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Mesenchymal stem cells modulate microglia activation via exosome-shuttled microRNA

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1. Introduction

1.1 Mesenchymal stem cells are multipotent stem cells with mesodermal origin

Stem cells are specialized cells with the capacity of self-renewal and differentiation in many cell lineages. They can be divided in three main categories: embryonic stem cells (ESC) (Thomson *et al.*, 1998), induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006) and adult stem cells (Maximow, 1909).

Mesenchymal Stem (or Stromal) Cells (MSC) are adult stem cells derived from the mesodermal layer. In the past, MSC were mistakenly considered to be able to repair and regenerate almost all tissues (Prockop, 1997). Actually, it is known that they are multipotent cells, belonging to the group of adult stem cells, that can generate only mesodermal lineage cells, such as adipocytes, chondrocytes and osteocytes (Nardi and da Silva Meirelles, 2006), while maintaining their potential ectodermal (Petersen *et al.*, 1999) and neuroectodermal (Beyth *et al.*, 2005) differentiation.

MSC were first isolated from bone marrow (Friedenstein *et al.*, 1974), but have been reported in numerous adult organs and tissues (da Silva Meirelles *et al.*, 2006), including adipose tissue, umbilical cord, amniotic fluid and endometrium (Ullah *et al.*, 2015).

Although there are no known markers specific for MSC, a cell is defined as mesenchymal stem if it exhibits adhesion properties *in vitro*, has a fibroblast-like morphology (Hematti, 2012), is recognized by the monoclonal antibody STRO-1 and is phenotypically identified by expression of surface stromal markers, including Cluster of Differentiation 44 (CD44), CD71, CD73, CD90, CD105, CD271 and Ganglioside GD2, concomitantly with the lack of expression of hematopoietic markers CD11b, CD14, CD19, CD34, CD45, CD79a, Major Histocompatibility Complex class II (MHCII), or costimulatory molecules CD80, CD86 and CD40 (Dominici et al., 2006; Chamberlain et al., 2007; Horwitz et al., 2005).

1.2 MSC have an anti-inflammatory effect on immune cells in vitro

In 2002, the demonstration that bone marrow-derived MSC were able to inhibit T-cell proliferation *in vitro* (Di Nicola *et al.*, 2002; Bartholomew *et al.*, 2002) set the basis for the studies of the significant therapeutic plasticity that characterizes this cell population. Indeed, MSC have the capacity to modulate effector functions of different cells of both innate and adaptive immunity (Lovato *et al.*, 2013), through a direct or indirect mechanism.

1.2.1 *MSC modulate the effector functions of T and B cells in vitro*

The direct inhibition of T cells by MSC depends on the release of soluble factors, including indoleamine 2,3-dioxygenase (IDO), prostaglandin 2 (PGE-2), interleukin 10 (IL-10), transforming growth factor β (TGF-β), through which MSC are able to make T cells anergic (Benvenuto *et al.*, 2007; Glennie *et al.*, 2005; Ghannam *et al.*, 2010) or to skew their phenotype to a regulatory one (Treg) (Melief *et al.*, 2013). A recent study showed that MSC promote the formation of $CD4^{+}CD25^{+}$ Treg cells, directly through production of TGF-β1, or indirectly through release of chemokine (C-C motif) ligand 18 (CCL18), which induces monocytes to acquire an anti-inflammatory phenotype and to promote Treg development (Melief *et al.*, 2013).

While some studies showed that MSC have a direct inhibitory effect on B-cell proliferation, maturation and antibody release *in vitro* (Corcione *et al.*, 2006), others demonstrated that MSC induce proliferation and differentiation of purified B cells (Traggiai *et al.*, 2008). Such controversy was resolved with the demonstration that MSC need T-cell signals for being able to modulate B cells effects (Rosado *et al.*, 2015). When MSC are stimulated with IFN-γ, released by effector T cells, they create more interactions with B cells through binding between programmed cell death 1 (PD-1), expressed on MSC, and its ligand (PDL-1), expressed on B cells. The cross-talk between MSC and B cells induces inhibition of B-cell receptor-mediated activation of follicular and marginal zone B cells isolated from lupus mice (Schena *et al.*, 2010).

MSC suppress B-cell maturation independently of cell contact and the soluble factors involved are still unknown, but the pathway of B lymphocyte–induced maturation protein-1 (Blimp-1), a transcriptional factor that regulates B-cell differentiation, is dysregulated (Asari *et al.*, 2009). Asari and co-workers confirmed that the MSC suppressive activity on B cells acts through paracrine mechanisms also *in vivo*; indeed, administration of MSC-conditioned medium (MSC-CM), supernatant of MSC culture, in mice results in a decrease of IgM and IgG production (Asari *et al.*, 2009). An *in vitro* study demonstrated that in the presence of MSC plasmablast development was decreased, but MSC induced the formation of CD19+CD24highCD38high IL-10-secreting B cells, known as regulatory B cells (Franquesa *et al.*, 2015).

MSC have immunomodulatory effects on T and B cells also through indirect mechanisms, influencing differentiation and functions of myeloid-derived cells, such as Dendritic cells (DC) and macrophages, related to the adaptive immune response.

1.2.2 MSC affect the activity of cells of the innate immunity in vitro

MSC inhibit differentiation of monocytes into immature DC (Nauta *et al.*, 2006; Spaggiari *et al.*, 2009). *In vitro* and *in vivo* studies showed that MSC impair Toll–like receptor (TLR) activation of DC, inhibiting their capacity to present antigen to T cells (Chiesa *et al.*, 2011), which results in decreased T-cell proliferation. Cytokine production by activated DC was blocked by MSC through release of soluble factors that weaken the activation of the mitogen-activated protein kinase (MAPK) cascade of the NF-κB pathway, upregulated upon TLR4–induced DC activation (Chiesa *et al.*, 2011).

Specifically, MSC influence DC to assume a suppressive phenotype through release of growth-regulated oncogene (GRO) chemokines (Chen *et al.*, 2013). When DC are activated by Gro-γ, they induce T cells to differentiate toward a tolerogenic phenotype, characterized by the production of high levels of anti-inflammatory cytokines, such as IL-10 and IL-4, and reduced levels of pro-inflammatory cytokine expression, such as IL-12 and IFN-γ (Chen *et al.*, 2013).

MSC exert their effect on DC not only through paracrine mechanisms: a direct contact MSC-DC is required to inhibit formation of active immune synapses by DC with T cells (Aldinucci *et al.*, 2010).

MSC have also been shown to interact with NK cells *in vitro* (Spaggiari *et al.*, 2006; Sotiropoulou *et al.,* 2006; Krampera *et al.,* 2006; Poggi *et al.,* 2005); MSC can block IL-2-mediated NK cell proliferation (Spaggiari *et al.*, 2006), and effector functions, such as cytotoxicity and cytokine secretion (Sotiropoulou *et al.*, 2005; Spaggiari *et al.*, 2008). This inhibitory effect of MSC is mediated by released IDO, PGE2, and TGF-β, that induce a down-regulation of the expression of specific NK receptors, NKp30, NKp44, and NKG2D (Spaggiari *et al.*, 2008). Unfortunately, the underlying mechanisms are yet unknown.

MSC play a suppressive role also on macrophages through a direct cell-to-cell contact, inducing pro-inflammatory macrophages (M1 phenotype) to acquire an anti-inflammatory profile (M2 phenotype), but the modulatory mechanism of activated macrophages by MSC is still unknown (Siniscalco *et al.*, 2011). When put in co-culture with LPS-activated alveolar macrophages (macrophages that acquire a pro-inflammatory phenotype also known as M1 macrophages), MSC cause a deregulation in the expression of inflammatory cytokines, such as IL-1α and IL-6, resulting in a skewing of M1 macrophages toward an anti-inflammatory phenotype (M2 macrophages) and an increase in the expression of anti-inflammatory factors (Jin *et al.*, 2013). Dayan *et al.* observed that pro-inflammatory macrophages were induced to assume an alternative activated profile by the overexpression of IL-10 by MSC (Dayan *et al.*, 2011). Moreover, MSC induce macrophages to acquire a regulatory phenotype, characterized by an increase in expression of IL-10 and reduced levels of molecules inducing T-cell activation, such as

TNF-α, IL-12, and co-stimulatory molecule CD86 (Maggini *et al.,* 2010). A recent study *in vitro* demonstrated that human MSC modulate both human and mouse macrophage activity through the inhibition of NLRP3 inflammasome activation in LPS or ATP-activated macrophages (Oh *et al.*, 2014). Kim and Hematti confirmed the capacity of MSC to promote an alternative activation of macrophages *in vitro*, as demonstrated by the expression of high levels of IL-6 and CD206, typical markers of alternatively activated macrophages, and IL-10, together with low levels of TNF-α and IL-12, specific markers of classical macrophage activation, in macrophages exposed to MSC (Kim and Hematti, 2009). This shift in phenotype was accompanied by an improved phagocytic activity of macrophages (Kim and Hematti, 2009).

As a consequence of the modulatory/suppressive effect of MSC on the peripheral immune cells *in vitro*, we postulated that MSC could affect also microglial cells, which represent the resident macrophages in the central nervous system (CNS).

1.3 Microglia are resident macrophages of the CNS

In 1920, Pío del Río-Hortega discovered a new type of cells belonging to neuroglia population in the CNS, microglia (Tremblay *et al.*, 2015).

Even if they are considered one of the four main cellular type of CNS, microglia do not arise from the same precursors as neurons, astrocytes and oligodendrocytes, but they are macrophages resident in the CNS originating from myeloid progenitor cells and sharing various markers with dendritic cells, monocytes and peripheral macrophages, such as CD11b, F4/80 (also known as EMR1 or Ly71) and CD45 (Prinz *et al.*, 2011).

Microglial cells originate in the yolk sac during early hematopoiesis (Ginhoux *et al.*, 2010). Later the hematopoietic precursors migrate to the brain and proliferate *in situ* (Alliot *et al.*, 1999). After their migration in the neural tissue, the cells resettle stably in the CNS and undergo self-renewal until the adult age (Ginhoux *et al.*, 2010; Greter and Merard, 2013). Microglia are ubiquitously present in brain and spinal cord and it was estimated that they represent about 10% of all cells found within the CNS (Lawson *et al.*, 1992).

At the beginning, microglia were considered as a population of inactivated and quiescent cells (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005), but through two-photon microscopy, it was observed that also in absence of damage, microglia are surprisingly dynamic (Schwartz *et al.*, 2006), playing an important role in the maintenance of tissue homeostasis and in defense mechanisms for the CNS against exogenous and endogenous threats; indeed, they are able to eliminate dead cells and harmful debris through phagocytosis and to produce cytokines and growth factors (Aloisi *et al.*, 2000), having a beneficial role for the CNS.

Microglial cells play a relevant role in neuroinflammation, including tissue repair and regeneration, immunosuppression and cytotoxicity; indeed, they are able to acquire different states or phenotypes of activation (Chhor *et al.*, 2013).

