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A NEW HOPE FOR ALZHEIMER'S DISEASE FROM PRECONDITIONED BONE MARROW MESENCHYMAL STEM CELL-DERIVED EXTRACELLULAR VESICLES: ANALYSIS OF THE IMMUNOMODULATORY EFFECTS

Losurdo Morris

Registration number: 809334

Tutor: Dr. Coco Silvia

Coordinator: Prof. Cavaletti Guido

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Ai miei Genitori, ad Ambra, a Graziella,
a Micol,
Sempre vicini in questo Percorso,
con Amore.

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ABSTRACT

Alzheimer's disease (AD), the most common form of age-related dementia, is characterized by a progressive degeneration of the central nervous system (CNS) that leads to a gradual decline of cognitive functions and memory loss. Neuropathological hallmarks of AD include extracellular β-amyloid plaques, derived from the altered processing of amyloid precursor protein (APP), neurofibrillary tangles (NFTs, intraneuronal aggregates of hyperphosphorylated and misfolded tau), dystrophic neurites, neuronal loss and glial activation. According to the "Amyloid cascade hypothesis" - the most validated theory in the field of AD for the past few decades - neuroinflammation was assumed to occur only in the late stages of the disease, being considered as a mere secondary response to Aβ-induced pathophysiological events. Recently, new preclinical, epidemiological and genetic studies have demonstrated a much earlier involvement of immune system-related actions, leading to a reassessment of the role of the principal innate immune entities of the brain, that are **microglia cells**. Since there is still no cure for AD, these studies motivated the design of innovative therapeutic strategies aiming at slowing down degenerative processes by targeting microglia cells, in virtue of their main recognized role in orchestrating neuroinflammatory process in neurodegenerative diseases, including AD. **Mesenchymal stem cells** (MSCs) are adult multipotent stem cells that over the last decades have been demonstrated to show improvement in various model of neurodegenerative pathologies, thanks to their paracrine ability that is largely dependent on the secretion of **extracellular vesicles** (EVs). EVs - membrane bound entities known to be important players in intercellular communication - have emerged as mediator of multiple MSC beneficial effects, including immunomodulation. Particularly, the concept that intrinsic immunomoregulatory abilities of MSCs are strongly influenced and strengthened by the environment, has led the scientists to design and optimize culture conditions (*preconditioning*) in order to enhance the anti-inflammatory properties of these cells and of their derived EVs. The aim of this study is to investigate the ability of preconditioned human bone marrow MSC-derived EVs (p-MSC-EVs) to immunoregulate microglia function in *in vitro* and *in vivo* AD contexts.

In *in vitro* studies we tested two different preconditioning protocols in order to isolate a highly immunomodulant MSC phenotype. Cytokine p-MSC-EVs were shown to switch microglia, previously polarized through inflammatory challenge to the M1 cytotoxic state, toward an anti-inflammatory phenotype. When we delved into the EV immunomodulatory potential in a triple transgenic AD (3xTg AD) mouse model, we observed a strong dampening effect on microglia activation and prevention of dendritic spine loss in hippocampus, entorhinal and prefrontal cortices of EV treated animals compared to controls. This suggests that an EV-dependent neuroprotective effect could be achieved through the modulation of microglia activation in this model.

In order to more selectively study the effect of EVs on microglia, we are taking advantage of a leech animal model (*Hirudo verbana*), because of its simple and well-characterized CNS structure (preliminary study). In conclusion, our results indicate that p-MSC-EVs may represent a possible therapeutic tool in AD by reducing chronic microglia activation and counteracting dendritic spine loss, which are traits typically observed both in AD transgenic animal models and patients.

INTRODUCTION

1. Alzheimer's Disease

Alzheimer's disease (AD) - the most common form of neurodegenerative disease - is characterized by gradual and inexorable neurodegeneration of the central nervous system (CNS) which leads to deterioration in memory, thinking and behaviour. Worldwide, around 50 million of people are affected by dementia, with AD accounting for 60-80% of all cases and predominantly hitting subjects over the age of 65, although there is a growing awareness of cases starting before that age (*World Health Organization, 2018*). The first case of AD was diagnosed by Alois Alzheimer in 1901, who described the histological markers, including "plaques" and "tangles" in the upper cortical layer, published in 1907 (O'Brien 1996). In the same year Oscar Fisher also published a study in which, for the first time, neurite plaques in the brain of 16 senile dementia subjects were delineated (Gouras et al.2005). Over the next 5 years, Alzheimer and Fisher collected additional cases correlating clinical symptoms with neuropathological findings. Discoveries in the history of dementia research were landmark because they defined the clinicopathological entity that is now known as Alzheimer's disease (AD). Although much had been written about Alzheimer's studies, only little is known about Fisher whose name had vanished from the history of AD until his relevant work was recognized by Michel Goedert in 2009 (Goedert 2009). However, even Alzheimer's works were rediscovered later by Maurer, Volk and Gerbaldo in 1995 and published only in 1997 (Maurer, Volk, and Gerbaldo 1997).

Alzheimer's disease first case was Auguste Deter, a 51 years old woman with an $\varepsilon 3/\varepsilon 3$ (see par. 1.4) Apolipoprotein E (APOE) genotype and presenilin 1 mutation (Graeber et al. 1998). Although the knowledge of the disease pathophysiology still remains fragmentary, it is now well accepted that inheritance of specific genes plays an important role in making susceptible to the onset and/or modifying the disease progression. The discovery of "risk genes" explains the dichotomy of familial (rare) vs. non-familial (common) forms, also known as "sporadic" or "idiopathic", even if a significative genetic background could also be involved in these latter forms.

Both the familial and sporadic forms of the disease begin with memory loss of recent events (short-term memory impairment), the inability to retain information of remote events later and finally robs patients of their sense of self. The gradual decline of memory slowly increases in severity until the symptoms become disabling and begin to involve other areas of cognition such as language, abstract reasoning and executive functions, such as decision-making. Changes in mood and affect as well as non-typically presenting traits like delusions and hallucinations accompany memory decline contributing to dramatically invalidate life at work or in social situations. Neurological symptoms, typically occurring later, comprise seizure, hypertonia, incontinence and mutism. Death commonly is caused by general inanition, malnutrition and pneumonia (Bird 2008). In addition, in order to anticipate the clinical diagnosis of AD before the declared stage of dementia,

a novel clinical construct, the “mild cognitive impairment” (MCI), was proposed as a new diagnostic entity that accompanies the transition between normal aging and AD dementia. Patients with MCI have already some cognitive disturbs, unlike as it occurs in dementia, but that do not interfere with their activities of daily life.

Nowadays there is no cure for AD and the drugs available are involved only in marginally improving symptoms.

1.1 Pathological hallmarks of Alzheimer’s Disease

AD neuropathology is thought to develop years before dementia appearance. Although the microscopic features are considered as more reliable hallmarks for the disease, some gross visual treats can be frequently detected in AD, even though they do not assume diagnostic value. However, a certain degree of cortical atrophy predominantly affecting the medial temporal lobes, the primary motor, sensory, and visual cortices, is identifiable in asymptomatic individuals nearly a decade before dementia appears, making this imaging parameter a potentially important biomarker of early neurodegeneration (Dickerson et al. 2011). Because of the pattern of cortical thinning, the lateral ventricles, particularly the temporal horns, can appear remarkably enlarged (*hydrocephalus ex vacuo*). This pattern is stereotypic and can be recognized early in the clinical course of the disease by MRI scan (Dickerson et al. 2011). Cerebrovascular disease, as result of chronic hypertension, is another condition that often accompany normal brain aging and also AD, in the form of cortical microinfarcts, lacunar infarcts in the basal ganglia, and demyelination of the periventricular white matter. Specifically, the presence of cortical microbleeds or lobar haemorrhages, particularly in the posterior parietal and occipital lobes, may be indicative of the cerebral amyloid angiopathy, also known as congophilic angiopathy. This is a pathological condition characterized by the deposition of β-amyloid in the tunica media of cortical capillaries, small arterioles and medium-size arteries, especially in the posterior regions of the brain.

The neuropathological hallmarks of AD include what have been defined as “positive” and “negative” features. Typical “positive” lesions are represented by amyloid plaques (Aβ plaques) and neurofibrillary tangles (NFTs), neuropil threads (axons and dendrites containing hyperphosphorylated and aggregated tau protein) and dystrophic neurites, that are typically represented by presynaptic terminal axons containing degenerating mitochondria, dense bodies and paired helical filaments (PHFs) (Crews and Masliah 2010), followed by astrocyte and microglia activation (Itagaki et al. 1989). Characteristic “negative” lesions are loss of neurons, neuropil and synaptic structure.

Aβ plaques are generated from the abnormal extracellular deposition of the amyloid-β peptide (Aβ) with 40 or 42 amino acids (Aβ40 and Aβ42), two normal byproducts of the metabolism of the amyloid precursor

protein (APP) after its sequential cleavage by the enzymes β- and γ-secretases in neurons (see par. 1.2). Due to its higher rate of fibrillization and insolubility, Aβ42 is more abundant than Aβ40 within the plaques. Classical histological staining of the plaques is performed with dyes specific for the β-pleated sheet conformation such as Congo Red and Thioflavin-S. The staining of Aβ plaques assumes important pathogenic relevance as it allows to distinguish between Thioflavin-S negative diffuse amyloid plaques, that are commonly found in the brains of cognitively normal elderly people, and Thioflavin-S positive dense-core plaques that are associated with synaptic loss, neuron loss and activation of both astrocytes and microglial cells (Itagaki et al. 1989). Ultrastructure analysis performed by electron microscopy of dense-core plaques revealed a central mass of extracellular filaments that radially prolong toward the periphery, where they interact with processes of astrocytic and microglial cells as well as dystrophic neurites, which represent the most notorious signs of Aβ-induced neurotoxicity (Su et al. 1993). In addition, neurofilament proteins, lysosomal and ubiquitinated proteins have been found in these dense core plaques.

Neurofibrillary Tangles (NFTs) are intraneuronal aggregates composed of highly phosphorylated forms of the microtubule-associated protein tau, which is involved in microtubule assembly and stabilization (Hyman et al. 1993) and in the regulation of the motor-driven axonal transport. Tau excessive phosphorylation driven by several kinases such as glycogen synthase kinase-3 (GSK-3) and cyclin-dependent kinase 5 (cdk5) leads to its dissociation from microtubules and accumulation in the aggregated form, with consequent microtubular destabilization (Mietelska-Porowska et al. 2014). These aggregates are made of ≈10 nm fibers that form PHFs with a helical tridimensional conformation at a regular periodicity of ≈65 nm (Wiśniewski et al. 1976). NFTs are argyrophilic and can be evidenced through silver impregnation methods such as the Gallyas technique (Braak and Braak 1991). Three morphological stages have been identified in the “maturation” of NFTs:

1. Pre-NFTs or diffuse NFTs, defined by a diffuse, sometimes punctate, tau staining within the neuron cytoplasm, with a centered nucleus and no dendrite alteration;
2. Mature or fibrillar intraneuronal NFTs (iNFTs), which are filamentous aggregates of tau that displace the nucleus toward the periphery of the soma extending to distorted-appearing dendrites and to the proximal segment of the axon;
3. extraneuronal “ghost” NFTs (eNFTs), derived from neuronal death and identified by the absence of nucleus and stainable cytoplasm.

The topographical distribution of amyloid plaques differs from that characterizing NFTs. As previously described by Braak and Braak in 1991, more recently Thal and colleagues proposed a model describing the descendent progression of amyloid deposition in five stages (Fig.1): 1) isocortical stage (stage 1); allocortical stage (stage 2), involving entorhinal cortex, hippocampal formation, amygdala, insular, and cingulated

cortices; 3) Stage 3, involving subcortical nuclei, including striatum, thalamus and hypothalamus; (4) Stage 4, characterized by the involvement of brainstem structures, such as red nucleus, substantia nigra, superior and inferior colliculi; (5) Stage 5, with the involvement of the pons and the molecular layer of the cerebellum) (Thal et al. 2002).

According to Braak and Braak the spatio-temporal pattern of NFT accumulation can be subdivided in 6 stages (summarized in 3 stages in Fig. 2): 1) The first NFTs consistently appear in the transentorhinal (perirhinal) region (stage I) and the entorhinal cortex, followed by the CA1 region of the hippocampus (stage II). Next, NFTs develop in limbic structures such as the hippocampal subiculum (stage III), amygdala, thalamus, and claustrum (stage IV). Finally, NFTs spread to all isocortical areas (isocortical stage), with the associative areas being affected prior and more severely (stage V) than the primary sensory, motor, and visual areas (stage VI).

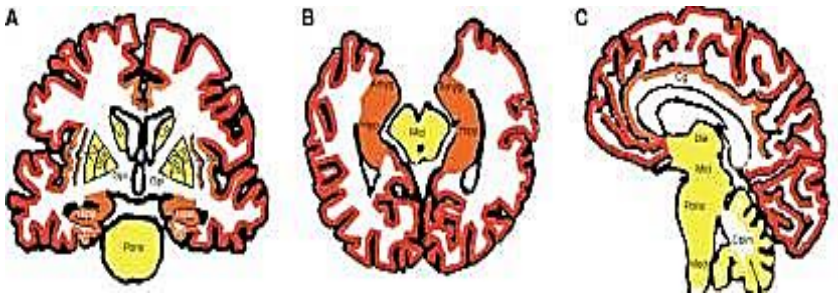


Figure 1. Spatio-temporal pattern of amyloid deposition. Coronal (A), axial (B), and sagittal (C) views of the brain. The five Thal's stages of amyloid deposition are here summarized in three stages. Amyloid deposits first accumulate in isocortical areas (stage 1, red), followed by limbic and allocortical structures, such as hippocampus, entorhinal cortex and amygdala, (stage 2, orange), and in a later stage, by subcortical structures including basal ganglia, selected nuclei in diencephalon and brainstem, and cerebellar cortex (stage 3 or subcortical, yellow) (Thal et al. 2002).

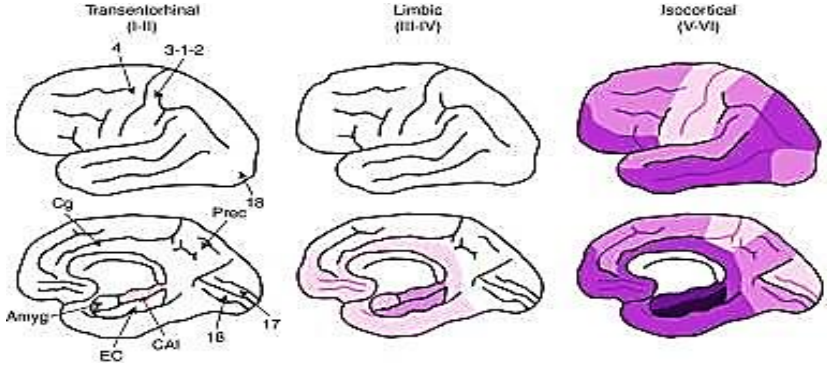


Figure 2. Spatio-temporal pattern of neurofibrillary degeneration. The Braak's six stages of NFTs accumulation are here summarized in 3 stages. NFTs start in transentorhinal structures and spread to limbic areas and finally to isocortical areas. Increased color intensity are referred to increased presence of NFTs (Arnold et al. 1991; Braak and Braak 1991).

Other typical AD features are represented by **glial activation, neuronal** and **synaptic loss**. Although reactive astrocytes and microglia are activated by amyloid-β in the first stages of the disease, their activation increase in temporal associative isocortex even after amyloid deposition has reached an early plateau. Serrazano-Pozo found a highly significant positive correlation between both gliosis and NFT burden but not between both

reactive glial cell types and amyloid burden, suggesting that glial responses are also related to NFTs (Serrano-Pozo et al. 2011).

Clinicopathological studies have revealed that the amyloid burden does not correlate with the severity or the duration of dementia. In fact in a region of early amyloid deposition such as the temporal associative isocortex, the amyloid burden reaches a plateau early after the onset of the cognitive deficits or even in the preclinical phase of the disease (Ingelsson et al. 2004; Serrano-Pozo et al. 2011). In addition, the size of the plaques do not increase significantly with the progression of the disease (Hyman et al. 1993). Regarding to the neuron cell death - which is a major contributor to cortical atrophy together with synaptic dysfunction - it was found that the regional pattern of neuronal loss matches that of NFTs, even if, within the same region, the entity of neuronal pauperization exceeds the number of NFTs, thus representing a better correlate of cognitive impairment than the number of NFTs (Gómez-Isla et al. 1996). However, it seems that synaptic loss represents the best correlate of cognitive deficits, as synaptic dysfunction may exceed neuronal loss within a particular area. In fact, it might occur that remaining neurons in a specific brain region become less efficiently connected to their synaptic partners than expected, just on the basis of the number of viable neurons (DeKosky and Scheff 1990).

1.2 APP processing

A role for Aβ plaques in the etiology of AD has been argued in the last twenty years from the the discovery of mutations in genes encoding proteins involved in APP processing (see par. 1.3).

APP is known to be one of three members of a larger family of related proteins that include the amyloid precursor-like protein (APLP1 and APLP2) in mammals, the amyloid recursor protein-like (APPL) in Drosophila, APP-related protein (APL-1) in Caenorhabditis Elegans. These are single-pass transmembrane proteins with large extracellular domains and are all processed in a similar manner to APP. However, only APP undergoes sequential proteolytic processing that generate fibrillar Aβ, because of the sequence divergence within Aβ sites. This protein is expressed in many tissues, especially in neurons. Although its function has been not fully clarified yet, studies have reported its involvement in cell adhesion, neuronal differentiation, neuronal migration, neurite outgrowth, synapse formation and neural plasticity (Priller et al. 2006; Zhou et al. 2011). APP is synthesized in the endoplasmic reticulum, undergoes post-transcriptional modification in the Golgi apparatus (N- and O-linked glycosylation, sulfation and phosphorylation), and is transported via secretory pathway to the cell surface from which it can be endocytosed and processed in the endosomal-lysosomal pathway (Koo and Squazzo 1994).

After sorting in the endoplasmic reticulum and Golgi apparatus, APP is transported - by fast axonal transport - to synaptic terminal (Koo et al. 1990). On the cell surface, APP can be proteolyzed directly through the so-called non-amyloidogenic pathway operated by α-secretase and then γ-secretase, or re-internalized in

clathrin-coated pits into endosomal compartment that contains the β-site APP-cleaving enzyme 1 (BACE1) and γ-secretase (amiloidogenic pathway) (Fig. 3).

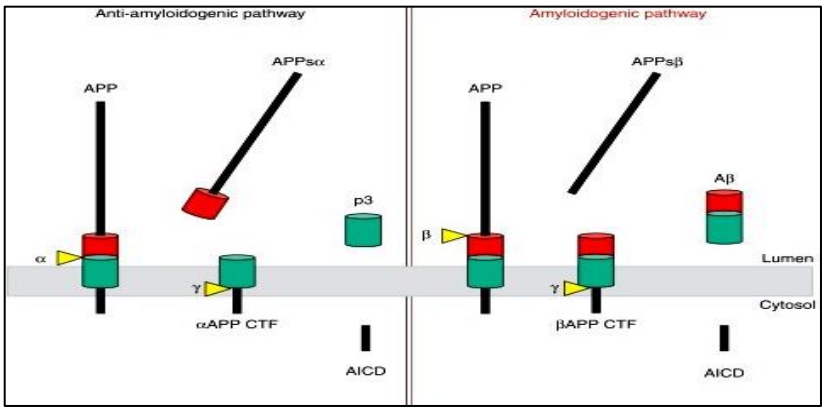


Figure 3. Sequential cleavage of APP through non-amiloidogenic and amiloidogenic pathway in trans Golgi network (TGN).
On the left: in the the non-amyloidogenic pathway APP is cleaved approximatley in the middle of Aβ region from a-secretase, thus the following cleavage of γ-sevretase generates a truncated Aβ peptide (p3) that is pathologically irrilevant. On the right: in the amyloidogenic pathway the APP-cleavage of b-secretase in a site that is more distant from the citosolic portion of APP generates an APP carboxy-terminal fragment (βAPP CTF) that is successively cleaved fro g-secretase to generate Aβ (Haass et al. 2012).

The first cleavage of APP by α- or β-secretase results in the generation of membrane tethered α- or β-C terminal fragments (αAPP-CTF and βAPP-CTF) and the release in the extracellular milieu of two soluble peptides, respectively sAPPα and sAPPβ.

The principal neuronal β-secretase (BACE1) is a transmembrane aspartic protease - normally localized at the Golgi/Trans-Golgi network (TGN) and endosomes - that cleaves APP within the ectodomain at +1 (prior to amino-acid 1) and +11 sites of Aβ, generating the N-terminus of Aβ (Vassar 2004). Full-lengh APP is not located for considerable lenght of time at the cell surface. In fact, because of a "YENPTY" motif located at the C-terminus of APP, the protein is efficiently internalized. After being delivered to late endosomes a fraction of endocytosed molecules is recycled to the cell surface while another fraction is directed to the lysosomes for degradation. Moreover, BACE1 optimal activity requires an acidic pH, at least *in vitro*. This would explain why the cleavage of APP typically occurs during the transportation to the acidic endocytic compartments, such as TGN.

On the other hand, α-cleavage of APP - attributed to ADAM (a disintegrine and metalloproteinase) (M. Asai et al. 2003) - occurs mostly at the cell surface, even if some activity has also been identified in TGN. α-secretase cleaves APP within Aβ domain (between residues Lys16 and Leu17 of the Aβ peptide), thus hindering the generation of intact Aβ. Noteworthy, therapeutic strategies have been pursued to increase the recruitment of α-secretase to the cell surface in order to favour the non-amyloidogenic APP processing. For example, the activation of protein kinase C has been shown to increase α-secretase cleavage of APP by augmenting its enzymatic activity as well as enhancing the transit to the cell surface (Hung et al. 1993) (Skovronsky et al. 2000).

The second proteolytic event in APP processing is characterized by cleavage of α-and β-CTFs by γ-secretase, that releases p3 (3 kDa) and Aβ (4kDa) peptides, respectively, into the extracellular environment.

