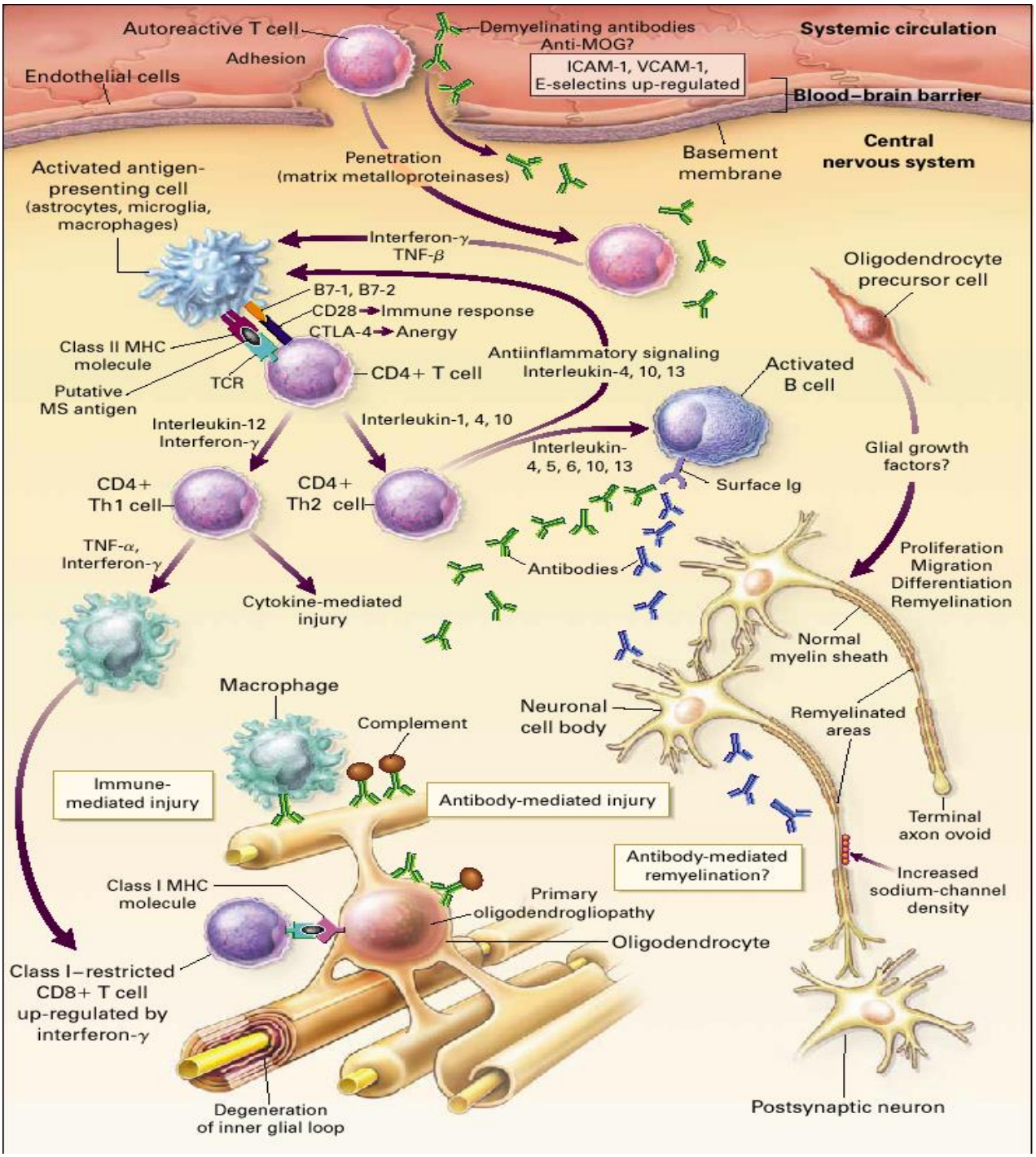


Figure C. Pathogenetic mechanisms in Multiple Sclerosis (Noseworthy et al., 2000).

1.10 - Diagnosis

Before 2001, the MS diagnosis, excluding other possible underlying causes of the neurological disorder, was placed in the presence of two neurological episodes involving different parts of the CNS and

distinct over time (the classic "Poser criteria"). After 2001, new diagnostic criteria began to be used (the so-called "McDonald's criteria") which are highly likely to recognize or exclude multiple sclerosis, especially using magnetic resonance imaging (MRI), in the months following the onset of the first disorder neurological (Brownlee et al., 2015). The latest version of the "McDonald criteria" is from 2017. The diagnostic process may sometimes be long and complex because MRI reports are uncertain or there are other elements that suppose the presence of other diseases. The diagnosis is based on clinical history and complaints of the patient (anamnesis) and on the physical examination of the patient (general and neurological); the diagnosis is the result of the convergence of clinical, instrumental and laboratory elements. Currently the most important instrumental examination for the diagnosis of MS is conventional MRI (Aliaga and Barkhof, 2014), useful for early diagnosis. The presence of demyelinating lesions compatible with those typical of MS, their location and the fact that they take contrast (i.e. change image characteristics after gadolinium administration) allows the MS diagnosis. However, other disorders could have similar MRI alterations (Bot et al., 2002) or even MRI signal alterations could be entirely non-specific and not directly correlated with the neurological disorder. Therefore, to do a correct diagnosis, the MRI must always be interpreted by the neurologist in the light of the disorders complained by the patient, the clinical history, the neurological physical examination and any other tests deemed necessary. Between these fall the search for anti-phospholipid antibodies (for the so-called Hughes syndrome), anti-nucleus antibodies (ANA) and other autoantibodies (e.g. ENA), the dosage of complement fractions, inflammation indices (to evaluate the presence of connective tissue diseases) and vitamin B12 (for deficiency syndromes). To improve MRI diagnostic accuracy, the CSF examination through lumbar puncture (rachicentesis) is considered useful (Freedman et al., 2005). The CSF examination is indicative for multiple sclerosis ("positive") if "oligo-clonal bands" (OCBs) are found thanks to a particular laboratory test (Arrambide et al., 2018; Dobson et al., 2013). OCBs are made of immunoglobulins which tend to be very similar to each other. If OCBs are found in the CSF but not in the blood, they are an expression of the immune system activation within the CNS and are an element in favor of the diagnosis of MS. Unfortunately, CSF analysis is not very convenient for patients because rachicentesis could be unpleasant and cause pain. Much effort

has been made to identify potential biomarkers for MS. Paul and colleagues classified MS biomarkers into clinically used, predictive and diagnostic biomarkers (Paul et al., 2019). In the last few years, osteopontin, basic myelin protein, KIR4.1, neurofilament light chain, GFAP and other proteins have been suggested as potential MS biomarkers. However, very often the clinical relevance of these potential biomarkers has proved to be quite low. For these reasons, nowadays, the goal of biomarker research in MS is to identify novel distinctive targets by studying more easily accessible biological fluids.

1.11 - MS therapies

Although to date there are no definitive therapies that completely resolve the pathology, different treatments which reduce MS attacks incidence and severity are available. The main goals of MS therapies are:

- to reduce relapses and its gravity (corticosteroids);
- to prevent relapses and disease progression (Disease Modifying Therapy, DMT).

In other words, MS treatments are used to prevent irreversible myelin and axonal damages, that already occur in early disease stages. The drugs currently used, based on their specificity, are distinct immunomodulators and immunosuppressants.

Immunomodulators as Interferon-β, IFN β-1a (Avonex and Rebif) and IFN β-1b (Betaseron and Extavia), and glatiramer acetate (GA; Copaxone) regulate lymphocytes activation (Mulakayala et al., 2013). In particular, IFN-β immunomodulatory effects have been attributed to inhibition of T cells activation and proliferation, auto-reactive T clones' apoptosis, Treg induction and inhibition of leucocytes migration across BBB (Dhib-Jalbut and Marks, 2010). IFN-β also acts on Th17 by modulating their vitality, differentiation and cytokines expression (Zhang and Markovic-Plese, 2010; Kurtuncu et al., 2012). GA is a synthetic polymer consisting of four aminoacids (L-alanine, L-glutamic acid, L-lysine and L-tyrosine) that works as altered peptide ligand since it competes with MBP, PLP and MOG for binding to MHC II expressed on APCs; it particularly induces a switch in lymphocytes differentiation from Th1 phenotypes

to Th2 GA-specific phenotypes that can produce neurotrophic factors (Shmidt, 2012). Natalizumab (Tysabri) is a humanized monoclonal antibody direct against α4-β1 integrin (VLA-4), a surface molecule expressed in all leucocytes except neutrophils. The antibody, by inhibiting the interaction between VLA-4 and VCAM-1 expressed on endothelial cells, decreases leucocytes adhesion and migration across BBB and therefore the entry in CNS, determining a marked reduction in inflammation (Putzki et al., 2010). Fingolimod (FTY720, Gilenya) is a non-selective agonist of the sphingosine 1-phosphate (S1P) receptor expressed by lymphocytes (Mulakayala et al., 2013). It prevents the lymphocytes exit from lymphnodes and their entrance in the CNS sites of inflammation binding, internalizing and inducing the degradation of this receptor. Recently a novel drug has been introduced: Cladribine. Cladribine is a chemotherapy and immunosuppressive drug used for the treatment of hairy cell leukemia (tricoloukemia) and relapsing-remitting multiple sclerosis. The tablet formulation of cladribine is an oral therapy indicated for the treatment of high activity RRMS, approved in Europe in 2017. As a purine analogue, it acts both as a chemotherapy and as an immunosuppressant. Chemically it mimics the adenosine nucleotide and inhibits the enzyme adenosine deaminase, which intervenes in the cells ability to process DNA. It is easily destroyed by cells except for blood cells, thus ensuring few side effects and great precision in the therapeutic target. Cladribine has been shown to exert a long-term effect by acting selectively and transiently on lymphocytes believed to be an integral part of the disease process of RRMS.

Chapter 2

Microglia

Microglia are brain-resident macrophages that represent 5-12% of the total brain cells. Under normal physiological conditions, microglia regulate brain development and continuously maintain homeostasis (Nimmerjahn et al., 2005). In response to injury or inflammatory stimuli, microglial cells are rapidly activated and promote neuroinflammatory processes through the secretion of various chemokines and cytokines. Microglia can acquire distinct phenotypes, pro-inflammatory (M1) or immunosuppressive (M2), in response to different signals in a process called polarization. Microglia display a more dynamic and varied spectrum of activation states between the M1 and M2 depending on the environment (Ransohoff, 2016).

2.1 - M1 microglia

M1 microglia are pro-inflammatory cells, classically activated by ligands such as interferon-γ (IFN-γ) alone or in combination with microbial stimuli or cytokines that bind Toll-like receptor (TLR). Generally, they act in the first line to defense tissue and promote the destruction of invading pathogens. However, M1 cells also induce neurotoxicity due to the release of pro-inflammatory cytokines (such as TNF-α, IL-1β, IL-6), chemokines (CCL4, CCL5, CCL8) and nitric oxide (NO) (Juhas et al., 2015).

2.2 - M2 microglia

M2 or alternatively activated microglia are induced mainly by IL-4 and IL-13 and produce anti-inflammatory cytokines (IL-10, IL-13, TGF- β) and neurotrophic factors (Gordon et al., 2003). They do not have any cytotoxic properties and contribute to tissue repair by inhibiting inflammation, remodelling tissue and promoting angiogenesis. The anti-inflammatory property of M2 cells is correlated to its lower NF-κB activation and higher phagocytic activity than M1 cells (Denney et al., 2012). M2-polarized microglia are further subdivided into M2a, M2b, and M2c (Sasaki et al., 2017). M2a cells, induced by IL-4/IL-13, are involved in wound-healing and anti-inflammatory processes and inhibit M1 activation by suppressing the nuclear factor-κB (NF-κB) signalling pathway. M2b cells are

generated following stimulation by immune complexes in the presence of a TLR ligand and show an inflammatory and modulatory phenotype. M2c cells are induced by IL-10, TGF-β, or glucocorticoids and display immunosuppressive properties (Chhor et al., 2013).

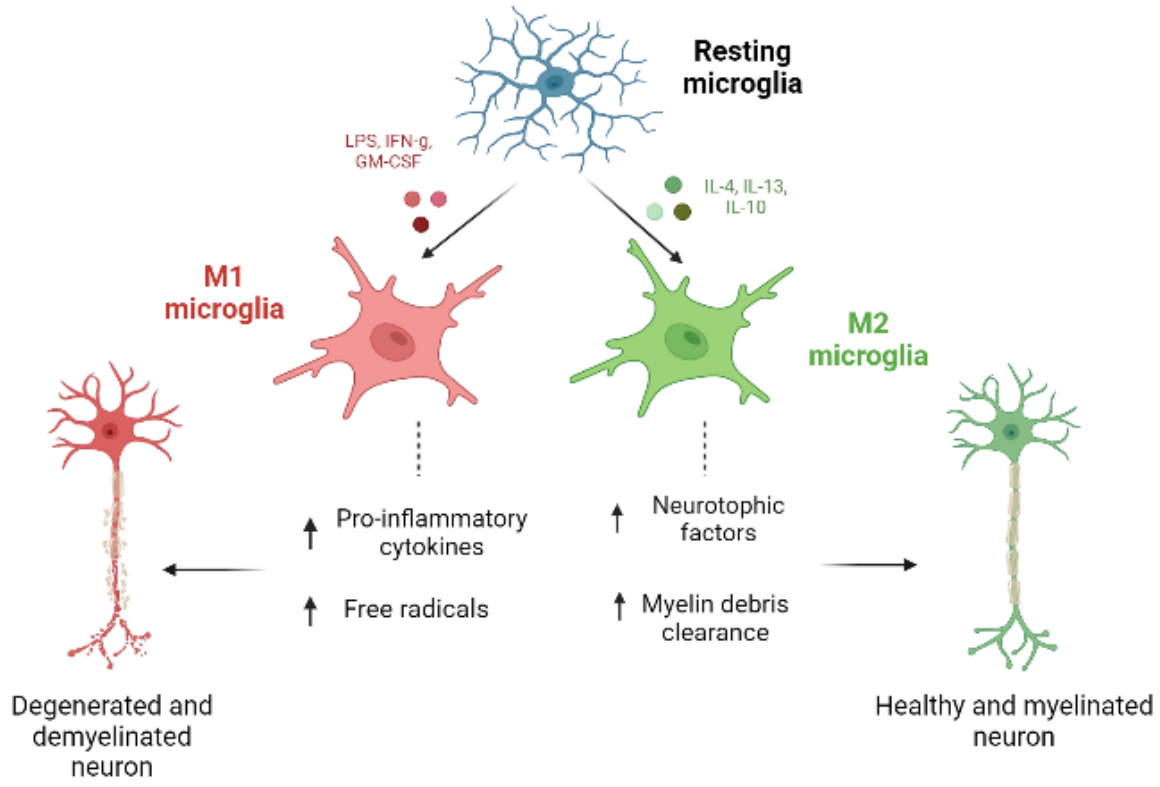


Figure D. M1/M2 microglia polarization. The presence of LPS and IFN-γ polarize microglial cells towards M1 phenotype, in contrast, IL-4 and IL-13 induce alternative activation of microglia to M2 phenotype. M1 microglia produce pro-inflammatory cytokines promoting neurotoxicity and demyelination. M2 microglia through neurotrophic factors production stimulate remyelination and axon regeneration.