1.3.1 Microglia can assume different phenotypes of activation on the basis of the signals received

In a healthy CNS, microglia assume a surveillance phenotype, defined as “resting”, characterized by a slow turnover rate, a low expression of functional markers, such as MHCII and CD45 (Lynch, 2009), and highly branched and very mobile processes which contact neurons and glial cells, while the soma stays fixed and stationary (Kalla *et al.*, 2001; Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). As soon as they detect an event that determines the loss of homeostasis or which can lead to tissue damage, such as the presence of some extraneous matter, damaged or apoptotic cells, DNA fragments, or plaques, microglial cells change their morphological, molecular and functional states, ranging from a resting to an ameboid intermediate form with short and thick processes to acquire a more active profile (Aloisi, 2001; Chhor *et al.*, 2013) characterized by a bigger, granular soma with cytoplasmic inclusions and shorter cellular processes (Frommann *et al.*, 1878; Colton and Wilcock, 2010). Furthermore, microglial cells become mobile and actively move following chemotactic gradients, causing a cellular thickening in the damaging site (Kettenmann *et al.*, 2011). Local microglial density also greatly increases due to an intense proliferative activity, in order to offer a more efficient defense mechanism (Kettenmann *et al.*, 2011). But when they are excessively and chronically activated, microglia lose their protective role and assume a neuroinflammatory, deleterious, phenotype.

The microglia activation process is characterized by the induction of expression or rearrangement of surface molecules that mediate cell-cell and cell-matrix interactions, by changes in intracellular enzyme activity, as well as by the release of a series of factors with pro-inflammatory and immunomodulatory effects (Kettenmann *et al.*, 2011).

The distinction of activated microglia phenotypes is based on the expression of different molecular markers; expression of pro-inflammatory receptors or cytokines with a cytotoxic effect distinguish M1-like phenotype or classical activated microglia, whereas anti-inflammatory receptors or cytokines involved in tissue repair and regeneration are overexpressed in an M2-like or alternative microglial activated phenotype (Guedes *et al.*, 2013; Chhor *et al.*, 2013).

Microglial cells assume a specific activation state on the basis of the nature, duration, and intensity of the received stimulus, and the microenvironment in which they are. Pro-inflammatory stimuli, such as lipopolysaccharide (LPS), interleukin-1 β (IL-1β) and tumor

necrosis factor α (TNF-α), increase genic expression of cytotoxic markers related to M1-like phenotype, for example cyclooxygenase-2 (Cox-2) and inducible nitric oxide synthase (iNOS), and release of factors, such as TNF-α, interleukin-6 (IL-6), and IL-1β (Chhor *et al.*, 2013). Furthermore, the classical microglial activation is associated with the production of reactive oxygen species (ROS) caused by a greater activity of NADPH oxidase. High levels of intracellular ROS in microglia amplify their pro-inflammatory cytokines expression contributing to microglia neurotoxicity (Block *et al.*, 2007).

Conversely, the alternative microglia activation is characterized by secretion at high levels of anti-inflammatory cytokines, such as IL-4, IL-5 or IL-10, and neurotrophic factors, like brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1) and TGF-β (Gomes-Leal, 2012; Boche *et al.*, 2013), which lead to a neuroprotective microglial phenotype and capacity to inhibit pro-inflammatory response.

More studies revealed the existence of different states of intermediate activation and evidenced that phenotypes M1- and M2-like can overlap, accordingly microglia are able to assume a wide spectrum of phenotypes which cannot be simplistically divided in discrete categories.

1.3.2 Microglia acquire an activated phenotype in response to the surrounding environment

The CNS milieu is full of molecules that modulate microglial activation; the nature of the surrounding tissue directs cells towards a particular phenotype rather than another one.

Microglial activation is controlled by two classes of signals: *off* and *on* signals (Biber *et al.*, 2007). The *off* signals are molecules constitutively present in brain environment; their absence triggers a microglia activation (Biber *et al.*, 2007). For instance, binding between CD200, expressed on neurons, and its receptor (CD200R), expressed on microglia (Hoek *et al.*, 2000), represents an *off* signal; indeed, CD200-CD200R interactions are essential to prevent microglial switch from the alternative neuroprotective phenotype to the classically activated state, thereby avoiding over-activation of microglia and consequent neurotoxicity (Zhang *et al.*, 2011). Another important factor produced by neural cells and involved in *off* signaling is fractalkine (CX3CL1), a C-X-C motif chemokine that acts through binding to its specific receptor, CX3CR1, expressed by M2-like microglia, preventing neurotoxic activity (Cardona *et al.*, 2006; Lee *et al.*, 2010). Even TGF-β is a relevant *off* signal for modulating microglia activation. Studies demonstrated that in presence of TGF-β in the microenvironment, microglia reduce their production of pro-inflammatory cytokines, while the lack of this factor determines a pathological microglial activation, leading to microgliosis and neuronal death (Brionne *et al.*, 2003).The *on* signals appear when cell damage occurs and they act by attracting microglia in the lesion site,

through the creation of a chemical gradient. Among the possible chemotactic factors, ATP (Kaufmann *et al.*, 2005) triggers the rapid microglial response migration to the lesion site (Davalos *et al.*, 2005). This phenomenon involves purinergic receptors (P2), as demonstrated by the decrease of microglial chemotactic response *in vivo* after administration of various P2 receptors inhibitors in the cortex (Davalos *et al.*, 2005), or through experiments in mice knock-out for metabotropic purinergic receptor P2Y12 (Haynes *et al.*, 2006), in which motility of microglia is reduced. Even an excessive release of neuronal glutamate, associated with neurodegenerative process, acts as an *on* signal for the activation and migration of microglia, presumably through the binding to specific ionotropic receptors for glutamate expressed on microglia surface; treatment with α-amino-3-hydroxy-5-methyl-4-isoxazolone propionate (AMPA), a glutamate agonist, induced microglia migration following glutamate concentration gradients (Liu *et al.*, 2009). However, the expression of N-methyl-D-aspartate (NMDA) receptor for glutamate remains uncertain in microglia.

The activation of microglia appears to have contradictory consequences, depending on inputs and feedback signals from the neural environment. On one hand, M2-like microglia activation facilitates the removal of degenerate neurons, inflammatory debris and protein aggregates, which are histopathological characteristic of numerous neurodegenerative disorders. On the other hand, neurotoxin secretion and pro-inflammatory activities associated with microglia M1-like phenotype may aggravate the disease by promoting the inflammatory process (Block *et al.*, 2007) and contributing to neurodegeneration. Therefore, depending on their state of activation, microglia play different functions in the neural tissue (Boche *et al.*, 2013).

The activation of microglia, whether or not accompanied by microgliosis, occurs practically in all diseases of the CNS. Although microglia appear to play a key role in controlling the efficiency of the nervous system in healthy subjects, and, in an alternately activated state, play a beneficial role in the early stages of the disease, a continued activation is implicated in the neurodegeneration process. Recent research has shown an involvement of microglial population in neural pathologies, considering it as one of the major causes of neuronal malfunction (Derecki *et al.*, 2014).

1.4 MSC can modulate activation of microglia in vitro

In vitro, MSC are able to modify the activation of microglia both in co-cultures (cultures where direct cell-cell contact occurs) and in transwell cultures (cultures in which the two different cell types are separated by a porous membrane that avoids a physical interaction between the two populations, but allows the cellular communication based on the release

of soluble factors). This most likely occurs through inhibition of the activation of mitogen-activated protein kinase (MAPK) p38, which is essential for microglial activation induced by TLR4 (LPS-binding receptor) (Zhou *et al.*, 2009; Pathak *et al.*, 2006). *In vitro* studies from our laboratory have shown that MSC are able to induce a switch of microglia from the M1-like phenotype to the classically activated phenotype (M2-like) by releasing CX3CL1, recognized by CX3CR1 on microglia. In particular, MSC cause a significant increase of expression and release of neuroprotective molecules by activated microglia, while significantly decrease the expression and release of various inflammatory factors and molecules involved in oxidative stress (Giunti *et al.*, 2012).

MSC therefore represent an efficient tool through which it might be possible to modulate the activation state of microglia, inducing a shift from a neurotoxic, pro-inflammatory phenotype to a neuroprotective one. It could partly explain the beneficial effects that are associated with MSC therapeutic plasticity in the various experimental models of neurological pathologies in which the inflammatory component plays a fundamental role (Uccelli and Mancardi, 2010; Uccelli *et al.*, 2011).

1.5 Administration of MSC to mice affected with amyotrophic lateral sclerosis-like disease decreases neuroinflammation

Many neurodegenerative diseases are characterized by a dysregulation of immune responses that sustains the neuroinflammation and neural degeneration progress. It has been observed that MSC adoptively transferred can migrate in the CNS injured site (Le Blon, *et al.*, 2014) and, with their immunomodulation potential on T cells, macrophages, and especially microglia, could provide remarkable therapeutic effects (Gordon *et al.*, 2008).

Recent evidences suggested the efficacy of MSC-based therapy in treating numerous neurodegenerative disease, including amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig’s disease) (Hajivalili *et al.*, 2016).

ALS is an adult-onset neurological disease characterized by degeneration of upper and lower motoneurons (MNs) in the motor cortex, brainstem and spinal cord (Bruijn *et al.*, 2004), causing their progressive loss of function, and eventually leading to death via respiratory muscle paralysis (Cozzolino *et al.*, 2008). The etiology of disease is still unclear, indeed most ALS cases (90%) are sporadic (SALS), defined as having no obvious genetic component (Brown, 1997); only 10% of cases are inherited in a dominant fashion (known as familial ALS; FALS). In both SALS and FALS there are similar pathological hallmarks, including weakness, atrophy, spasticity (Mulder, 1982) and accumulation of phosphorylated neurofilaments in MN axons (Ince *et al.*, 1998). In addition, SALS and

FALS show the same overactivation and overproliferation of astrocytes and microglia (Uccelli *et al.*, 2012). Twenty percent of ALS are related to mutations in the Cu/Zn superoxide dismutase (SOD1) gene. SOD1 is a ubiquitously expressed cytosolic enzyme consisting of 153 aminoacids. Binding one zinc and one copper atom, it catalyzes the conversion of superoxide radicals into oxygen and hydrogen peroxide (Sea *et al.*, 2015). More than 125 mutations on SOD1 gene have been observed but only 114 of them provoke disease, inducing a toxic gain-of-function of the enzyme (Turner and Talbot, 2008; Ilieva *et al.*, 2009; Rothstein, 2009).

Among others, an important SOD1 point mutation in which a glycine substitutes the alanine at residue 93 ($SOD1^{G93A}$) is commonly studied in ALS research. The transgenic mice expressing many copies of human $SOD1^{G93A}$ were developed by Gurney and collaborators, and this animal model shows a progressive adult-onset motor phenotype comparable to that seen in patients (Gurney, 1994). In agreement with these data, Reaume et al., created mice deficient for SOD1 ($SOD1^{-/-}$) and observed that those animals did not develop motor neuron disease (Reaume *et al.*, 1996). Moreover, transgenic mice overexpressing wild-type human SOD1 form were completely healthy (Wong *et al.*, 1995). Although the mechanisms underlying the ALS are still unknown, neuroinflammation plays an important role in ALS pathogenesis. Microglial activation was observed in the brain and spinal cord of ALS patients (Engelhardt and Appel 1990; Henkel *et al.*, 2004; McGeer *et al.*, 1991; Troost *et al.*, 1993; Turner *et al.*, 2004) and in the spinal cord of mutant SOD1 mice (Hall *et al.*, 1998; Henkel *et al.*, 2005; Kriz *et al.*, 2002). Henkel et al. observed a previous microglia activation that was followed by loss of MNs (Henkel *et al.*, 2006). Other studies confirmed a role for microglia in the pathogenesis of ALS. Forcing activation of microglial cells by administrating LPS to SOD1 transgenic mice exacerbated the disease (Nguyen *et al.*, 2004), and silencing the expression of CX3CR1 on microglia of mutated SOD1 mice accelerated neuronal loss (Cardona *et al.*, 2006).