Similarly to β-secretase, the majority of the mature components of γ-secretase multiprotein complex are found in the intracellular organelles such as Golgi apparatus/TGN and TGN late endosomes. The complex is composed of two integral membrane proteins [presenilin 1 (PS1) and presently 2 (PSE2)], a type I transmembrane glycoprotein [nicastrin (Nct)], and two multipass transmembrane proteins: [anterior pharynx-defective 1 (in humans, APH-1a or APH-1b) and presenilin enhancer 2 (PEN-2) (Bergmans and De Strooper 2010)]. Two aspartic residues within transmembrane domains 6 and 7 of PS1 and PS2 are critical for γ-secretase activity. APH-1 and PEN-2 seem to be involved in the stabilization of the γ-secretase complex while Nct is thought to mediate the recruitment of APP-CTF to the catalytic site, corresponding to positions 40 and 42 of Aβ.

In the amyloidogenic pathway more than 90% of secreted Aβ is represented by Aβ40, with Aβ42 accounting for only 10% of total Aβ.

Although the standard model for Aβ generation suggests that Aβ42 is normally secreted outside the cell to be deposited as extracellular insoluble amyloid plaques, there is also evidence for the presence of insoluble Aβ42 within endoplasmic reticulum (ER) (Hartmann et al. 1997; Greenfield et al. 1999). Indeed, *in vitro* experiments in transfected cell lines has suggested that significant amounts of APP is processed intracellularly to Aβ (Greenfield et al. 1999). This finding can be explained by the more frequent endosomal localization of BACE1 and correlate with the high levels of intracellular Aβ found in neurons from patients with early AD (LaFerla et al. 2007).

Finally, in both amyloidogenic and non-amylodogenic pathways, the cleavage generates also a cytoplasmic peptide, APP intracellular domain (AICD), whose fate is not fully resolved.

1.3 Early-Onset Familial Alzheimer's Disease (EOFAD)

The "risk genes" that show an association to AD display an intricate pattern of interaction with each other and with non-genetic factors. Moreover - unlike classical Mendelian ("simplex") disorders - they are not characterized by a simple or single mode of inheritance.

Only 5% of all AD cases can be attributable to early-onset familial AD (Tanzi 1999). These familial forms of the disease - rare, but with very penetrant mutations in *APP*, *PSEN1*, *PSEN2* genes - are often transmitted as an autosomal dominant trait with an onset that typically is below 65 years of age. Mutations in these genes might result in alteration of amyloid-β production (both Aβ40 and Aβ42) leading to apoptosis of neurons and dementia (Sorbi et al. 2001). The *APP* gene - located on chromosome 21q21 - contains 18 exons for encoding a 695-amino-acid protein the APP, with the Aβ peptide being encoded by exons 16 and 17. The transcripted mRNA is then processed by alternative splicing which produce at least five isoforms of APP protein (APP695,

APP714, APP751, APP770, APP563). Each isoforms contains the Aβ peptide sequence (Konig et al. 1992) and is differently expressed on the basis of the tissue type. APP695, APP751 and APP770 represent the three most relevant isoforms to AD, with the first being expressed only in CNS and the latter in both the peripheral and CNS tissues (Kang et al. 1987).

However, alterations of *APP* gene represent a very rare risk factor for AD, as only 21 and 3 mutations have been identified at exon 17 and 16, respectively. The first missense mutation in APP gene was discovered in 1991 (Goate et al. 1991). Since then, many different missense mutations in *APP* gene have been reported, but they only account for about one tenth of all individuals with EOFAD, including the *Swedish* mutation (KM670/671NL), *Flemish* mutation (A692G), *Arctic* mutation (E693G), *London* mutation (V717I) and *Indiana* mutation (V717F). Noteworthy, most of these variants occur in proximity to the cleavage of the APP processing enzymes α, β and γ-secretase. Interestingly, missense mutations near the γ-secretase cleavage site at residues 714 and 715 of *APP*, decrease the secretion of Aβ40, while mutations at codon 716-717 increase the production of Aβ42 (De Jonghe et al. 2001). This underlines the different and relevant role of mutations near the γ-secretase cleavage in driving an altered proteolysis of Aβ peptide in the onset of the AD. Importantly, triplication of chromosome 21 – as it occurs in Down syndrome – results in triplication of the APP gene leading to enhanced APP expression and Aβ production. Noteworthy, as evidenced in figure 4 showing a timeline of AD onset according to the age, Down syndrome patients have been reported to develop AD pathology earlier than those without Down syndrome (Prasher et al. 2004). These findings suggest that AD pathology might be strictly related to overexpression of APP and have been cited, among others, as proof to confirm the *amyloid hypothesis* as the explanation for the pathogenesis of AD (see par.1.5).

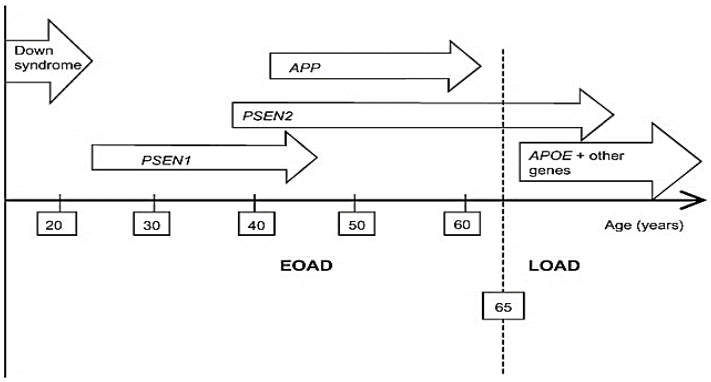


Figure 4. The age onset of AD, depending on the different involvement of genes. Typical dementia symptoms can occur at an earlier stage in Down syndrome patients than in AD patients without trisomy; *EOAD*: early-onset AD; *LOAD*: late-onset AD (Bagyinszky et al. 2014).

PSEN1 and *PSEN2*, with a homology of 67%, have been also implicated in the onset of AD. As described before, these genes encode proteins that are the major components of the multimeric atypical aspartyl protease complex that mediate the γ-secretase cleavage of APP.

PSEN1 (on chromosome 14q24.2) was the first gene in which AD-causing mutations were discovered (Sherrington et al. 1995). Mutation in this *PSEN1* gene are the most common cause of EOFAD (18%-50% of autosomal dominant EOFAD) (Theuns et al. 2000). It consists of 12 exons that encode a 467-amino-acid protein, which possesses 9 transmembrane domains. The membrane portions of these domains forms the catalytic core of γ-secretase complex and plays a crucial role in mediating intramembranous, γ-secretase processing of APP to produce Aβ. Although PSEN1 is an integral membrane protein normally located at the membrane surface, it may also be found in the Golgi apparatus, endoplasmic reticulum and mitochondria (De Strooper et al. 1998). In addition to APP, many type-I transmembrane proteins are cleaved by γ-secretase complex, including Notch, a protein involved in the embryonic development of numerous tissue types and cell-to-cell signaling. Therefore, the pleiotropic action of this protein might explain why *PSEN1* knock out mice are not viable (Shen et al. 1997). The γ-secretase inhibitor LY-411, for example, is able to reduce the production of Aβ in the brain but alters T- and B-cells maturation (Barten et al. 2005).

After the discovery of the pathogenic implication of *PSEN1* in AD, it appeared obvious that mutations in this gene as well as APP could not explain all cases of the autosomal dominant form of the disease. Infact, database analysis revealed that mutations in another gene - subsequently named *PSEN2* (protein: PS2) - of presenilin gene family, were associated to a small number of all EOFAD cases. Interestingly, individuals with mutations in *PSEN2* exhibit a later age of onset and a slower disease progression with respect to carriers with mutated *APP* or *PSEN1. PSEN2* gene is located on chromosome 1 (1q42.13) and is expressed in a variety of tissues, including the brain, where it is expressed predominantly in neurons. It consists of 12 exons (10 translated exons) that encode a 448-amino-acid peptide, which is characterized by nine transmembrane domains and a large loop structure between the sixth and seventh domain. *PSEN2* mutations - to date, as many as 14 mutations have been identified - are much rarer cause of familial AD than *PSEN1* and have been reported to increase the ratio Aβ42 to Aβ40 in mice and humans (Kang et al. 1987). Like *PSEN1, PSEN2* undergoes intense alternative splicing, giving origin to protein isoforms that strongly and differently influence the proteolytic processing of APP.

1.4 Late Onset Alzheimer's Disease (LOAD)

In late-onset Alzheimer's disease (LOAD), several genes have been described as potential risk factors, but non-genetic factors may also be implicated in disease's progression (Bertram and Tanzi 2005). The *APOE* gene, located on chromosome 19, is an important genetic risk factor for LOAD. Apolipoprotein E (ApoE) - which is predominantly expressed by astrocytes and strongly up-regulated by microglia in the context of Aβ pathology (Krasemann et al. 2017) - is the major cholesterol carrier in the brain, which is involved in neuronal maintenance and repair. On the cell surface, ApoE binds to several receptors which are involved in lipid

transport, glucose metabolism, neuronal signaling, and mitochondrial function. In addition, ApoE has been reported to bind to Aβ peptide, playing a role in its clearance (Bu 2009).

Two polymorphic sites, located at codon 112 and 158, have been described in the human *APOE* gene and three main variations of the *APOE* gene have been identified, referred to as "ε2, " "ε3," and "ε4" alleles. ε3 was defined as a normal allele with *Cys* at codon 112 and *Arg* at codon 158, while the ε2 and ε4 alleles carry Arg158Cys and Cys112Arg polymorphisms, respectively (Rihn et al. 2009; Green et al. 2009). Six different genotypes (3 homozygous and 3 heterozygous) have been identified, with the ε2 allele - that may be involved in neuronal maintenance and repair - being suggested to be protective against AD (Mahley and Huang 2006), and the ε4 allele associated with increased risk of AD in both homo- and heterozygosus phenotype (Bu 2009). In the ε4 allele, the altered orientation of Arg61 in the C-terminal portion, promotes different interaction between C- and N-terminal domains, driving conformational changes of ApoE protein, which may eventually leads to neuronal cell death.

In addition to ApoE, new genes associated with LOAD have been identified by genome-wide association studies (GWAS). They include:

- *clusterin (CLU)*, located on chromosome 8 and encoding for an inflammatory-related protein with role in regulation of apoptosis, cell damage and oxidative stress and found to be upregulated in brains of AD patients (Calero et al. 2000);
- *complement receptor 1 (CR1)*, located on chromosome 1 and encoding for the receptor C3b complement protein. CR1 is a polymorphic glycoprotein which functions regulating the complement system and, in phagocytes, the process of endocytosis of foreign particle. In the context of AD it is involved in the clearance of Aβ (Lambert et al. 2009);
- *phosphatidylinositol binding clathrin assembly* gene (*PICALM* or *CALM*), located on chromosome 11, (Xiao et al. 2012) encode a protein that was found to be expressed in neurons and co-localized with APP throughout the cortex and hippocampus in APP/PS1 mice. Several single nucleotide polymorphisms (SNPs) in this gene were significantly associated with AD, suggesting a role in APP endocytosis and Aβ generation;
- *Sortilin-related receptor* (*SORL1*) on chromosome 11q23-24, encodes a multifunctional endocytic receptor, that may be implicated in the uptake of lipoproteins and proteases. It is involved in APP trafficking to and from the Golgi apparatus (Rogaeva et al. 2007);
- *Bridging Integrator 1* (*BIN1*), located on chromosome 2, encodes a widely expressed adaptor protein that partecipate in clathrin-mediated endocytosis and endocytic recycling. In the brain it is involved in the retrieval of synaptic vesicles, while ubiquitous isoforms of BIN1 regulates apoptosis, inflammation and calcium homeostasis (Cruchaga et al. 2011).

Other genes that genetic linkage studies have associated to LOAD are the lo*w-density lipoprotein receptor-related protein 6* (*LRP6*)**,** *cadherin-associated protein alpha 3* (*CTNNA3*), *growth factor receptor-bound protein 2-associated-binding protein 2 (GAB2)*, *A disintegrin and metalloproteinase domain-containing protein 10* (*ADAM10*)*,* ATP-binding cassette transporter A7 (*ABCA7*). In addition, *triggering receptor expressed on myeloid cells 2 (TREM2)* and *Cluster of differentiation 33 (CD33),* encoding for proteins mainly expressed from cells belonging to myeloid lineage, such as monocytes and macrophages, have underlined the role of the immune system dysfunction in the pathogenesis of AD (Bagyinszky et al. 2014) (see 2.1.5).

1.5 Hypotheses for Alzheimer's Disease pathogenesis

The "Amyloid hypothesis" has been the mainstream explanation for the pathogenesis of AD for over 25 years (Hardy and Allsop 1991). According to this hypothesis, the accumulation of Aβ in the brain is the primary feature driving AD pathogenesis. The other pathological hallmarks, such as NFTs, synaptic dysfunction and neuronal loss, are proposed to result from an imbalance between Aβ production and Aβ clearance (Fig. 5).
The cloning of the APP gene and the discovery of its localization on chromosome 21 (Kang et al. 1987) along with the finding that trisomy 21 (Down's syndrome) increase the risk of developing AD (Olson and Shaw 1969), paved the way for the proposal that Aβ accumulation was the primary event in AD pathogenesis. Evidence derives also by the fact that most of the mutations associated to a higher risk of developing familiar forms of the disease cluster at or very near to the sites of APP gene, or involve proteins that are directly associated to APP metabolism that favour the production of amyloidogenic Aβ, such as PS1 and PS2 (Scheuner et al. 1996). These genetic studies contributed to increase the reliability of the "Amyloid hypothesis". In addition, other four findings can be cited in support of the role of Aβ in the pathogenic cascade: 1) *tau* mutations cause frontotemporal dementia with parkinsonism (Poorkaj et al. 2004), extensive NFTs formation, but not amyloid deposition. This suggests that NFTs are not sufficient to initialize amyloid deposition and are likely to have been deposited after Aβ formation (Hardy et al. 1998); 2) in contraposition to mice overexpressing tau alone, transgenic mice overexpressing both mutant human APP and mutant human tau are characterized by increased formation of tau-positive tangles. However, the structure and number of their amyloid plaques remain unaltered (Lewis et al. 2001), suggesting that the dysregulation in APP processing may worsen tau pathology; 3) the lack of apoE gene in APP transgenic mice reduces the cerebral Aβ deposition in the offspring (Bales et al. 1997). Importantly, apoE ε4 allele - that was found to markedly increase AD risk - leads to excess of Aβ aggregation and typical downstream processes of AD neuropathology. This underlines the pathogenic role of genetic variability at the human apoE locus and its involvement in Aβ processing; 4) evidence indicates that genetic variability in Aβ catabolism and clearance may contribute to the risk of late-onset AD (Kang et al. 2000). Although the number of amyloid plaques have been shown to not correlate with cognitive impairment, more often these deposits are almost exclusively

diffuse forms of amyloid plaques, with no evidence of surrounding neuritic and glial pathology. Moreover, the severity of dementia correlates much better with Aβ assayed biochemically than with histologically analysed plaques, and the soluble Aβ oligomers - which are not detected by immunohistochemistry - have been described as the most toxic species, which better correlate with cognitive impairment (Hardy and Selkoe 2002).

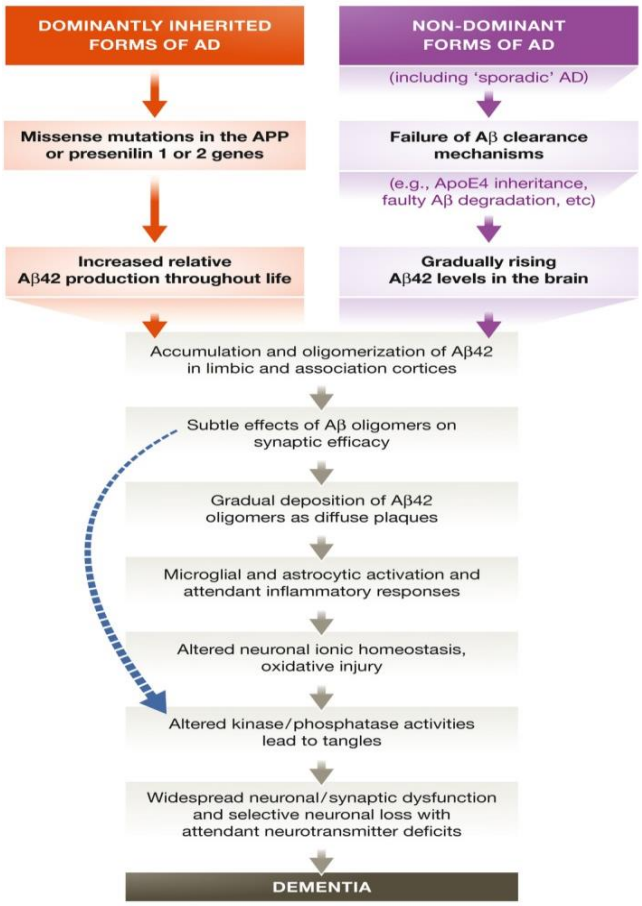


Figure 5. Sequence of pathogenic events leading to AD proposed by the Amyloid hypothesis. The curved blue arrow indicates that Aβ oligomers may directly injure synapses and neurites in neurons, in addition to gradually deposit as diffuse plaques, thus activating microglia and contributing to neuronal dysfunction (Selkoe and Hardy 2016).

Taken together, these findings are consistent with the notion that cerebral Aβ accumulation is the initial factor in AD driving the rest of the disease and explained all the clinical trials testing medications that targeted the production, accumulation and persistence of amyloid-β in the brain. However, all of the Aβ - targeted phase 3 clinical trials in AD have ended in failure leading scientists to wonder which putative other mechanisms may be targeted to block or slow down AD progression.

More recent findings have led to a redefinition of the role the "Amyloid hypothesis". Decreased synapse number has long been considered as the strongest quantitative neuropathological correlate of dementia in AD. Indeed, a consistent decrease of synaptic proteins is observed in AD patients (Davies et al. 1987). Therefore, it has been suggested that Aβ, which is present at early stages in mouse model of AD, may

underline synaptic dysfunction (William et al. 2012). However, although Aβ oligomers obtained from AD patients' brains have been reported to impair synaptic plasticity and memory learning after injection into hippocampus of healthy adult rats (Shankar et al. 2008), in other cases overexpression of Aβ42 in a mice model of AD prompted amyloid deposition and soluble oligomer formation, but no neuronal loss and cognitive decline (Kim et al. 2013). This suggests a dissociation between brain Aβ accumulation and memory impairment. In addition immunotherapies targeting Aβ are effective in decreasing amyloid deposits but do not lead to improvement of cognitive deficits or tau pathology (Salloway et al. 2014). Finally, the brain of elderly non-demented patients, the amount of non-senile plaques is as wide as that of AD patients (Chételat 2013), indicating that amyloid deposition should be considered as a normal process of aging, with no relation with AD. Considering these facts, other hypotheses have been advanced involving the role of tau pathology or the innate immune system. At the basis of tau hypothesis for AD there are recent PET studies showing that the spatial temporal patterns of a tau tracer binding correlate with the pattern of neurodegeneration and clinical symptoms in the brain of AD patients. This suggests that the spreading of tau pathology is robustly associated to the cognitive impairment (Johnson et al. 2016; Okamura and Yanai 2017).

According to these studies, progression of AD seem to be associated with tau pathology, rather than Aβ amyloid accumulation.

Finally, genetic evidences describing the role of inflammatory processes in AD has led to a redefinition of the role of immune system and neuroinflammation. As stated by "Glial Dysfunction Hypothesis", AD has its cause on dysregulated activation of resident innate immune cells (microglia) which become increasingly cytotoxic releasing pro-inflammatory and pro-oxidant mediators and decreasing their protective effects (von Bernhardi et al. 2015). In fact, experimental and clinical evidence have demonstrated the increased synthesis of pro-inflammatory cytokines as well as the upregulation of their receptors in AD brain (Sastre et al. 2006). As mentioned before, amyloid plaques appear a decade or two before clinical symptoms in AD, but it is tau pathology and synapse loss that best correlate with cognitive impairment during disease progression. Since microglia has been shown to directly mediate synapse loss (Wu et al. 2015) and exacerbate tau pathology (Leyns and Holtzman 2017), it may represent an appealing therapeutic target in AD. Non-steroidal anti-inflammatory drug (NSAID) epidemiology and clinical trial results appeared to be promising, but not without conflicting results that were likely to depend on the fact that microglia phenotypes and, in general, glia responses are highly context- and stage-dependent (Heneka et al. 2015). In this sense, ligands for PET or other imaging modalities that enable to discriminate different states of microglia activation *in vivo* could help to better understand the role of neuroinflammation in the human CNS.

To conclude, since in its first formulation, the Amyloid hypothesis has been supported by several studies. The fail of treatment strategies targeting Aβ fomented several optional perspectives on the pathogenesis of AD

but an alternative hypothesis that has as much experimental support as the Aβ hypothesis has not emerged yet.

1.6 Mice models of Alzheimer's Disease

The aforementioned genetic mutations and neuropathological features have served as basis for the creation and validation of mouse model of AD. Over 100 genetically engineered mouse lines that recapitulate some typical aspects of AD neuropathology have been generated, each one with its own advantages and limitations. Numerous AD models have successfully reproduced amyloid plaque deposition, generally by deriving mice with high expression of APP gene carrying different human mutations, as well as the inclusion of a mutant PS1 that accelerate both deposition rate and disease progression. However, since different models may be the most appropriate for investigation of specific topics, no one model should be considered the best.

hAPP transgenic models - the first mouse model of AD - are characterized by the expression of human APP (hAPP). Despite the low incidence of familial forms in all AD cases, these mutations serve as basis for most AD models. These lines develop a robust amyloid pathology, synaptotoxicity (even if no significant neuron loss is present), memory deficits and differ for promoters driving hAPP expression, the hAPP isoform(s) and mutation(s) expressed, and the background strain.