2.3 - Microglia in MS

Because the activated microglia produce a plethora of neurotoxic cytokines, chemokines and reactive oxygen species, they are now considered key players in the pathogenesis of MS (Friese et al., 2014). Reactive microglia exert neurodestructive or neuroprotective effects in MS, which contribute to the relapsing-remitting nature of the disease. In the CNS of MS patients an activation of resident microglia is prominent and frequently observed in active MS plaques (Lucchinetti et al., 2000; Sriram et al., 2011). However, the presence of activated microglia is not restricted to lesion areas but also in normal-appearing white matter of MS patients in the proximity of plaques (Zrzavy et al., 2017). There is

evidence demonstrating the harmful effects of microglia in the demyelinated lesion. These detrimental mechanisms may include the T cells recruitment and reactivation, neurotoxicity due to the release of the pro-inflammatory cytokines and ROS, and oligodendrocyte precursor cells (OPCs) toxicity through excitatory amino acids (Murphy et al., 2010; Takeuchi et al., 2005; Merrill et al., 1991). It is clear that microglia have a variety of beneficial effects promoting remyelination and axonal regeneration through the clearance of myelin debris and secretion of beneficial growth factors that promote neuronal repair (Kotter et al., 2005; Yin et al., 2006).

During the acute phase of the disease, activated microglia polarize towards the M1 phenotype and potentially release pro-inflammatory cytokines inducing tissue damage, demyelination and neuronal death in the CNS (Almolda et al., 2011; Chunyun Liu et al., 2012; Jiang et al., 2012). Furthermore, microglia are more important for myelin antigen presentation in the early stage of disease. While M2 microglia increase gradually during the inflammation until the peak of disease, where the amount of M1 cells is decreased (Shin et al., 2012). During the later phase, M2 cells are predominant in the CNS and can release anti-inflammatory cytokines that mediate inflammation resolution and tissue repair (Laria et al., 2016). M2 microglia also can promote Th2 and regulatory T cells differentiation.

Chapter 3

Mesenchymal stem cells (MSCs)

MSCs are adult multipotent cells capable of self-renewal in vitro derived from various human tissues mainly bone marrow (BM), adipose tissue (AD), placenta and amniotic fluid. In the last few years, MSCs caught the attention of regenerative medicine due to their ability to expand in vitro while preserving their multipotent potential (Pittenger et al., 1999). It has been demonstrated a wealth of paracrine actions of stem cells trough their secretome, bioactive factors (i.e., soluble proteins, nucleic acids, lipids, and extracellular vesicles) that regulate immune system, angiogenesis, apoptosis, oxidative stress, cell differentiation, and extracellular matrix composition (Fuentes et al., 2015). In neurodegenerative disease, MSCs have therapeutic potential contributing to neuronal regeneration, neurotrophic factor (NF)-mediated protection, increase of neurogenesis, modulation of inflammation, and abnormal protein aggregate clearance (Volkman et al., 2017). MSCs can also affect microglia status through diffusible molecules inhibiting proliferation and pro-inflammatory molecules release and inducing their phagocytic capability and neuroprotective molecules production (Giunti et al., 2012; Jose et al., 2014; Yan et al., 2013). They are also able to skew the microglial phenotype from M1 to M2 (Le Blanc et al., 2012). The immunomodulatory effect of MSCs could induce a shift in T cells from a pro-inflammatory to an anti-inflammatory state, inhibit naïve and memory T cell proliferation and maturation, pro-inflammatory cytokines secretion, B cells proliferation and antibodies production supporting their therapeutic potential in MS (Prasanna et al., 2010; Magatti et al., 2018). Clinical trials demonstrated that MSCs injection stabilized MS symptoms in 70% patients and, in some cases, improved EDSS as well as the quality of life (Bonab et al., 2012; Llufriu et al., 2014; Fernández et al., 2018).

The amniotic membrane is a source of large quantities of MSCs that could be cost-effectively collected without invasive procedures and ethical concerns because placenta is spontaneously expulsed post-partum and it is considered a medical waste (Miyamoto et al., 2017). Human amniotic mesenchymal stem cells (hAMSCs) could potentially be applied in various neurological disorders due to their ability to differentiate to neuron and glial cells (Sanluis-Verdes et al., 2017). hAMSCs are suitable for

transplantation because they have low immunogenicity and can pass the BBB and enter the CNS (Wang et al., 2015; Smith et al., 2015).

Unfortunately, preclinical studies and clinical trials using MSCs have produced mixed outcomes because of the limited understanding of their mechanisms of action. It has been demonstrated that, following systemic transplantation, an amount less than 1% of MSCs reaches and engrafts at the target sites because they are quickly entrapped in the lung vasculature bed due to their large size (Fischer et al., 2009). The limited clinical efficacy of MSCs might be attributed to short-life engraftment duration, limited in vivo transdifferentiation, and restricted accessibility to damaged sites (Yousefi et al., 2019).

Chapter 4

Extracellular vesicles (EVs)

EVs are small particles (30-1000 nm) with lipidic bilayer secreted by different cell types and released in culture medium and biological fluids. They are divided into apoptotic bodies, microvesicles (MVs) or ectosomes, and exosomes (EXOs) with different biogenesis, release pathways, size, content, and function (Doyle et al., 2019; Zaborowski et al., 2015). EVs cargo consists of nucleic acids, lipids, plasma membrane and cytosolic proteins. Several studies report the importance of EVs in central nervous system (CNS) activities. Glia and neurons secrete EVs and the recent literature shows that intercellular communication by EVs has a versatile functional impact in the CNS homeostasis, including myelin formation, metabolic support, and immune defense (Frühbeis, 2013). Moreover, EVs have been associated with neurogenesis, modulation of synaptic activity and nerve generation. This may be due to the proteins and RNAs cargo that possibly modify the target cell phenotypes. However, their contents and complete functions still need to be fully characterized. The implication of EVs in the pathogenesis of neurological disorders, such as Alzheimer's, Parkinson's diseases and stroke, has been already reported (Lai, 2012; Ngolab, 2017; Lööv, 2016; Couch, 2017). In CNS inflammatory demyelination like Multiple Sclerosis (MS), EVs showed both protective and damaging functions respectively by inducing the maturation and migration of oligodendrocyte precursor cells, and by promoting transendothelial migration of lymphocytes and monocytes that spreads the inflammation into CNS (Sáenz-Cuesta, 2014a).

4.1 - Apoptotic bodies

Apoptotic cells release membrane vesicles, named apoptotic bodies, into the extracellular space. They are large particles up to 5000 nm in diameter (Borges et al., 2013). Apototic bodies contain intact organelles, chromatin, and high levels of nuclear, mitochondrial, Golgi apparatus and endoplasmatic reticulum-associated proteins (Kerr et al., 1972) similar to proteomic profile of cell lysate.

4.2 - Microvesicles

MVs are large vesicles (100 nm - 1 µm in diameter) arise from outward protrusion and fission of the plasma membrane. Because MVs form by a budding of cytoplasmatic membrane, their content is mainly represented by cytosol and plasma membrane-associated proteins (Escola et al., 1998). Other protein, such as cytoskeletal proteins, heat shock proteins, integrins, and glycosylated and phosphorylated proteins have been identified in MVs (Heijnen et al., 1999).

4.3 - Exosomes

EXOs are small vesicles from 30 nm up to 150 nm in diameter of endocytic origin secreted by all cell types and found in biological fluids like serum, saliva, breast milk, urine, CSF (Keller et al., 2007; Gallo et al., 2012; Saman et al., 2012). Exosome biogenesis begins in the endosomal system with the formation of endocytic vesicles from specialized regions of the plasma membrane. These vesicles detach from the plasma membrane, and they fuse with the early endosomes. The latters mature into late endosomes: during this process the endosomal membrane is invaginated to generate intraluminal vesicles (ILVs) in the lumen of the organelles (Huotari et al., 2011). The last ILVs-containing endosome is also called multivesicular body (MVB). MVBs could be sent to the lysosomes to be degraded or to fuse with the plasma membrane releasing ILVs into the extracellular space in the form of exosomes (Van Niel et al., 2006). The regulation of EXOs biogenesis seems to be through the endosomal sorting complexes required for transport (ESCRT) pathway (Wollert et al., 2010). Some studies indicate there is another mechanism, an ESCRT independent mechanism, dependent on sphingomyelinase enzyme (Stuffers et al., 2009, Van Niel et al., 2011). Regarding the protein cargo, ESCRT proteins and their accessory proteins (Alix, TSG101, HSC70, and HSP90β) have been found in exosomes (Morita et al., 2007). Transmembrane proteins of tetraspanin family (CD63, CD9, CD81) and other plasma membrane proteins are commonly enriched in exosomes compared to the cell lysate (Sinha et al., 2014). Exosomes are secreted in both normal and pathological conditions and they participate in cell–cell communication, cell maintenance, and tumor progression (Chaput et al., 2011). In the CNS, it has been observed that, on the one hand, exosomes could promote myelin formation, neurite growth, and

neuronal survival (Krämer-Albers et al., 2007; Wang et al., 2011), on the other hand, they contain pathogenic proteins aiding disease progression (Rajendran, 2006; Emmanouilidou et al., 2011).

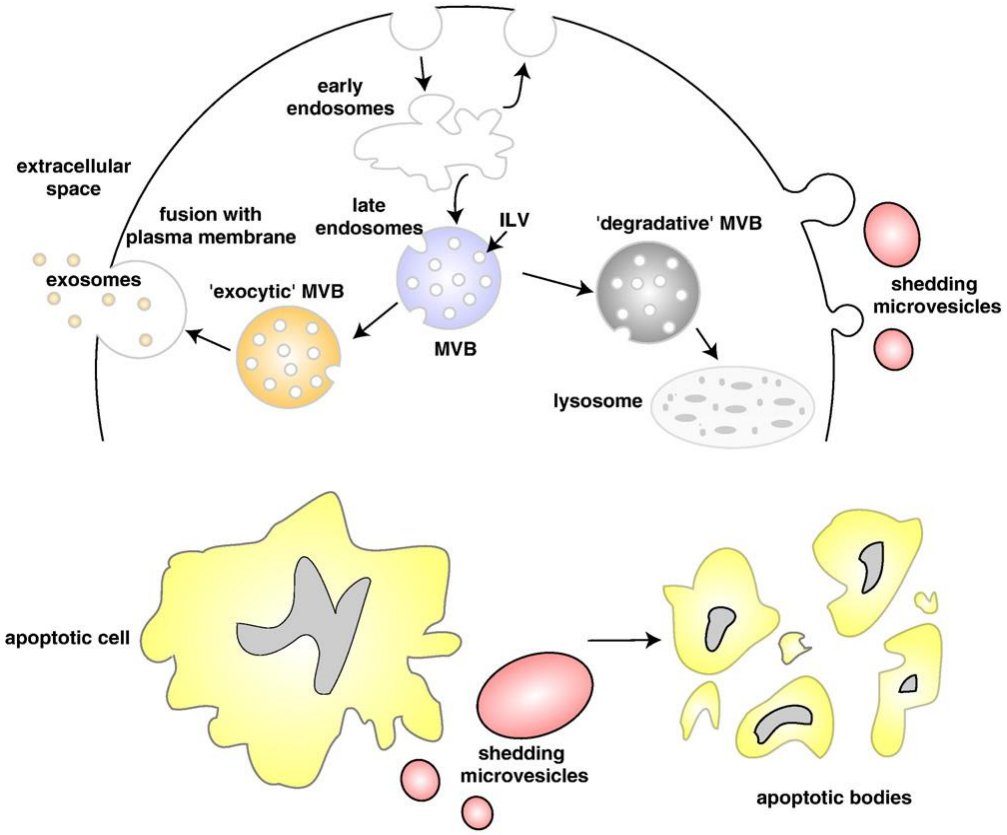


Figure E. Schematic representation of the EVs released into the extracellular space. EXOs and MVs release and apoptotic bodies release by apoptotic cells (Mathivanan et al., 2010).

4.4 - MSCs-derived EVs

EVs are proposed today as the main mediators of MSCs' paracrine beneficial effects (Joo et al., 2020). MSC-EVs have been studied in various human diseases, such as brain injury, Alzheimer's disease, liver and kidney injury, wound healing, and angiogenesis (Zhang et al., 2017; Cui et al., 2018; Zou et al., 2016; Zhang et al., 2015). Recently, it has been demonstrated that MSC-EVs possess therapeutic effects in autoimmune diseases such as progressive MS (Laso-Garcia et al., 2018). EVs are safer and more specific than stem cells, thanks to their ability to pass the BBB (Ochocinska et al., 2017) and their low immunogenicity due to the lack of MHC-II and low levels of MHC-I (Klyushnenkova et al., 2005). They

are stable, easier to store and deliver, and have longer half-life and shelf life than MSCs (Rani et al., 2015; Maumus et al., 2020; Lou et al., 2017). The therapeutic and anti-inflammatory effects of MSC-EVs are due to their content (nucleic acids and proteins). Interestingly, this cargo does not necessarily reflect the parental cell content, but it is affected by environmental stimuli (Valadi et al., 2007). Indeed, preconditioning of MSCs with hypoxia or IFN-γ influences the cargo of EVs by improving their immunomodulatory properties (Liu et al., 2020; Riazifar et al., 2019; Giunti et al., 2021) (**figure F**). In EXOs from BM- and umbilical cord (UC)-MSCs, a repertoire of anti-inflammatory mRNAs such as indoleamine 2,3-dioxygenase 1 (IDO-1), thymosin beta 10 pseudogene 1 (TMSB10P-1), and CD74 molecules, have been identified particularly overexpressed in EXOs from MSCs primed with IFN-γ (IFN-γ-EXOs) compared to EXOs from unprimed MSCs (Native-EXOs) (Riazifar et al., 2019). MSC-EVs also contain many noncoding RNAs (miRNAs, tRNAs, lincRNAs and antisense RNAs). Interestingly, miRNAs involved in niche maintenance, proliferation, differentiation and homing of stem cells are highly enriched in MSC-EVs (Clark et al., 2014; Kim et al., 2012; Li et al., 2013). Vilaça-Faria et al. identified in MSC-EXOs a set of miRNAs (miR-17, miR-18a, miR-19a/b, miR-20a, and miR-90a) that promote CNS recovery by modulating neurogenesis and stimulating axonal growth (Vilaça-Faria et al., 2019). Priming with IFN-γ causes an overexpression of miRNAs with anti-inflammatory effect on M1-activated microglia (miR-467f and miR-466q) in MSC-EVs (Giunti et al., 2021). Also under hypoxic conditions, it has been demonstrated an up-regulation of miR-216a-5p, a regulator of M1/M2 microglial shift in vivo and in vitro, in EXOs from BM-MSCs (Liu et al., 2020). Regarding protein cargo, MSC-EVs contain several immunomodulatory and/or neuroprotective factors, including transforming growth factor-β (TGF-β), growth factors (e.g., HGF, VEGF, FGF), IDO-1, anti-inflammatory interleukins (IL-10), IL-1 receptor antagonist (IL-1Ra), and prostaglandin E2 (PGE2) (Harrell et al., 2019). Interestingly, MSC-EVs encapsulate proteins involved in neural development, synaptogenesis, and angiogenesis, such as nestin, neuro-D, growth-associated protein 43, synaptophysins, VEGF, and FGF (Chopp and Li, 2002). Moreover, MSC-EVs express factors with immunosuppressive effects on T cells; among these, galectin-1 and PD-L1 promote Treg proliferation (Kilpinen et al., 2013). Riazifar et al. showed that preconditioning of MSCs with IFN-γ causes the release of EXOs enriched in protein, with anti-

inflammatory or neuroprotective properties, such as laminin subunit beta-2, macrophage inhibitory cytokine 1 (MIC-1), gremlin-1, annexin A4, etc. (Riazifar et al., 2019).