In vivo studies in our laboratory have shown that intravenous administration of MSC in $SOD1^{G93A}$ mice with overt disease results in a slowdown of pathology progression and an improvement of motor function. The beneficial effect is related to the decrease of oxidative stress, the inhibition of glutamate-mediated excitotoxicity, but also to a reduction in microglial activation and hence of inflammation affecting MNs (Uccelli *et al.*, 2012). Immunohistochemical analysis of the spinal cord showed that MSC treatment did not induce differences in expression of iNOS markers, insulin-like growth factor 1 (IGF1), CX3CR1, and nuclear receptor related 1 protein (Nurr1), but rather a significant down-regulation of RNA expression for TNF-α and IL-1β pro-inflammatory cytokines in MSC-treated mice compared to controls (Uccelli *et al.*, 2012). Similarly, Vercelli *et al.* observed that ALS mice transplanted with hMSC into the spinal cord showed a decreased microglial

activation and ameliorated motility (Vercelli *et al.*, 2008). Another research group systemically administered hMSC in ALS mice and obtained a disease progression slowdown with decrease microglial activation and reactivated astrogliosis (Sun *et al.*, 2014).

1.6 Exosomes mediate intercellular communication transferring their content, including microRNAs (miRNA), to target cells

MSC are among the stem cells most studied for their potential therapeutic capacity, but their mode of action in vivo is still unclear. Indeed, administration of MSC intravenously results in less than 1% of transplanted MSC reaching the damaged site, the rest being trapped in lungs, spleen and liver (Djouad *et al.*, 2003). Accordingly, and on the basis of their in vitro effect and, more recently, of the demonstrated therapeutic impact of their secretome, their therapeutic effect is likely predominantly attributable to paracrine mechanisms (Uccelli *et al.*, 2008).

A possible paracrine mechanism through which MSC could affect their target cells is the release of vesicles, in particular of exosomes (Ludwig and Giebel 2012). Exosomes are nanovesicles with a diameter of 40-100 nm of endocytic origin that exhibit a set of preserved proteins, including ALIX and CD9, CD63 (Lai *et al.*, 2011).

Many research groups are studying the capacity of exosomes to mediate paracrine effects of the cells that release them. Chen *et al.* showed that MSC-derived exosomes alter production of pro-inflammatory cytokines, TNF-α and IL-1β, on T cells, inducing them to secrete anti-inflammatory mediator TGF-β *in vitro,* and promote a shift to anti-inflammatory T cells, with the transformation of T helper type 1 (Th1) into T helper type 2 (Th2) cells and the reduced differentiation of T cells to interleukin 17-producing effector T cells (Th17) (Chen *et al.*, 2016). Teng *et al.* observed an anti-inflammatory effect when exosomes isolated from MSC conditioned medium are in culture with T cells (Teng *et al.*, 2015). Another group demonstrated that MSC affect macrophage activation through microRNAs-containing exosomes that suppress Toll-like receptor (TLR) expression and inflammatory signaling both *in vitro* and in an *in vivo* model of lung injury (Phinney *et al.*, 2015). Exosomes can affect the fate of the receiving cell through the transfer of their content, which includes proteins, mRNA and microRNA (miRNA) into the cellular lumen (Valadi *et al.*, 2007).

MiRNA are small regulatory sequences of non-coding RNA consisting of 20-22 nucleotides whose purpose is to regulate gene expression at post-transcriptional level (Bartel, 2004). They are transcribed by RNA polymerase II as precursors, called primary miRNA (pri-miRNA). Pri-miRNA assumes an asymmetric structure, partly single- and partly double-

stranded with intramolecular interaction. Subsequently, it is processed by RNAse III Drosha in the nucleus to form the premature miRNA (pre-miRNA) (Han *et al.*, 2004). The pre-miRNA arrives in the cytoplasm of the cell where another RNAse III, Dicer (Bernstein *et al.*, 2001), generates the mature form of miRNA (Filipowicz *et al.*, 2008).The mature miRNA are mainly expressed in the cytoplasm where they can recognize and target specific mRNA, generally preventing their translation or promoting their degradation (Shyu *et al.*, 2008).

The post-transcriptional mechanism mediated by miRNA plays an important role in events that determine changes in cell fate, such as differentiation and activation. Several studies confirm that miRNA may act as modulators of microglia activation state. One of the first described pro-inflammatory miRNA associated with the microglial M1-like phenotype is miRNA-155. This particular miRNA is overexpressed in microglial cells in response to various pro-inflammatory stimuli, such as LPS, IFN-γ and TNF-α (Cardoso *et al.*, 2012; Bala *et al.*, 2011; Wang *et al.*, 2010). In this regard, it has recently been shown that miRNA-155 decreases the expression of some microglia anti-inflammatory proteins, such as suppressor of cytokine signaling 1 (SOCS-1), and causes upregulation of various inflammatory mediators characteristic of the M1-like phenotype, including iNOS, IL-6 and TNF-α (Cardoso *et al.*, 2012). Interestingly, there is a feedback mechanism that controls immune response, indeed the over-expression of miR-155 increases the expression of Interferon β (IFN-β), which in turn increases the expression of SOCS-1 and IL-10, two major anti-inflammatory mediators (Benveniste *et al.*, 2007; Dalpke *et al.,* 2008).

Several other miRNA have been directly linked to the alternative activation state of microglia. Recently, the role played by miRNA-200b in microglial inflammatory response has been analyzed. MiRNA-200b influences various aspects of the microglia inflammatory process, particularly reducing pro-inflammatory cytokine secretion, NO production, and inducing a neuroprotective activity, and it does this through the modulation of MAPK pathway (Jadhav *et al.*, 2014).

Moreover, it has recently been shown that several miRNA play an important role in the control of neuroinflammatory mechanisms. Specifically, miRNA-365 and miRNA-125b are over-expressed in the primary microglia obtained from murine model of ALS ALS and they contribute to increase TNF-α transcription through IL6-STAT3 pathway repression on microglia (Parisi *et al.*, 2013).

MiRNA-124 is specifically expressed in microglial cells, but not in peripheral monocytes or macrophages (Ponomarevet *et al.*, 2011). An overexpression of miRNA-124 in microglia induces activated cells to assume a quiescent resting phenotype, characterized by low expressions of CD45 and MHCII (Ponomarev *et al.*, 2011). In EAE mice, animal model of Multiple Sclerosis (MS), miR-124 is downregulated in M1-like microglia, and its

administration deactivates microglia and reduces activation of T cells (Ponomarev *et al.*, 2011).

Since miRNA affect the expression of different proteins and the signaling pathways in which those proteins are involved, inducing an over-expression or down-regulation of specific miRNA could be a useful therapeutic approach. Moreover, it has been shown that MSC-derived exosomes are enriched of miRNA with specific pattern, suggesting the existence of a finely regulated mechanism of accumulation and transfer of miRNA that mirror the cell state (Collino *et al.*, 2010).

It is well known from the literature that MSC play modulatory roles on innate and adaptive immunity mostly through paracrine mechanisms, which include soluble factors or release of exosomes. For these reasons MSC are considered a possible alternative therapy for neurological disease associated with neuroinflammation, such as ALS. The objects of our study were to ascertain whether or not MSC-derived exosomes could affect microglia activation through shuttling of specific miRNA, to identify genes concerned, and to understand whether such a mechanism could account, at least in part, for the effect of MSC treatment in ALS-affected mice.

2. Materials and Methods

2.1 N9 microglia cell line culture

The microglial cell line N9 results from the immortalization of cultures from encephalus of mouse embryo at the thirteenth day via retrovirus 3RV containing an activated v-myc oncogene. The N9 are plated in 75 cm^2 cell culture flasks at a concentration of 5-6x10^5 in 15 ml RPMI (Sigma-Aldrich) medium with 10% of Fetal Bovine Serum (FBS) (Lonza Cat No. DE14-801F), penicillin 100 U/ml, streptomycin 100 µg/ml, maintained at 37° C and 5% CO^2 in incubator. For microglial activation, 1 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich, cat. no. L2137-5MG) was added to the culture medium for 24 hours.

2.2 MSC culture

MSC were derived from bone marrow obtained from tibia and femur of 6-8 weeks old female C57Bl/6J mouse. After withdrawal from the animal, tibia and femur were transferred to a Petri dish in which, with an insulin syringe, 1 ml of sterile medium (RPMI, Sigma-Aldrich) was injected into the channel of each bone to eject the bone marrow content. The marrow fragments, deposited on the bottom of the Petri, were minced with the needle of the syringe. The suspension was transferred to a 15 ml Falcon tube and brought to a final volume of 10 ml with RPMI. Two washings were made through centrifugation at 1500 rpm for 5 minutes at room temperature and the obtained pellet was resuspended in 1 ml of MesenCult MSC Basal Medium (mouse) (Stem Cell Technologies Inc., cat. n. 05401) and counted in Burker's chamber.

The cells were then plated in 75 cm^2 cell culture flasks at a total concentration of 20-25x10^6 in 15 ml of mouse-specific Mesencult and maintained at 37° C and 5% CO^2 in incubator, changing MesenCult medium every 3/4 days for 4/5 weeks, time to reach about 80% of confluence. Cell culture were maintained in 75 cm^2 flask, plating 3x10^5 cells and splitting them upon reaching confluence with a 0.05% trypsin solution containing 0,02% ethylenediaminotetraacetic acid (EDTA, Sigma-Aldrich). Mature cells were obtained after 4-5 culture steps and were defined as having the ability to inhibit proliferation of splenocytes in vitro and their expression of stromal markers such as CD9, Sca-1, CD73 and CD44, but not of hematopoietic markers, such as CD45, CD34 and CD11b.