Promoters. hAPP has been expressed from numerous promoters, especially under the promoter of platelet-derived growth factor B-chain (PDGF), thymocyte differentiation antigen 1 (Thy-1), and prion protein (PrP) genes. Each promoter guides different levels, and spatial/temporal patterns of expression; for example, the PDGF promoter drives the expression primarily in the brain and selectively in neurons, while the PrP promoter cause the strongest expression but it is less selective, as it drives the expression of hAPP also in extraneural tissues.

APP isoforms. The *APP* mRNA undergoes alternative splicing of exons 7 and 8, resulting in three isoforms that differ in the number of amino acids in the final product: APP695, APP751, and APP770. In the brain, APP695 is expressed at high levels while the APP751 and APP770 isoforms are expressed at significantly lower levels. The two longer include a Kunitz protease inhibitor (KPI) domain - which mediates specific protein-protein interactions - and their proportion has been reported to increase with age, becoming also more prevalent in AD brain (Moir et al. 1998). KPI-positive APP isoforms are more likely to undergo amyloidogenic β-secretase cleavage (Ho et al. 1996), assuming an important pathogenic relevance.

Mutations. Different hAPP transgenic lines express different AD-associated mutations. Some of the earliest lines have a mutation only at the γ-secretase cleavage site (e.g., PDAPP), while the most currently used mouse lines express the K670N/M671L Swedish double mutation at the β-secretase cleavage site. Some lines

express only the Swedish mutation (e.g., Tg2576), while others combine two or more mutations and are characterized by a more rapid and extensive formation of amyloid plaques (Cheng et al. 2004).

Background strain. Background strain can affect the phenotype of AD models at several levels, with effects also on anxiety and activity. Some strains are inclined to hearing and vision issues, which may affect performance on behavioural tests. Other strains differ in susceptibility to hAPP/Aβ effects, excitotoxicity, inflammation, neurodegeneration, and memory/learning abilities (Hall and Roberson 2012).

The first single transgenic model - the NSEAPP mouse, with the human APP 751 complementary DNA and the rat neuron-specific enolase (NSE) promoter - was described by Quon and colleagues in 1991 (Quon et al. 1991). However, the majority of the deposits in these mice are diffuse plaques with only rare evidence of dense-core plaques as found in AD brain. Then, other human APP mouse models were developed, including PDAPP (Indiana mutation), Tg2576 (APP695 with Swedish mutation), TgAPP23 (APP751 with Swedish mutation), TgCRND8 (APP695 with Swedish and Indiana mutation), and J20 (APP with Swedish and Indiana mutation). Most APP transgenes utilizes a cDNA encoding APP695 isoform, which is the most expressed in the brain, or APP751. All these mouse models develop Thioflavin-S positive plaques that strongly resemble those found in AD brain, even if with a time course and pathological traits that may change also considerably.

AD mouse models can also be generated by combining *APP* mutations with one or multiple FAD mutations in presenilin genes, with the advantage of accelerating the pathogenesis rate compared to monogenic models. PS/APP line was one of the earlier double transgenic model. It was generated by crossing Tg2576 mice overexpressing human APP with the Swedish mutation driven by the hamster prion protein gene promoter, with mice overexpressing human PSEN1 with the M146L mutation under the PDGF-β promoter. These mice develop plaque pathology earlier than the Tg2576 mice, producing also higher amount of Aβ (Duff et al. 1996). It suggests the critical role of the PS1 mutation in driving increased production of Aβ in respect to transgenic Tg2576 line containing only the Swedish mutation.

Other common double transgenic AD models are represented the APPswe/PS1ΔE9 mice - combining the Swedish mutation in APP gene and the ΔE9 mutation in PS1 gene - and the 5xFAD, which is generated by combining five AD-related mutations: the Swedish, Florida (I716V), and London (V717I) mutations in hAPP and the M146L and L286V mutations in PS1 (Oakley et al. 2006). Although at different ages, both these models develop Aβ plaques and cognitive deficits, with 5xFAD mice having a more rapidly and severe pathological course (amyloid plaques within cortical layers as early as 1.5 months of age and intraneuronal Aβ by approximately 45 days of age).

1.6.1 Modeling the role of Tau - the triple transgenic (3xTg) mice

Developed by Oddo and colleagues in order to create a more accurate model of AD, 3xTg-AD were generated by co-injecting hAPP with the Swedish mutation and human tau with P310L mutation into embryonic cells -

of a Tg2576 background - homozygous for the mutant versions of the presenilin 1 (*PS1$_{M146V}$*)(Oddo et al. 2003). This model developS an age-related and progressive neuropathological phenotype characterized by both plaque and tangle pathology. Extracellular Aβ deposits become apparent in 6 month-old mice in the frontal cortex (prominently in layers 4 to 5) becoming consistent by 12 months, also in other cortical regions and in the hippocampus. Tau pathology is first evident in the hippocampus of 12-15 month-old 3xTg mice, especially within pyramidal neurons of CA1 subfield, and then progresses to involve cortical structures. Importantly, the regional pattern of the neuropathological progression closely mimic that observed in AD. In fact, Aβ pathology firstly hits cortical regions and later hippocampus, while NFTs formation typically initiates in brain limbic regions and then spreads to cortical areas.

This model is also characterized by the presence of a strong intraneuronal Aβ reactivity, that is one of the earliest pathological manifestation in 3xTg AD mice, first detectable in neocortical regions (3 and 4 months of age) and subsequently (by 6 months of age) in CA1 pyramidal neurons. The role of intracellular Aβ in driving pathogenic processes has been documented in AD (Gouras et al. 2000), even if its pathophysiological relevance remains unclear. However, the precocious - before Aβ plaque and NFTs appearance - synaptic dysfunction in 3xTg mice seem to provide compelling evidence for a role of intraneuronal Aβ as trigger for cognitive deficits, already apparent at 6.5 months of age (Stover et al. 2015).

Notably, various inflammatory processes have been shown to correspond to the presentation of early (< 6 months of age) intracellular Aβ pathology in 3xTg-AD mice (Janelsins et al. 2005) and the expression pattern of pro-inflammatory cytokines has been shown to correlate with increased numbers of microglia in this model as compared to age-matched, non-transgenic control mice. Moreover microglial cells increase in both frontal and entorhinal cortices at a stage in which both Aβ plaques and NFTs are not present, yet (Mastrangelo and Bowers 2008b), suggesting a potential role of inflammatory processes in driving pathogenic cascade in this model.

Finally, the progressive development of both plaques and tangles in an age - and region-dependent manner has important clinical implications. In fact, besides being a model that represents more carefully the AD neuropathology, it could help to investigate if a therapeutic intervention against one of the AD hallmarks may be more effective in slowing down or halting the progression in respect with another.

2. Microglia

Microglia belong(s) to glial cells. The term "*glia*" "derive from the ancient Greek word "*glía*" meaning "glue" in English. In fact, before the identification and the characterization in the 19th century by neuroscientists including Rudolf Virchow, Santiago Ramón y Cajal and Pío del Río-Hortega, glia was thought to solely function as so-called "*Nervenkitt*", the German word for nerve glue. With time, scientists started to speculate about additional putative roles of these cells (Glass et al. 2010). Several studies have been performed to unravel

these further functions. Initially, general ablation approaches based on cytotoxic substances such as ethidium bromide (EtBr), that induces general irreversible DNA damage - leading also to neuronal cell death - were used for the depletion of mature brain cells that were already integrated in an established network *in vivo* (Yajima and Suzuki 1979). The application of high doses of X-irradiation (Kalderon and Fuks 1996) or the mitotic blocker arabinofuranosyl cytidine (AraC) (Doetsch et al. 1999) were also used to specifically target and, subsequently, ablate cycling cells. With the advancement in the field of genetic manipulation, cell ablation techniques have been improved. The use, for example, of "suicide" genes has resulted in the advantage to specifically deplete distinct cell types because of few side effects for surrounding cells or other tissues. These ablation methods have led to a better understanding of the functions of glia, making it clear that these cells are more than just mere "*Nervenkitt*". Importantly, glial cells are anything but a minor cellular fraction, as they constitute - depending on the mammalian species - between 33% and 66% of the total brain mass (Azevedo et al. 2009).

The total brain glial cell population can be subdivided into four major groups: microglia, astrocytes, oligodendrocytes and their progenitors NG2-glia.

Microglia are the resident and phagocytic immune cells of the brain and spinal cord. These cells represent 10-15% of the total population of cells within the brain (5-20% of total glial cells) and exhibit distinct morphologies and functions across different anatomical regions. Nissl and Robertson first identified them at the end of the nineteenth century. In 1932, Pio del Rio Hortega introduced for the first time the concept of "microglia" as a defined cellular element of CNS in a book entitled "Microglia" and introduced some basic characteristics of the cells that are still valid today (Kettenmann et al. 2011). In his first studies, published in a series of scientific articles between 1919 and 1927, Del Rio-Hortega used a modified silver carbonate impregnation to label microglia obtaining excellent images of the cells (Fig. 6). For many decades after the identification and first characterization of microglia, their importance for CNS physiology remained largely underappreciated. The modern era of microglia research started in the 1960s when Georg Kreutzberg introduced the facial nerve lesion model, which opened the chance to study microglial responses to injury in tissues with intact blood brain barrier (BBB), as well as to distinguish the behaviour of resident microglia and invading monocytes (Blinzinger and Kreutzberg 1968). His studies helped to define the concept that microglial cells are key players in both degeneration and regeneration of the brain. The introduction of *in vitro* cultures of microglia described by Costero in 1930 (Costero 1930), as a wide-spread tool to study microglial properties and functions, led to an explosion of studies on cultured microglia. As expected, cell culture environment generates modifications in cell behaviour with the consequence that all these *in vitro* studies may not faithfully reflect properties of microglia in the normal, non-pathological brain. The combination of advanced imaging techniques (luminescence imaging, PET, multi-photon or two-photon laser

scanning microscopy) with the use of genetically based cell-specific markers has allowed investigations of microglia in the non-perturbed tissue (Davalos et al. 2005).

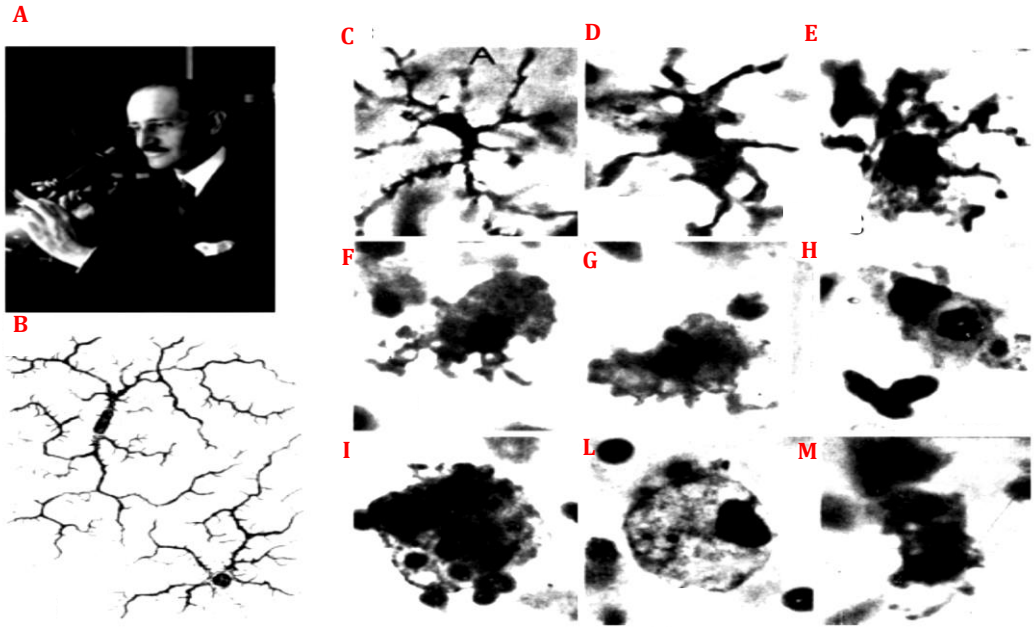


Figure 6. Microglial cells discovered by Pio del Rio-Hortega. ***A***: Pio del Rio-Hortega (1882–1945). ***B***: images of ramified microglial cells drawn by Hortega. *C-M*: evolution of microglia during its phagocytic activity. ***C***: cell with thick, rough prolongations; ***D***: cells with short prolongations and enlarged cell body; ***E***: hypertrophic cell with pseudopodia; **F**,**G**: amoeboid and pseudopodic forms; ***H***: cell with phagocytosed leukocyte; ***I***: cell with numerous phagocytosed erythrocytes; ***L***: fat-granule cell; ***M***: cell in mitosis (Photomicrographs from (Del Rio-Hortega 1932).

2.1 Microglia: origin and development

Although the origin of microglia has been long an area of debate, consensus about the the precise nature of the microglial progenitors has been recently reached. With respect to the ectodermal origin of the other glial cells, microglia derive from primitive myeloid precursors that differentiate in mesodermal yolk sac of mammals, birds and zebrafish before the onset of blood circulation and seed the brain during early foetal development (Alliot et al. 1991; Lichanska and Hume 2000). The yolk sac origin of microglia was proved using a range of different strategies, including a fate-mapping mouse model that expressed a fluorescent protein exclusively in yolk sac progenitors and their progeny, such as yolk sac macrophages (Ginhoux et al. 2010). By these studies Ginhoux and colleagues revealed a minimal, if any, contribution of definitive hematopoiesis to the development of adult microglia providing evidence that these cells derive from unique embryonic precursors (yolk sac macrophages). These primitive macrophages - that differentiate in the yolk sac blood islands around E8.0 - spread into the embryos at the onset of blood circulation (around E8.5) and migrate to the neuroepithelium from E9.0/9.5, giving rise to embryonic microglia (Ginhoux and Prinz 2015).

Since the formation of BBB starts at E13.5, it may reduce the contribution to microglial population of foetal liver and, later, bone marrow haematopoiesis, which give rise to monocytes, macrophages, and lymphocytes from E10.5 (Cumano and Godin 2007; Ginhoux and Prinz 2015).

However, shortly after birth in rodents, microglia population increases dramatically (Francoise et al. 1999), suggesting that the proliferation of embryonic cells alone could not account for this robust cell expansion and that an influx of cells from other compartments might contribute to resident microglia. Infact, as suggested by Pio-Hortega, blood monocytes have been described as capable of entering the CNS in the perinatal period and giving rise to microglia, replacing the embryonic microglial cells. Studies employing the PU.1 (a key myeloid transcription factor) knockout (KO) mouse model lacking embryonic microglia, have also demonstrated the capacity of blood-borne cells to contribute to the post-natal microglial population (Beers et al. 2006). Regarding the adult brain, although there is a very little exchange between blood and brain parenchyma making the exchange of microglial cells almost negligible, in pathological conditions BBB may be damaged, allowing bone marrow-derived progenitors to enter the CNS and transform into microglia (Mildner et al. 2007).

2.2 Microglia activation: the M1 and M2 phenotype

Once invaded the brain parenchyma, microglial cells change their morphology acquiring a ramified phenotype. This phenotype is different from a classical macrophage and it has been associated with microglial “resting” state. Loss of brain homeostasis induced by infection, trauma, ischemia or neurodegenerative diseases generate rapid and profound changes in the microglial morphology, gene expression and the functional behaviour, which summarily is defined as “microglial activation”. Morphologically, the complexity of cellular processes is reduced as they become less ramified and increased in thickness, size soma increases and, in some cases, microglial revert to an amoeboid appearance (Fig. 6). The process of activation involves also the induction of surface molecules, release of cytokines, chemokines and neurotrophic factors and the acquisition of a phagocytic activity to clear tissue debris, damaged cells or microbes. Signaling immunoreceptors such as Toll-like receptors (TLRs), scavenger receptors (SRs), nucleotide binding oligomerization domains (NODs) and NOD-like receptors, regulate microglia response to any changes of the brain microenvironment (Ransohoff and Brown 2012).

Moreover, microglia become motile and actively move to the injured site (virus-infected cells, bacteria, protein aggregates) following chemotactic gradients. At the site of injury microglia proliferate and release factors in order to recruit more cells to counteract whatever is the brain homeostasis-disrupting agent and to prevent further damage after an injury, promote tissue protection and regeneration. In this sense, the activated population with a determined chosen scheme may eventually convert to a repair/restoration-oriented program for tissue regeneration.

The stages of microglial activation represent phases of a highly regulated process by which microglia assume specific morphological, molecular and functional features. However, the term "activation" may appear misleading. In fact the transition from a "resting" to the "executive" states represents more a shift in activity rather than an "activation" per se, as it underlines the concept that these cells may pass through periods of inactivity, that is wrong, since microglia continuously patrol CNS, release factors and actively interact with other cells in unchallenged brain. Moreover, the term "activation" does not contain any information about the functional orientation. The two fundamental polar states of microglia, the so-called M1 and M2 phenotypes, are associated with several functions and differently evolve during an inflammatory process (Graeber 2010). When classically activated, microglia acquired the **M1 phenotype**, characterized by the release of pro-inflammatory and pro-killing molecules such as interleukin-1β (IL-1β), IL-6, IL-12, IL-17, IL-18, IL-23, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), nitric oxide (NO) and chemokines like CCL2 (Subramaniam and Federoff 2017). Specific markers as inducible NO synthase (iNOS), cyclooxygenase-2 (COX2), major histocompatibility complex class II (MHC-II), CD86 (cluster of differentiation marker 86), reactive oxygen species (ROS) and prostaglandin E2 are also induced in M1 phenotypic state (Chhor et al. 2013). All these molecules are induced as the first line of defence in order to eliminate pathogens and to elicit T-cell adaptive and specific immune response.

The M1 phenotype of microglia can be experimentally induced using microbe-associated molecular pattern molecules such as lipopolysaccharide (LPS), an endotoxin found in the cell membranes of Gram-negative bacteria. Other inducers are represented by IFNγ and TNFα (Chhor et al. 2013). The former is a pleiotropic cytokine produced by Th1 cells, $CD8^+$T cells, NK cells, and, in macrophages, it triggers chemokine synthesis, enhanced oxygen radical generation, NO production and induction of major histocompatibility complex II (MHCII).

Activation type/function	Source	Substances produced
M1 (classical activation): pro-inflammatory and pro-killing	LPS, IFN-γ	*Cytokines*: IL-1β, IL-6, IL-12, IL-17, IL-18, IL-23, TNF-α *Markers*: CD86, MHC-II *Chemokines*: CCL2 *Metabolic enzyme/redox molecules*: iNOS, COX-2, reactive oxygen species and reactive nitrogen species prostaglandin E2
M2a (alternative activation): tissue repair and phagocytosis	IL-4, IL-13	*Cytokines*: IL-10 *Markers*: CD206, SR-A1, SR-B1, Arg1, Ym1, Fizz1 *Others*: extracellular matrix proteins, PPAR
M2b (alternative activation): recruitment of regulatory T cells	Fcγ receptors, TLRs and immune complexes (IgG)	*Cytokines*: IL-1β, IL-6, IL-10, TNF-α *Markers*: CD86, MHC-II *Others*: SOCS3, COX-2, Sphk
M2c (alternative activation): anti-inflammatory and healing	IL-10, TGF-β and glucocorticoids	*Cytokines*: IL-10, TGF-β *Markers*: CD163

Figure 7. Microglial polarization states with respective produced factors. Pro-inflammatory cytokines such as IFN-γ or LPS are potent experimental inducers of classically activated phenotype, while the anti-inflammatory cytokines IL-4 and IL-13 are commonly used to induce alternatively activated M2 phenotype. Other microglial alternative activated phenotypes are M2b (immunoregulatory

phenotype) and M2c (acquired-deactivating phenotype), induced by Toll-like Receptor ligands, immune complexes and IL-10, Transforming Grow Factor-β (TGF-β),respectively (Modified by Subramaniam and Federoff 2017).

Specifically, M1 activation by IFN-γ occurs through activation of IFN-γ receptors 1 and 2 (IFN-γR1/2) that leads to Janus kinase 1/2 (JAK1/2) activation, phosphorylation and the nuclear translocation of signal transducer and activator of transcription 1 (STAT1) along with interferon regulatory factors (IRFs). This signaling cascade causes the expression of M1-associated pro-inflammatory cytokines, chemokines, and other genes (Boche et al. 2012). TNFα is a cytokine released by a variety of cells including macrophages, monocytes, endothelial cells, neutrophils, activated lymphocytes, astrocytes and it is known to be a central mediator of a broad range of biological activities such as protective immune responses against infectious pathogens (Turner et al. 2014). The alternative activated microglial **M2 phenotype** is characterized by the release of anti-inflammatory cytokines, extracellular matrix proteins and other substances involved in different processes including immunoregulation, inflammation, repair and injury resolution. As for macrophages, M2-microglia produce high levels of IL-4, IL-10, Arginase 1 (Arg1), chitinase-like protein (Ym1), Found in inflammatory zone (Fizz1) and peroxisome proliferator-activated receptor (PPAR) (Michelucci et al. 2009). As described by Mantovani and colleagues, M1 and M2 phenotypes represent only the extremes of a wide spectrum of possible forms of the macrophage activation (Mantovani et al. 2002). The same nomenclature has also been adopted for the characterization of microglial functional states. Infact, the M2 phenotype has been sub-classified into M2a (alternative), M2b (type II) and M2c (deactivated) activation states (Fig. 7). The M2a state is induced by IL-4 and IL-13 and it is associated with tissue repair and phagocytosis. In the signaling cascade, the binding of IL-4 to its receptor stimulates JAK1 and JAK3 and activates STAT6 which, in turn, leads to the transcription of specific M2a-related genes, such as CD206 and the suppressor of cytokine signaling 3 (SOCS3). The M2b activation state is prompted by the stimulation of Toll-like Receptors (TLRs) and IL-1 receptor, and it is involved in the recruitment of regulatory T-cells. In fact, the engagement of TLRs causes its interaction with Fcγ receptors, which then bind to IgG produced by B cells. M2b activation state is characterized by increased expression of MHC-II and CD86, a protein typically expressed by antigen-presenting cells and that provides costimulatory signals necessary for T-cell activation. M2c phenotype is induced by IL-10 and glucocorticoid hormones, and is involved in anti-inflammatory functions. By IL-10R1 and IL10R2-mediated activation of JAK1, IL-10 induces the translocation of STAT3 into the nucleus and the down-regulation of the expression of different genes associated to M1-phenotype (Franco and Fernández-Suárez 2015).