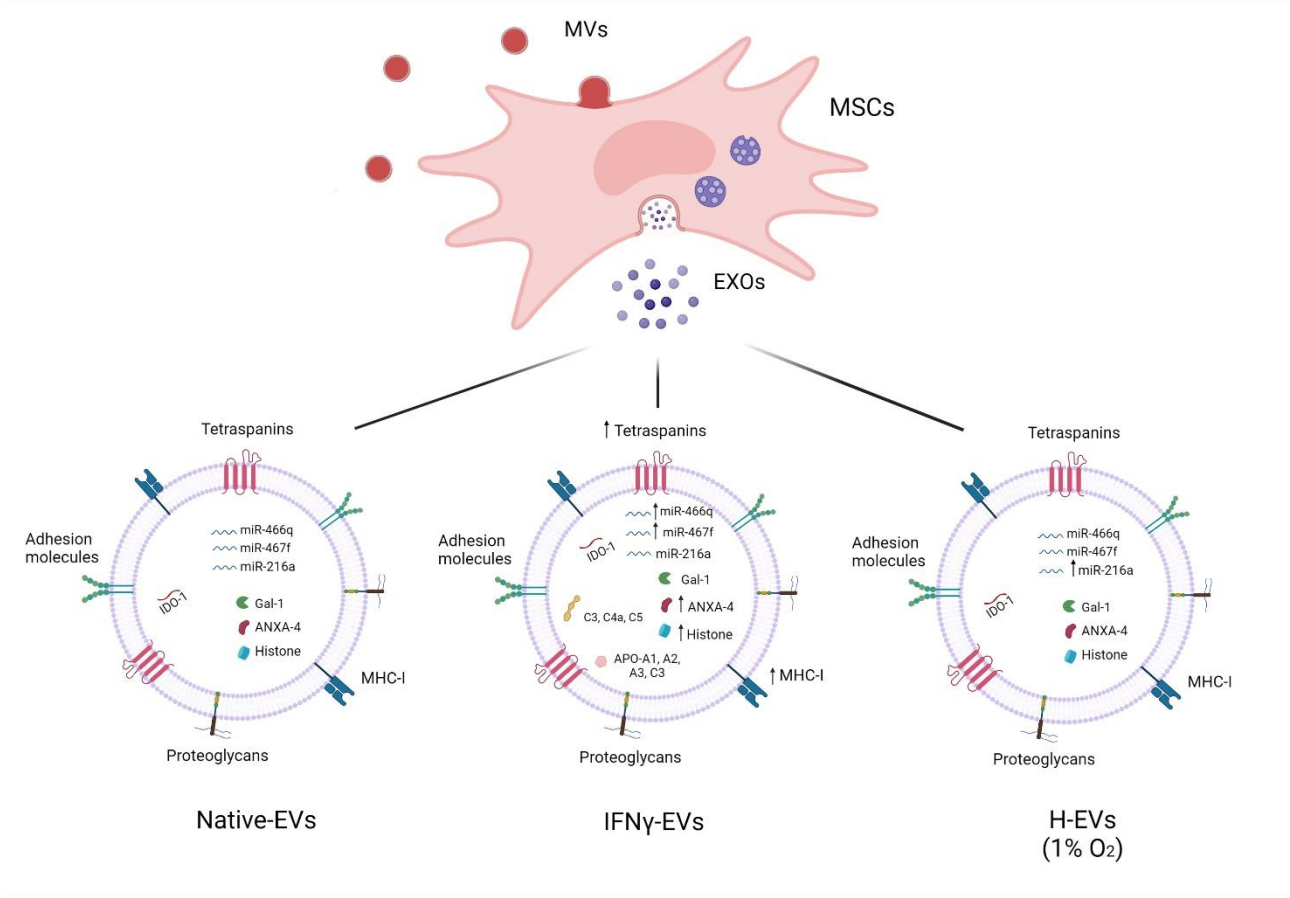


Figure F. Molecular cargo of EVs from unstimulated (Native-EVs) and IFN-γ (IFN-γ-EVs and hypoxia (H-EVs)-primed MSCs. Treatment with IFN-γ and hypoxia implicates the high expression of specific immunomodulatory molecules, indicated by an upward arrow. Preconditioning of MSCs with IFN-γ causes an overexpression of indoleamine 2,3- dioxygenase 1 (IDO-1) mRNA, miR-467f, miR-466q, annexin A4 (ANXA4), and histones, while hypoxic conditions cause an increase in miR-216a. Moreover, IFN-γ-EVs contain several apolipoproteins and complement-related proteins (Allegretta et al., 2022).

4.5 - MSC-Derived EVs in Autoimmune Demyelinating Diseases

The features described above make MSC-EVs suitable for the treatment of CNS diseases. MSC-EVs are an ideal choice for the treatment of neuroimmune disorders due to their roles in immune modulation, neuroprotection, and anti-inflammatory mechanisms. In vivo studies using an animal model of MS, experimental autoimmune encephalomyelitis (EAE), demonstrated that injection of MSC-EVs, from different source, enhances motor skills and reduces brain atrophy, neuroinflammation and

demyelination, by increasing Tregs and reducing macrophages/microglia and pro-inflammatory T cells within the spinal cord. Additionally, it has been found a reduction in peripheral blood mononuclear cell (PBMC) proliferation and pro-inflammatory Th1 and Th17 cytokine levels (Riazifar et al., 2019; Li et al., 2019; Giunti et al., 2021; Farinazzo et al., 2018; Jafarinia et al., 2020). In vitro studies have elucidated some molecular mechanisms underlying the effects of MSC-EVs on neuroinflammation, immunomodulation, microglial activation, demyelination, and astrogliosis. It has been demonstrated that MSC-EVs, on the one hand, reduce auto-reactive T cells, pro-inflammatory cytokines production and mitochondrial dysfunction, on the other hand, they enhance Tregs, anti-inflammatory cytokines levels and neurite outgrowth (Mokarizadeh et al., 2012; Riazifar et al., 2019; Li et al., 2019; Xian et al., 2019; Kumar et al., 2019). MSC-EVs' in vivo and in vitro effects are summarized in **figure G**.

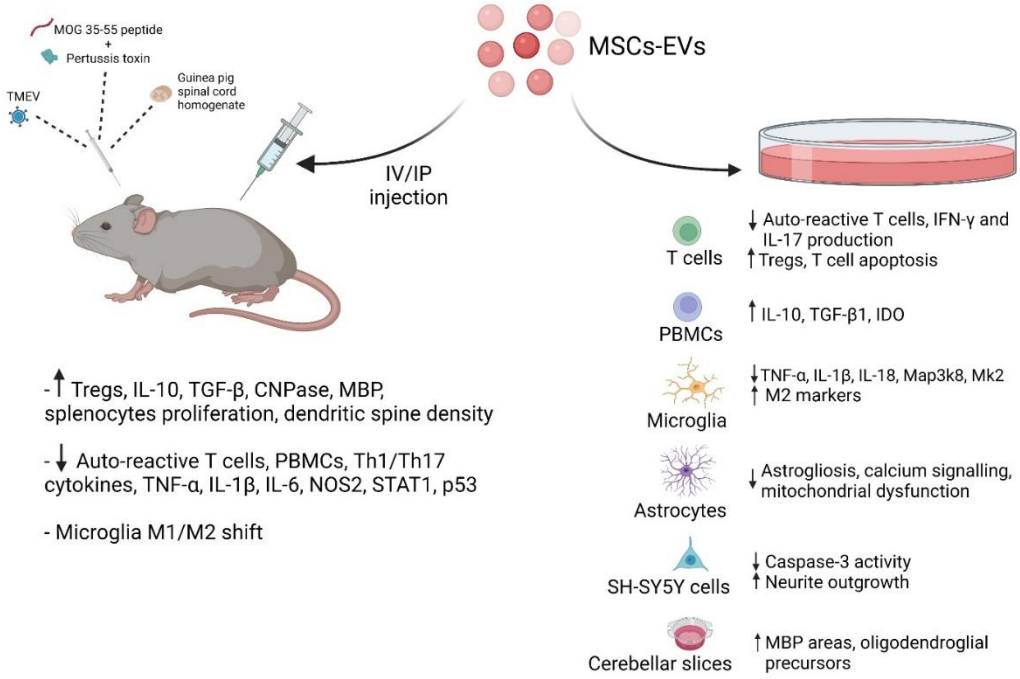


Figure G. MSCs-EVs treatment showed their immunomodulatory, neuroprotective and anti-inflammatory role by enhancing Tregs number and neurite outgrowth, and diminishing pro-inflammatory cytokines (TNF-α, IL-1β) in favor of anti-inflammatory ones (IL-10, TGF-β), in EAE animal models (injecting MOG35-55 peptide/pertussis toxin, or TMEV, or spinal cord homogenates) and in different cell types cultures (Allegretta et al., 2022).

Chapter 5

MicroRNAs

MicroRNAs (miRNAs) are small single stranded non-coding RNAs of 21-23 nucleotides which have recently been discovered to be post-transcriptional modulators of gene expression, either by targeting mRNA degradation or by stopping protein translation. In the most cases, they are transcribed by RNA polymerase II as long primary miRNAs (pri-miRNAs) in the nucleus. The pri-miRNA is cleaved by Drosha RNAse III endonuclease forming a pre-miRNA, which is exported into the cytosol by exportin-5 and further processed by another RNAse III endonuclease Dicer into mature miRNAs, which is double stranded. This miRNA is unwound, and a strand is incorporated into the RNA-induced silencing complex (RISC), which mediates mRNA translational inhibition or mRNA degradation (Bartel, 2004). An individual miRNA can control the expression of more than one target mRNA and each mRNA may be regulated by multiple miRNAs. miRNAs are important in several biological processes, such as cell proliferation, differentiation, metabolism, apoptosis, inflammation and immunity. Their expression could also affect several important human diseases, including cancer, neurodegeneration and autoimmunity (Fenoglio et al., 2012). miRNAs can be secreted into extracellular space and detected in several body fluids, including plasma, serum, CSF, breast milk, urine, tears, semen, and saliva (Cortez et al., 2011). They are highly stable in the blood thanks to their resistance to circulating ribonucleases and severe conditions such as extended storage, freeze-thaw, and extreme pH due to their packaging in exosomes, binding to RNA-binding protein, and association with high-density lipoprotein (Chen et al., 2008; Mitchell et al., 2008).

5.1 - MiRNAs in Multiple Sclerosis

Emerging evidence supports a critical role of miRNAs in the pathogenesis of autoimmune diseases, including MS (O'Connell et al., 2010; Junker et al., 2011). MiRNAs are involved in neurogenesis, oligodendrocyte differentiation, neurite outgrowth, and myelin formation (Dugas et al., 2010; de Faria et al., 2012; Zhao et al., 2010). Dysregulated expression of miRNAs associated with the MS phenotypes has been found previously in blood and CSF (Søndergaard et al., 2013; Gandhi et al., 2013; Fenoglio et

al., 2013). A comprehensive miRNA expression analysis in MS lesions showed an up-regulation of miR-155, miR-326 and miR-34a in active MS lesions compared to inactive lesions or normal brain white matter (Junker et al., 2009). miRNAs control several aspects of immunity, so a dysregulated miRNA expression has been observed in immune cells of MS patients. In Th17 cells isolated from RRMS patients an increase of miR-326 has been found. miR-326 is associated with Th-17 cell development by inhibiting Ets-1, a negative regulator of Th-17 differentiation (Du et al., 2009). A differentially expression of miR-17-5p, miR-92, miR-193a and miR-497 has been identified in T and B cells of MS patients. The targets of these miRNAs are genes involved in lymphocyte development, activation, and survival (Lindberg et al., 2010). Guerau-De-Arellano et al. suggested increased expression of miR-128 and miR-27b in naïve CD4+ T cells and miR-340 in memory CD4+ T cells of patients with MS. These miRNAs are involved in suppressing Th2 differentiation and in predisposition to the development of an autoimmune Th1 response (Guerau-De-Arellano et al., 2011).

MiR-223

MiR-223 has diverse roles in modulating inflammatory processes (Haneklaus et al., 2013) and its expression is upregulated in both MS patient peripheral blood mononuclear cells (PBMCs) (Keller et al., 2009) and early active, demyelinating MS lesions (Junker et al., 2009).

MiR-223 seems to promote plasticity from the M1 to M2 phenotype by decreasing M1 markers (such as iNOS-2) and production of pro-inflammatory cytokines, and by increasing M2 markers expression (such as Arg-1) (Tran et al., 2016).

Galloway et al. observed elevated miR-223 levels in MS patient monocytes and M2 myeloid cells. MiR-223 deficiency delays EAE onset but it is not necessary for severity and M1 polarization. Furthermore, miR-223 is required for efficient M2 polarization and phagocytosis in myeloid cells, and miR-223 knock-out mice display impaired CNS remyelination (Galloway et al., 2019). These results suggest a complex, but overall beneficial role for miR-223 in regulating CNS inflammation and remyelination. However, it is currently unknown how miR-223 contributes to repair and remyelination in the CNS following demyelination.