2.3 Primary microglia isolated from wtSOD1 or SOD1^{G93A} mice

Primary microglia were obtained from brain of 135-day old wtSOD1 mice (transgenic mice that overexpress the human SOD1 wt) or SOD1^{G93A} (transgenic mice that overexpress mutated human SOD1) mice. Brains were chopped in a Petri dish and transferred into a

15 ml Falcon tube with 2 ml of activated papain solution (2 ml of Papain stock solution (composed by 10 ml raw papain (Roche cat. no. 10108014001), 0.4 ml 0.5 M EDTA (2 mM final), 10 ml 10x HBSS Ca^{2+} and Mg^{2+} free (1x final), 2.5 ml 1 M HEPES (25 mM final), 77.1 ml ddH2O) with 10 µl 14.3 mM β-Mercaptoethanol (BME)) for 30 minutes at 37° C in the water bath, pipetting every 10 minutes. 500 µl of leupeptin (R&D Systems, cat. no.EI002) were added to the suspension and mixed thorougly for 2 minutes. 8 ml prewarmed Dnase (Sigma, cat. no.D5025) were added to the samples and incubated for 10 minutes at 37° C. Suspensions were filtered with a 100-250 µm filter. Samples were centrifuged at 450 x g at 4° C for 5 minutes. Supernatants were aspirated and the pellets were resuspended in 9 ml of RPMI with 25 mM Hepes with 10% FBS and 3.6 ml of 100% Percoll (Sigma-Aldrich cat. no.P1644-500ML) (composed of 9 parts of Percoll and 1 part of 10x HBSS). 1 ml of 10% FBS in RPMI was stratified over the Percoll and cell suspension. Samples were centrifuged at 800 x g at 4° C for 15 minutes without brake. Pellets were resuspended and cells were counted. After determining the cell count, primary microglia were purified using CD11b (Microglia) MicroBeads (Miltenyi Biotec, cat. no. 130-093-634), following the instructions provided.

2.4 Transfection

$1X10^5$ cells were plated in 24-well plate in 500 µl of RPMI and transfected using the HiPerFect® Transfection Reagent (Qiagen), according to the instruction, with mimics specific for each miRNA (miRNA Mimic miRNA, Qiagen) for 48 hours to analyze the ability of miRNA to modulate microglial phenotype, with mimics specific for each miRNA (miRNA Mimic miRNA, Qiagen) and with MISSION miRNA Mimic Negative Control, a synthetic miRNA mimic (Sigma-Aldrich cat. no. HMC0002) which does not recognize any gene target in cells, and for 1 hour to evaluate in which pathway miRNA could have a modulatory role on microglia cells.

2.5 Isolation of MSC-derived exosomes

MSCs were cultured in 75 cm^2 flasks at a concentration of $5\text{-}6x10^5$ in 15 ml of MesenCult MSC Basal Medium and allowed to expand.

Before they reached confluence, MSCs were stimulated with IFN-γ at concentration of 10 ng/ml and incubated for 24 hours. After stimulation, the cells were washed with DPBS (Lonza cat no. BE17-512F) 3 times, and 10 ml of RPMI 1640 medium containing penicillin/streptomycin and L-glutamine were added to the culture which was incubated for 24 hours. In order to increase the production of exosomes by MSC, the cells were stimulated for 20 minutes with ATP (Sigma-Aldrich, cat. no A6419-1G) 1 mM at 37° C

(Bianco et al., 2005). Subsequently the supernatant was centrifuged at 2,000 x g at 4° C for 20 minutes to eliminate all cells and debris. To recover the exosomes, the supernatant was incubated overnight at 4° C with 0.5 volume of Total Exosome Isolation Kit (Invitrogen, cat. no.4478359) and centrifuged at 10,000 x g at 4° C for 1 hour. The pellet containing the exosomes was then resuspended in various solutions based on experimental needs.

2.6 Microglia exposure to IFN-γ primed MSC-derived exosomes

$1x10^5$ LPS-activated N9 cells or $3x10^5$ primary microglia were plated in a 24-well plate with 1 ml RPMI in presence or absence of exosomes derived from IFN-γ-stimulated MSC. The amount of exosomes put in culture was based on the number of MSC from which the exosomes derived, maintaining the ratio microglia:MSC = 1:3. Cells were incubated at 37° C and 5% CO^2 for 24 hours, then processed for the extraction of RNA.

2.7 RNA extraction, mRNA retrotranscription in cDNA and real time PCR

RNA was isolated through the phenol-chloroform extraction procedure, using QIAzol Lysis Reagent (Qiagen, cat. no.79306). The purity of the RNA was evaluated by spectrophotometric analysis at the absorbance 230, 260 and 280 nm, to exclude the presence of peptides, phenols, aromatic compounds, or carbohydrates and proteins.

The cDNA was synthesized according to the instructions provided by the kit used (Transcriptor First Strand cDNA synthesis kit, Roche Diagnostics, Germany).

Quantification of the gene expression was evaluated in duplicate, in a final volume of 20 µl containing 50 ng cDNA, 1 µl of each pair of forward and reverse primers (20 µM) (synthetized by Tib Molbiol) and 10 µl of FastStart Essential DNA Green Master (Roche, cat. no.06402712001), using the LightCycler 480 (Roche). The amplification of the 3-phosphate dehydrogenase glyceraldehyde gene (GAPDH) was adopted as housekeeping gene to normalize data.

Primer sequences used: Tnf-α forward (5′-TCTTCTCATTCCTGCTTGTGG-3′) and reverse (5′-GGTCTGGGCCATAGAACTGA-3′); Il1β forward (5’-AGTTGACGGACCCCAAAAG-3′) and reverse (5′-TTTGAAGCTGGATGCTCTCAT-3′); Cx3cr1 forward (5′-AAGTTCCCTTCCCATCTGCT-3′) and reverse (5′ - CAAAATTCTCTAGATCCAGTTCAGG - 3′); Nr4a2 forward (5′-TCAGAGCCCACGTCGATT-3′) and reverse (5′-TAGTCAGGGTTTGCCTGGAA-3′); Cd206 forward (5′-CCACAGCATTGAGGAGTTTG-3′) and reverse (5′-ACAGCTCATCATTTGGCTCA-3′); Mk2 forward (5’-AGTGCAGCTCCACCTCTCTG-3’) and reverse (5’-CAGCAAAAATTCGCCCTAAA-3’); Map3k8 forward (5’-

TTCCAGTGCTCATGTACTCCA-3') and reverse (5'-GGACTGCTGAACTCTGTTTGC-3'); GAPDH forward (5'-ATGGTGAAGGTCGGTGTGA-3') and reverse (5'-AATCTCCACTTTGCCACTGC-3').

2.8 miRNA retrotranscription in cDNA, Microarray analyses and real time PCR

RNA was extracted from MSC which were activated or not with IFN-γ (10 ng/ml) for 24 hours or exosomes isolated therefrom, according to manufacturer's instructions (miRNeasy Mini Kit, Qiagen, Netherlands). The purity of the RNA was evaluated by spectrophotometric analysis at the absorbance 230, 260 and 280 nm, to exclude the presence of peptides, phenols, aromatic compounds, or carbohydrates and proteins.
Microarray analyses were performed on 5µg of RNA by LC Science LLC (Houston, TX). The cDNA was obtained from 200 ng of total miRNA according to manufacturer's instructions (miScript II RT Kit, Qiagen, Netherlands).
MiRNA expression was analyzed by RT-PCR using a suitable kit (miScript SYBR Green PCR Kit, Qiagen, cat. no. 218073) in a final volume of 25 µl containing 2.5 µl of cDNA, 2.5 µl of the specific forward primer (10 pmol / µl) (miScript miRNA Mimic, Qiagen), 12.5 µl Universal Primer, and 12.5 µl Master Mix, using the LightCycler 480 (Roche). The Scarna-17 (Qiagen, cat. no. MS00014014) miRNA amplification was used as housekeeping gene to normalize data.

2.9 Western Blot on Exosomes

To verify the purity of the exosomes, exosomes released by IFN-γ-stimulated MSC culture were lysed in RIPA buffer containing a protease inhibitors cocktail (Roche, Indianapolis, IN). Proteins were quantified through Pierce BCA Protein Assay kit (Thermo Fisher Scientific, cat. no. 23227) following provided instructions. 15 µg of proteins were loaded on a precast gel with polyacrylamide from 4% to 12% gradient (Life Technologies, cat. no.NW04120BOX), using the Bolt® Mini Gel Tank (Life Technologies) system. Protein were then transferred on a nitrocellulose membrane (BioRad, Hercules, CA) using XCell II™ Blot Module (Life Technologies). After blocking in BSA 5% in PBS + 0.1% Tween-20 for 1 hour, the membrane was incubated overnight at 4° C with primary rabbit anti-ALIX (1: 1000, Merck Millipore, Milan, Italy) and anti-CD9 (1:1000, BD Pharmigen, cat. no.553758) antibodies in BSA 2% in PBS + 0.1% Tween-20. Membranes were washed three times with PBS + 0.1% Tween-20 for 5 minutes and incubated with goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1: 5000, Merck Millipore, cat. no. 12-348) in BSA 2% in PBS + 0.1% Tween-20 for 1 hour. Membranes were washed

three times with PBS + 0.1% Tween-20 and then developed using the ECL Plus kit (Thermo Fisher Scientific, Rockford, IL).

2.10 Electronic microscopy analyses on exosomes

To observe the exosomes at the electronic microscope, the samples were fixed according to a published protocol (Théry *et al.*, 2006). The exosomes were resuspended in a volume of 50-100 µl of paraformaldehyde (PFA) at 2%. 5 µl of resuspended pellet was allowed to adhere to electron microscopy screens (Formvar-Carbon) for 20 minutes at 42° C. Subsequently, the screens were washed 2 times with 100 µl of PBS for 3 minutes, after with 1% glutaraldehyde for 5 minutes. Finally, the screens were washed with 100 µl of distilled water for 2 minutes for seven times. For the contrast phase, the screens were transferred to 50 µl of 2% uranyl acetate (UA) solution for 5 minutes and then transferred to 50 µl of methylcellulose (MC) and UA (9 ml MC + 1 ml UA 4%) for 10 minutes in ice. Finally, the screens were dried on a filter paper and then in the air. The sections were visualized using a FEI CM10 microscope and acquired via a Leo912ab camera.

2.11 Western Blotting of LPS-activated N9 cells transfected with miRNA mimics

LPS-activated N9 transfected were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Proteins were quantified through Pierce BCA Protein Assay kit (Thermo Fisher Scientific, cat. no. 23227) following manufacturer's instructions. 15-30 µg of proteins were loaded on precast 4% to 12% polyacrylamide gradient gels (Life Technologies, cat. no.NW04120BOX) and the gels were run using the Bolt® Mini Gel Tank (Life Technologies) system. Proteins were then transferred on a nitrocellulose membrane (BioRad, Hercules, CA) using XCell II™ Blot Module (Life Technologies). After blocking in BSA 5% in PBS + 0.1% Tween-20 for 1 hour, the membrane was incubated overnight at 4° C with anti-phosphorylated p38 (1:2000, Cell Signaling, cat. no.4511) antibody in BSA 2% in PBS + 0.1% Tween-20. The membrane was washed three times with PBS + 0.1% Tween-20 for 5 minutes and incubated with anti-IgG secondary antibody conjugated with horseradish peroxidase (1: 5000, Merck Millipore, Milan, cat. no. 12-348) in BSA 2% in PBS + 0.1% Tween-20 for 1 hour. The membrane was washed three times with PBS + 0.1% Tween-20 and then developed using the ECL Plus kit (Thermo Fisher Scientific, Rockford, IL). The concentration of the phosphorylated form of p38 was normalized on the level of expression of total p38.