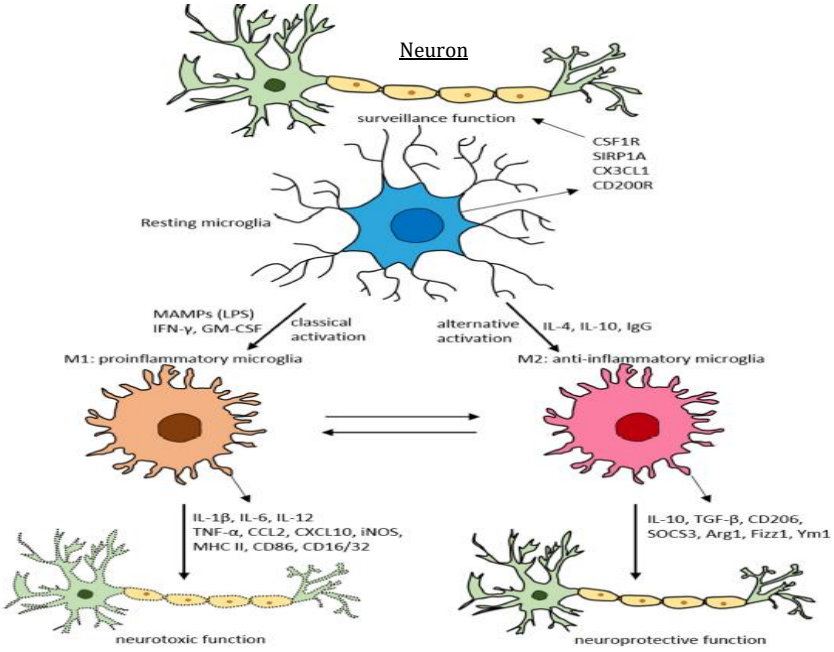


Figure 8. Schematic representation of microglia polarization and functions. In normal physiological conditions microglia acquire a surveillance phenotype in order to patrol and control CNS environment. To maintain this surveillance state, microglia secrete several factors including colony stimulating factor 1 receptor (CSF1R), chemokine CX3CL1 and CD200R. Upon classical activation induced by LPS, IFN-γ, or GM-CSF, microglia acquire M1 pro-inflammatory phenotype that, under chronic inflammation, contributes to neurotoxicity by secreting several pro-inflammatory mediators such as TNF-α, IL-6, iNOS, CD86. When activated alternatively by IL-4, IgG, or IL-10 microglia assume the M2 anti-inflammatory state prompting neuroprotection through secretion of variety of substances IL-10, TGF-β, Arg1, Ym1 (Modified by Subramaniam and Federoff 2017).

The concept of activation associated to macrophage polarization was proposed for the first time 1962, extrapolating results from *in vitro* and *in vivo* experiments. This concept underlines two precepts: first, that there is a two-dimensional spectrum comprising of all macrophage activation states; second, that the extreme poles of this spectrum can be modelled by an inflammatory macrophage (M1) at one end and anti-inflammatory macrophage (M2) at the other. This paradigm has been recently reconsidered, as M1 and M2 macrophage signalling pathways are found to overlap suggesting that stable polarized states cannot be identified. Network analysis of stimulus-dependent human macrophage transcriptomes indicate that these states fail to align along a linear spectrum but rather were distributed as points within an apparent “sphere” (Martinez and Gordon 2014). In this “sphere” there are not stable subsets, but different cellular pathways interact to form complex, even mixed, phenotypes. Moreover, transcriptomic analysis have evidenced that the more stimuli were studied the more “polarized states” for macrophages were found (Xue et al. 2014), suggesting that it is the time to abandon this too simplified paradigm also for microglia characterization. These data may explain why, although different studies have striven to find a correlation between microglia morphology and functional profiles, a univocal correspondence is still lacking and remain widely debated.

2.3 Microglia-neuron interaction: role in synaptogenesis and synaptic plasticity

Microglial cells play a crucial role - both in early embryonic stages and into adulthood - in sustaining the cerebral homeostasis, neuronal integrity, synaptic plasticity and setting functional brain connectivity by modulating synaptogenesis, synaptic pruning and wiring (Paolicelli et al. 2011). In particular, the pruning of excess or dysfunctional synapses takes on a relevant activity by which microglia provide to the synaptic maintenance in steady-state conditions (Tremblay et al. 2012). These processes, that are critical for the correct brain development, are mainly achieved by three different mechanisms that involve (i) the complement system, (ii) the chemokine pathway and (iii) the activity-dependent signaling. The complement system is recruited in antigen-driven phagocytosis. Complement proteins are highly expressed in neurons and glia, and selectively label unwanted synapses (Stevens et al. 2007). By complement C3 receptor (known as CD11b, Itgam and Mac-1) microglia is able to recognize activated C3 fragments that tag immature or even dysfunctional synapses, thus initiating phagocytosis (Ransohoff and Perry 2009). The chemokine pathway regulates microglia-synaptic pruning during brain circuit maturation and it is based on the fractalkine (CX3CL1) signaling, which is activated through the microglial chemokine receptor CX3CR1. By the binding to the neuronal chemokine this receptor participates in regulating the balance of excitatory/inhibitory synapses, as evidenced by the fact that *CXCR1* KO mice are characterized by a reduction in microglia number leading to an excess of excitatory synapses and spine density (Paolicelli et al. 2011). On the other hand, the enhanced expression of the fractalkine by developing neurons acts as a potent chemoattractant for microglia, that is recruited to the developing synapses to control their maturation (Hoshiko et al. 2012). The role of microglia in participating in the shaping of neuronal mature network has been demonstrated to rely also on neuronal activity and to persist in the adulthood (Tremblay et al. 2010), suggesting a role both in physiological and pathological conditions. In fact, altered synaptic pruning, as it occurs in certain disease conditions, results in microglia-mediated synapse loss (Hong et al. 2016). Immune-electron microscopy (EM) has revealed that almost all microglial processes (94%) directly contact synaptic elements in the visual cortex of adolescent mice, in physiological conditions (Tremblay et al. 2010) but also throughout adulthood and normal aging (Tremblay et al. 2012). 3D reconstruction of EM serial sections shows that a single microglial process can contact several synaptic elements at multiple synapses simultaneously, sometimes with morphological specializations such as finger-like protrusions wrapping around dendritic spines and axon terminals (Fig. 9).

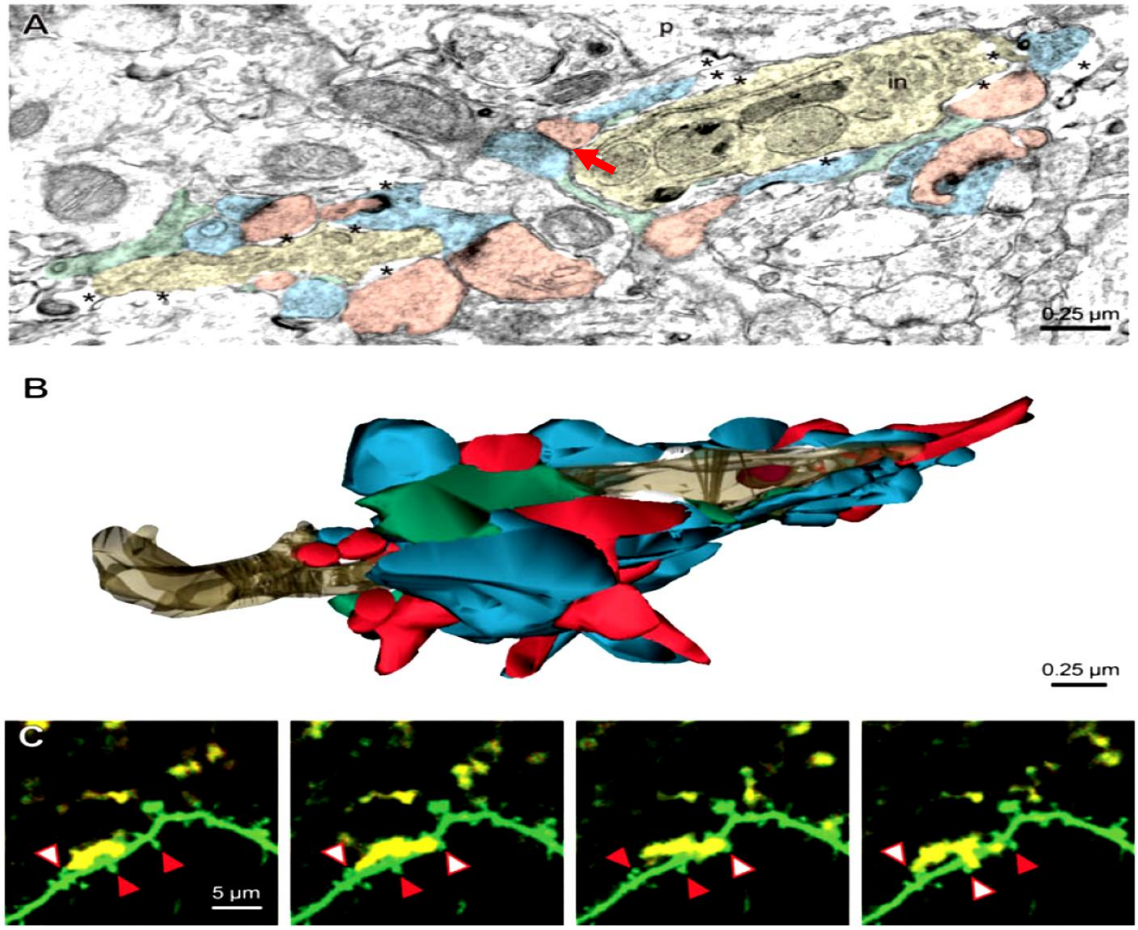


Figure 9. **Microglia - synapse interaction in the visual cortex of a healthy brain**. **A**. Electron micrograph from a series of sections shows a proximal microglial process (transverse section, *yellow*) with a distal protrusion (cut longitudinally) contiguous to a neuronal perikaryon (p), and making direct contacts with dendritic spines (*pink*), axon terminals (*blue*), synaptic cleft (*red arrow*), and peri-synaptic astrocytic processes (*green*) at postnatal day 28. *Asterisks:* extracellular space pockets surrounding the microglia; *in*: inclusions **B.** Serial section 3D reconstruction of the microglial process and protrusion shown in **A**, contacting axon terminals (*blue*), dendritic spines (*red*), and peri-synaptic astrocytic processes (*green*) at multiple excitatory synapses. Extracellular space pockets (*white*) and a microglia phagocytic inclusion (*purple*) are also shown. **C**. Two-photon time-lapse micrographs showing a microglial process (*yellow*) interacting with three dendritic spines (*green*) over the course of 20 min in the visual cortex of a $CX3CR1^{GFP/-}$ /Thy1-YFP mouse. *Red arrowheads* indicate non-targeted dendritic spines, and *white arrowheads* the targeted ones (Tremblay et al. 2010).

Moreover, phagocytic inclusions, identified as axon terminals and dendritic spines, based on their ultrastructural features (synaptic vesicles, post-synaptic densities) or immunostained for specific markers, have also been found inside the cytoplasm and lysosomes of microglial cell bodies and processes, during postnatal development, adolescence, adulthood and normal aging, in mouse hippocampus, visual cortex and thalamus, and auditory cortex. Importantly, a study investigating plasticity in the visual system has evidenced that these microglial phagocytic structures become more evident during light deprivation, still persisting after re-exposure to light. This suggests a role in the activity-dependent remodeling of neuronal circuits in layer II/III of primary visual cortex during adaptation to a novel environment (Tremblay et al. 2010).

The mechanisms by which microglia is able to modulate cortical plasticity throughout the lifetime have been investigated also by Lim and colleagues. They found that microglia-mediated release of IL-10 was able to increase dendritic spine density (Lim et al. 2013). The anti-inflammatory cytokine appears to interact with IL-

10 receptors expressed on cultured primary hippocampal neurons of early developmental stage, thus increasing the number of excitatory and inhibitory synapses. Conversely, the IL-1β antagonized the effects of IL-10 when endogenously released from microglia or applied as recombinant proteins.

This unexpected role of CNS immune cells in the formation of synapses was also investigated by Parkhurst and colleagues (Parkhurst et al. 2014). In order to selectively manipulate gene function in microglia, researchers generated a CX3CR1^{CreER} mice expressing tamoxifen-inducible Cre recombinase fused to estrogen receptor (CreER) under the control of endogenous *CX3CR1* promoter. By using *CX$_3$CR1^{CreER}* to drive diphtheria toxin receptor expression in microglia, they found that microglia depletion - driven by diphtheria toxin administration - induced deficits in multiple learning tasks in mice and a significant reduction in motor learning-dependent synapse formation.

In order to specifically remove in microglia the brain-derived neurotrophic factor (BDNF) - a critical mediator of neuronal survival, differentiation and plasticity - they crossed a CX3CR1^{CreER} mice with mice containing a floxed allele of BDNF (*BDNF^{flox}*). Interestingly, the Cre-dependent removal of BDNF from microglia recapitulated the effects of microglia depletion, by decreasing the formation rate of dendritic spines and suggested the important physiological function of this neurotrophic factor on synaptic plasticity. Finally, microglia has been demonstrated to participate also in strengthening neuronal connections and long term potentiation (LTP) through Hebbian plasticity (Penn et al. 1998). All these results indicate that microglia can regulate synaptic plasticity and contribute to spinogenesis in healthy brain by physically interacting with neuronal cells, as well as releasing cytokines and neurotrophic factors whose receptors are expressed on early postnatal neurons.

2.4 Other microglia functions

Microglia contribute to synaptogenesis and synaptic plasticity in the early embryonic stages until the adulthood by serving as phagocytic cells. However, their phagocytic activity do not run out at the synapses. Infact microglia are known to actively phagocytose biological waste and a broad range of pathogens, including apoptotic bodies, cellular debris, and other exogenous particles, through different and specialized phagocytic pathways (Fu et al. 2014). For phagocytosis, for example, of apoptotic neurons it has been proposed a four-step model: apoptotic cells first release “find-me” signals (ATP, UTP, fractalkine, sphingosine-1-phosphate) attracting microglia, followed by “eat-me” activity carried on through receptor-mediated recognition of “eat me” signals (e.g. phosphatidylserine) expressed by targets, the “digest me” phase, resulting in degradation of internalized materials, and finally, the post-phagocytic phase, which is characterized by the release of anti-inflammatory cytokines and chemokines (Ravichandran 2010). The fractalkine signaling and the triggering receptor expressed on myeloid cells 2 (TREM2) has been also shown to mediate microglia phagocytosis of apoptotic cells, with the latter being involved also in microglia internalization of Aβ (Sierra et al. 2013; Yuan et al. 2016). Moreover, TLRs in complex with CD14 complex

have been associated to α–synuclein phagocytosis (Venezia et al. 2017). Importantly, it has been recently evidenced a role of microglia also in initiating cell death by phagocytosis of viable cells, a process known as "phagoptosis" (Brown and Neher 2012). These findings indicate that microglia phagocytosis may have protective but also detrimental effects, with profound implications in the pathogenesis of different neurodegenerative diseases. Another important function associated to microglia is its ability to act as antigen presenting-cell (APC) in response to immune-related insults (Gottfried-Blackmore et al. 2009). In inflammatory conditions and neurodegenerative diseases microglia upregulate the expression of MHC-II and travel to the meninges and the choroid plexus coming into contact with peripheral APCs, mediating the activation of the adaptive immune T-cells (Louveau et al. 2015).

The capacity of these cells to behave as APCs is strictly associated to their highly dynamic behaviour and chemotaxis, defined as the ability of a cell to move in response to chemical factors. The pathways involved in mediating chemotaxis are different and highly complex, depending on interaction between microglia-receptors and chemokine/cytokine released by cells, in both physiological and pathological conditions. Specifically, pathogen associated molecular patterns (PAMPs) expressed by microorganisms and danger associated molecular patterns (DAMPs) released by damaged cells, represent crucial factors in mediating microglia chemotaxis and cell activation in neuroinflammatory conditions. LPS is a PAMP that exerts its function by TLR4 and prompts microglia to release a variety of pro-inflammatory cytokines (Lu et al. 2008). NOD-like receptors (NLRs), purinergic receptors activated by DAMP molecules such as ATP, and NLRP3 can also mediate microglia activation and the production of inflammatory molecules (Koizumi et al. 2012; Kigerl et al. 2014). In addition to these receptors that enables microglia to sense the damage and activate, these cells express also neurotransmitter receptors, by which they sense, for example, glutamate released by neurons and participate in the modulation of synaptic transmission. Importantly, activation of microglial group III mGlu receptors (mGluR3) have been reported to protect neurons against microglial neurotoxicity, while stimulation of mGluR2 induced microglial TNFα release, which contributed to neuron apoptosis through neuronal TNF receptor 1 (TNFR1) and caspase -3 activation (Taylor et al. 2005). Moreover, activated microglia has been shown to release glutamate, which induces neurotoxicity and may contribute to pathogenesis of several neurodegenerative diseases (Takeuchi et al. 2006).

All the microglia functions described above underline the critical role exerted by these cells in driving brain development and contributing to homeostasis in the adulthood. However, in aging, which is the predominant risk factor for all neurodegenerative diseases, microglia undergo several morphological and functional changes that impair phagocytic activity, chemotaxis and inflammatory response. For example, in aged brain, microglia has been described to present a reduced cell soma volume, thinner and fewer distal branched processes containing spheroids (probably derived by myelin fragmentation within the major ones), a less homogeneous distribution and dynamic behaviour in the brain (Damani et al. 2010; Von Bernhardi et al. 2015; Safaiyan et al. 2016). All these traits are typically associated to the so-called "dystrophic microglia", a

phenotypic state that is normally present in elderly. Moreover, typical age-related changes, such as chronic increased levels of the pro-inflammatory markers IL-1β, IL-6, CD68, CD11b, TLRs and the decreased level in anti-inflammatory cytokines, such as IL-10 and IL-4 might strongly prime microglia and polarize the cells towards an highly reactive phenotype (Maher et al. 2004; Maher et al. 2005). Recently a new microglia state, referred to as "dark microglia" has been identified (Bisht et al. 2016). This phenotype - characterized by typical signs of oxidative stress, such as highly electron-dense cytoplasm and nucleoplasm and a pronounced remodeling of nuclear chromatin - is rarely present in steady-state conditions, but becomes abundant during chronic stress, aging, or in contexts of fractalkine signaling deficiency (CX3CR1 KO mice) and AD pathology (APP/PS1 mouse model). Dark microglia appear to be more active than the normal microglia encircling axon terminals and dendritic spines with their highly ramified and thin processes, indicating a putative role of this phenotype in the pathological remodeling of neuronal circuits. Overall, in the aged brain, microglial phenotype shift from homeostatic to pathological, potentially leading to harmful inflammatory responses and further promoting neurodegeneration, as it is typically observed in many neurodegenerative diseases, like AD.

2.5 Evidence for microglia involvement in LOAD

A recent network-based integrative analysis has shown that the immune/microglial gene network had the strongest association with AD neuropathology (Zhang et al. 2013). In the last 10 years, genome-wide association studies (GWASs) using single-nucleotide polymorphisms (SNPs), have identified over 20 genetic loci that strongly associate with AD risk (Lambert et al. 2013). A stunning feature of the identified AD risk genes is that the majority of them are selectively or preferentially expressed in microglia, with respect to other cell types in the brain (Srinivasan et al. 2016). As described before, E ε4 allele - which confers a threefold increased risk of developing AD with respect to the most common ε3 allele - has been shown to reduce the clearance of Aβ and to increase plaque deposition (Fleisher et al. 2013). Importantly, apoE is principally released by astrocytes and microglia in the brain. Although the precise mechanisms remain to be elucidated, this lipoprotein has been shown to facilitates preoteolytic degradation of soluble Aβ, both in microglia and in the extracellular milieu, through the action of enzymes like neprylisin (NEP) and insulin-degrading enzyme (IDE) (Q. Jiang et al. 2008).

A common variant in *SPI1* gene, associated with reduced gene expression and AD risk (Huang et al. 2017), encodes the PU.1 transcription factor that exerts a critical role in microglial development (Schulz et al. 2012). In addition to the common variants, rare genetic variants have been found by GWAS to be associated with AD. A mutation in *TREM2* (triggering receptor expressed in myeloid cells 2) gene - R47H in less than 0.5% of most populations - has been found to increase the risk of AD threefold (Guerreiro et al. 2013; Jonsson et al. 2013). This gene is highly expressed by microglial cells encoding a cell surface protein that takes part in the

regulation of microglia phagocytosis and inhibition of pro-inflammatory cytokine release (Paradowska-Gorycka and Jurkowska 2013). In particular, the variant is a loss-of-function mutation, thus reducing the ability of microglia to engulf Aβ and leading to systematic inflammation and neuronal death (Jay et al. 2017). Transmembrane glycoprotein cluster of differentiation 33 (*CD33*) has been also shown to strongly associate with AD risk (Bradshaw et al. 2013). The gene encodes a transmembrane protein which belongs to the sialic-acid-binding immunoglobulin-like lectins (SIGLECs) expressed by monocytes, macrophages and myeloid progenitor cells (Crocker et al. 2012) and plays a role in cell-cell interactions, cytokine release, modulation of endocytosis and possibly Aβ clearance (D. G. Walker et al. 2015). Two single-nucleotide polymorphisms (SNPs) – rs3865444 and rs38266656, have been suggested to be related to LOAD (Jiang et al. 2014). Regarding the rs3865444 SNP, the allele rs3865444A was thought to reduce AD risk while rs3865444C allele was demonstrated to increase AD risk (Bradshaw et al. 2013; Villegas-Llerena et al. 2016). In fact, the protective effect of the first allele is associated to an increased isoform of *CD33* lacking exon 2 that encodes the IgV domain. This domain mediates the interaction between SIGLECS family members and sialic acid, thus leading to the inhibition of phagocytosis (Villegas-Llerena et al. 2016). This explains the reduced amyloid plaque burdens found in the brain of patient bringing this allele compared to the subjects with rs3865444C allele.