MiR-146a

MiR-146a is an important molecular brake that blocks the autocrine IL-6– and IL-21–induced Th17 differentiation pathways inhibiting differentiation of autoreactive CD4 T cells into pathogenic Th17 cells. MiR-146a deficiency promotes the production of pro-inflammatory Th1 and Th17 cytokines (e.g., IFN-γ and IL-17A) and suppresses the production of anti-inflammatory Th2 cytokines (e.g., IL-4) (Li et al., 2017). MiR-146a has been reported overexpressed in the CSF of MS patients associated with radiological variables and clinical disability (Muñoz-San Martín et al., 2019), in active MS lesions (Junker et al., 2009) and in PBMCs of relapsing-remitting MS patients compared to controls (Fenoglio et al., 2011). Fenoglio and colleagues suggested that this upregulation was specific to the acute phase of MS and contribute to the differentiation and regulation of CD4+T cells (Fenoglio et al., 2011).

MiR-21

MiR-21 may modulate T cell activation and apoptosis, Treg function and development, or Th17 differentiation (Wang et al., 2016). MiR-21 upregulation in PBMCs of RRMS patients in remission comparing with RRMS patients in relapse was observed (Baulina et al., 2018). Furthermore, miR-21 was upregulated in CD4+ T cells in RRMS patients (Fenoglio et al., 2011), but downregulated in SPMS patients (Sanders et al., 2016). As observed for miR-146a, miR-21 was overexpressed in the CSF of MS patients associated with radiological variables and clinical disability (Muñoz-San Martín et al., 2019), in active MS lesions (Junker et al., 2009) and in PBMCs of relapsing-remitting MS patients compared to controls (Fenoglio et al., 2011).

MiR-155

MiR-155 is an encephalitogenic miRNA involved in Th17 cells development (Mycko et al., 2015). It seems to drive myeloid cell polarization toward a pro-inflammatory state (Moore et al., 2013; Butovsky et al., 2015). miR-155 deletion also causes increased expression of targets that block myeloid cell

proliferation and survival, Th17 development, oxidative stress, and BBB permeability (Zhang and Braun, 2015; Hu et al., 2013; Koval et al., 2013; Lopez-Ramirez et al., 2014).

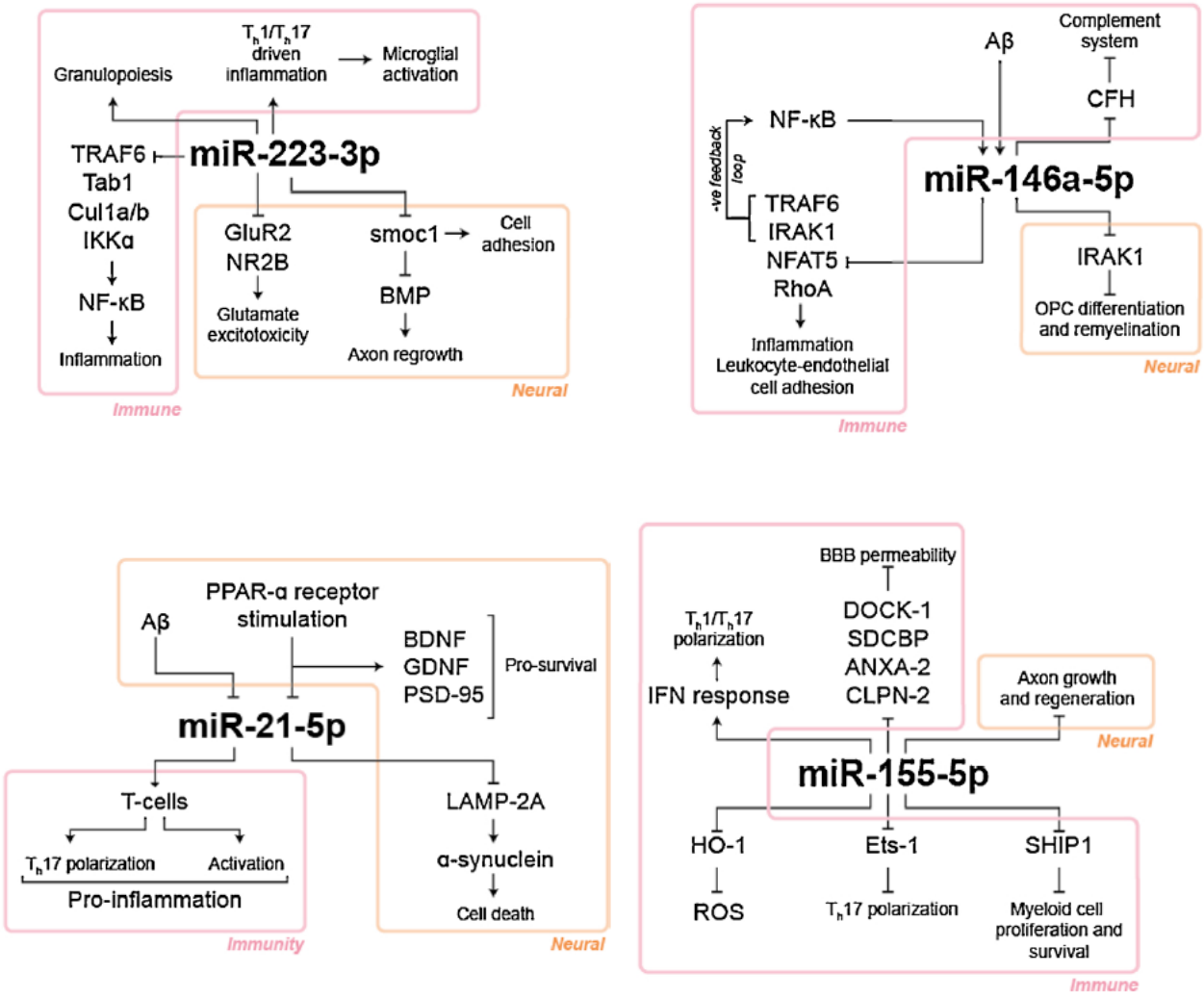


Figure J. Summary flow chart of the neural and immune components regulated by miR-223-3p, miR-146a-5p, miR-21-5p and miR-155-5p (Juźwik et al., 2019).

AIMS

AIM 1

Recent research has demonstrated that miRNAs are involved in several crucial biological functions, as well as in a broad spectrum of human diseases such as neurological disorders. In recent years, there has been a significant focus on understanding the role of miRNAs in the pathogenesis of neurodegenerative and autoimmune diseases. miRNAs are released by cells and can circulate in body fluids, packaged inside EVs, where they are emerging as promising biomarkers. Despite the biological role of extracellular miRNAs having just started to be explored, several reports demonstrate their involvement in intercellular communication in the immune system. The potential use of EVs-associated miRNAs as reliable biomarkers for MS could positively affect the development of highly sensitive diagnostic strategies for rapid, noninvasive monitoring of the pathologic condition of MS patients (Fabian, 2010). For this purpose, a set of specific miRNAs, involved in myelin production pathways, oligodendrocyte differentiation and pro- and anti-inflammatory processes, has been evaluated in EXOs from serum of MS patients and HDs.

AIM 2

Several studies support the role of EVs in the crosstalk between immune cells and the brain, suggesting that they may be used as sensitive biomarkers of these processes. Evidence has demonstrated that EVs have both protective and detrimental functions that may lead to spreading inflammation in the CNS but also support brain health. Current literature, besides studying the content of EVs, is trying to investigate the effects that circulating EVs could have on neuronal cells such as microglia. Although whether and how EVs are transported bidirectionally across the BBB is a question still under consideration, growing evidences suggest that EVs have an important role in regulating intercellular communication in the whole body including the brain, but it remains unclear how EVs are transferred between circulating blood and brain (Matsumoto et al., 2017). We aimed to better characterize the impact and the role of serum derived EXOs in MS in order to provide further insight into their function as mediators of the different processes of the disease. Therefore, we studied the effect of serum EXOs

from MS patients on human microglia cultured cells, analyzing the mRNA expression levels of pro-inflammatory and anti-inflammatory cytokines.

AIM 3

The therapeutic potential of EXOs, especially those derived from mesenchymal stem cells, in CNS demyelinating disorders has been widely discussed (Allegretta et., 2022). The anti-inflammatory and immunomodulatory effect of MSCs-EXOs is due to their mRNA, miRNA and protein cargoes. Therefore, we hypothesized that EXOs isolated from human amniotic mesenchymal stromal cells could provide a better course of treatment for MS by investigating whether hAMSCs-EXOs treatment would reduce pro-inflammatory cytokines expression in human microglia cells.

MATERIALS AND METHODS

Study population:

This investigation was performed on twenty-one patients with clinically definite relapsing-remitting MS (RRMS), ten patients in the active disease phase and eleven in the not-active one, followed at the Neurology Unit, Department of Medical and Surgical Sciences, University of Foggia and recruited within 10 years of the disease onset. As controls, thirteen age-matched HD were submitted to the same procedure for blood isolation. Venous blood samples (20 ml) were obtained from all subjects. Study approval was sent to the Ethics Committee of the Ospedali Riuniti of Foggia; written informed consent was signed by the recruited subjects.

Serum collection:

Peripheral blood samples were collected from MS and HDs subjects in Vacutainer blood collection tubes containing no anticoagulant, centrifuged at 3.000 xg for 7 min to separate the serum fraction from blood clot.

Serum EXOs isolation:

EXOs from each serum sample were separated and collected by subsequent differential centrifugation steps in the following way: the starting biological samples were centrifuged at 2.000 xg for 30 min at 4°C. The supernatant was filtered by gravity through 0.8 μm filters to remove particles >800 nm and centrifuged at 12.000 xg for 45 min at 4°C. The supernatant was collected and filtered through 0.2 μm filters. EXOs were pelleted by ultracentrifugation at 120,000 xg for 2 h at 4°C. EXOs pellets were re-suspended in PBS, and stored at -80°C.

Serum EXOs characterization:

Western Blot (WB)

EXOs were analysed by SDS–PAGE on 12% Tris–glycine gels (Life Technologies, Carlsbad, CA, USA). After electrophoresis, the proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA,

USA) and incubated in 5% milk. Appropriate primary antibodies were used for characterization of EVs: anti-CD63 (sc-59286), anti-CD81 (sc-166029), anti-Alix (sc-53540) and anti-TSG-101 (sc-13611) (Santa Cruz Biotechnology, Inc.) using a 1:1000 dilution. The blots were developed with Clarity Max Western ECL (Biorad) using ChemiDoc Imaging System (Biorad).

Dynamic laser light scattering (DLS)

Serum EXOs size and zeta potential were measured by laser doppler electrophoresis (LDE) using a Zeta sizer Nano ZS and in diffusion barrier mode with water (Trapani et al., 2018). Three different exosomes samples have been analysed and at least three measurements were acquired for each sample.

Total miRNA Isolation and Quantitative Real-Time PCR miRNAs Analysis:

To perform miRNAs analysis, total miRNAs were isolated from EXOs using miRNeasy Serum/Plasma Kit, according to the manufacturer's instructions. Concentration of miRNAs was determined by measuring the absorbance at 260 nm with a NanoDrop 1000 Spectrophotometer. miRNAs were reverse transcribed into cDNA using miRCURY LNA RT Kit (Qiagen). The resulting cDNA transcript was used for PCR amplification using miRCURY LNA SYBR Green PCR Kit (Qiagen) and miRNA specific primer set for miR-223-3p, miR-146a-5p, miR-21-5p, and miR-155-5p. The relative miRNA levels were calculated using the comparative Ct method, with U6 snRNA as endogenous control.

Cell culture:

Experiments were performed in human microglia clone 3 cell line (HMC3) (ATCC®CRL-3304) and in human amniotic mesenchymal stromal cells (hAMSCs).

HMC3 were maintained in MEM (Corning, New York, NY, USA) supplemented with 10% FBS (Euroclone, Milan, Italy), L-glutamine (Euroclone) and penicillin/streptomycin (Euroclone) at 37 °C, with 5% CO_2.

hAMSCs were isolated from the amniotic membrane of term placenta and grown in advanced DMEM supplemented with 10% FBS, 55 μM β-mercaptoethanol, 1% L-glutamine, 1% penicillin/streptomycin and 10 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich, Milan, Italy). For exosome collection, 80%

confluent cells, unstimulated and stimulated with 10 ng/ml IFN-γ (IFN-γ-primed) to increase their immunomodulatory features (Giunti et al., 2021), were cultured in serum-free medium for 48 h.

The usage of hAMSCs was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Ospedali Riuniti of Foggia (n. 138/CE/2020 issued on 30 November 2020). Informed consent was obtained from all women included in the study.

hAMSCs-EXOs isolation:

The conditioned media from IFN-γ-primed and unprimed hAMSCs were harvested and centrifugated at 300 ×g for 10 min, 2,000 ×g for 10 min, 10,000 ×g for 30 min, and 100,000 ×g for 70 min twice using an ultracentrifuge, as previously described by Li et al. (Li et al., 2019). The resulting pellets were resuspended in PBS and stored at -80°C.

hAMSCs-EXOs characterization:

Dynamic laser light scattering (DLS)

The size and zeta potential of EXOs isolated from IFN-γ-primed and unprimed hAMSCs were measured by laser doppler electrophoresis (LDE) using a Zeta sizer Nano ZS and in diffusion barrier mode with water (Trapani et al., 2018). At least three measurements were acquired for each sample.

Transmission Electron microscopy (TEM)

The TEM analysis was done by a method previously described with modification (De Leo et al., 2021). In brief, samples were prepared by dropping the EXOs aqueous suspension (2 µL) on a 400-mesh amorphous carbon-coated Cu grid (Agar Scientific Ltd, Stansted, UK, Cat# S160-4) and letting the solvent evaporate. Sample on the grid was left to dry overnight and finally stored in a vacuum chamber until analysis. The samples were stable under the electron beam and did not degrade within the typical observation times. No staining was used in these experiments. Micrographs were recorded using a Jeol JEM-1011 microscope working at an accelerating voltage of 100 kV and acquired by an Olympus Quemesa Camera (11 Mpx). Size statistical analysis (average size and size distribution) of each sample

was performed on 200 nanostructures by means of a freeware Image J analysis program (National Institutes of Health, USA).

HMC3 M1 polarization:

HMC3 were treated for 4 hours and 24 hours at 37 ◦C with PBS, 50 ng/mL IL-17A (Peprotech), 50 ng/mL IFNγ (Peprotech), 30 ng/mL TNFα (Peprotech), 50 ng/mL IL-17A + 50 ng/mL IFNγ, 30 ng/mL TNFα + 50 ng/mL IFNγ or 50 ng/mL IL-17A + 30 ng/mL TNFα + 50 ng/mL IFNγ.