The membrane was stripped for reprobing following the Harsh Stripping protocol (AbCAM). The membrane was incubated in a pre-warmed solution containing 20 ml SDS 10%, 12.5

ml TrisHCl pH 6.8 0.5 M, 67.5 ml ultra pure water and 0.8 ml β-mercapto-ethanol at 50° C for 45 minutes with some agitation. The membrane was rinsed under running water tap for 1-2 hours and washed for 5 minutes in PBS+ 0.1% Tween-20. After blocking in BSA 5% in PBS + 0.1% Tween-20 for 1 hour, the membrane was incubated overnight at 4° C with the anti-p38 (1:2000, Cell Signaling, cat. no.9212) in BSA 2% in PBS + 0.1% Tween-20. The membrane was washed three times with PBS + 0.1% Tween-20 for 5 minutes and incubated with goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1: 5000, Merck Millipore, Milan, Italy) in BSA 2% in PBS + 0.1% Tween-20 for 1 hour. The membrane was washed three times with PBS + 0.1% Tween-20 and then developed using the ECL Plus kit (Thermo Fisher Scientific, Rockford, IL).

2.13 Bioinformatics analysis of miRNA 466q and miRNA 467f

Online softwares miRWalk, MicroT4, miRanda and Targetscan were consulted to predict specific target genes of miRNA 466q and miRNA 467f. Pathways in which miRNA 466q and 467 might have a modulation were predicted in-silico using Kyoto encyclopedia of genes and genomes (KEGG) Pathway database which predicts on the basis of miRNA sequences. In order to lower the false positive results, Panther database was consulted to predict pathways in which target genes identified through miRWalk are involved.

2.14 Statistical analysis

The results are represented as mean ± standard error (SEM). Statistical analysis was performed on independent experiments using Student's test using the Prism 5 program (GraphPad Software, La Jolla, CA). In all analyses, it is considered statistically significant $p < 0.05$.

3. Results

3.1 Microarray analysis shows an up-regulation of nine miRNA in immunosuppressive IFN-γ-stimulated MSC

As already known from the literature, MSC can assume immunomodulatory properties in inflammatory conditions (Krampera *et al.,* 2006; Sivanathan *et al.,* 2014). In particular, IFN-γ was shown to license the ability of MSC to acquire an immunosuppressive profile, inhibiting T cells proliferation and this occurs mostly through the activation of IDO that catalyzes the catabolism of tryptophan, essential amino acid for T cell growth (Munn *et al.*, 1999; Krampera *et al.,* 2006; Meesuk *et al.*, 2016). We have speculated that at least part of the immunomodulatory role of MSC on microglia could be exerted via exosome-shuttling of specific miRNA that affect inflammatory pathways in the target cell. To assess if the switch to an immunosuppressive phenotype was associated with changes in miRNA content in the MSC, we analyzed, through microarray (LC Sciences), the expression of miRNA on MSC stimulated with IFN-γ compared to unstimulated ones. We identified nine microRNA (listed in Table 3.1) that are significantly upregulated in IFNγ-activated MSC compared to their expression in unstimulated MSC, and validated their upregulation in immunosuppressive MSC through qRT-PCR.

We speculated that at least some of these upregulated miRNA could be involved in the immunosuppressive activity of MSC.

Upregulated miRNA	p value
miRNA 466i 3p	p<0.05
miRNA 669c 3p	p<0.05
miRNA 5126	p<0.05
miRNA 466q	p<0.05
miRNA 467g	p<0.05
miRNA 467f	p<0.05
miRNA 466m 5p	p<0.05
miRNA 466i 5p	p<0.05
miRNA 3082 5p	p<0.05

Table 3.1 *miRNA resulted upregulated in the MSC stimulated with IFN-γ (modulatory MSCs) compared to unstimulated MSC. p< 0.05 t test.*

3.2 All nine miRNAs are present in MSC-derived exosomes, some are more abundant in immunosuppressive MSC-derived exosomes

We have proposed that the suppressive mode of action of MSC could be mediated, at least in part, through the transfer of miRNA to target cells, via exosome shuttling. Accordingly, we assessed whether or not miRNA upregulated in immunosuppressive MSC were contained in exosomes released in the culture medium by MSC stimulated with IFN-γ. To isolate MSC-derived exosomes, we used Total exosome isolation kit (Invitrogen), which permits the precipitation, and purification of exosomes from MSC supernatant. We characterized the isolated exosomes through electron microscopy (Figure 3.1a) and western blot analyses (Figure 3.1b). Electron microscopy analysis revealed a preparation composed of purified nanovesicles, with a diameter ranging from 30 to 100 nm, as expected for exosomes. Moreover, we performed western blotting experiments on the isolated vesicles and we detected the expression of ALIX and CD9, which are exosomal markers, confirming the good yield of exosomes collected.

To ascertain whether or not exosomes released by IFN-γ-treated MSC contain miRNA upregulated upon licensing of the immunosuppressive activity of the MSC, we measured the miRNA content in exosomes from MSC primed or not with IFN-γ through RT-PCR analyses (Figure 3.2).

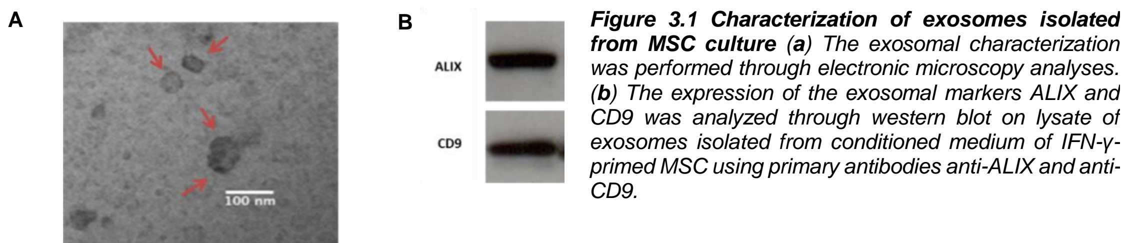

Figure 3.1 Characterization of exosomes isolated from MSC culture *(**a**) The exosomal characterization was performed through electronic microscopy analyses. (**b**) The expression of the exosomal markers ALIX and CD9 was analyzed through western blot on lysate of exosomes isolated from conditioned medium of IFN-γ-primed MSC using primary antibodies anti-ALIX and anti-CD9.*

As seen in Figure 3.2, all the nine miRNA overexpressed in immunosuppressive MSC are present in MSC-derived exosomes, albeit at different levels. Only some of the miRNA, namely miR-466q, -466m 5p, -466i 3p and -467f, are significantly upregulated in exosomes released by IFN-γ-primed MSC. These outcomes may suggest a possible active role played by these specific miRNA in the immunomodulatory capacity of MSC.

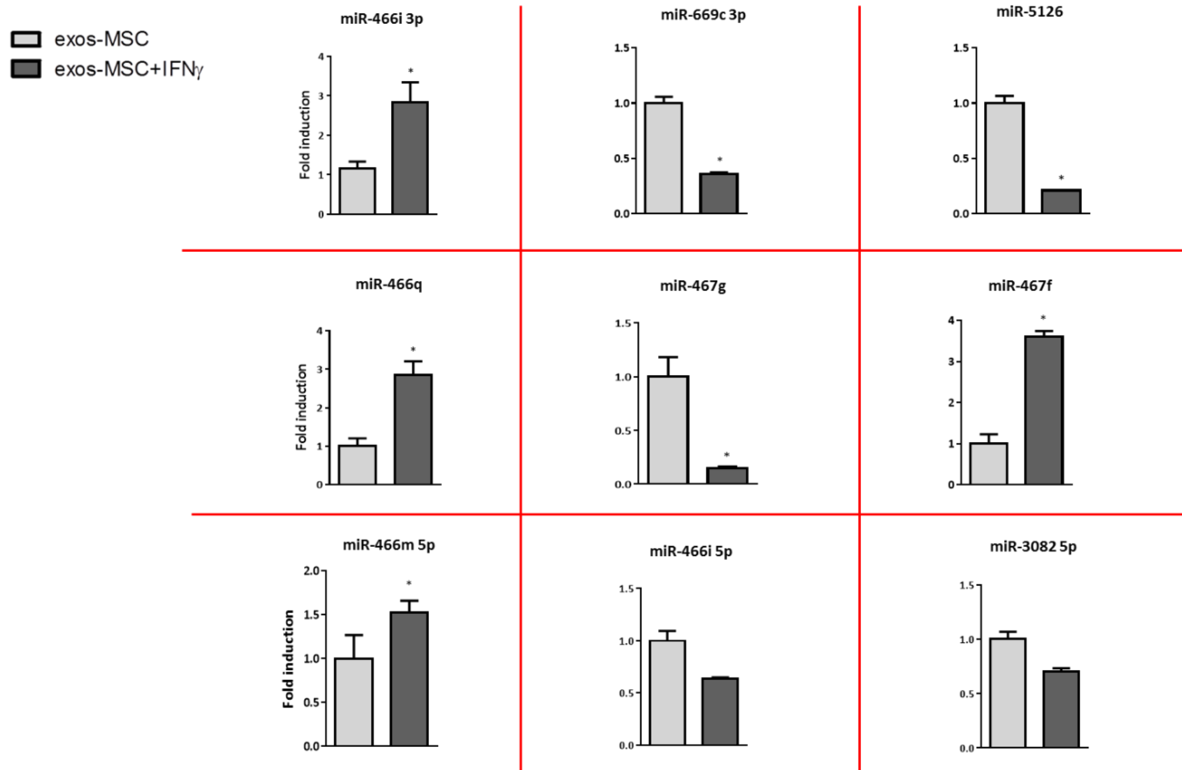

Figure. 3.2 Exosomes derived from MSC primed or not with IFN-γ are differentially enriched in miRNA. *miRNA upregulated in MSC are present in the derived exosomes, but not all at the same level and only some are significantly more expressed in the exosomes derived from immunomodulatory MSC. (RT-PCR analysis). *P < 0.05*

3.3 miR-466q and -467f affect the expression of pro-inflammatory cytokines, showing an anti-inflammatory potential

In order to investigate whether the miRNA overexpressed in immunosuppressive MSC show a modulatory role in activated microglia upon transfer, our prerequisite was that microglial N9 cells, upon activation with LPS, did not themselves overexpress any of the nine selected miRNA shown to be upregulated by modulatory MSC. RT-PCR analyses demonstrated that there is a basal expression of miRNA in unstimulated microglia which does not increase when the cells are activated (Figure 3.3).