Many other genes that are preferentially expressed by microglia have been associated to increased AD risk such as *INPP5D*, *MS4A6A* and *PLCG2*, underlining the key role of neuroinflammation as a driving factor for AD (Hansen et al. 2018).

In addition to the aforementioned genetic studies, epidemiological evidence supports the role of inflammation in AD. Several immune molecules, including complement factors, eicosanoids, chemokines and pro-inflammatory cytokines, have been found to be upregulated in AD animal models as well as in the brain and cerebrospinal fluid (CSF) of AD patients (Heppner et al. 2015). Although the *Amyloid cascade hypothesis* states that immune-system activation would follow Aβ deposition, correlative analysis of clinical symptoms in MCI patients and inflammatory changes (in CSF), have indicated a much earlier involvement of the immune system (Tarkowski et al. 2003), suggesting a putative role of neuroinflammation in driving, rather following, the pathogenic cascade. The early changes of inflammatory markers and microglia activation have been investigated as predictive biomarkers of dementia by exploiting imaging techniques. In particular, [^{11}C]DAA1106 and [^{11}C]PK11195 ligands, that bind the peripheral benzodiazepine receptor expressed on activated microglial cells, have assumed a prognostic role by detecting early microglia activation before the onset of clinical symptoms of dementia. This has helped in the identification of patients affected by MCI who will probably develop full AD in a certain period of time (Cagnin et al. 2001; Yasuno et al. 2012).

On the other hand, although prolonged treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to reduce the risk of developing AD (in't Veld et al. 2001), randomised trials with NSAIDs seemed to be not completely successful, probably due to the need of the adequate pharmacological treatment

according to the specific stage of the disease. All these studies have redefined the role of neuroinflammation as an active contributor in AD showing that the immune system has the capacity to facilitate or trigger the pathophysiology of AD.

2.6 The role of microglia in Alzheimer's Disease

In AD, the balance between Aβ production and removal seems to determine the amyloid burden (Bradley et al. 2018). Importantly, a dysregulated Aβ clearance rather than Aβ production has been linked to the pathogenesis of sporadic form of AD (Mawuenyega et al. 2010).

Since microglia represent the principal phagocytic component of the CNS, it is primarily responsible for the phagocytic clearance of Aβ, or its local degradation achieved by the release of Aβ degrading enzymes (Heneka 2017). In fact, in AD, microglia mount an acute immune response against Aβ deposits. Specifically, pattern recognition receptors (PRRs) present on the surface of microglial cells recognize and bind both PAMPs and DAMPs, such as Aβ. Moreover, TLR2 and TLR4, receptor for advanced end glycation products (RAGE), scavenger receptors have been reported to mediate microglia interaction to Aβ. Importantly, these receptors are upregulated in AD-microglia and mediate the pro-inflammatory effects of Aβ (Arancio et al. 2004). Increased microglia proliferation is another factor that has been often described in the brains of AD patients (Gomez-Nicola et al. 2013) as well as several murine AD models (Kamphuis et al. 2012). In a mouse model of AD, the increase in microglia number has been correlated with the disease severity (Olmos-Alonso et al. 2016). In normal brain microglia constantly extend and retract processes to scan the brain environment (Nimmerjahn et al. 2005). However, in the presence of Aβ deposits, microglia processes may become static, showing a stable association with plaques over days or weeks. The clustering of microglia around the plaques is mediated by the immune cell modulator TREM2 and its obligate intracellular adaptor DAP12 (Yeh et al. 2016). However, the function of this highly proliferative microglia and reactive microglia around the plaques is not clear. Some studies suggest that microglia form a protective barrier around Aβ deposits compacting amyloid fibrils into a tightly packed and potentially less toxic form, counteracting the outward plaque expansion, by preventing the seeding of new Aβ onto existing plaques and reducing neuritic dystrophy (Condello et al. 2015). Since a halo of soluble, oligomeric Aβ (which are the more neurotoxic species) is likely present around amyloid plaques, this compaction of protofibrillary Aβ, that is mediated by TREM2 and apoE (Yuan et al. 2016), could be a protective mechanism that limits the damage of amyloid deposits (Fig. 10). These results indicate that, at least at an initial stage of the pathology, microglia can protect from the accumulation of Aβ species by their uptake, clearance, compaction and induction of an activated state.

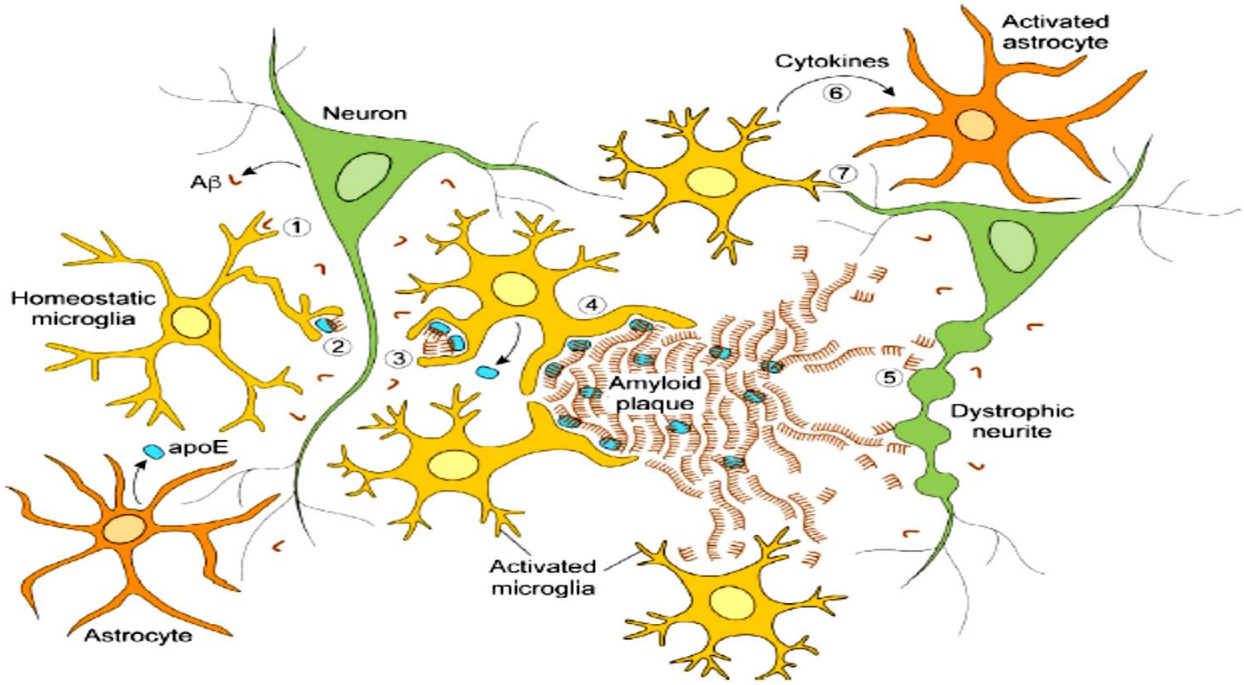


Figure 10. Depiction of microglial cellular activities related to β-amyloid pathology. On the left side protective microglial activities that limit the progression of the disease. Microglia may clear Aβ peptides via macropinocytosis of soluble Aβ [**1**; (Mandrekar et al. 2009)], uptake of lipoprotein-associated Aβ (**2**), or phagocytosis of fibrillar Aβ deposits (**3**). Microglia also help corral larger deposits of Aβ in plaques (**4**), minimizing damage to the adjacent neuropil. On the right side: the disease states when microglial containment mechanisms are defective or outstripped. Aβ fibrils on the outskirts of the plaque act as substrate for additional amyloid fibrillization and a reservoir of toxic Aβ species that induce neuritic dystrophy (**5**). Microglia can secrete factors that activate astrocytes (**6**) and participate in amyloid-dependent synapse loss (**7**) (Hansen et al. 2017).

As in normal aging, microglia in AD experience a specific change in phenotype that may compromise their protective functions. In fact, human microglia in aged brains exhibit a dystrophic and fragmented morphology, further suggesting that AD develops in a context of reduced neuroprotective microglial function (Streit et al. 2009). By using a gene expression profile, a recent study has defined a new microglia phenotype – beyond the M1 and M2 states - called "disease-associated" microglia (MGnD) (Fig. 11). These microglia cells are the result of chronic exposure to disease pathology and revealed a reduction in the expression of 68 homeostatic microglial genes and up-regulation of 28 inflammatory molecules (Krasemann et al. 2017). Specifically, *APOE*, *TREM2* genes were upregulated in the presence of the plaques. A strong increase in miR-155 is also observed in MGnD. This miRNA is typically upregulated in different conditions of brain homeostasis disruption and has been demonstrated to prompt the release of pro-inflammatory cytokines IL-6, IL-1β, iNOS and TNFα (Woodbury et al. 2015).

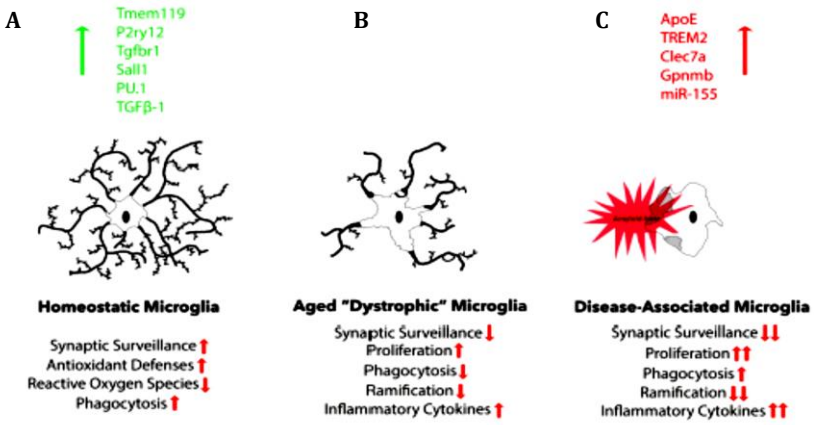


Figure 11. Schematic representation of microglia phenotypes. **A**. Resting microglia are found in the adult brain under non-infectious, non-diseased, and non-aged conditions, exhibiting robust expression of homeostatic microglial markers: Tmem119, P2ry12, TGF-βR1, and transcription factor Sall1. **B**. During normal aging, homeostatic markers gradually undergo downregulation, resulting in reduced proliferation, phagocytosis, dendritic process branching and cytokine secretion. **C**. Microglia phenotype that is associated with neurodegeneration and characterized by a more exacerbated dystrophic phenotype. MGnD is specifically associated with plaques and dystrophic neurites that cause neurodegeneration (Clayton et al. 2017).

An unbridled microglia activity has been demonstrated to contribute to the pathogenesis of AD. Although microglia activation is a necessary process in response to harmful stimuli, chronic activation – that can arise from excessive neuronal or immune-related damage - diverts their physiological and beneficial functions. This can lead to a prolonged release of pro-inflammatory cytokines and ROS, which, in turn, exacerbate the primary damage (Fig. 12). In fact, a moderate increase in inflammatory mediators is generally considerate as part of the physiological brain aging, but large increases, as observed in AD, lead to neurotoxicity (Glass et al. 2010). In turn, neurotoxicity leads to further microglia activation, generating a harmful loop of inflammation-neuronal damage termed "reactive microgliosis", which compromise the physiological functions of microglia.

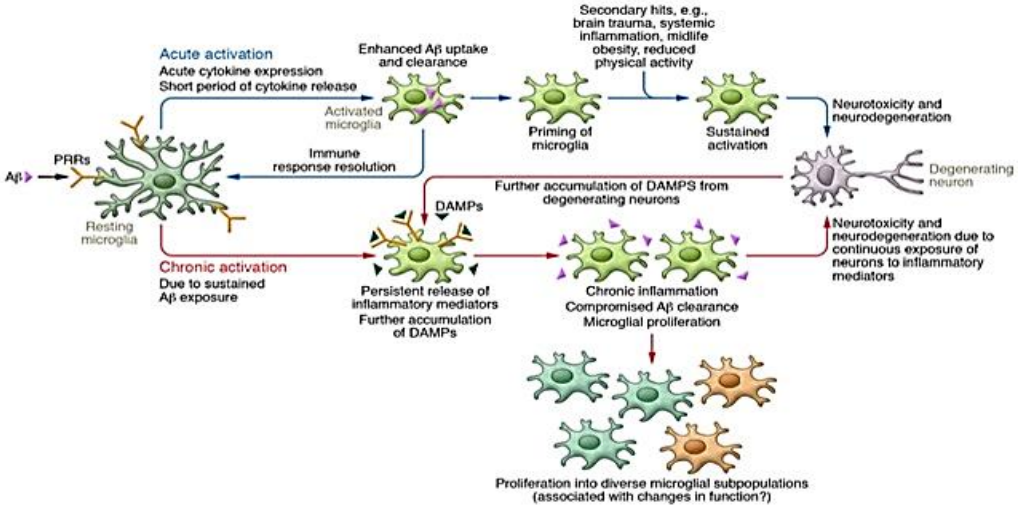


Figure 12. Acute and chronic activated microglia in AD. Aβ binds to PRRs, leading to activation of resting microglia. Acutely activated microglia produce cytokines, which drive enhanced phagocytosis, uptake, and clearance of Aβ. Long-term activation of microglia drives proliferation as well as a chronic inflammatory state that causes neurotoxicity and neurodegeneration. Sustained activation of microglia, induced by secondary hits such as brain trauma or systemic inflammation, exacerbate neurotoxicity and neurodegeneration. DAMPs that arise from these processes further activate microglia, thus compromising Aβ phagocytosis and propagating chronic inflammation (Sarlus and Heneka 2017).

In this context, characterized by sustained high levels of inflammation, the regulation of glial cell activation is impaired (Ramírez et al. 2008), as it occurs normally in aged brain. This enhanced activation under chronic inflammatory conditions could result in amplified neurotoxicity making the neuroinflammatory state a promoting factor for the development of neurodegenerative disorders, such as AD (Von Bernhardi et al. 2010). An example of how age-related changes may predispose to the development of AD is provided by TGF-β1-Smad3 signaling. This pathway mediates the regulatory effect of TGF-β1 that is largely secreted by astrocytes in response to inflammatory conditions and limits the temporal and spatial extent of neuroinflammation by regulating microglia activation, reducing the release of inflammatory cytokines and reactive species (Herrera-Molina et al. 2013). In AD patients and transgenic mice models of AD, TGF-β levels are increased in both CSF and plasma, but Smad3 signaling is reduced (Tesseur et al. 2006), likely due to age and inflammation driven altered expression of Smad3 protein (Tichauer et al. 2014). In fact, while 2 month-old mice show a robust increase of Smad3 in the hippocampus after a systemic inflammatory stimulus, 12-month-old animals maintain the amount of the protein at the same increased levels. Moreover, the phosphorylation (activation) of Smad3 is not induced by inflammation in old animals (Tichauer et al. 2014). This may be due to the fact that activation of Smad pathway in young animals is responsive to inflammation-driven TGF-β1 elevation, while in old animals the physiological increased basal levels of TGF-β1 - probably induced by chronic inflammatory status characterizing aging - maintain the amount of Smad3 protein and its phosphorylated/activated form (Smad3p) at such elevated levels that no further increase can be induced by a new inflammatory stimulus. Therefore, the inhibition on the activation of Smad, as well as other inflammatory pathways, alters the TGF-β1 regulatory effect on microglia cells, likely resulting in their pathological activation and potentially compromising their several functions. The fact that aged microglia possess decreased ability to phagocytose Aβ in comparison to young microglia (Floden and Combs 2011) may underline alterations in these regulatory pathways.

Dysregulated neuron-microglia communication - such as that observed in AD transgenic mice deficient for CX3CR31 - has been implicated in AD, by affecting microglia accumulation, clustering and phagocytosis (Lee et al. 2010). Pro-inflammatory cytokines, such as TNF-α and IL-1β, which are strongly released upon PAMP or DAMP binding to PRRs, have been reported to compromise microglia Aβ phagocytosis (Wang et al. 2015) as well as directly contributing to neuronal dysfunction by altering synaptic proteins (Rao et al. 2012). Recently, inflammatory factors released by classically activated microglia have been shown to contribute to neuronal demise also by inducing A1 reactive astrocytes (“A1” and “A2” astrocyte respectively in analogy to the “M1”/“M2” macrophage nomenclature) that in turn help to drive neuronal death through release of toxic molecules, reduced trophic support and clearance capacity (Liddelow et al. 2017).

Finally, microglia has been implicated in the progression of tauopathy. Tau protein has been shown to spread from the entorhinal cortex to the hippocampal region early in AD. Recently it was found that microglia spread

tau via exosomes secretion and the inhibition of exosome synthesis was able to reduce tau propagation both *in vitro* and *in vivo* (Asai et al. 2015).

These results suggest that in AD context microglia undergo different changes in morphology, markers expression, and signaling pathways polarizing towards a pro-inflammatory phenotype. Any therapeutic approach aiming at modulating microglia response in AD has to be taken with caution. TNF receptor deficiency in 3xTg-AD mice was associated with increased amyloid deposition and enhanced tau phosphorylation (Montgomery et al. 2011), while the inhibition of pro-inflammatory response by knocking-out NLRP3 inflammasome, IL-12 and IL-23 was shown to reduce amyloid pathology in APP/PS1 AD mice (Vom Berg et al. 2012). In another study the overexpression of the anti-inflammatory cytokine IL-10 in APP mice (TgCRND8) increased the APOE expression and exacerbated memory impairment (Chakrabarty et al. 2015). *In vivo* imaging shows that microglia activation precedes the onset of AD (Hamelin et al. 2016) and correlate with increased levels of inflammatory molecules in CSF (Brosseron et al. 2014), indicating that inflammation occurs early in AD pathogenesis. In patients with MCI, reduction in microglia activation was observed after a 2-year follow-up, while in AD these cells progressively increased activation (Z. Fan et al. 2017). Specifically, the presence of activated microglia positively correlated with the grey matter volume and cognition in MCI patients and glucose hypometabolism in AD patients, suggesting in the latter case an altered ability to face neuron energy demand (Hamelin et al. 2016). These results suggest a bimodal distribution and function of microglia activation in AD, with a peak that assumes a protective role at the prodromal phase and a peak that is characterized by a pro-inflammatory phenotype mediating synapse loss at the clinical stage.

Finally, the double-edged sword of microglial function in AD complicate the therapeutic approaches aiming at targeting these cells, as stimulation of microglia activity may be helpful at an early stage, but detrimental later, when inflammatory processes have become consistent. Understanding of the phenotypic state responsible for mediating neuroinflammatory damage would be of great relevance for driving potential therapeutic interventions that target deleterious activities while leaving the beneficial ones unhindered. Consequently, any immunomodulatory therapy aiming at dampening inflammatory process will have to take into account both the precise time window of the start of the therapy and the disease phase.

2.7 Leech CNS: a valuable model to study microglia

As described before, microglia are regulators of tissue homeostasis in the developing and adult CNS, rapidly responding to alterations in the brain and orchestrating the innate immune response. Microglia activation is a highly regulate process by which these cells assume a variety of different phenotypes which are capable of eliciting a wide range of different responses that may finally promote regeneration/repair or exacerbate the primary damage. The fact that, under certain circumstances, vertebrate CNS is supported by two different

macrophage populations, that are resident microglia and CNS-infiltrating macrophages, furtherly increases the complexity of microglia research (Le Marrec-Croq et al. 2013).

In this scenario, the leech represents a valuable model for the study of microglia role in neuroinflammation and regeneration, thanks to the simple and tightly defined structure of its CNS: it is located in the ventral blood sinus and is enveloped in the outer capsule that is covered outside by a visceral layer of the endothelium.

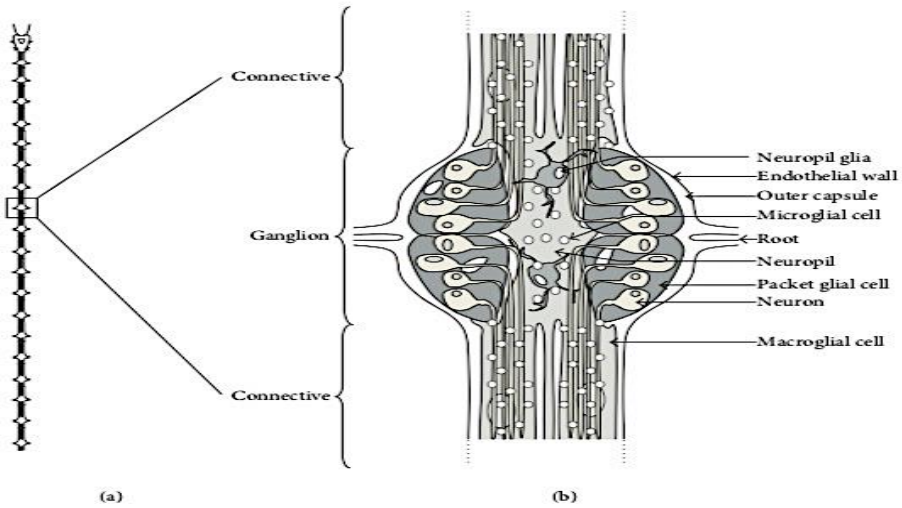


Figure 13. Leech CNS structure. **a**. Leech CNS contains a head ganglion, 21 body ganglia, and 7 fused tail ganglia joined by connectives. **a**. The dorsal view of the ganglion presents packet glial cells that envelope neuron cell bodies and two giant glial cells (neuropil glia). The axonal processes passing through the neuropil are prolonged into connectives where they are enveloped by two connective glial cells (macroglial cells). Thousands of microglial cells are distributed in ganglia and connectives. The CNS is enclosed in the outer capsule which is covered on the outside by a visceral layer of the endothelium (lining the ventral blood sinus) (Modified by Tahtouh et al. 2009).