Serum EXOs and HMC3 experiments set:

Cells were treated with PBS (negative control), 30 ng/mL TNFα + 50 ng/mL IFNγ (positive control) or serum exosomes from MS patients (in active or inactive phase of the disease) at different concentrations (1, 5, 10, 50, 100 μg/mL). Four different protocols were set up:

1. EXOs treatment for 4 h;
2. TNFα+IFNγ and EXOs co-treatment for 4 h;
3. TNFα+IFNγ treatment for 4 h and EXOs treatment for 24 h;
4. EXOs treatment for 24 h and TNFα+IFNγ treatment for 4 h.

hAMSCs-EXOs and HMC3 experiments set:

HMC3 were treated for 4 h with PBS, 30 ng/mL TNFα + 50 ng/mL IFNγ or exosomes isolated from hAMSCs (unprimed and IFNγ-primed cells) at different concentrations (0.2, 1.0, and 2.0 μg/mL). Three different protocols were set up:

1. hAMSCs-EXOs treatment for 4 h;
2. TNFα+IFNγ treatment for 4 h and hAMSCs-EXOs treatment for 24 h;
3. hAMSCs-EXOs treatment for 24 h and TNFα+IFNγ treatment for 4 h.

RNA Purification and Quantitative Real-Time PCR:

HMC3 were lysed and total RNA was isolated from using Direct-zol TM RNA MiniPrep kit (Zymo Research), according to the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260 nm with NanoDrop 1000 Spectrophotometer. RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Trascription Kits (Applied Biosystem). Quantitative real-time PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad). The primers used for amplification were: human IL1β forward: 5'-AGCTACGAATCTCCGACCAC-3', human IL1β reverse: 5'-CGTTATCCCATGTGTCGAAGAA-3'; human TNFα forward: 5'- CCTCTCTCTAATCAGCCCTCTG -3', human TNFα reverse: 5'- GAGGACCTGGGAGTAGATGAG-3'; human iNOS forward: 5'- TTCAGTATCACAACCTCAGCAAG-3', human iNOS reverse 5'-TGGACCTGCAAGTTAAAATCCC-3'; human IL-10 forward: 5'-TCTCCGAGATGCCTTCAGCAGA-3', human IL-10 reverse: 5'- TCAGACAAGGCTTGGCAACCCA-3'; human β-actin forward: 5'-CACCATTGGCAATGAGCGGTTC-3', human β-actin reverse: 5'- AGGTCTTTGCGGATGTCCACGT-3'. The relative mRNA levels were calculated using the comparative Ct method, β -actin as endogenous control.

Statistical analysis:

Statistical comparison of miRNAs expression between different groups was performed using the non-parametric Mann Whitney U test as post-hoc test. P value <0.05 was considered significant. All analyses were performed using GraphPad Prism version 5.0.

Statistical comparison of IL-1β, TNFα, iNOS and IL-10 gene expression was performed using the non-parametric Kruskal-Wallis test and Dunn's Multiple Comparison Test as post-hoc test. P value <0.05 was considered significant. All analyses were performed using GraphPad Prism version 5.0.

RESULTS

Serum EXOs characterization

Western blot

Western blot analysis revealed the expression of exosomal markers (Alix, CD63, CD81, TSG101) in HD serum exosome samples, absent in cell lysate samples (LC) (**Figure 1**). The expression of β-actin has been used as control to demonstrate the presence of cell lysate samples in second lane.

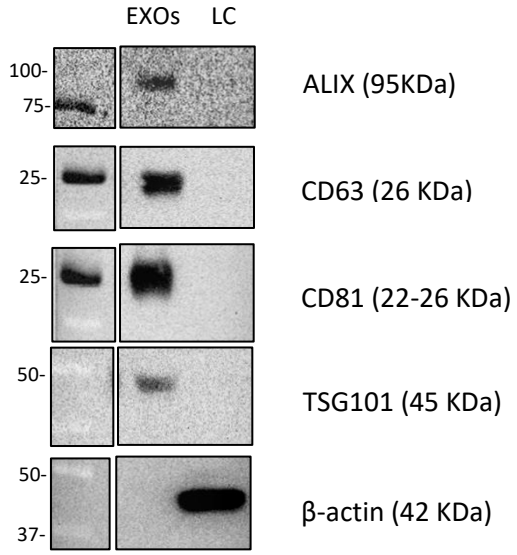


Figure 1. Characterization of serum EXOs by Western Blot.

Dynamic laser light scattering

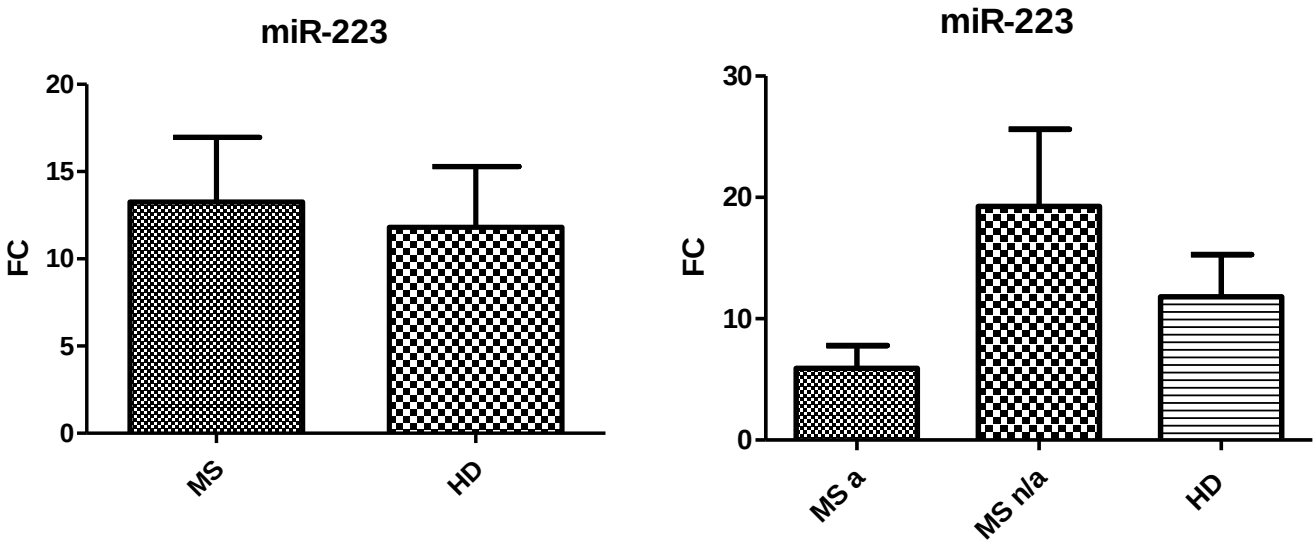
Size distribution and Zeta potential measurement by using DLS technique revealed that serum EXOs are nanosized and exhibit a fair degree of polydispersity (**table 2**). The average size measured by statistical analysis was about 30 nm, while zeta potential values were negative as expected.

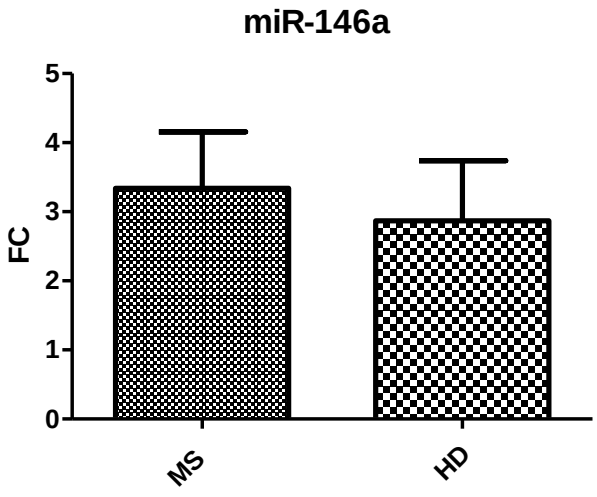
Sample	Size (nm)	Size SD	PDI	PDI SD	Z potential (mV)	Z potential SD
EXOs 1	30,42	0,46	0,412	0,006	-8,84	0,7
EXO 2	33,97	0,48	0,461	0,021	-8,11	0,62
EXO 3	26,55	0,77	0,454	0,003	-6,61	0,33

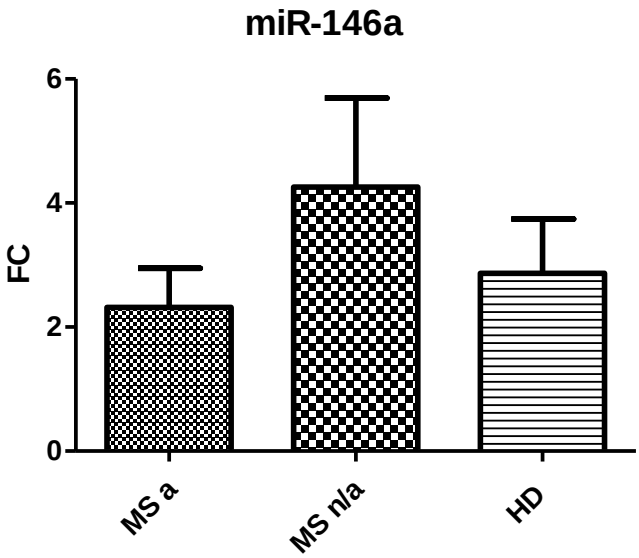
Table 2. Size and zeta potential of three serum EXOs samples.

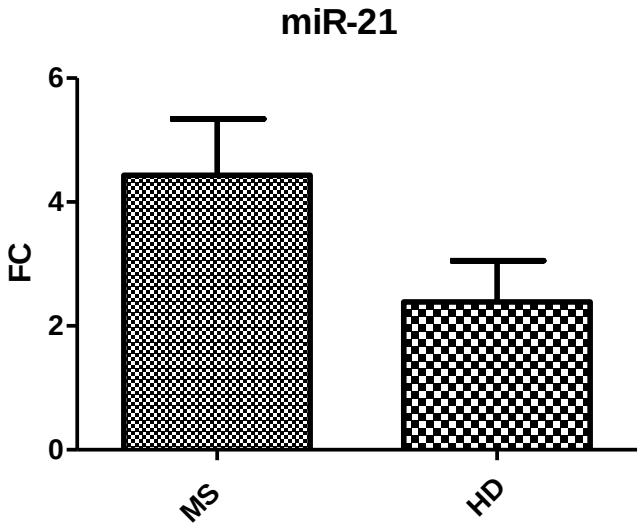
MiRNAs expression in serum EXOs

MiRNA expression analysis was performed in serum EXOs isolated from ten active and eleven not-active MS patients and thirteen HD by real-time PCR. The expression of miR-223-3p, miR-146a-5p, miR-21-5p, and miR-155-5p was evaluated. We observed not statistically significant difference in the expression of all these miRNAs between MS patients and HD, except for miR-155 expression significantly up-regulated in MS patients. Nevertheless, can be appreciated higher levels of these miRNAs in MS patients in the not-active disease phase compared to those in the active phase and donors, but statistically significant difference was found only for miR-155-5p between active patients and controls (**figure 2**).




miR-146a
FC
0
1
2
3
4
5
MS
HD


miR-146a
FC
0
2
4
6
MS a
MS n/a
HD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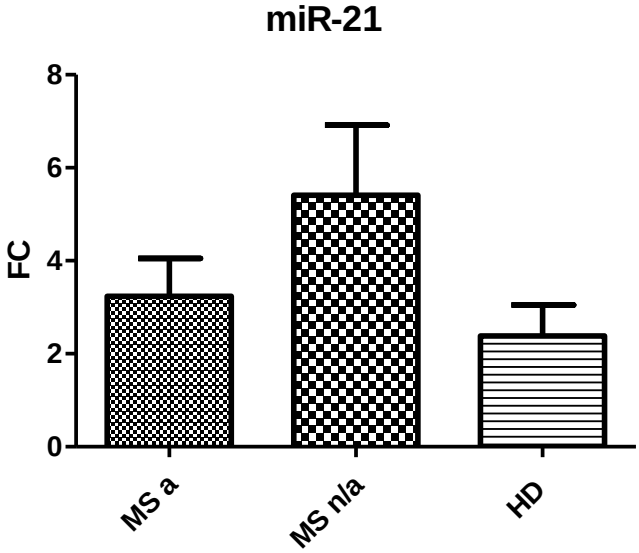
6
MS
HD