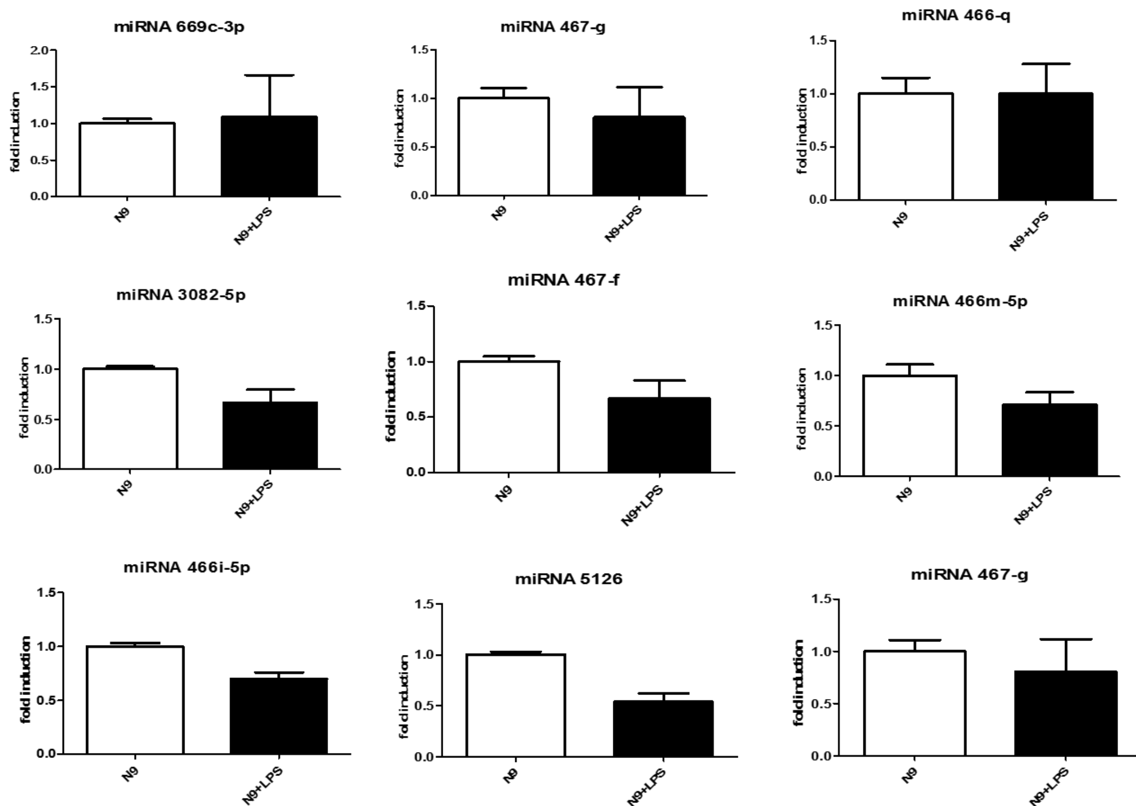

Figure 3.3 Activated microglia N9 cells do not overexpress any of the nine miRNA upregulated in immunomodulatory MSC. *There is no difference in the expression of the nine miRNA upregulated in immunosuppressive MSC in stimulated and unstimulated N9. (RT-PCR)*

With the purpose of understanding if miRNA upregulated in immunosuppressive MSC could affect the molecular phenotype of activated microglia, we used mimics (synthetically generated oligonucleotide of identical sequence with endogenous miRNA) of the miRNA upregulated in immunosuppressive MSC to transfect immortalized microglial N9 cells activated with a pro-inflammatory stimuli, lipopolysaccharide (LPS). Then we evaluated if these miRNA have an effect on the microglial profile by measuring the mRNA expression of TNF-α and IL-1β, as pro-inflammatory phenotype markers, and CX3CR1, as a marker of alternative microglia activation (Figure 3.4). As expected from our previous and other studies, activation with LPS induced microglia to overexpress the main pro-inflammatory cytokines and to downregulate CX3CR1 expression. Transfection with mimics demonstrated that some of the nine miRNA could regulate pro-inflammatory pathways, with miR-466q and -467f inducing down-regulation of TNF-α and IL-1β and miR-466m 5p inducing a reduced expression of IL-1β, while others (miR-5126, -466m 5p and -3082 5p) upregulated the expression of CX3CR1.

Interestingly, miR-466q and -467f, which appear involved in the regulation of expression of inflammatory genes, were significantly overexpressed in the exosomes released by immunosuppressive MSC, as shown above (Figure 3.2). Results obtained suggest a selective role of specific miRNA in microglia phenotype modulation, in particular these two

miRNA could be able to modify microglia activation state from a pro-inflammatory (M1-like) to an anti-inflammatory/neuroprotective (M2-like) phenotype. For this reason, we focused our further studies on miRNA 466q and miRNA 467f.

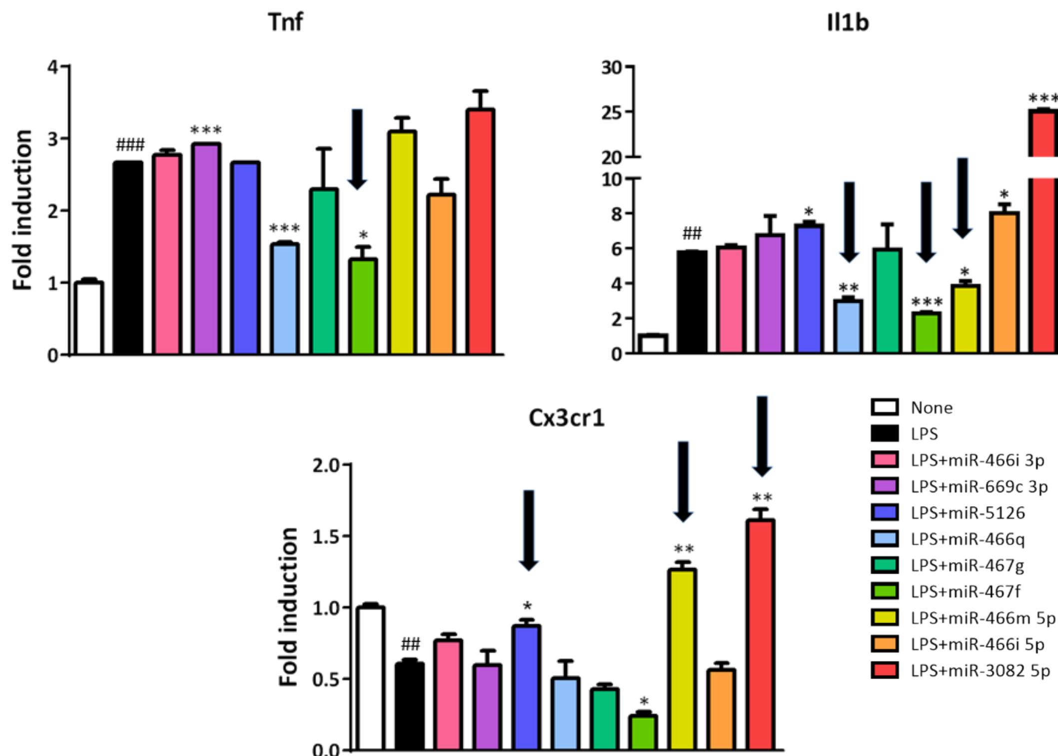

Figure 3.4 Transfection with specific miRNA mimics induces changes in molecular phenotype of activated N9 microglia. *When stimulated with LPS, N9 are activated, as demonstrated by the significant increase of the expression of pro-inflammatory cytokines and the significant decrease of Cx3cr1, a marker of alternative activated microglial phenotype. Transfection of LPS-activated N9 with synthetic mimics of the nine miRNA upregulated in modulatory MSC demonstrates the effect of miR-466q and miR-467f that significantly reduce the expression of TNF and IL1b (pro-inflammatory markers), whereas miR-466m 5p, miR-5126 and miR-3082 5p affect the mRNA expression of Cx3cr1 ("neuroprotective" marker). (RT-PCR analysis). (None vs LPS-activated N9) ## P< 0.01; ### P< 0.001; (LPS-activated N9 vs transfected LPS-activated N9) *P < 0.05; **P < 0.01; ***P<0.001*

3.4 miR-466q and -467f could modulate MAPK signaling pathway by affecting their target gene expression

miRNA are sequences of regulatory RNA that promote, in most cases, the inhibition or degradation of their mRNA targets. We hypothesized that modulatory MSC induce alternative microglia activation through exosome-shuttling of miRNA that would affect gene expression by microglia cells.

To predict possible miRNA targets, we used miRWak online database, which provides information on predicted miRNA target genes, calculated through algorithms based on different parameters, such as complementary and pairing sequences, AU content and binding miRNA-mRNA energy. Through miRWalk database, we predicted 241 target

genes of miR-466q and 494 target genes for miR-467f. To better understand the role of miRNA in the modulation of microglia activation, we analyzed in-silico pathways in which target genes of miR-466q and -467f could be involved. We used two different parameters to predict pathways modulated by miRNA 466q and 467f. We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, which predicts pathways based on miRNA itself, and Panther Classification System, which predicts pathways on the basis of predicted target genes we identified through miRWalk database. By combining data from these two databases, we predicted a number of pathways, listed in Table 3.2a, which specific miRNA might modulate. Of these, we have chosen to focus on the MAPK signaling pathway, which could be regulated by both miR-466q and miR-467f, because it plays an important role in inflammatory cytokine expression. Accordingly, the miRNA could play an anti-inflammatory role in activated microglia by preventing the activation of MAPK pathway via inhibition of the expression of some of its component.

A

miR-467f	
Pathways in cancer	Dorso ventral axis formation
TGF beta signaling pathway	Adipocytokine signaling pathway
Endometrial cancer	Vascular smooth muscle contraction
Acute myeloid leukemia	Wnt signaling pathway
Chronic myeloid leukemia	Regulation of actin cytoskeleton
Colorectal cancer	Ubiquitin mediated proteolysis
MAPK signaling pathway	Melanoma
B cell receptor signaling pathway	Glioma
ErbB signaling pathway	Gap junction
Prostate cancer	Basal cell carcinoma
Renal cell carcinoma	Fc epsilon RI signaling pathway
Chemokine signaling pathway	Jak STAT signaling pathway
T cell receptor signaling pathway	GnRH signaling pathway
Sphingolipid metabolism	

miR-466q
Pathways in cancer
Neurotrophin signaling pathway
Colorectal cancer
Endometrial cancer
Amyotrophic lateral sclerosis ALS
MAPK signaling pathway
Thyroid cancer
VEGF signaling pathway
Fc epsilon RI signaling pathway

B

MAPK signaling pathway

Target of miR-466q	**Target of miR-467f**
MAPK11/p38beta	Eif4ebp1
MAPKAPK1c	MAPKAPK3/MK3
MAPKAPK2/MK2	IL1R type 1
	MAPK3/ERK1
	MAP3K8

Table 3.2 miR-466q and -467f might have a role in the modulation of MAPK signaling pathway through affecting their target genes

*(**a**) Bioinformatic analysis predicted regulation of MAPK pathway by miR-466q and miR-467f. (**b**) Bioinformatic analysis predicted target genes of miR-466q and miR-467f whithin the MAPK signaling pathway.*

To further understand the possible effect of miR-466q and -467f on MAPK signaling, we analyzed their predicted target genes that are components of this pathway. Of the several components of MAPK pathway which could be regulated by miRNA-466q and -467f (Table 3.2b), we focused on MAPKAPK2 (or MK2), target of miR-466q, and MAP3K8, target of miR-467f, important steps of activation of the p38 pathway (Figure 3.5), which plays a key role in neuroinflammation. In addition, an overactivation of the p38 MAPK signaling pathway has also been observed in degenerating MN and reactive astrocytes in ALS patients (Bendotti *et al.*, 2004) and in post-mortem human ALS brain tissues (Sama *et al.*, 2017). For its implications in ALS pathology, we decided to further analyze the effect of the immunomodulatory miRNA on this pathway.