Leech CNS is composed by head ganglion, 21 body ganglia and 7 fused tail ganglia (Fig. 13), which are tied together by connectives, consisting of two lateral bundles of nerve fibers (containing several thousand axons) and a thin medial one (containing about 100 axons). Each segmental ganglion consists of about 400 neurons connected to the others through thousands of axons (connectives). Other cell types are found in leech CNS: two connective glial cells (macroglial cells) that envelope the axons, two giant glial cell whose cell bodies are located in the neuropil (neuropil glia) and six packet glial cells, that enclose the neuron cell bodies. Thousands of microglial cells are present in ganglia and connectives (Fig. 13b). The simple structure of the nervous system in leech has simplified the study of regeneration, as these animals are able to restore the complete function of CNS after injury. Particularly, since specific depletion of both connective ensheathing glial cells was not shown to affect damaged axon reconnection to their target, studies started to focus on the role of microglia to explain this efficient regeneration (Elliot and Muller 1982).

Although phylogenetically different, the leech microglial cells are very similar to mammalian counterparts. Indeed, after injury, leech microglia have been shown to increase in number, express cytokines, change morphology, shift from resting to activated state upon different stimuli (cell plasticity), exhibit phagocytic activity and migrate at the lesion site. Here, the cells can interact with neurons, thus allowing neurite

outgrowth and axon sprouting (Le Marrec-Croq et al. 2013). Importantly, neither astrocytes nor oligodendrocytes are found in leech CNS (the other glial cells are extremely less represented than in the vertebrate CNS). Moreover, the absence of blood vessels within CNS as well as the very low level of infiltration of blood cells that was observed in injured CNS - even surrounded by the blood sinus - make the leech an ideal animal model to specifically study microglia, without the contribution of any other "contaminating" cells.

Since microglia migration to the lesion site was discovered to be a critical process for regeneration in leech, several studies have been focused on the role of signals and molecular processes necessary for this recruitment. Importantly, these studies have contributed to reveal the similarities between mammalians and leech, as evidenced by the interaction of leech immune molecules with human immune cells, and vice versa. As in vertebrates, ATP and NO were described as potent chemotactic factors for microglia in leech (Yuanli et al. 2010). A molecule homologous to the human complex p43/endothelial monocyte-activating polypeptide II (EMAPII) (Schikorski et al. 2009), has been reported to exert a chemotactic effect on both leech and human microglia. Noteworthy, since this molecule is strongly upregulated in activated microglia of injured brain, it may be involved in inflammatory and neurodegenerative diseases (Mueller et al. 2003). Functional similarities have been observed also between human IL-16, a well-known pro-inflammatory cytokine in mammalian, and leech *Hm*IL-16, which is rapidly induced in neurons after a lesion and promote the recruitment of microglial cells to the lesioned axons (Croq et al. 2010). Both these molecules represent potent chemotactic stimuli for leech microglial cells. *Hm*C1q is another molecule with human counterpart: its vertebrate homologous is C1q, a molecule that is part of the complement system and has been described to be involved in different human neurodegenerative diseases, such as AD (Bergamaschini et al. 2001). Meaningfully, in chemotaxis assays, recombinant human C1q was demonstrated to induce leech microglial cell chemotaxis (Tahtouh et al. 2012). In addition to these chemotactic molecules, Drago and colleagues (Drago et al. 2014) recently discovered that leech microglia express a specific activation marker homologous to vertebrate Iba-1, also known as Allograft Inflammatory Factor 1 (AIF-1). Through immunochemistry and gene expression analysis, the authors found that *Hm*Iba1, like its vertebrate counterpart, was constitutively express in leech CNS and was upregulated by mechanical injury or chemical stimuli (ATP).

To conclude, although there are evident differences between human and invertebrate models, all these studies aiming at characterizing microglia in leech CNS, showed an intriguing functional convergence with mammalian cells. Moreover, the absence of other main glial cells (astrocytes and oligodendrocytes) and infiltrating macrophages makes the resident microglia the only player orchestrating inflammatory response. Taking into account these considerations, it becomes evident how this simple animal model may represent a powerful tool to dissect out the role of resident microglia in inflammatory and healing events.

2.8 Drug treatment for Alzheimer's Disease

There is currently no cure for AD. Treatments under research include compounds that act on the pathological hallmarks of the disease: extracellular Aβ plaques, intracellular NFTs, inflammation and oxidative stress. Most of the drugs currently available for the treatment of AD merely alleviate the symptoms of the disease and slow down its progression. The treatment is linked to the evidence for both *cholinergic* and *glutamatergic* hypothesis in the etiology of AD today. According to the *cholinergic* hypothesis Acetylcholine (Ach) neurotransmission in the basal forebrain are affected early in the disease process, including loss of Ach neurons, loss of enzymatic function for Ach synthesis and degradation, resulting in memory loss and deterioration of other cognitive and non-cognitive functions such as neuropsychiatric symptoms (Bartus et al. 1982). In order to enhance the cholinergic transmission by slowing down the degradation of Ach in the synaptic cleft, three approved cholinesterase (AChe) inhibitors are commonly used for the treatment of mild to moderate AD (Birks 2006): *donepezil* (Pfizer, New York, NY, USA), *rivastigmine (*Novartis, Basel, Switzerland) and *galantamine* (Janssen, Beerse, Belgium).

According to the "*glutamatergic hypothesis*", the cognitive decline in AD patients is related to neuronal damage resulting from the destructive chain of events arised from exaggerated calcium release within the following persistent overactivation of NMDA receptor. Therefore, a further therapeutic option for moderate to severe AD is *memantine* (Lundbeck, Valby, Denmark) which is an uncompetitive, moderate-affinity N-methyl-D-aspartate (NMDA) antagonist believed to protect neurons from excitotoxicity. Studies on parallel groups of patients with moderate to severe AD showed that the combined therapy of memantine and donepezil had significant beneficial effects on cognitive functions, language, behaviours and global state over the placebo group (memantine and placebo) (Howard et al. 2012). However, such benefit was not demonstrated in patients with mild to moderate AD.

In light of this evidence, it becomes clear how a pressing need to develop a disease-modifying treatment is required, in order to directly tackle the pathogenic process of AD, by slowing down the disease progression, but also restoring the functions of damaged brain.

3. Stem cells

The history of stem cell research began in the mid 1800' with the discovery that the cells were basically the building blocks of life and that some cells had the ability to produce new ones. James Thomson and colleagues derived for the first time a human embryonic stem cell line at the University of Wisconsin-Madison in 1998 (Thomson et al. 1998), almost two hundred years later.

Stem cells are defined as unspecialized cells that are capable of self-renewal through replication (symmetric division), as well as commitment into specific cell lineages that make up the different tissue types (asymmetric division).

Based on the source they are classified in:

- *Embryonic stem cells* (ESCs), which derived from the embryos at a developmental stage before the time that implantation would normally occur in the uterus (pre-implantation blastocyst). These stem cells were first isolated from the inner cell mass of the blastocyst-stage embryo (Evans and Kaufman 1981).
- *Extra-embryonic Stem Cells*, which give rise to extra embryonic tissues, such as placenta, umbilical cord, amnion;
- *Adult stem cells* (ASCs), that are postnatal derivatives of ESCs located throughout the body. These cells maintain the co-expression of at least three of the four transcription factors typical of ESCs (OCT4, KLF4, and SOX2) and show high expression of ATP binding cassette (ABC) transporters and alkaline phosphatase. However, it is not yet known how many markers are common to all ASCs or present organ specificity.
- *Induced pluripotent stem cells* (IPSCs), which were generated by the introduction of only four embryonic transcription factors (Oct4, Sox2, Klf4, and c-Myc) into a mouse somatic cell (fibroblast). These pluripotency factors were able to *reprogram* mouse fibroblast to an embryonic stem cell-like by inducing the expression of genes critical for maintaining the properties of ESCs (Takahashi and Yamanaka 2006).

Based on the potency stem cells can be categorize in:

- *Totipotent Stem Cells*: generate all lineages of the organism, including extraembryonic cell types (e.g. zygote and the first division-derived blastomeres up to the stage of the morula);
- *Pluripotent Cells*: generate all body cells including germ cells, e.g. Embryonic Stem Cells (ESCs), induced pluripotent stem cells (iPSCs).
- *Multipotent Cells*: generate all tissue cells, e.g. adult stem cells (ASCs), such as Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs);
- *Unipotent Cells*: generate a single cell type, which belongs to the tissue from which it originates, e.g. Spermatogonial Stem Cell (SPCs).

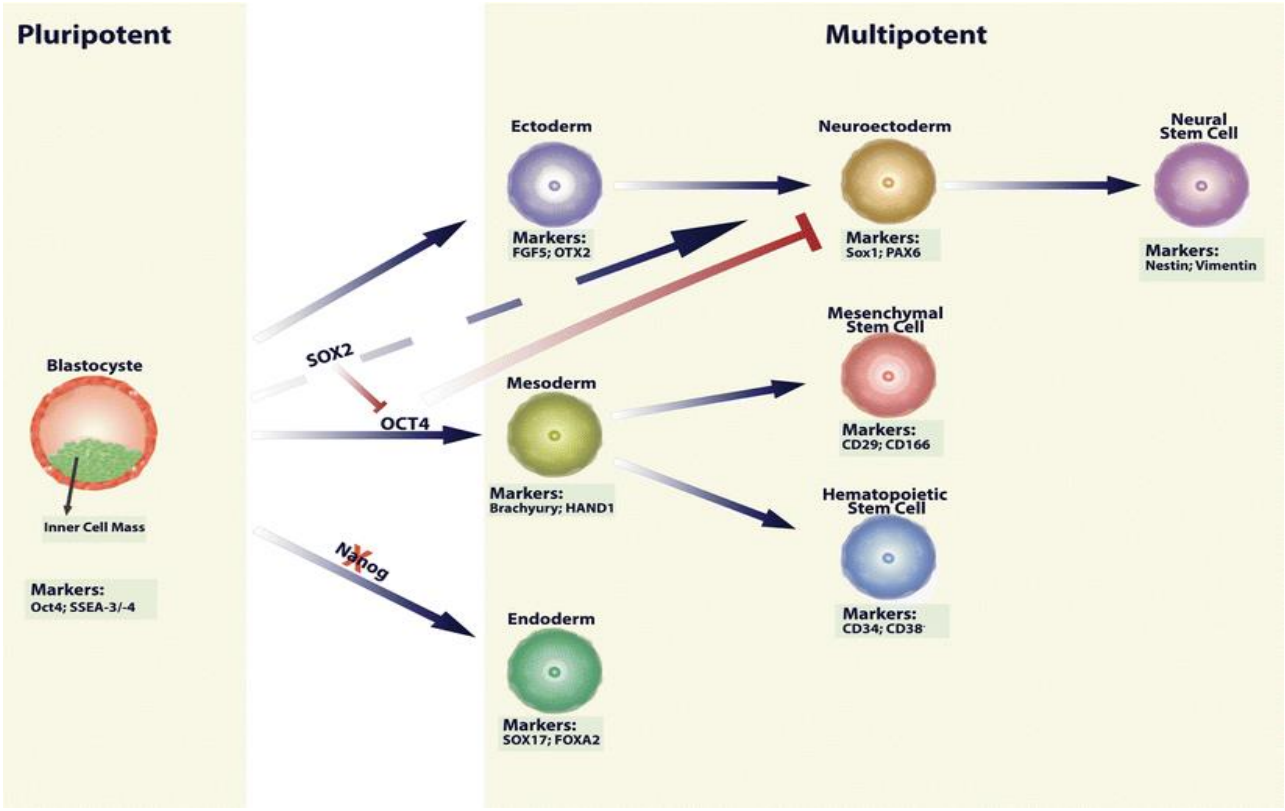


Figure 14. **Progeny of tissue-specific stem cells from ESCs**. ESCs of inner cell mass of the blastocyst, characterized by pluripotency marker expression, differentiate into three germ layers. Differentiation into neuroectoderm giving progeny to neural stem cells (NSCs) depends on increased SOX2 expression and inhibition of OCT4 expression. When OCT4 expression is upregulated, differentiation into mesodermal lineages occurs. Complete suppression of Nanog expression is required for differentiation into endodermal tissue-specific stem cells (Ulrich et al. 2015).

3.1 Stem cell niche

Stem cells typically reside in the stem cell niche, an *in vivo* highly dynamic microenvironment in which they receive stimuli and release factors that determine their fate. These stimuli comprise cell-to-cell contact, cell-matrix interactions, as well as the release of molecules/vesicles that activate and/or switch off the expression of specific genes. Because of these interactions, stem cells are maintained in a quiescent state, induced to self-renewal or differentiate into specific cell lineages.

The first characterization of the niche components was carried on in the invertebrate model of *Drosophila Melanogaster* and *Caenorhabditis Elegans* gonads. The study of these simple systems has conducted to a more deep understanding of the more complex mammalian cytoarchitecture (Xie and Spradling 1998). In fact, both anatomical components and molecular pathways of the stem cell niche are extremely conserved among different species, even if their respective functions and roles might exhibit a few variations. Common niche components are associated with similar functions.

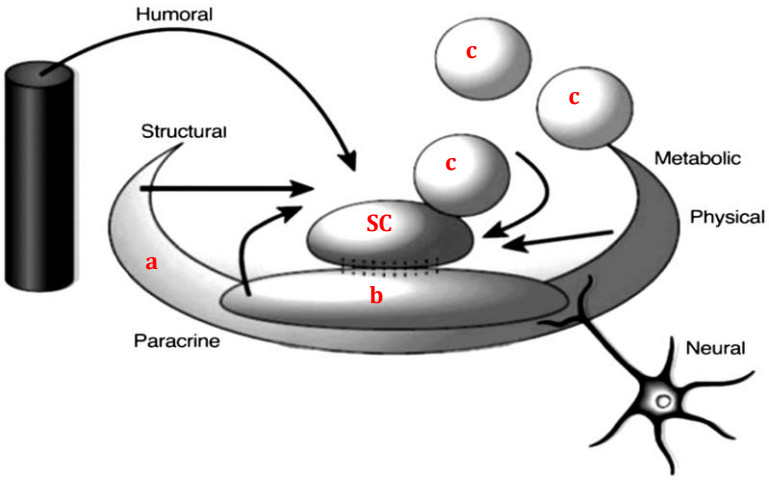


Figure 15. Schematic representation of a stem cell niche. Heterologous cell types (a, b, c), humoral, neuronal, paracrine, physical and metabolic cues interact with each other to regulate stem cell (SC) fate (adapted from Scadden 2006).

In the stem cell niche (Fig. 15) a strong interplay between resident stem cells and heterologous cell types - the niche cells - takes place.

Niche components are represented by:

- Stromal support cells, including cell-cell adhesion molecules and soluble factors, which are found near to stem cells;
- Extracellular matrix (ECM) proteins, which act as a mechanical scaffolding structures that anchor stem cells and transmit the cell signaling;
- Blood vessels, which carry different signals to the niche and take part in recruiting circulating stem cells from and to the niche;
- Neural inputs, by which signals from different tissues and organs are processed and integrated, thus stimulating the mobilization of stem cells.

Several niches have been identified in many mammalian tissues such as hematopoietic system, skin, gut, muscle and brain. For example, in the trabecular bone marrow, hematopoietic stem and progenitor cells (HSPs) situate along the endosteal surface in proximity to the osteoblastic cells (Calvi et al. 2003) and to the blood vessels (Kiel et al. 2005). In the muscle, satellite stem cells are placed along the muscle fiber bundles and the basal lamina may be considered the niche for these stem cells (Mauro 1961). In adult mammals, the neural stem cells (NSCs) are mainly confined in the “neurogenic” areas, represented by the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, where new granular neurons are generated, and the subventricular zone (SVZ) of the lateral ventricles. In SVZ new neuroblasts are continuously produced and migrate through the rostral migratory stream (RMS) to the olfactory bulb (Bellenchi et al. 2013). Interestingly, in SGZ and SVZ reside cells with characteristics and markers that are typical of embryonic radial glia

(Kriegstein and Alvarez-Buylla 2009). These cells undergo a differentiation process characterized by different cell types (*transient amplifying cell*, *neuroblast*, *immature neuron*, and, finally *mature neuron*) each characterized by stage-specific markers. The newly formed neurons - positive for the NeuN marker - integrate into existing circuits and are essential for specific brain functions.

Finally, each of these niches is characterized by ECM that acts as a scaffolding system in which stem cells, stromal cells and molecular cues are located. Importantly, the mechanical characteristic of ECM, such as the grade of stiffness and elasticity, contribute to drive stem cell fate by regulation of self-renewal or lineage commitment. Each perturbation of the niche environment may alter the highly dynamic equilibrium of the niche causing dysfunction in stem cells behaviour as it is seen in aging or neoplastic transformation (Conboy et al. 2005).

3.2 Adult stem cells and plasticity

Adult stem cells (ASCs) are multipotent stem cells derived from postnatal tissues.

The best characterized ASCs that are currently been used in several preclinical and clinical studies are:

- Hematopoietic Stem Cells (HSCs), that differentiate into hematopoietic cells;
- Mesenchymal Stem Cells (MSCs), that give rise to mesenchymal tissues;
- Neural Stem Cells, that are able to generate astrocytes, oligodendrocytes and neurons (Altman 1962);
- Epithelial and skin stem cells, giving rise to components of digestive tract and epidermis, respectively.

All these cell types possess specific properties that make them suitable for current regenerative medicine. First, their main function is the maintenance/repairing of adult body tissues by providing the cell turnover necessary to maintain cellular homeostasis and participate in tissue repair in response to traumatic events (Ulrich et al. 2014); this is why they possess a potential clinical relevance. Secondly, they can be largely expanded and manipulated *ex vivo* even if numerous numbers of passages make them undergo growth arrest process, known as replicative senescence, linked to a progressive shortening of telomeres (Lodi et al. 2011). ASCs are mainly present in tissues with high cellular turnover, such as bone marrow, muscle, adipose tissue, respiratory tract, gut, blood, epidermal system. These observations led to the hypothesis that a strong stem cell function could be found only in highly regenerative tissues. However, the majority of cells and tissues in the adult mammals exhibit a very low turnover under normal circumstances and organs once thought as non-renewing, like brain and hearth, have been shown to exhibit a very appreciable cell turnover (Altman and Das 1966; Kajstura et al. 1998). These observations paved the way for the study of their potential regenerative role to be exploited for the intervention in different pathological contexts.

Currently, cell therapy using ASCs is being investigated as a therapeutic tool for the treatment of many pathologies that are not necessarily related to lymphohematopoietic system, such as blood (all cell lineages),

gut, hearth, skeletal muscle, kidney, liver, lung, neurons/glia. The wide range of clinical settings in which ASC therapeutic potential is being investigated may be largely attributable to their plasticity.

Cell plasticity is the process by which ASCs, extracted by their natural niche, expanded *ex vivo* or not, and transplanted to another physiological environment, is able to produce cell lineages that are different from those that are genetically coded in its developmental programme. Different mechanisms have been implicated in this process, including *dedifferentiation*, *transdifferentiation*, and *reprogramming*. Cell transdifferentiation is one of the mechanisms by which stem cells potentially contribute to cell types of different lineages. The lineage conversion was proposed to occur directly – through the activation of a dormant differentiation program – or involve a dedifferentiation process of a tissue-committed cell and subsequent redifferentiation along a new cell lineage. These distinct mechanisms that drive stem cell behaviour might depend on cells intrinsic factors, paracrine signalling in the niche microenvironment, as well as "remote" signals such as hormonal effects, circadian effects, cytokines and neural regulation. In the hematopoietic system, for example, stem cells normally leave the bone marrow and enter the circulation before returning to their niche. This exit is under the control of the suprachiasmatic nucleus in the brain through the release of noradrenaline from nerve endings in the bone marrow. Here, the neurotransmitter induced the transcription of the chemockine CXCL12 that drive HSCs migration (Méndez-Ferrer et al. 2008).

Besides their role in cell compensation, ASCs, like other stem cells, are also able to release growth factors, cytokines, chemokines, micro-RNAs and extracellular vesicles. These entities - known as "secretome" - have been shown to control not only the homeostatic balance between self-duplication and differentiation but also the behaviour of different immune cells including macrophages, dendritic cells, neutrophils, NK cells, T and B cells. In fact, the cell secretome is strongly regulated by local inflammatory cues, highlighting its role in stem cell-based therapies.

To conclude, ASCs have evolved a high variable repertoire of behaviours in order to meet tissue-specific requirements. In particular, the ability to adapt their responsiveness to the dynamic changes in microenvironment makes these cells suitable also for restoring tissue homeostasis. This takes on a remarkable clinical relevance.

3.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are adult stem cells that were first described by Friedenstein and colleagues, who identified bone marrow-derived fibroblastoid clonogenic cells that supported haematopoiesis with multipotent differentiation capabilities (Friedenstein et al. 1968). These cells are self-renewable, multipotent and easily accessible with no ethical issues associated to their use. Importantly they can be expanded *in vitro* for many passages without accumulating genomic alterations. These characteristics make MSCs ideal candidates for cell-based therapy, regenerative medicine and tissue repair.

MSCs were officinally named more than 25 years ago (Caplan 1991) to indicate a class of cells that could be isolated and expanded in culture while maintaining their *in vitro* capacity to be committed towards a variety of mesodermal lineages and tissues. Caplan chose this term because the "mesenchyme", which derives from the Greek word "*meso*" meaning "middle", refers to the ability of mesenchymatous cells to migrate in early embryonic development between the ectodermal and endodermal layers. This migratory property underlie the MSC ability to reach damaged tissues and promote repair. The term "stem cell" was chosen in order to provocatively appeal to orthopaedic community, since these cells were capable of serial transplantation, unlimited doublings and lineage-specific differentiation (Fig.16).