miR-21
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6
8
MS a
MS n/a
HD

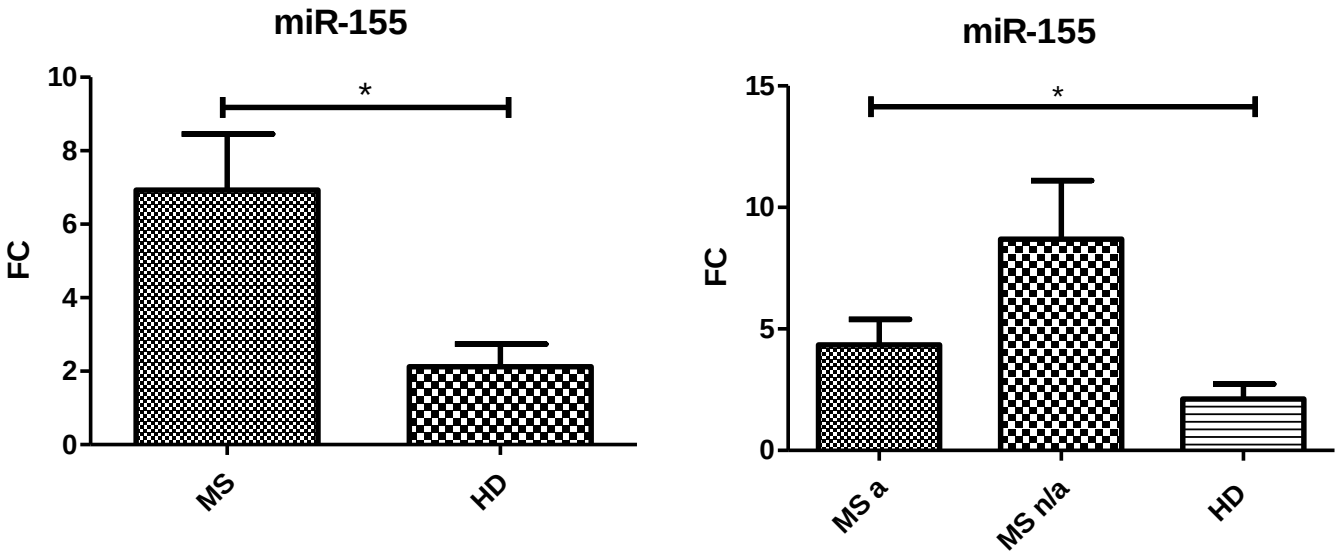


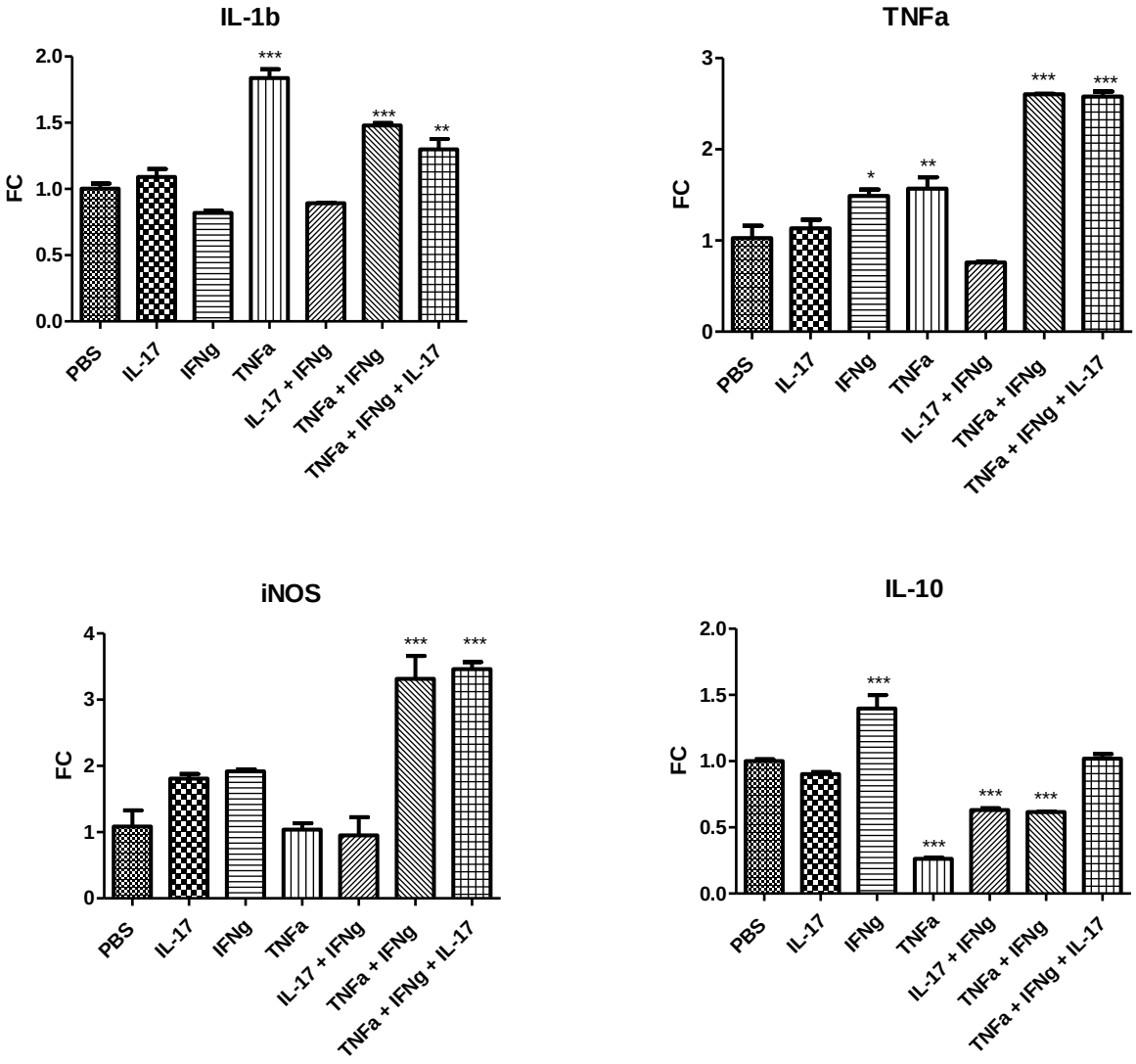
Figure 2. miRNAs expression levels in serum EXOs of MS patients in active (MS a) or not-active (MS n/a) disease phase and HD by quantitative Real-Time PCR. The relative expression levels were calculated using the comparative Ct method, with U6 snRNA as endogenous control. Data are expressed as mean ± SD of fold change (FC) values (*$p < 0.05$).

Human microglia cells M1 polarization

Before studying the effect of serum EXOs on human microglia cells, we preliminarily have evaluated which could be the best stimulus and the time point for an optimal pro-inflammatory polarization. To stimulate microglia, we used different pro-inflammatory cytokines alone or in combination (IL-17A, IFNγ, TNFα, IL-17A+IFNγ, TNFα+IFNγ or IL-17A+TNFα+IFNγ) and we assess the polarization status analysing IL-1β, TNFα, iNOS and IL-10 gene expression by quantitative real-time PCR at two different time points, 4 hours and 24 hours. Figure 3 demonstrates that TNFα+IFNγ cocktail is the optimal stimulus for a pro-inflammatory polarization at 4 hours (**figure 3A**) with a statistically significant increase in the pro-inflammatory gene expression (IL-1β, TNFα and iNOS) and a significant decrease in anti-inflammatory one (IL-10). At 24h we observed a significant increase of pro- and anti-inflammatory gene expression following TNFα+IFNγ treatment (**figure 3B**), so we can speculate that, after 24h of pro-inflammatory cytokines treatment, microglia cells react to this inflammatory milieu by increasing anti-

inflammatory cytokines production. All these reasons have led us to consider TNFα+IFNγ combination as the best choice to estimate M1 gene expression in human microglia at 4 hours.

A



B

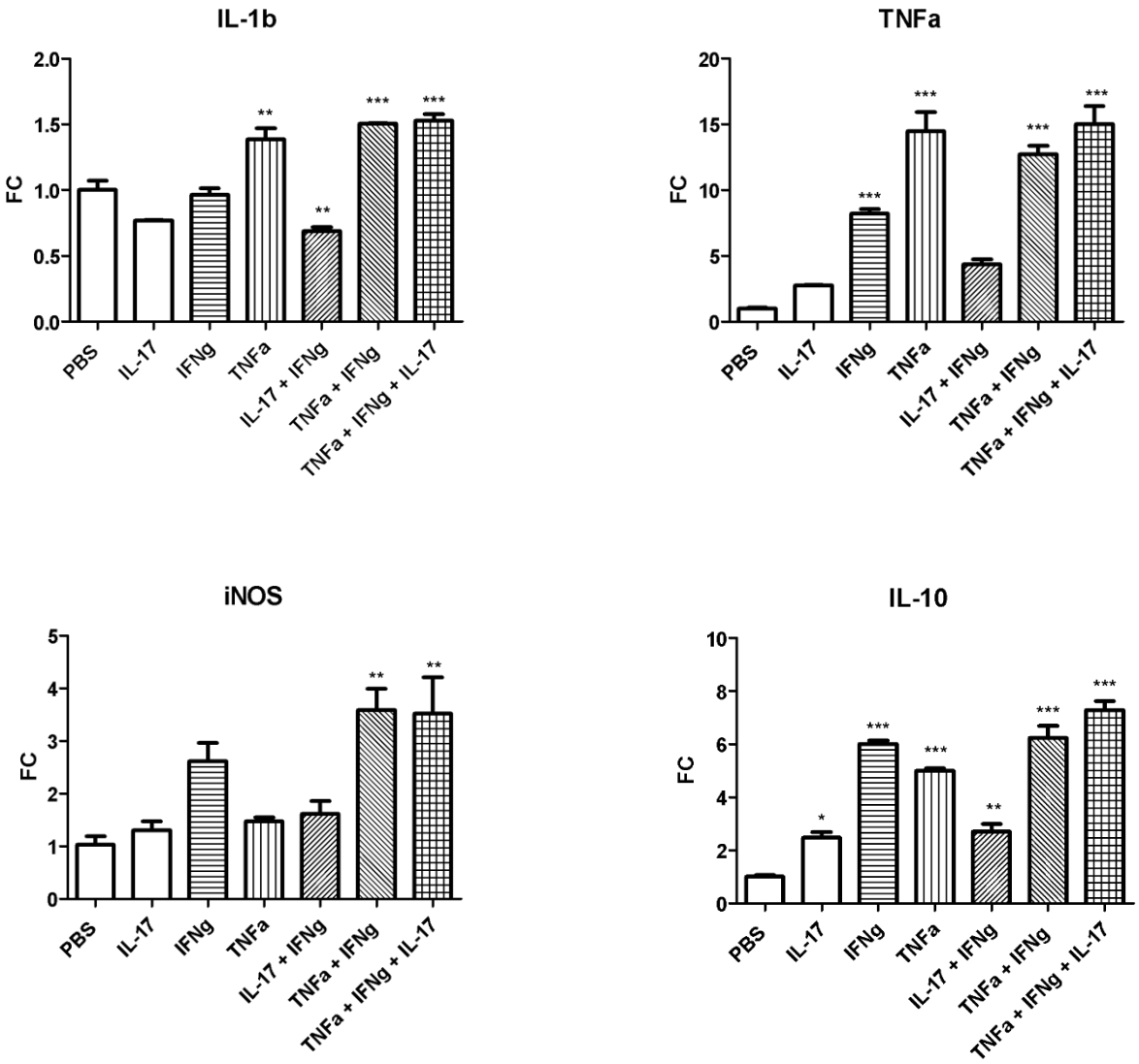


Figure 3. Human microglia cells M1 polarization. Different cytokines, alone or in combination, have been used to stimulate HMC3 and IL-1β, TNFα, iNOS and IL-10 gene expression has been evaluated by Real-Time PCR after 4h (**A**) and 24h (**B**) of treatment. Data are expressed as mean ± SD of fold change (FC) values of two experiments for each condition (*p < 0.05; **p < 0.01; ***p < 0.001 vs. PBS controls).

Serum EXOs effect on human microglia cells

HMC3 were treated with serum EXOs isolated from MS patients (in active and not-active disease phase) according to the protocols mentioned above and the polarization status was assessed analysing IL-1β, TNFα, and IL-10 gene expression by quantitative real-time PCR.

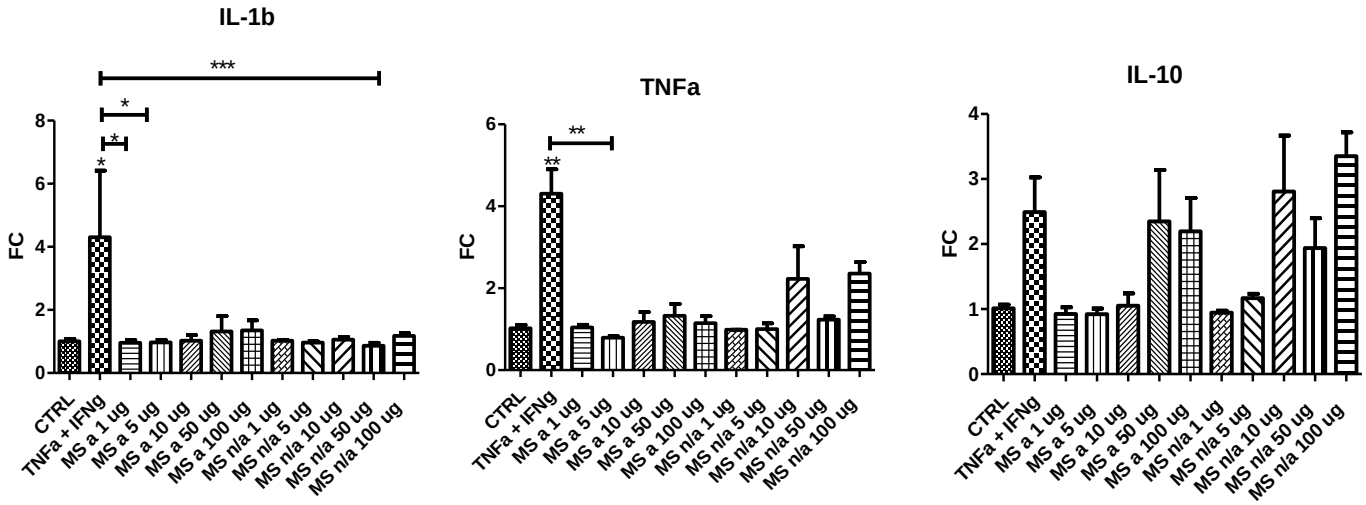
Cells treatment with serum EXOs at different concentrations for 4h didn't result in a significant alteration in the cytokines expression compared to the negative control. At 5 ug/mL concentration,

serum EXOs from active patients significantly reduce both IL-1β and TNFα levels compared to positive control. Moreover, a statistically significant decrease in IL-1β expression has been noted in microglia treated with serum EXOs from active patients at 1 ug/mL concentrations and from not-active patients at 50 ug/mL compared to positive control (**figure 4A**).

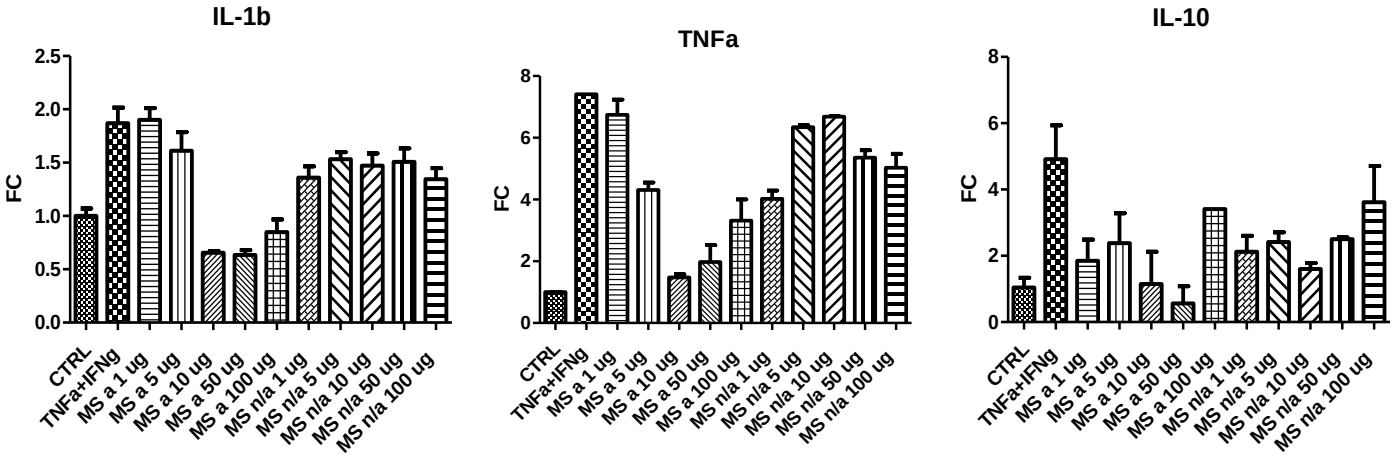
In co-treatment experiments we observed no additive effect of serum EXOs in increasing pro-inflammatory cytokines and lower IL-10 levels in co-treated cells compared to positive control (**figure 4B**). Therefore, we decided to test another protocol: TNFα + IFNγ treatment for 4h following by EXOs treatment for 24h. Even in this case, no significant effects of serum EXOs from both active and not-active patients have been appreciated except a significant increase of IL-10 expression in HMC3 treated with serum EXOs from not-active MS patients at 10 ug/mL concentration (**figure 4C**). Lastly, we treated microglia cells with serum EXOs (5, 10, 50 ug/mL) for 24h and TNFα + IFNγ for 4h and no significant increase and/or decrease in IL-1β, TNFα, and IL-10 expression has been detected (**figure 4D**).