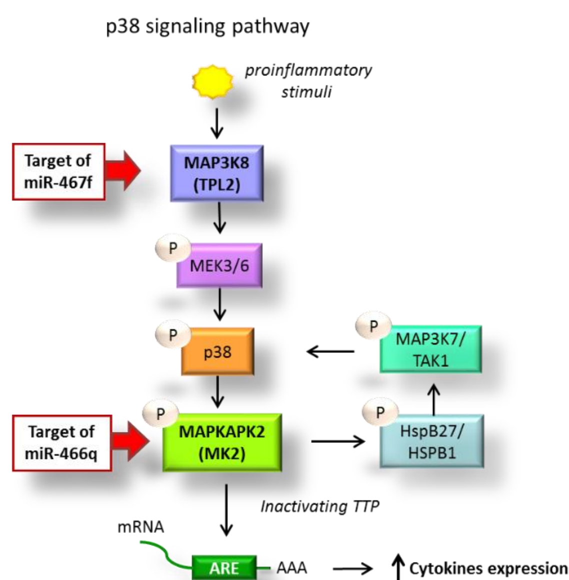

Figure 3.5 p38 MAPK signaling might be modulated by miR-466q and -467f. *In the p38 pathway, miR 467f targets MAP3K8 and 466q targets MK2. The effect of both miRNAs on their targets affects the phosphorylation of p38.*

To validate whether MK2 and MAP3K8 are really targets of miR-466q and -467f in microglia, we performed RT-PCR for MK2 and MAP3K8 mRNA on LPS-activated N9 and transfected with the relevant mimics. When stimulated with LPS, the cells show an upregulation of both these genes. As we expected, miR-466q induced a downregulation of its direct target, Mk2, affected also by miR-467f, whose upstream effect on its target, Map3k8, affects the whole pathway. Interestingly, the expression of Map3k8 was inhibited by both miRNA, in particular miR-467f inhibits its translation by directly targeting it, while miR-466q apparently does this through an indirect mechanism still unknown. Our results validated that miR-466q and -467f modulate the expression of these two genes involved in the p38 MAPK pathway (Figure 3.6).

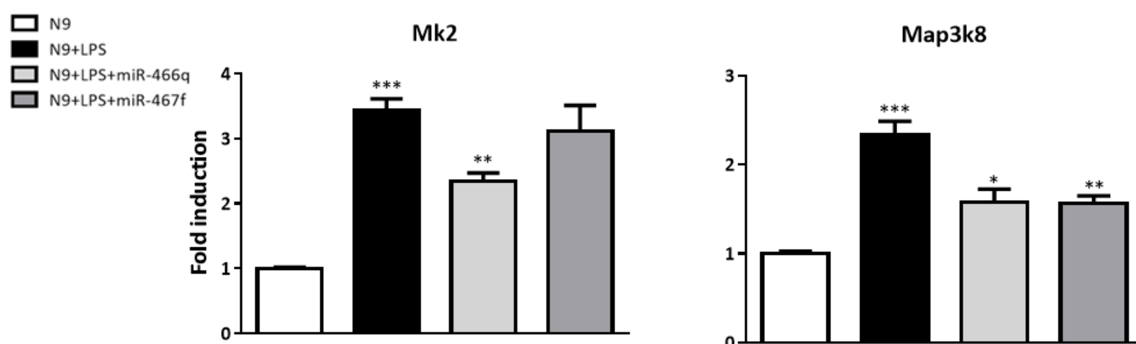

*__Figure 3.6__ RT-PCR analysis on transfected LPS-activated N9 confirmed that Mk2 and Map3k8 are target genes of miRNAs 466q and 467f. *P<0.05; **P<0.01; ***P<0.001*

3.5 miR-466q and -467f affect the p38 MAPK pathway

To evaluate how miRNA modulation affects the p38 MAPK signaling pathway, we assessed phosphorylation of p38 compared to the total p38 expressed through WB analyses of LPS-activated N9 cells transfected with mimics of miR-466q and -467f. We observed an expected increase in the phosphorylated form of this situation. This effect could be specifically attributed to these miRNA, as transfection with a negative control (a synthetic miRNA mimic (Sigma-Aldrich) which does not recognize any gene target in cells) did not have any effect (Figure 3.7).

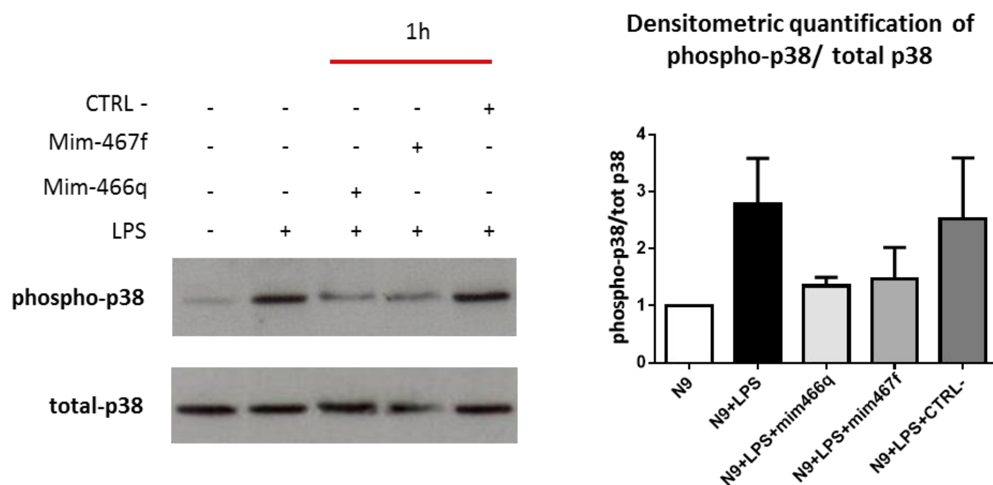

Figure 3.7 miR-466q and -467f are able to affect the phosphorylation of p38, inhibiting the activation of p38 MAPK pathway *Transfection of LPS-stimulated N9 with mimics of miRNA 466q and miRNA 467f decreases p38 phosphorylation.* (Western Blot).

3.6 Exosomes derived from IFN-γ-primed MSC affect genes related to the inflammatory and neuroprotective phenotype of microglia

We had demonstrated that the exosomes released by immunomodulatory MSC contain miRNA upregulated in IFN-γ-primed MSC. We had observed that these specific miRNA have an effect on the molecular phenotype of activated microglia, and in particular miR-466q and -467f that are significantly more abundant in the immunomodulatory MSC-derived exosomes compared to exosomes released by unstimulated MSC, and have an anti-inflammatory role. To have the proof of concept that the immunosuppressive MSC-derived exosomes, containing modulatory miRNA, can mediate the paracrine effect of MSC on activated microglia, we exposed LPS-activated N9 cells to the IFN-γ-primed MSC-derived exosomes. Results obtained confirmed the role of exosomes in mediating the immunomodulatory effect of MSC on the molecular phenotype of activated microglia. Indeed, N9 cell activation to a pro-inflammatory profile, as demonstrated by the overexpression of Tnf and downregulation of the expression of alternative activated microglia phenotype marker, was reverted upon exposure to IFN-γ-primed MSC-derived exosomes, with downregulation of TNF expression and upregulation of the expression of markers of alternative microglial activation, Cx3cr1 and Nr4a2 (Figure 3.8).

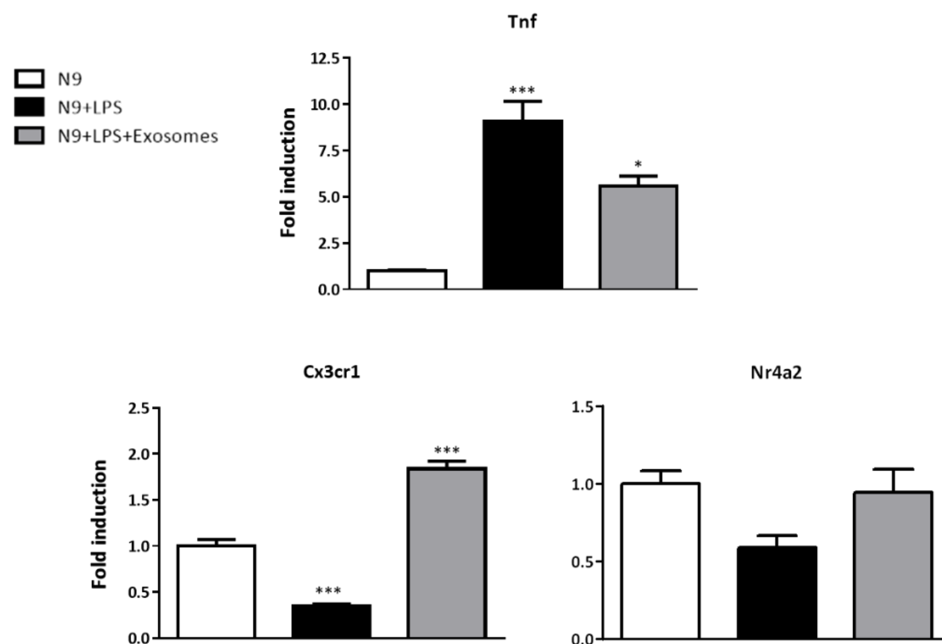

Figure 3.8 Exosomes derived from immunosuppressive MSC are able to affect the molecular phenotype of activated microglia. *Exposure to exosomes derived from modulatory MSC results in downregulation of the pro-inflammatory gene (Tnf) and upregulation of genes associated with anti-inflammatory/neuroprotective phenotype (Cx3cr1 and Nr4a2) in LPS-activated immortalized microglial cell line. (RT-PCR analysis). *P < 0.05; ***P<0.001*

3.7 Immunosuppressive MSC-derived exosomes affect the molecular phenotype of end-stage mSOD1-microglia

We have postulated that the beneficial effect of MSC in the murine ALS model could be mediated, at least in part, by exosome shuttling of specific miRNA inducing a shift of microglia profile from classical to alternative activation with upregulation of the neuroprotective phenotype. To evaluate whether the exosomes released by immunosuppressive MSC were able to affect the activation of microglia of SOD1^{G93A} mice, we exposed primary microglia isolated from mice at the end stage of ALS, which show an over-activated molecular phenotype (Figure 3.9), to exosomes derived from immunosuppressive MSC.