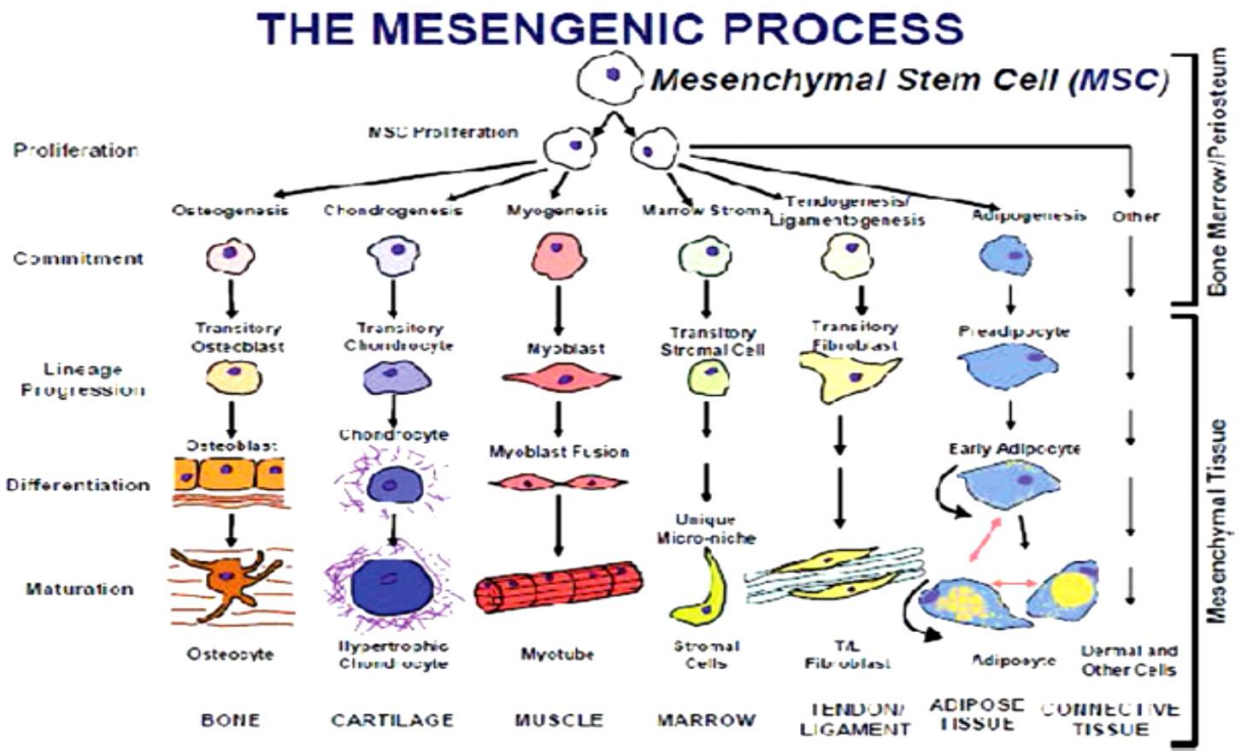


Figure 16. The mesengenic process. This hypothesis was originally verbalized in crude form in 1988 and refined as a figure in 1990, with its current format published in 1994. All of the proposed lineage pathways to bone, cartilage, muscle, etc., have been verified by Caplan A. and others using inductive cell culture conditions (Caplan 1994).

The stromal cell system - firstly described by Maureen Owen in 1985 (Owen 1985) - includes the hematopoietic stem cell (HSC) population and MSCs. The formers are located close to endosteum and generate blood and immune cells, while MSCs, sited around the vascular system, contribute to maintain a level of self-renewal and give rise to cells that are able to differentiate into various connective tissue lineages. A standard *in vitro* method for examining mesenchymal tissue potential is the colony-forming unit fibroblast (CFU-F) assay, in which cells obtained from bone marrow are plated at low density, expanded as an adherent population and quantified by scoring individual foci or colonies that were presumed to be derived from a single precursor. Using this assay, different groups estimated the number of MSCs in bone marrow to be

approximately to one in 10^4 to one in 10^5 marrow mononuclear cells. Moreover, taking advantage of *in vitro* assays for evaluating differentiation ability of individual loci, several groups demonstrated the existence of subpopulations within mesenchymal stromal cells (Castro-Malaspina et al. 2017), with some showing osteogenic potential only and others that maintained the ability to be committed also towards adipogenic and chondrogenic lineages.

3.3.1 Characterization of MSCs

Since the first description of hMSC from bone marrow, they have been isolated from almost all tissues such as adipose tissue, amniotic fluid, amniotic membrane, dental tissues, endometrium, limb bud, menstrual blood, peripheral blood, placenta and foetal membrane, salivary gland, skin and foreskin sub-amniotic umbilical cord lining membrane, synovial fluid, and Wharton's jelly (Ullah et al. 2015).

Still there is neither a single definition nor a quantitative assay to help in the identification of MSCs in mixed population of cells.

Although there are different protocols in terms of isolation, characterization and expansion, as well as source (BM-MSCs are considered the best cell source and taken as standard for the comparison of cells derived from other sources), all MSCs have to exhibit the three minimal requirements proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al. 2006):

- The ability to adhere to plastic surface when maintained in culture;
- The expression of specific set of surface markers. More than 95% of the MSC population must express CD73, CD90, CD105, as measured by flow cytometry. Genetically encoded by the NT5E gene, CD73 is used as a typical marker to characterize MSCs, although it is highly expressed onto the lymphocyte membranes (Resta et al. 1998) as well. CD73 is an ecto-5'-nucleotidase, that converts AMP substrates into adenosines. CD90 is the ultimate stemness marker; it is also known as Thy-1, being firstly described on membranes of thymocytes (Ades et al. 1980), the T-cell precursors in the thymus. It is considered one of the gold standard markers for the identification of MSCs and stem cells. CD105, also known as Endoglin, is a type I glycoprotein that identifies hematopoietic progenitor cells and belongs to the TGF-βreceptor complex, together with betaglycan, interacting with TGF-βIII and TGF-βI receptors (Barbara et al. 1999). It is involved in stem cell regulation of cell cycle and progression, as well as in other cellular pathways that involve TGFβ responses, such as cellular migration and localization (Guerrero-Esteo et al. 2002). In addition to these markers, human MSCs obtained from different tissues, have been reported to exhibit expression of CD29, CD44, CD146, CD140b, specific to the tissue of origin. Additionally, MSCs must lack expression of CD34 (marker for hematopoietic primitive progenitors and endothelial cells), CD45 (a pan leukocyte marker), CD14 or CD11b (markers that are prominently expressed on monocytes and macrophages), CD79αor CD19 (marker of B cells) and human leucocyte antigen-DR (HLA-DR), that

is not expressed by MSCs unless stimulated (e.g. by IFN-γ);

- The ability to differentiate into adipocytes, osteocytes and chondrocytes, confirmed by the production of oil droplet, formation of mineralized matrices and expression of type II collagen respectively.

The goal of these requirements is to encourage MSC investigators to adopt minimal universal criteria in an effort to standardize the cell preparations thus allowing a comparision (and reproducibility) of scientific results among laboratories.

Based on technical feasibility, promising curative effects, reduced economic costs, circumvention of ethical issues, as well as the possibility to be used for autologous transplantation, MSCs have become the principal cell-source in cell-based treatment. Although the great advantages over other stem cells, their clinical application might be hindered by some problems. Infact, although the ease of isolation and long-term expansion in respect to other multipotent stem cells (e.g. NSCs), these cells have been reported to lose their potency after reaching high passages due to aging. Infact, It has been reported that MSCs become senescent during long-term culture, as evidenced by decline in differentiation ability, shortening of the telomere length and morphological alterations (Bonab et al. 2006). The *in vitro* senescence of MSCs is related to the decreased activity of telomerase (Kassem 2004). Moreover, even if MSCs are less prone to develop genetic abnormalities, their *in vitro* culturing for a long time may result in an increase in the probability to undergo chromosomal alteration with consequent malignant transformation (Røsland et al. 2009).

3.3.2 Therapeutic properties of MSCs: mechanisms of action

The therapeutic effects of MSCs in various life-threatening human diseases, including cerebral spinal cord injury, haematological disorders, cardiovascular diseases, diabetes, immune diseases, graft versus host diseases (GvHDs), and cancer, are well documented (Kim and Cho 2013). Nonetheless, the precise mechanisms by which MSCs act remain a matter for debate and exploration. The generally putative concepts cover transdifferentiation, cell fusion, mithocondrial transfer and paracrine effects, such as the release of soluble factors and microvesicles/exosomes (Fig. 17).

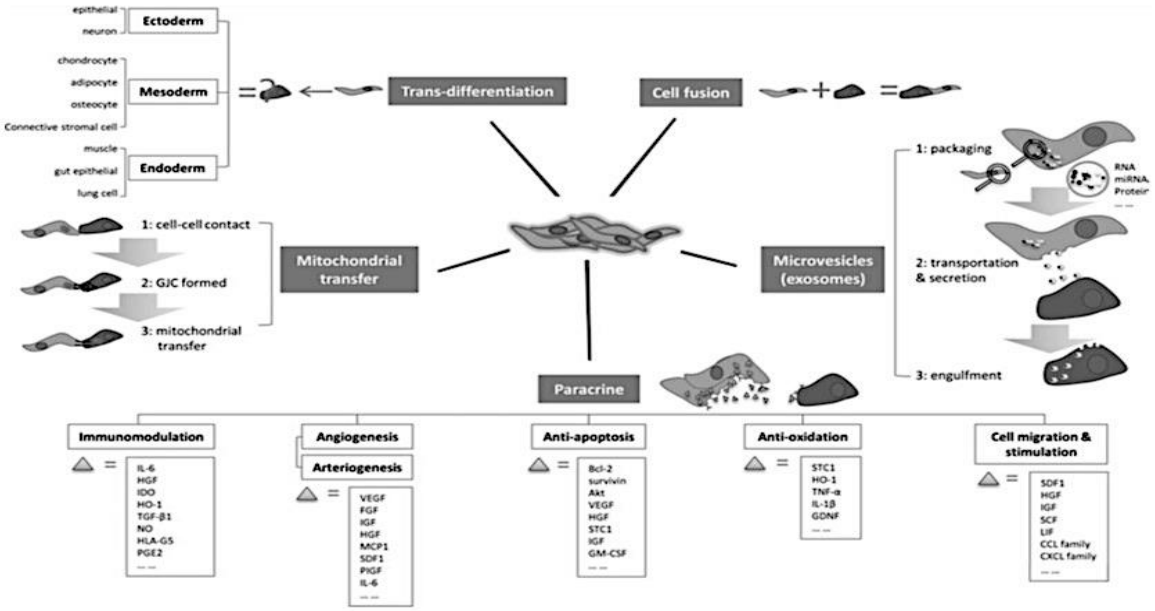


Figure 17. Action modes of MSCs. Trans-differentiation: MSCs possess the ability to differentiate into another cell types, from all germ layers - ectoderm, mesoderm and endoderm. Cell fusion: MSC fuse with another cell to form a multinuclear cell, known as syncytium. Mitochondrial transfer: MSC make contact with the adjacent cells and a gap junctional channel (GJC) is built. MSC transfer their mitochondria to the impaired cells through this GJC. Exosomes/Microvesicles (MVs): MSC release exosomes and MVs containing RNAs, microRNAs (miRNAs), lipids and proteins to the microenvironment. The cell nearby engulfs these extracellular vesicles through different mechanisms, including endocytosis. Paracrine: MSC secrete bioactive molecules that act on immunomodulation, angiogenesis/arteriogenesis, anti-apoptosis, anti-oxidation, and cell migration/stimulation (Liang et al. 2014).

Originally, MSC therapeutic properties were thought to be due to their engraftment in the injured organ, where they could directly lead to the regeneration process (Fig. 17). Despite the fact that MSCs are described as multipotent cells, *in vivo* studies have reported that these cells are endowed with a cross-lineage differentiation potentiality, known as *transdifferentiation* (Jiang et al. 2002; LaBarge and Blau 2002; Zhao et al. 2002), thus displaying a high plasticity. This enormously enlarges the field of their therapeutic action. Transdifferentiation is a cellular mechanism that allows cells to differentiate across lineages beyond their own classical commitment, thus acting as a progenitor cells for other lineages, such as ectoderm and endoderm. Both epigenetic modifications and the alterations of gene expression patterns, led by environment, can drive cells towards a new differentiation. In determining MSC therapeutic properties, the role of environment, in particular, becomes relevant, by virtue of the natural MSC property, referred to as *homing*, to migrate across the damaged tissue through the interaction between chemokines released by the injured tissue and chemokine receptors expressed on stem cells. Notably, the ability of MSCs to migrate to the site of injury is restricted to damaged sites: in fact, in non-injured context, the majority of intravenous injected MSCs tend to migrate to the bone marrow, while the remaining becomes entrapped in the lungs and liver or, to a lesser extent, in the capillary bed (Monsel et al. 2014). Such ability might account for the success of MSC engraftment that, then, might modulate *in loco* the pathological status, possibly by

transdifferentiation, although, up-to-date, this option is still debated in the field of regenerative medicine.

Relevant studies concerning the hepatic and pancreatic regeneration support the idea that another process, next to transdifferentiation, could occur: the *cell fusion* (Fig.17). BM-MSC cell fusion has been demonstrated after irradiation of the host or during ischemia *in vivo* (Yang et al. 2012). Actually, there are some protocols, by physical (electric pulses), chemical (polyethylene glycol, PEG) or biological methods (inactivated virus) that take advantage from this spontaneous event (Aurich et al. 2007; Azizi et al. 2016). *Mitochondrial transfer* to host cells is another mechanism by which MSCs try to regain the tissue homeostasis (Fig.17). With live imaging techniques, the authors observed that instilled MSCs attached to alveoli and transferred functional mitochondria by gap junctional channels (GJCs) to alveolar epithelium, as evidenced by increased ATP concentration in the recipient cells (Islam et al. 2012). However, this successful delivery also compromises the energetic balance of MSCs and, as for transdifferentiation and cell fusion, seems to occur at a very low frequency, thus the latter opportunities do not appear the best ones from which regenerative medicine may take advantage. In fact, many evidences suggest that *paracrine signaling* represents, at least as far as we currently know, the elective method to foster regenerative processes in chronic pathological contexts.

The secretion of cytoprotective factors by MSCs was reported for the first time by Gnecchi and colleagues (Gnecchi et al. 2005). The authors found that genetically modified MSCs overexpressing v-akt B (Akt-MSCs) could prevent ventricular remodeling reestablishing hearth function in less than 72 hours following surgical induction of myocardial infarction (MI) and cell transplantation. Given the extremely brief time, the authors raised the possibility that an action different from myogenic differentiation may be implicated in myocardial recovery. Moreover previous studies have pointed out that the limited frequency of the transplanted stem cell-derived cardiomyocytes (CMCs) was unlikely to be the major contributor that drove protection and repair after ischemic insult (Alvarez-Dolado et al. 2003; Kajstura et al. 2005; Laflamme et al. 2007). Therefore, a new mechanism of action was proposed in which transplanted MSCs might release a range of molecules that were responsible for the observed therapeutic effects. This hypothesis was then confirmed by the discovery that the injection of conditioned medium (CM) collected from hypoxic MSCs could restore the cardiac function. The *in vitro* experiments also demonstrated that the CM was able to protect CMCs against apoptosis after hypoxic challenge. Moreover, by comparing the composition of the CM collected from Akt-MSCs and control-MSCs, the authors observed an upregulation of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and thymosin β 4 (Tβ4) in medium of genetically modified cells. The paracrine mechanism of MSCs emerged as an original mechanism of action, exploited by these cells to restore tissue homeostasis. Subsequent studies have been carried on to characterize the specific composition of MSC-CM, by identifying the soluble factors responsible for the therapeutic effect in different pathological contexts.

Caplan himself proposed to change the name of MSCs to "*Medicinal Signaling Cells"* to more accurately reflect the fact that these cells home in on sites of injury or disease and secrete bioactive factors with immunomodulatory, trophic (regenerative) functions, meaning that these cells are able to produce therapeutic drugs that are medicinal. It is, indeed, the patient's own tissue-specific resident stem cells that construct the new tissue as stimulated by the bioactive factors secreted by the exogenously supplied MSCs (Caplan 2015).

Among the factors released/expressed by MSCs that have been reported to drive regeneration and repair can be mentioned:

- *angiogenic* and *arteriogenic* factors, such as VEGF, bFGF, HGF, TGF-β, Platelet-Derived Growth Factor (PDGF), Placental Growth Factor (PGF), IL-6, leading to increased vascular density and recovery of blood supply in damaged areas (Huang et al. 2009; Shabbir et al. 2009; Dong et al. 2012; Kwon et al. 2014);
- *antioxidant* factors, such as staniocalcin 1 (STC1), heme oxygenase-1 (HO-1), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), (Ohkouchi et al. 2012; Li et al. 2012; Inan et al. 2017) by which MSCs are able to prevent the accumulation of oxidative by-products referred to as ROS (reactive oxygen species), including oxygen ions, oxygen free radicals, and peroxides, that have been implicated in the pathogenic processes driving a variety of diseases, such as carcinogenesis, immune disorders, inflammation, neurodegeneration (Kahles et al. 2007; Kamata 2009; Fisher 2005; Lull and Block 2010).
- *antiapoptotic* factors, such as B-cell lymphoma, 2 (Bcl-2), survivin, and Akt (Okazaki et al. 2008; Wang et al. 2012). In addition to release anti-apoptotic factors, MSCs were reported to synthesize cytokines that either neutralize apoptotic pathway by reducing the expression of apoptotic proteins, such as Bax, caspase-3 (Pan et al. 2012) or enhance survival, such as VEGF that participate in the anti-apoptotic process through activation (by posphorylation) of focal adhesion kinase (FAK), an important prosurvival signal that acts by suppressing p53-mediated apoptosis (Ilić et al. 1998).
- *chemoattractant receptors*, that play a role in MSC *homing*, defined as the ability of these cells to sense specific signaling molecules and migrate to the sites of inflammation and tissue damage, which is typically associated with cytokine outburst (Sohni and Verfaillie 2013). Among these molecules it can be mentioned the chemokine receptor CXCR4 and its binding partner Stromal-Derived Factor 1 (SDF-1) as well as other chemokine receptors like CCR1, CCR3, CCR7, CCR10, CCR9, CXCR5, CXCR6, CXCR1, CXCR2, CXCR4, CCR1, CCR2 (Zhang et al. 2008; Honczarenko et al. 2009), receptors for growth factors such as PDGF, PGF as well as for Macrophage Inflammatory Protein 1α (MIP-1α), Monocyte Chemoattractant protein 1 (MCP-1) and IL-8 (Ringe et al. 2007; Boomsma and Geenen 2012; Cipriani et al. 2013; Park et al. 2014). Migration and homing require that cells can attach to and migrate

between endothelial cells to enter the target tissue. MSCs express a number of adhesion molecules, including selectins and integrins, involved in these steps such as VLA-4 (or integrin-beta1 and integrin-alpha4 dimer) expressed on MSCs and interacting with VCAM-1 found on endothelial cells (Rüster et al. 2006). In addition MSCs have been reported to secrete matrix metalloproteinase-2 (MMP2) and membrane type 1 metalloproteinase (MT1-MMP) that contribute to MSC transendothelial migration capacity by degradation of collagen IV - a major component of basement membrane - and activation of different molecules including pro-MMP2 molecules, respectively (Son et al. 2006; De Becker et al. 2007).

- *Immunomodulatory factors*, such as IL-6, HGF, TGF-β1, indoleamine 2,3-dyoxigenase (IDO), HO-1 heme-oxygenase 1 (HO-1), prostaglandin E2 (PGE2), have been reported to regulate the behaviour of immune cells in order to create an environment favourable to repair and regeneration. (Liang et al. 2014).

In addition to this plethora of soluble factors by which MSCs play an influential role in the regeneration of injured tissues in various diseases, these cells are known to release high amount of *Extracellular vesicles (EVs)* that have emerged as important contributor to MSC paracrine action and responsible for their therapeutic effect, largely mediated by their immunomodulatory activity, in a plenty of animal disease models (par.5).

3.3.3 Immunomodulatory properties of MSCs

MSC immunomodulatory functions were reported for the first time by Bartholomew and colleagues who observed a dose-dependent inhibitory effect on cell proliferative response of allogenic mitogen-stimulated lymphocytes in mixed lymphocyte culture (MLC) (Bartholomew et al. 2002). Later, Di Nicola and colleagues attributed the observed immunosuppressive effect to the release of soluble factors with no involvement in cell-cell communication, as evidenced by the persistence of the inhibitory effects on T-cells proliferation in a transwell system (Di Nicola et al. 2002). Several studies have shown that MSCs are endowed with potent anti-inflammatory activities and immuno-modulating properties over T-cell activation, proliferation, differentiation and effector function (Wan and Flavell 2009). Once the T cells are activated, they can perform their function by differentiating into different pro-inflammatory subpopulations, including T helper type 1 (Th1), Th2, Th17 cells, cytotoxic T lymphocytes (CTLs), or anti-inflammatory and immunoregulatory subtypes, such as T regulatory (Treg) cells.

The differentiation of T helper cells into effector cells depends largely on the cytokine milieu present at the time of antigen presentation and activation. Several studies have suggested that MSCs modulate the differentiation, function and balance of these subpopulations and foster the development of anti-inflammatory immune response, by increasing the expression of IL-10 as well as the number and the activity of Treg cells. These cells - characterized by the expression of IL-2 receptors on the surface and intercellular

transcription factor FOXP3 - play an important immunosuppressive role in the activation, differentiation of other Th cell subtypes through the release of soluble factors and cell-to-cell contact (Wan and Flavell 2009). Moreover, MSCs directly suppress the activity of Th1 - important for macrophage recruitment to sites of inflammation through release of TNF-α and IFN-γ (Wan and Flavell 2009) - and that of Th2, Th17 pro-inflammatory lymphocytic phenotypes as well as cytotoxic T lymphocyte (CTL)-associated cell lysis (only if contact between CTLs and MSCs occurs during the primary stimulation phase)(Rasmusson et al. 2003; Di Ianni et al. 2008; Gonzalez-Rey et al. 2010; Wang et al. 2012).