In all performed experiments no dose dependent effects and no statistical difference in cytokines gene expression has been noticed in microglia treated with serum exosomes isolated from both active and not-active MS patients.

A



B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2.5
2.0
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1.0
0.5
0.0
CTRL
TNFa+IFNg
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MS a 5 ug
MS a 10 ug
MS a 50 ug
MS a 100 ug
MS n/a 1 ug
MS n/a 5 ug
MS n/a 10 ug
MS n/a 50 ug
MS n/a 100 ug
TNFa
FC
8
6
4
2
0
CTRL
TNFa+IFNg
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MS a 5 ug
MS a 10 ug
MS a 50 ug
MS a 100 ug
MS n/a 1 ug
MS n/a 5 ug
MS n/a 10 ug
MS n/a 50 ug
MS n/a 100 ug
IL-10
FC
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TNFa+IFNg
MS a 1 ug
MS a 5 ug
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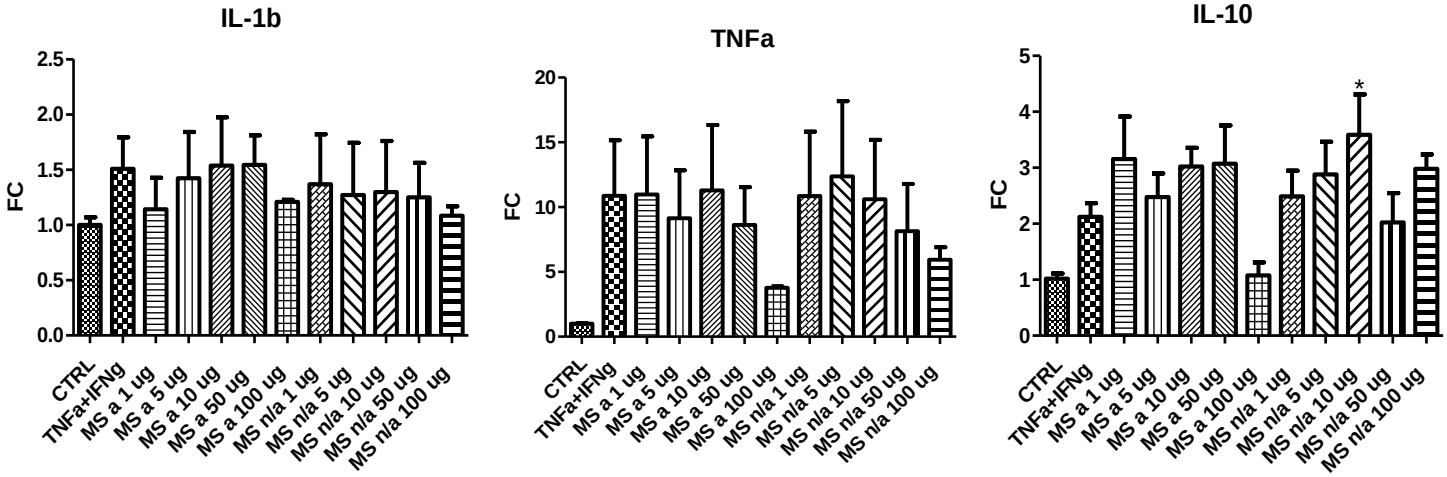
MS n/a 100 ug

C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MS n/a 1 ug
MS n/a 5 ug
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TNFa
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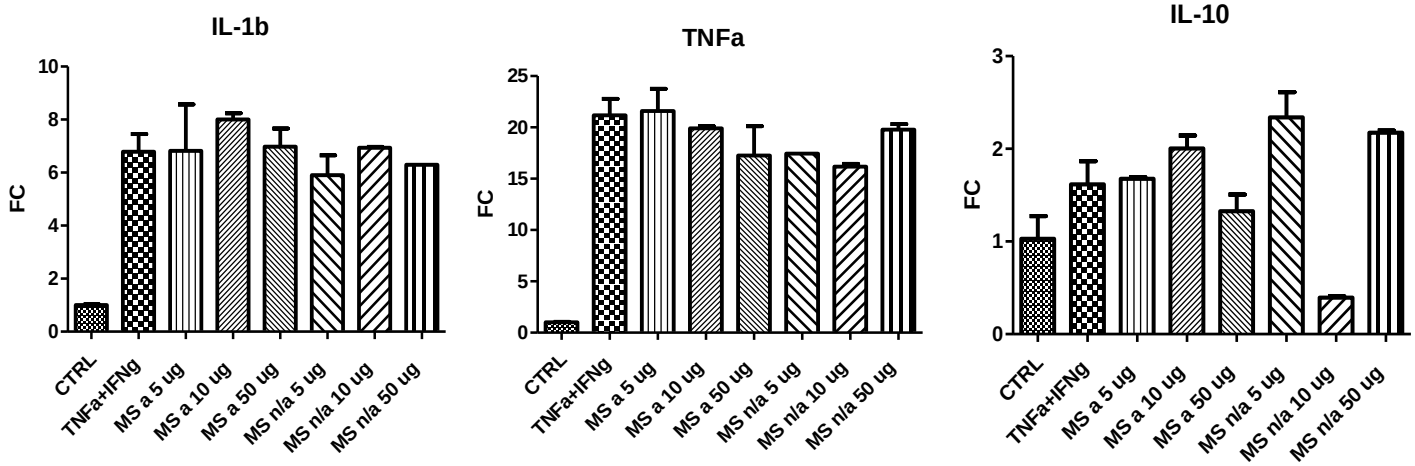
D


IL-1b
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MS n/a 10 ug
MS n/a 50 ug
TNFa
FC
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TNFa+IFNg
MS a 5 ug
MS a 10 ug
MS a 50 ug
MS n/a 5 ug
MS n/a 10 ug
MS n/a 50 ug
IL-10
FC
3
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1
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CTRL
TNFa+IFNg
MS a 5 ug
MS a 10 ug
MS a 50 ug
MS n/a 5 ug
MS n/a 10 ug
MS n/a 50 ug

Figure 4. Serum exosomes effect on HMC3. Serum exosomes from MS patients in active (MS a) and not-active (MS n/a) disease phase have been used to stimulate HMC3 and IL-1β, TNFα, and IL-10 gene expression has been evaluated by Real-Time PCR following EXOs treatment for 4h (**A**), TNFα+IFNγ and EXOs co-treatment for 4h (**B**), TNFα+IFNγ treatment for 4h and EXOs treatment for 24h (**C**) and EXOs treatment for 24h and TNFα+IFNγ treatment for 4h (**D**). Data are expressed as mean ± SD of fold change (FC) values of three experiments for each condition (*p < 0.05; **p < 0.01; ***p < 0.001 vs. PBS controls).

hAMSCs-EXOs characterization

Dynamic laser light scattering

Size measurement by using DLS technique revealed that EXOs isolated from medium of IFN-γ-primed and unprimed hAMSCs are nanosized and exhibit a fair degree of polydispersity (**Table 3**). The average size measured by statistical analysis was about 160 nm for EXOs from unprimed hAMSCs and 200 nm for those from IFN-γ-primed hAMSCs.

Sample	Size (nm)	Size SD	PDI	PDI SD
EXOs from unprimed hAMSCs	162	24	0,32	0,4
EXOs from IFN-γ-primed hAMSCs.	219	31	0,6	0,08

Table 3. Size of EXOs from conditioned medium of IFN-γ-primed and unprimed hAMSCs.

Transmission electron microscopy

TEM analysis showed the morphology, integrity, and size of EXOs derived from unprimed (**figure 5**) and IFN-γ-primed (**figure 6**) hAMSCs.

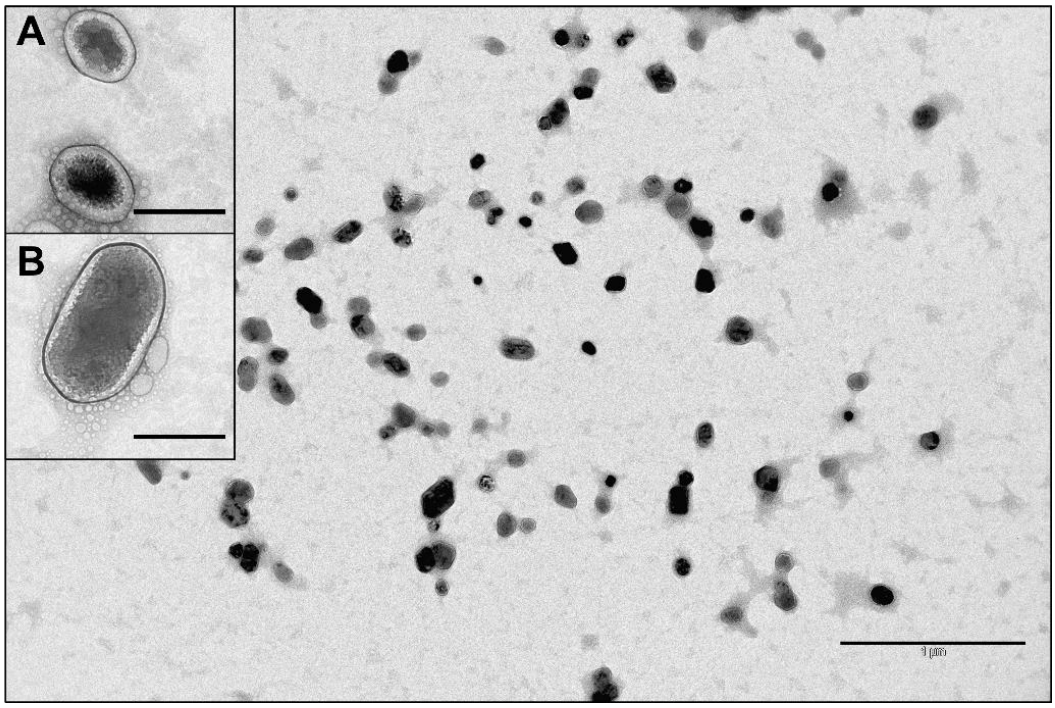


Figure 5. Representative TEM images of EXOs from unprimed hAMSCs. The scale bars (**A-B**) indicate 200 nm.

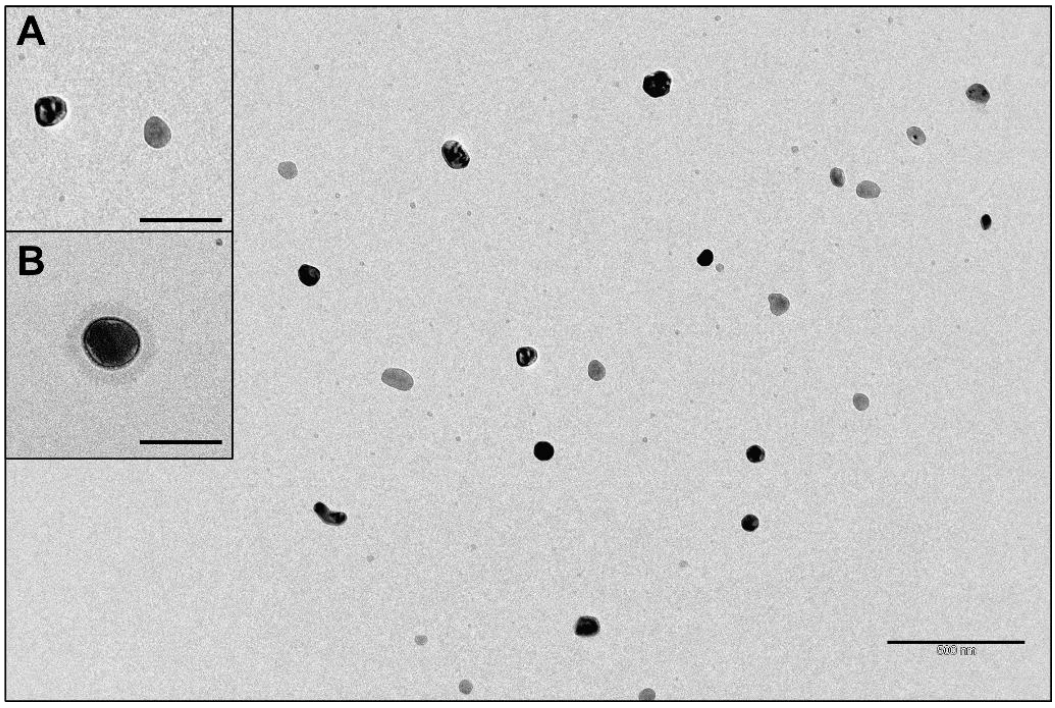


Figure 6. Representative TEM images of EXOs from IFN-γ-primed hAMSCs. The scale bars (**A-B**) indicate 500 nm.