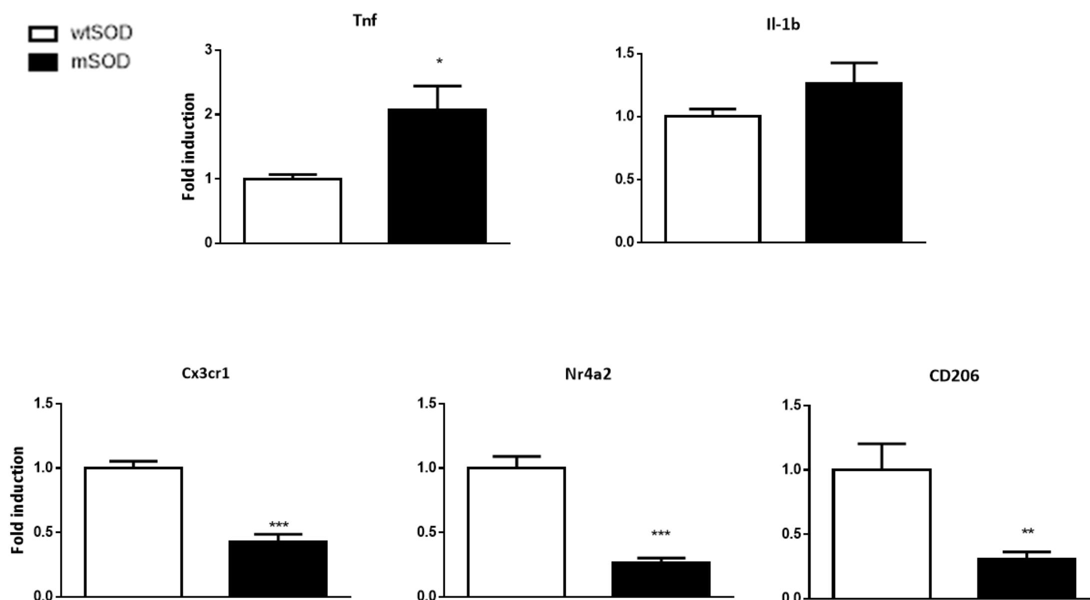

Figure 3.9 mSOD1-microglia show an over-activated molecular phenotype at the end-stage of ALS. *mSOD1-microglia show up-regulation of genes associated with a proinflammatory (Tnf and Il1b) phenotype and down-regulation of genes associated with an anti-inflammatory/neuroprotective phenotype (Cx3cr1, Nr4a2 and Cd206) at the end stage of disease (day 135). (q-RT-PCR analysis) *P < 0.05, **P < 0.01.*

As can be seen in Figure 3.10, exposure of SOD1^{G93A} microglia to IFN-γ-primed MSC-derived exosomes led to downregulation of TNF and upregulation of M2-like molecular phenotype markers (Figure 3.10).

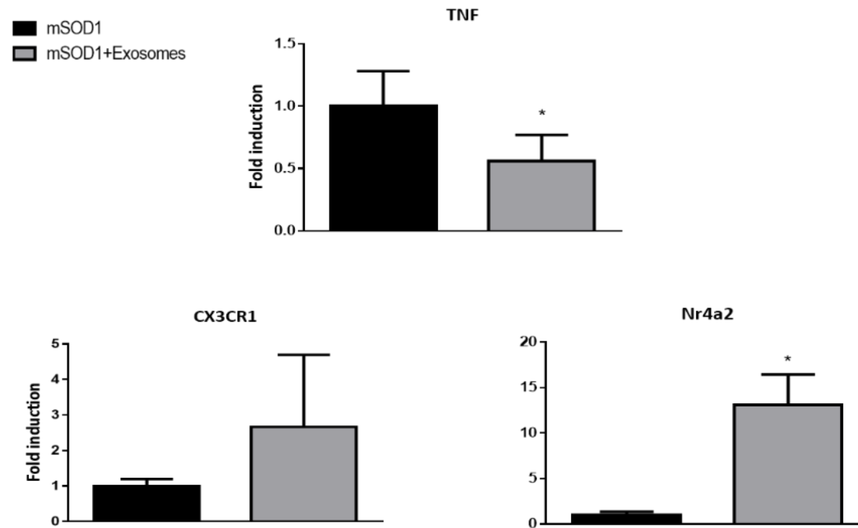

Figure 3.10 *Exposure to exosomes derived from modulatory MSCs results in downregulation of the pro-inflammatory gene (Tnf) and upregulation of genes associated with anti-inflammatory/neuroprotective phenotype (Cx3cr1 and Nr4a2) in microglia isolated from end-stage mSOD1 mice. (RT-PCR analysis).*

These data suggest that specific miRNA shuttled by exosomes derived from modulatory MSC are able to alter microglial gene expression, inducing a switch from neurotoxic to neuroprotective phenotype, and that this occurs, at least in part, through the regulation of p38 MAPK signaling pathway involved in the expression of inflammatory mediators.

4. Discussion and conclusion

The microglia act as guardians of brain homeostasis until they perceive an activating signal (Amor, 2010). In most cases, immune responses in the CNS lead to repair and regeneration (Neumann *et al.*, 2009), but there is a delicate balance between pathogenic and restoring process, which can be triggered by immune responses. Modulation of microglia activation from the M1-like phenotype to the M2-like one represents a therapeutic target in research on neurological diseases characterized by a strong inflammatory component.

While research aimed at modulating neuroinfiammation with classical immunomodulatory factors were not convincing, stem cell therapy, particularly the administration of MSC, which showed a potent neuroprotective, modulatory and probably also neurogenic potential in vitro (Zappia *et al.,* 2005), is getting more and more considered as an alternative approach for treatment of neurodegenerative diseases in which the inflammatory component plays a key role.

Using MSC could be a logical solution, since, in addition to carrying out an important immunomodulatory function, they are able to migrate where they can support local neurogenesis, providing support for precursors, e.g. neural stem cells, to differentiate (Uccelli *et al.*, 2008).

The use of bone marrow-derived MSC has several advantages over conventional neural, embryonic and hematopoietic stem cells: they can be obtained from adult bone marrow, are easily cultured and expandable *in vitro*, can be administered in an autologous manner without using immunosuppressive anti-rejection drugs, and lastly are apparently not subject to genetic change despite repeated culture steps, thus possessing a low level of risk of tumorigenesis compared to other types of stem cells (Kassis *et al.*, 2008). But the therapeutic effectiveness of MSC depends on their ability to modify the damaged tissue microenvironment. These events take place through the release of inflammatory cytokines, anti-apoptotic molecules and neurotrophic factors that promote repair and protection of damaged tissues (Uccelli *et al.*, 2008).

In this work, we demonstrated the presence of specific miRNA in exosomes derived from immunomodulatory MSC, which are able to influence the activated molecular phenotype of microglia *in vitro*, leading them to acquire an alternative activated phenotype characterized by the expression of anti-inflammatory / neuroprotective molecules. We observed that in inflammatory conditions, simulated in our *in vitro* experiments by stimulation with IFN-γ, MSC showed a different expression of endogenous miRNA, compared to MSC at basal conditions, and in particular we evidenced the significant over-expression of nine specific miRNA. This interesting aspect suggests that at least some of

the nine overexpressed miRNA in the MSC could be involved in the immunosuppressive activity of the IFN-γ-primed MSC, through modulation of gene expression in target cells (Krampera *et al.,* 2005; Sivanathan *et al.,* 2014; Rasmusson *et al.,* 2003).

Our aim was to investigate whether specific miRNA-shuttled by exosomes derived from immunomodulatory MSC could account for the modulatory paracrine action of these MSC on microglial cells. We confirmed the presence of all the nine miRNA in the exosomes released by IFN-γ-treated MSC. Of interest, we observed a differential enrichment of specific miRNA in the exosomes derived from immunosuppressive MSC compared to the parental cells, with some of the miRNA being present in significantly greater amount in the exosomes derived from modulatory MSC, thus supporting the demonstration of Squadrito *et al.* that the exosomal content does not reflect the cytoplasm of the cell from which they originate, but miRNA are sorted into the exosomes depending on the state of the cells (Squadrito *et al.,* 2014). Accordingly, exosomes could mediate the paracrine action of MSC by transferring specific miRNA, that might have a role in the target cells.

The data obtained *in vitro* with immortalized N9 microglial cells support the idea that MSC can also exert their neuroprotective effects on microglia. In addition to our previous study indicating that exposure of microglia to MSC significantly increases the production of molecules potentially involved in anti-inflammatory / neuroprotective mechanisms (Giunti *et al.,* 2012), we show here that MSC are also able to inhibit microglia activation and the production of inflammatory mediators. Transfection of selective miRNA in activated microglia showed that some miRNA are able to affect microglia activation, inhibiting the expression of pro-inflammatory cytokines or upregulating the expression of M2-like marker. Interestingly, we noted correlations between the "beneficial" anti-inflammatory effect displayed by some modulatory miRNA when transferred to activated microglia through transfection of mimics or through exosome shuttling, in particular miR-466q and -467f, and their significant over-expression in the exosomes derived from immunomodulatory MSC. Indeed, it is well known that exosomes are modulators of neuroinflammation (Gupta and Pulliam, 2014) by shuttling soluble factors, mRNA and miRNA. In particular, we proposed that these two miRNA can attenuate M1-like microglia functions acting on the p38 MAPK pathway, which is involved in the neuroinflammation process. Many groups (Morfini *et al.*, 2013; Holasek *et al.*, 2005; Lee *et al.,* 2013; Tortarolo *et al.*, 2003; Nahirnyj *et al.,* 2013; Lee and Kim, 2017; Sama *et al.*, 2017; Bendotti *et al.,* 2004) have reported the increase of activation of p38 MAPK both in $SOD1^{G93A}$ mice and in human sporadic and familial ALS cases (Bendotti *et al.,* 2004; Picher-Martel *et al.,* 2015), suggesting the crucial role of this pathway in ALS pathology. In this context, the possible inhibitory role of p38 MAPK signaling pathway played by miR-466q and -467f

could be of relevant importance for all pathology in which there is an inflammatory component, including ALS.
On the basis of results obtained *in vitro*, we aimed at demonstrating the effect of modulatory miRNA in controlling microglial activation in the animal model of ALS.
Although microglia assume a neuroprotective phenotype at the clinical onset of murine ALS, they switch to a proinflammatory phenotype towards the end stage of pathology (Zhao *et al.*, 2013); accordingly, we analyzed the clinical stage of the disease in the SOD1^{G93A} mice in which the primary microglia assume an over-activated and more deleterious phenotype. Our results on primary microglia reflect the modulatory effect of exosomes obtained *in vitro*, indeed we observed a reduction in microglia activation. Although exosome functions are still not well defined, it is clear that they play an important role in intercellular communication and our results confirmed that exosomes can mediate paracrine effects exerted by MSC on microglia cells, and that their content can modulate microglia activation. The usefulness of exosomes depends on their ability to cross the blood-brain barrier (BBB), highly impenetrable to most drugs, and their potential as a therapeutic vehicle in inflammatory neurological disorders has been demonstrated *in vivo* with intra-nasal administration in the animal model of the EAE (Zhuang *et al.,* 2011). The importance of the exosomes as a therapeutic tool also includes their capacity to reach specific tissues and deliver their cargo into particular cells, in which they have to act (Bryniarski *et al.,* 2013).
The therapeutical use of exosomes would provide several advantages compared to classical cell therapy, mainly linked to the ability to mitigate risks associated with cell transplantation and the ability to transport soluble factors, transcription factors, genes and miRNA (Lai *et al.,* 2011; Andaloussi *et al.,* 2013).
The results obtained from this study support the starting experimental hypothesis, that MSC can exert their modulatory effect on activated microglia by the transfer of specific miRNAs contained in their exosomes.
We are planning to perform pre-clinical studies in SOD1^{G93A} mice to verify the efficacy of MSC-derived exosomes administered intra-nasally, a route that has been demonstrated to be the preferential exosomal route to efficiently reach the neural tissue and deliver their cargo in microglia cells (Zhuang et al., 2011).
Overall, this study will provide us important information about the innovative use of miRNA derived from MSC as a therapeutic tool for controlling neuroinflammation not only in ALS but also in other neurodegenerative diseases in which neuroinfiammation plays a fundamental role in the disease progression.

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