hAMSCs-EXOs effect on human microglia cells

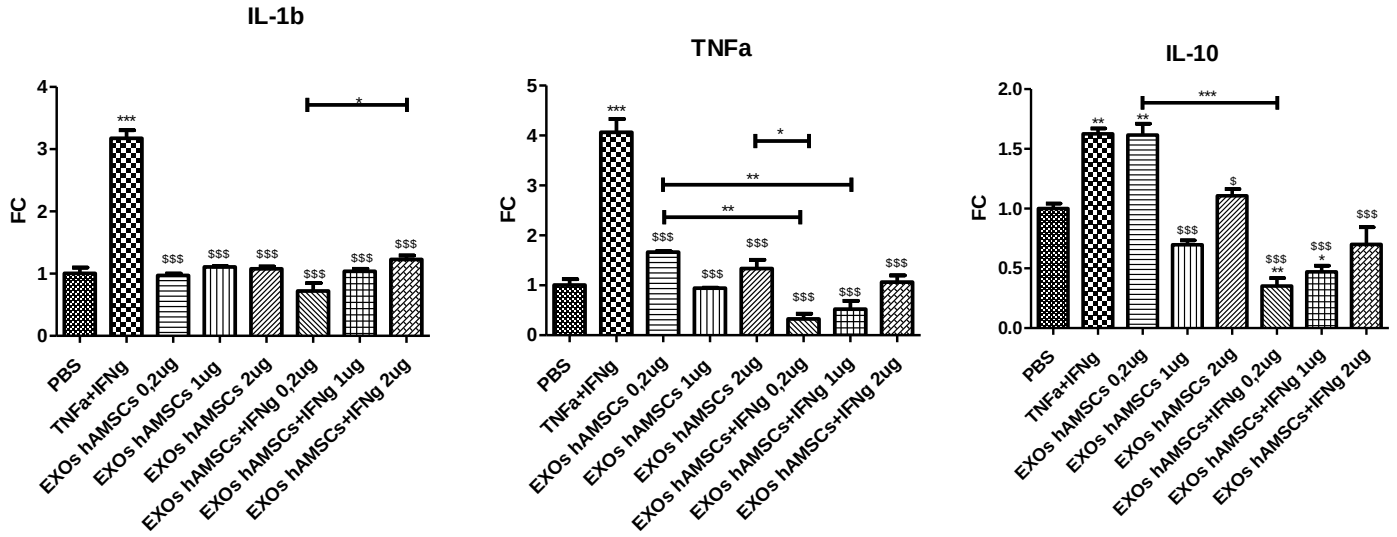
HMC3 were treated with EXOs isolated from conditioned medium of unprimed and IFN-γ-primed hAMSCs according to the protocols mentioned above and the polarization status was assessed analysing IL-1β, TNFα, and IL-10 gene expression by quantitative real-time PCR. Cells treatment with hAMSCs-EXOs at different concentrations for 4h resulted in a significant decrease of IL-1β, TNFα and IL-10 expression levels in cells treated with hAMSCs-EXOs (unprimed and IFN-γ-primed) compared to positive control (TNFα+IFNγ). Moreover, a statistically significant increase in IL-10 levels has been observed following EXOs from unprimed hAMSCs treatment at 0,2 μg/mL concentration, while a significant decrease in IL-10 expression has been noticed in cells treated with EXOs from IFN-γ-primed hAMSCs at 0,2 and 1 μg/mL concentration compared to negative control (PBS) (**figure 7A**).

In TNFα + IFNγ treatment for 4h following by EXOs treatment for 24h no significant effects of EXOs from both unprimed and IFN-γ-primed hAMSCs on pro- and anti-inflammatory cytokines mRNA levels have been appreciated (**figure 7B**).

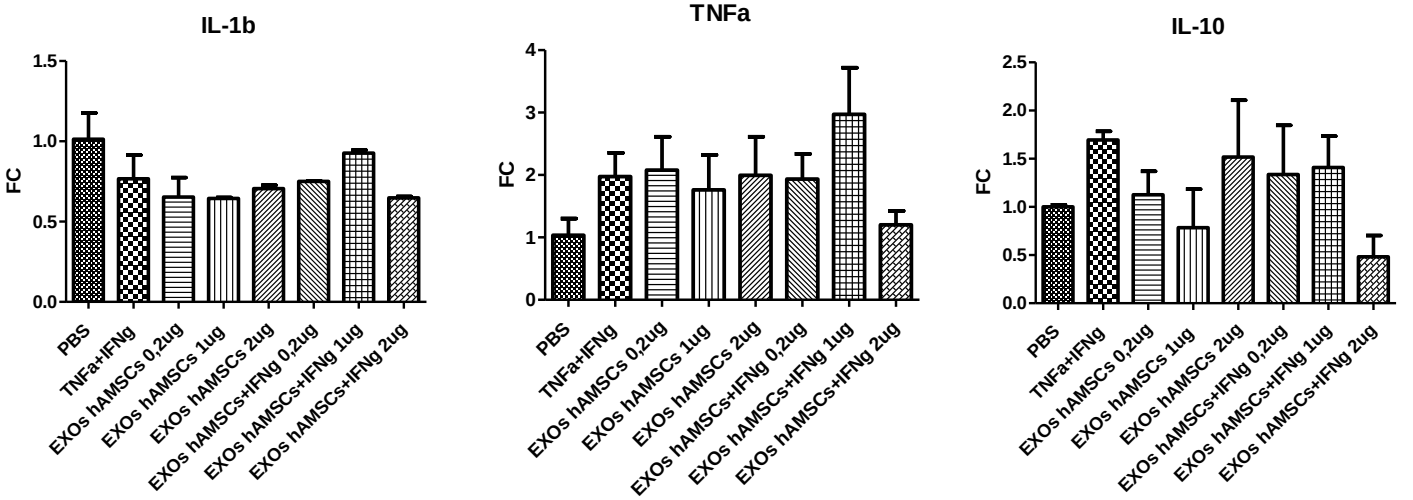
Lastly, we treated microglia cells with hAMSCs-EXOs for 24h and TNFα+IFNγ for 4h observing a significant increase of IL-1β and TNFα levels compared to negative control. Furthermore, a downward trend of TNFα expression has been found in cells pre-treated with hAMSCs-EXOs compared to positive control, but significant difference only following treatment with EXOs from IFN-γ-primed hAMSCs at 1 μg/mL. A little increase, not statistically significant, in IL-10 levels following treatment with EXOs from unprimed hAMSCs at 1μg/mL and those from IFN-γ-primed hAMSCs at 0,2 – 1μg/mL concentration has been appreciated (**figure 7C**).

In all performed experiments no dose dependent effects in cytokines gene expression have been noticed in microglia treated with exosomes isolated from unprimed and IFN-γ-primed hAMSCs.

A


IL-1b
TNFa
IL-10
FC
PBS
TNFa+IFNg
EXOs hAMSCs 0,2ug
EXOs hAMSCs 1ug
EXOs hAMSCs 2ug
EXOs hAMSCs+IFNg 0,2ug
EXOs hAMSCs+IFNg 1ug
EXOs hAMSCs+IFNg 2ug

B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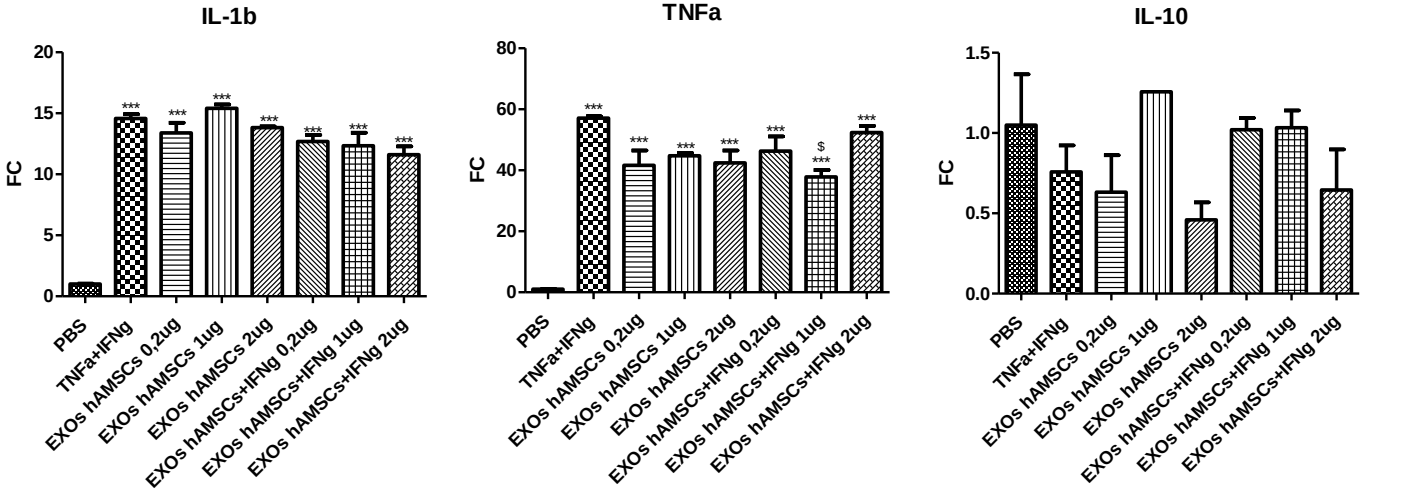
C


IL-1b
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EXOs hAMSCs 2ug
EXOs hAMSCs+IFNg 0,2ug
EXOs hAMSCs+IFNg 1ug
EXOs hAMSCs+IFNg 2ug

Figure 7. EXOs-hAMSCs effect on HMC3. Exosomes from unprimed and IFN-γ-primed hAMSCs conditioned medium have been used to stimulate HMC3 and IL-1β, TNFα, and IL-10 gene expression has been evaluated by Real-Time PCR following EXOs treatment for 4h (**A**), TNFα+IFNγ treatment for 4h and EXOs treatment for 24h (**B**) and EXOs treatment for 24h and TNFα+IFNγ treatment for 4h (**C**). Data are expressed as mean ± SD of fold change (FC) values of two experiments for each condition (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. PBS controls; $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ vs. TNFα+IFNγ controls).

DISCUSSION AND CONCLUSION

In the last few years, there has been a growing attention on the extracellular vesicles as biomarkers and therapeutic mediators in several human diseases. The potential role of EVs in MS pathogenesis has already suggested. An increase of EVs has been reported in serum and CSF of MS patients compared to controls. Recently, miRNAs have attracted interest in the context of the investigation of molecular pathways involved in the development and progression of MS. In particular, the potential use of EVs-associated miRNAs as reliable biomarkers for MS could positively affect the development of highly sensitive diagnostic strategies for rapid, non-invasive monitoring of the pathologic condition of MS patients.

Previous studies conducted by my colleagues have demonstrated that the expression of miR-146a, miR-223, miR-125a, miR-30c and miR-23a were increased in relapsing-remitting MS patients compare to healthy controls in peripheral circulating monocytes (Amoruso et al., 2020). Then, we focused on the expression of a specific set of miRNAs (miR-223, miR-146a, miR-21, and miR-155) involved in MS immune-related processes and macrophage polarization and plasticity (Self-Fordham et al., 2017) in serum EXOs from HD and MS patients.

Regarding miR-223, its expression increased, but not statistically significant, in MS patients in not-active disease phase compared to those in active phase and healthy controls. MiR-223 is highly expressed in myeloid cells and is involved in inflammatory response via NF-kB pathway (Ridolfi et al., 2013) and via STAT5 (Fenoglio et al., 2016). MiR-223 is responsible for M2 polarization in monocyte/macrophages (Essandoh et al., 2016) and its delivery by EVs can modulate monocyte/macrophage differentiation (Ismail et al., 2013). Galloway et al. suggested that miR-223 constrains neuroinflammation while also promoting repair, a finding of important pathophysiological relevance to MS (Galloway et al., 2019).

As concerning miR-146a, we observed an increment of expression levels in not-active disease phase compared to active phase and healthy donors, but not significant difference has been found. MiR-146a has emerged as anti-inflammatory molecule by suppressing the NF-κB and MAPKs pathways (Li et al.,

2016) and by inhibiting the differentiation of autoreactive CD4 T cells into pathogenic Th17 cells (Li et al., 2017). Furthermore, Prada and colleagues demonstrated that rat microglia-derived MVs enriched in miR-146a induce important alterations of synaptic structure and function in neurons downregulating Syt 1 and Nlgn 1 expression (Prada et al., 2018).

Moreover, we found a not significant increase of miR-21 expression levels in MS patients compared to HD especially in not-active phase patients. MiR-21 promotes Th17 differentiation and may modulate T cell activation and apoptosis, Treg function and development (Wang et al., 2016).

Of the most studied miRNAs implicated in MS, miR-155 is an encephalitogenic, Th17 subset-associated miRNA, described as a master regulator of the immune response, specifically driving myeloid cell polarization to a pro-inflammatory state (Moore et al., 2013; Butovsky et al., 2015). MiR-155 promotes demyelination acting indirectly on astrocytes and oligodendrocytes (Jagot and Davoust, 2016). We observed a statistically significant up-regulation of miR-155 expression in MS patients compared to healthy controls.

In summary, our results showed an upregulation of the studied miRNAs in MS not-active phase compared to both healthy donors and MS active phase that may indicate their contribution to the pathogenesis of the disease. This observed phenomenon could be due to the coexistence of pro- and anti-inflammatory processes in not-active patients that are seeking to counteract the harmful activity of pathogenic cells, promoting suppression of active inflammation, and maintaining stable RRMS course. However, their role in MS has yet to be fully clarified. We will aim to better understand the role of these miRNAs in the disease course with possible future enrolment of treated patients to analyse if disease modifying treatments (DMT) can affect the expression of these miRNAs. Furthermore, it would be interesting to understand whether these exosomes could impact neuroinflammation in MS by analyzing their pro- and anti-inflammatory cytokine content.

Then, to deepen the hypothetical pathophysiologic functions of serum exosomes in MS, we studied the effects of these vesicles on CNS cells like microglia. Recent studies about serum exosomes have demonstrated that they are capable to induce in vitro and in vivo microglia activation toward a pro-inflammatory phenotype in a model of autism spectrum disorder (Tsilioni et al., 2018) and Parkinson's

disease (Han et al., 2019). So, we decided to evaluate the effect of serum exosomes isolated from MS patients in active or not-active disease phase on human microglia cell line. Preliminarily we identified the treatment with TNFα+IFNγ for 4 h as best pro-inflammatory stimulus and subsequently proceeded with the patients' serum exosomes treatment to understand if EXOs are able to increase or decrease inflammation in microglia. Contrary to expectations, in this study we found no role of serum EXOs from MS patients in triggering or increasing microglia pro-inflammatory polarization in vitro, likely suggesting no relationship between circulating exosomes and neuroinflammation in MS disease. In future studies we could investigate the effects of serum exosomes on SNC cells such as neurons or oligodendrocytes.

Lastly, we hypothesized that EXOs isolated from human amniotic mesenchymal stromal cells could provide a better course of treatment for MS by investigating whether hAMSCs-EXOs treatment would reduce inflammation in human microglia cells. Despite the therapeutic potential of EXOs derived from MSCs has been already demonstrated (Riazifar et al., 2019; Giunti et al., 2021), we did not observe a clear anti-inflammatory effect of EXOs derived from unprimed and IFN-γ-primed hAMSCs on human microglia cells in all three performed protocols. Nevertheless, data show that hAMSCs-EXOs are not pro-inflammatory by themselves. However, further investigations are needed to elucidate the immunomodulatory effect of hAMSCs-EXOs and other sources of MSCs should be investigated in this context.

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