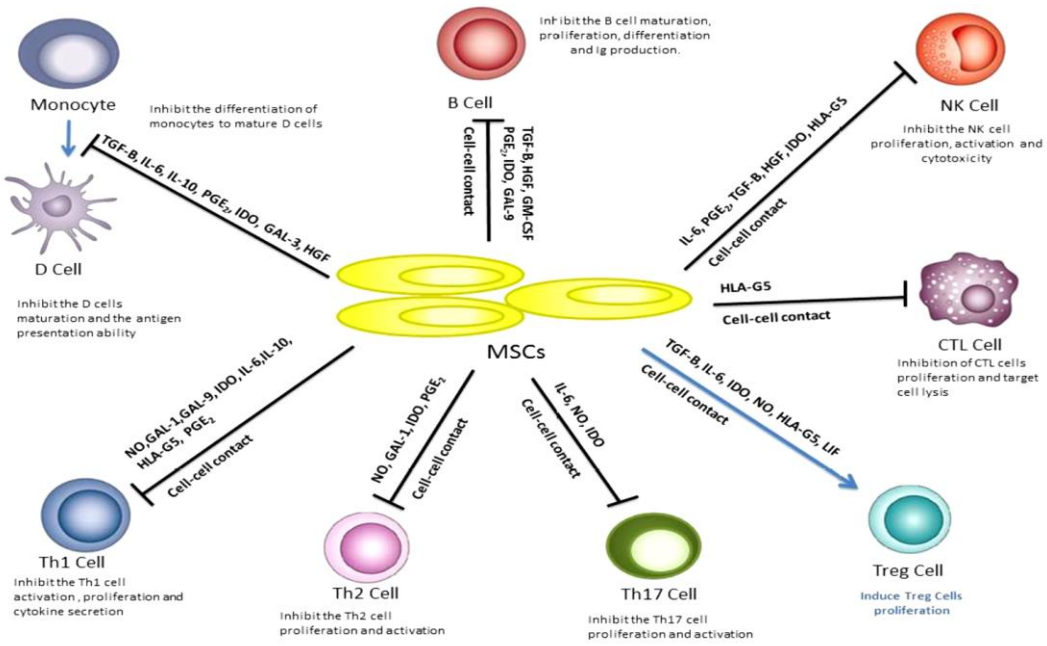


Figure 18: Immumodulatory effects of mesenchymal stem cells (MSC) on immune cells. MSCs inhibit monocyte differentiation into dendritic cells (DCs), suppress the activation and proliferation from B, Th1, Th2 and Th17 cells, induce the activity of T regulatory (Treg) and inhibit the proliferation and cytotoxicity of natural killer (NK) cells and cytotoxic T lymphocytes (CTL) through cell-cell contact mechanisms and through the release of soluble factors.

A limited number of studies have been published regarding the regulatory effect of MSCs on B cells in humans (Franquesa et al. 2012). However, MSCs inhibit the proliferation of B cells by arresting cell cycle G0/G1 without inducing apoptosis and differentiation in plasma cells with consequent reduced production of IgG, IgA, IgM (Tabera et al. 2008).

Another MSC reported function is the ability to modulate the cells of the innate immunity. Dendritic cells (DCs) are the most important APCs specialized in the uptake, transport, antigen processing and presentation to memory T cells, B lymphocytes and Natural killer cells (NKCs). DCs play a key role in the initiation of primary immune response, which depends on the activation and maturation stage of the cells (Banchereau and Steinman 1998). MSCs affect the recruitment, maturation and function of DCs. Co-culture studies showed that MSCs induce the polarization of mature DCs towards an immature phenotype, as evidenced by reduced expression of HLA-DR, CD1a, CD80 and CD86, and down-regulated IL-12, that is associated with tolerance

and anergy of T cells. Moreover, MSCs were able to induce an anti-inflammatory and tolerant phenotype in LPS-treated mature DCs, by increasing their release of IL-10 (Aggarwal and Pittenger 2005).
MSCs exert also inhibitory effects on NK cells – a lymphocytic subtype critical for the innate immune response against viral infections and tumors (Trinchieri 1989) - by reducing both proliferation and cytotoxic potential, through the release of IDO and PGE-2, TGFβ, IL-10, HGF (Spaggiari et al. 2008).

3.3.4 MSC-macrophage crosstalk: polarization toward M2 phenotype

In addition to DCs and NK cells, MSCs have been reported to strongly affect immunosuppressive functions of macrophages, considered as major partakers in inflammatory and regenerative processes. In a study evaluating the crosstalk between MSCs and macrophages, the authors found that PGE-2 play a pivotal role in MSC-modified macrophage polarization towards the M2b-immune regulating phenotype. The immunologically relevant synthesis of PGE-2 depends mostly on ciclooxygenase-2 (COX-2) - but not ciclooxygenase-1 (COX-1) - enzyme activity, with COX-2 being the inducible and COX-1 the constitutively active isoforms (Kalinski 2012) respectively. In the study, the inhibition of COX-2, but not COX-1, resulted in an increase of TNFα and in the decrease of IL-10 production in a concentration-dependent manner. Moreover, the exogenous application of PGE2 to macrophages acted similarly to MSCs, suggesting that PGE-2 is an important molecule in the immunomodulatory crosstalk between MSC and macrophages (Kudlik et al. 2016).
In a mice model of acute colitis, intraperitoneally-administrated MSCs were able to reduce the severity of the disease by polarizing macrophages towards the alternatively activated M2 phenotype and increasing serum levels of IL-10. Specifically, in a mouse model of colitis, pharmacological inhibition of MSC Galectin-3 (Gal-3) - a molecule important for macrophage polarization and function (Yubin Li et al. 2008) - enhanced the presence of colonic M2-macrophages as well as IL-10 serum concentration (Markovic et al. 2016). In another study, MSCs were able to inhibit the production of TNF-α by cocoltured LPS-stimulated macrophages, preventing their polarization towards M1 phenotype and increasing the levels of Arg-1, TGF-β and IL-10 (Zheng et al. 2018).
Among the many recent studies linking MSCs to resolution of the inflammatory processes, several demonstrate that MSCs can modulate the pathways of inflammation through two feedback loops (Fig.19), that can be explained by adopting the concepts of *Inducer, Sensor, Mediator, and Effector,* proposed by Medzhitov in 2008 to describe the players involved in a generic inflammatory cascade (Medzhitov 2008). One negative feedback loop is initiated by the proinflammatory molecules (*Mediators*) released from the *Sensors* (resident macrophages/microglia and mast cells expressing receptors for the *Inducers*) after interaction with *Inducers*, that may be represented by products of micro-organisms or injured cells. The released *Mediators* activate MSCs, inducing the upregulation of different molecules, such as COX-2 other

components of the arachidonic acid pathway. As a result, MSCs increase the secretion of PGE-2, that, in turn, drives the transition of the *Sensors* (resident macrophages, microglia) from the classic pro-inflammatory M1 phenotype toward the M2 phenotype - associated with repair and regeneration - characterized by the release of factors, such as IL-10 and and interleukin-1 (IL-1) receptor antagonist, that finally exert an anti-inflammatory action on the *Effectors* (different cell types in the tissue).

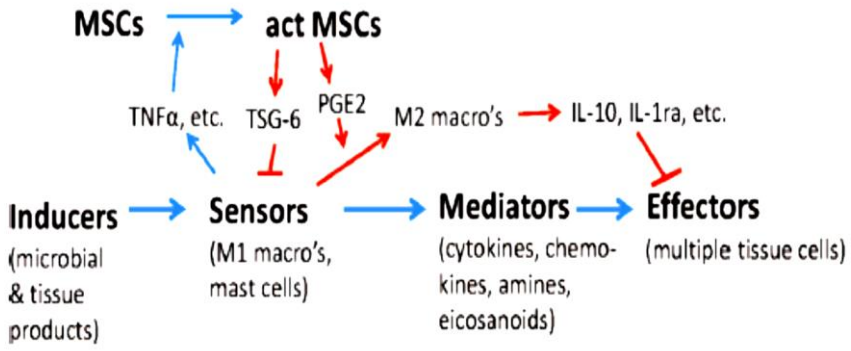


Figure 19. Schematic negative feedback loops of TSG-6 and PGE2 into inflammatory responses by MSCs. After interaction with *Mediators* released by M1 pro-inflammatory macrophages/microglia MSCs activate (act MSCs) and release factors that switch off inflammation by polarizing innate immune cells toward an anti-inflammatory phenotype. Abbreviations: act MSCs, activated mesenchymal stem cells; PGE-2, prostaglandin E2; TNF-α, tissue necrosis factor alpha; TSG-6, TNF-α stimulated gene/protein 6 (Prockop 2013).

In the second negative feedback loop MSCs release factors that, instead of polarizing innate immune cells *(Sensors*) toward an anti-inflammatory phenotype, inhibit specific inflammatory pathways within the *Sensors*, thus avoiding their release of pro-inflammatory mediators. Infact, after activation by *Mediators* released by *Sensors*, MSCs increase the expression of a number of genes, including TNF-α stimulated gene/protein 6 (TSG-6), a protein that has been reported to exert multiple anti-inflammatory as well as tissue remodeling activities (Wisniewski and Vilček 2004; Milner et al. 2006). Among its many effects, TSG-6 was able to interact with CD44 on resident macrophages, either directly or in a complex with hyaluronan, to dissociate CD44 from Toll-like receptor (TLR2) and thereby limit TLR2 driven NFκ-B signaling. The result is a decrease in the secretion of TNF-α and other pro-inflammatory *Mediators* that target *Effectors*. The negative feedback loop driven by MSCs was observed for the first time in a model of sepsis induced by cecal ligation and puncture (CLP) (Németh et al. 2009). The authors observed that intravenous injection of BM-MSCs beneficially modulated the response of the host immune system to sepsis, reducing TNF-α and IL-6 and increasing IL-10 serum levels, and improving survival. Importantly, the treatment with MSCs significantly increased the number of IL-10–producing monocytes and macrophages in lungs, compared to control mice. The antibody-mediated neutralization of IL-10 in mice before CLP prevented the beneficial effect of MSC injections, suggesting the key role of IL-10 in MSC therapeutic effect. By combining the *in vivo* results with the coculture *in vitro* studies to understand the molecular basis of the MSC-macrophage interaction, the

authors proposed the hypothesis accordingly to which specific molecules, such LPS and TNF-α, interact with TLR-4 and TNF-R1 respective receptors on MSCs inducing NF-κB translocation to the nucleus. This leads to increased release of activated MSC PGE-2 that acts on the EP2 and EP4 receptors of the macrophages and stimulates the release of the anti-inflammatory cytokine IL-10. This feedback loop may explain the therapeutic effect exerted by MSCs in the CLP model of sepsis. In another study, the anti-inflammatory effects of MSCs were investigated in a model of zymosan-induced peritonitis. The authors found that the decreased inflammation observed in MSC-treated mice were at least in part due to activation of hMSCs by the initial inflammatory environment. In particular, zymosan induced the TLR2-mediated activation of macrophages and secretion, via NF-κB signaling, of pro-inflammatory cytokines that, in turn, induced activated MSCs to secrete TSG-6. This anti-inflammatory protein negatively regulated the TLR-2 mediated responses through CD44 receptor expressed on resident macrophages, thus limiting the release of pro-inflammatory mediators and the infiltration of other immune cells, such as neutrophils (H. Choi et al. 2011).
Multiple groups have examined the role of the secretome of MSCs upon TLR ligation, given that different activities of these cells, including immunomodulation (Tomchuck et al. 2008; Waterman et al. 2010), is greatly regulated by the stimulation of these receptors. Importantly, eleven TLRs (TLR1-11) have been identified in human cells (Yu et al. 2010) and several have been reported to be expressed by hMSC at different levels depending on tissue of origin. For example, BM-MSCs have been reported to express TLR1-2 (Tomchuck et al. 2008), TLR3 (Tomchuck et al. 2008; Waterman et al. 2010), TLR4 (Tomchuck et al. 2008; Waterman et al. 2010; Lu et al. 2015), TLR5-6 (Romieu-Mourez et al. 2009; Raicevic et al. 2011), TLR7 (Tomchuck et al. 2008; Romieu-Mourez et al. 2009) and TLR10 (Hwa Cho, Bae, and Jung 2006).
Particularly, several studies reported that the stimulation of TLR3 or TLR4 on BM-MSCs and ASCs with their respective agonists, LPS and polyinosinic:polycytidylic acid [poly(I:C)], promoted the release of cytokines and chemokines with different roles in immunomodulation, such as CXCL10 (Tomchuck et al. 2008), IL-6 (Tomchuck et al. 2008; Waterman et al. 2010), IL-8 (Tomchuck et al. 2008; Lombardo et al. 2008; Waterman et al. 2010) CCL5 (Raicevic et al. 2011), IL-12 (Tomchuck et al. 2008; Raicevic et al. 2011), IL-27, IL-23 (Raicevic et al. 2011), IL1β (Romieu-Mourez et al. 2009), TNFα (Tomchuck et al. 2008), CCL2 (Hwa Cho et al. 2006). Therefore, referring to nomenclature used for macrophage polarization, MSCs have been classified into two phenotypes: pro-inflammatory MSC1 phenotype and immunosuppressive MSC2 phenotype (Waterman et al. 2010) (Fig. 20).

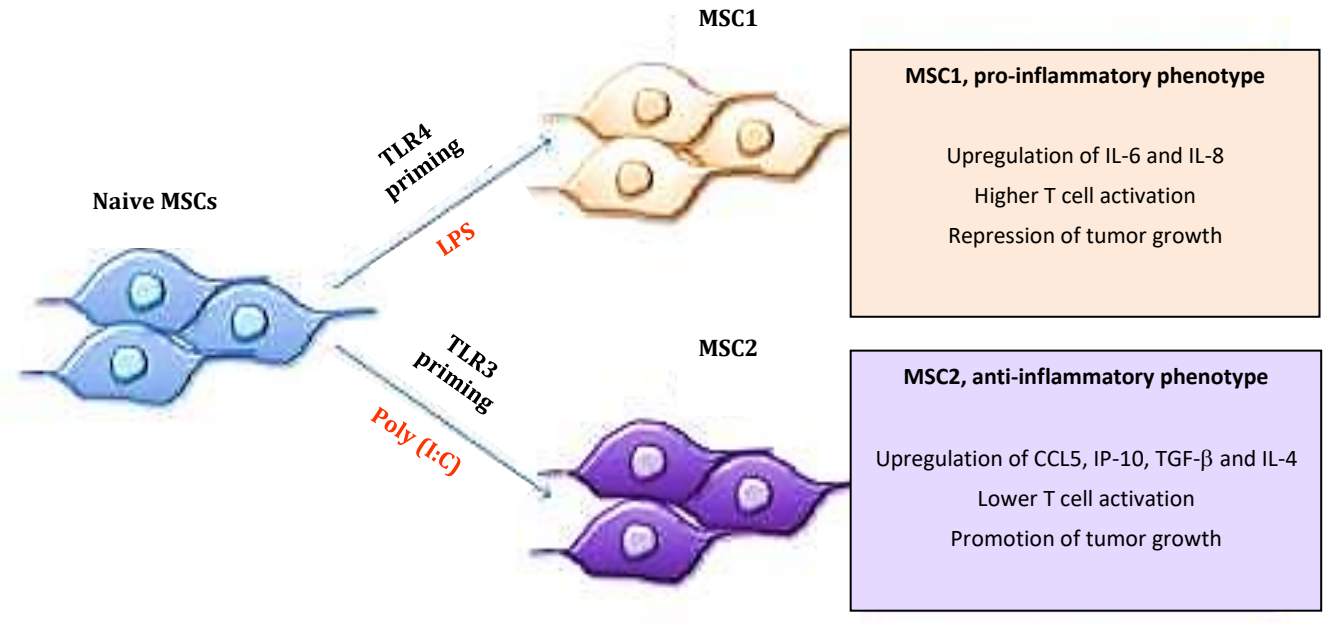


Figure 20. **Interactions of MSCs with environmental cues leading to polarization**. MSCs are polarized toward MSC1 pro-inflammatory and MSC2 anti-inflammatory phenotype by LPS and poly (I:C), respectively (Modified by Rivera-Cruz et al. 2017). This nomenclature was defined following the description of MSCs abilities to induce the polarization of macrophages (see the text).

MSC1 is characterized by an increased synthesis and secretion of proinflammatory cytokines and chemokines, such as IL-6 and IL-8, while MSC2 has increased production of immunosuppressive mediators such as Interferon gamma-induced protein 10 (IP-10) and CCL5.

All these results highlight the importance of environmental cues for eliciting MSC immunoregulatory activity. Therefore, the developing of engineering or specific *ex vivo* preconditioning strategies that lead to a controlled and purposeful cell polarization could enhance the therapeutic potential of these cells as well as their derived products.

3.3.5 Preconditioning to enhance MSC immunomodulatory functions

Although MSCs are widely applied in cellular transplantation, the low survival rate and high apoptosis rate reduce their therapeutic effects. In fact, when they are injected *in vivo,* they migrate into damaged tissues or organs, where they encounter a harsh environment characterized by high levels of pro-inflammatory mediators, ROS and death molecules that can compromise their functions. One of the approaches to improve the ability of MSCs to survive in the harsh environment is to *precondition* cells *ex vivo* in a specifically designed/engineered environment, enriched with different physical or chemical parameter(s)/factor(s) (Fig. 21).

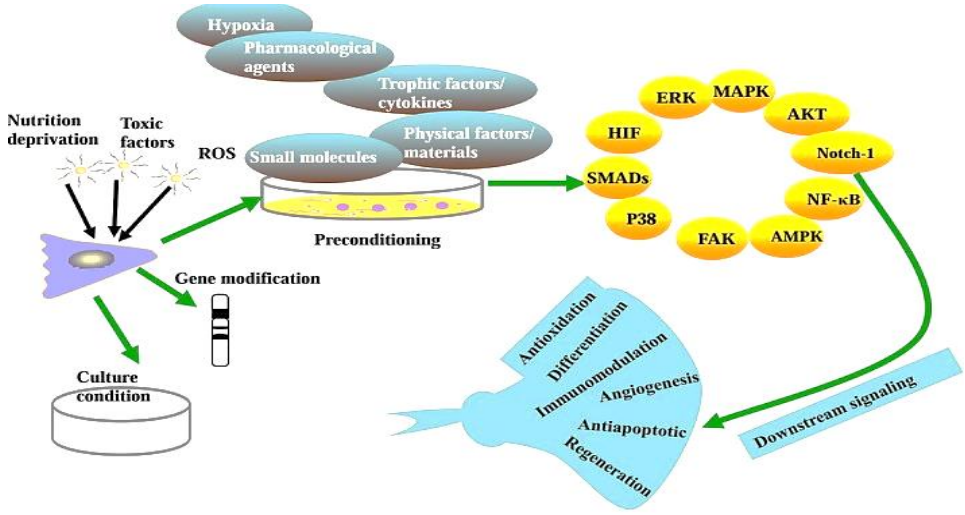


Figure 21. Schematic representation of *ex vivo* strategies to improve MSC function. Preconditioning, genetic modification, nutrition deprivation and optimization of MSC culture conditions are able to activate specific cellular signaling pathways that confer enhanced properties (e.g. anti-oxidation, anti-apoptosis, immunomodulation) on the cells (Hu and Li 2018).

Preconditioning of MSCs by hypoxia, pharmacological/chemical agents, trophic factors, cytokine have been investigated as key strategies to prepare the cells to survive in the harsh environment (Saparov et al. 2016). Moreover, considering the natural interaction between MSCs and immune cells, preconditioning may represent an important tool for driving MSC cell polarization towards a specific immunoregulatory phenotype, as well as endowing MSCs with enhanced abilities to regulate innate immune response. Importantly, if MSCs can build up a constructive immune response after injury by regulating the function of inflammatory cells that actively contribute to exacerbate primary damage - as it occurs, for example, in neurodegenerative diseases - this would be likely to enhance tissue healing. Hypoxia/Oxygen deprivation (H/OD) has been described as a strategy to enhance MSC expression of anti-apoptotic proteins, angiogenic factors (Bader et al. 2015), as well as increase migration capacity (Rosová et al. 2008) and ability to regulate the innate immune response. Chen and colleagues investigated if the paracrine functions of BM-MSCs were favourably affected by hypoxia (2% O_2) to enhance their ability to stimulate cutaneous wound healing in mice. They found that the medium from preconditioned cells contained significantly higher amounts of bFGF, vascular endothelial growth factor A (VEGF-A), IL-6, IL-8, compared to medium derived from MSCs grown in normal culture conditions (20% atmospheric O_2). Moreover mice treated with preconditioned cells evidenced a higher infiltration of macrophages - generally regarded as beneficial to wound healing - in site of injury, suggesting that they may take part in accelerated skin wound contraction, compared to mice treated with normoxic cells (Chen et al. 2014). In another mice model of skin wound, intravenously infusion of 24h hypoxic stimulated human gingival–derived mesenchymal stem cells (hGMSCs) was found to accelerate skin wound closure compared to mice infused with non-preconditioned cells. In particular, hypoxic treatment of preconditioned MSCs reduced the recruitment of peripheral blood mononuclear cells (PBMCs)

at the skin wound, decreased the peripheral blood concentration of pro-inflammatory cytokine TNF-α, and increased anti-inflammatory cytokine IL-10 (Jiang et al. 2015). In another study, Wei and colleagues investigated the effects of intravenous administration of hypoxia preconditioned BM-MSCs in a model of ischemic stroke induced by 90-min occlusion of right middle cerebral artery (Wei et al. 2012). They found that 48h sub-lethal hypoxia (0.5% O_2) preconditioning of MSCs (H-BMCs) upregulated the expression of a variety of trophic factors, including BDNF, GDNF, VEGF, angiotensin (Ang-1) with respect to normoxic cells (N-BMCs). In addition to regenerative factors, 24h hypoxia preconditioning and 24h re-oxygenation were able to significantly reduce the H-BMCs expression of a broad range of pro-inflammatory genes, such as *CC3*, *CC5*, *CC17*, *CCL4*, *CXCR3*, known to be potent attractor for monocytes, macrophages and T-cells (Dufour et al. 2002). The number of OX-42-positive cells (activated microglia) in the ischemic cortex of H-BMCs administered rats was noticeably lower and neurogenesis was increased compared to stroke-only and N-BMCs rats. Moreover, the authors observed significant locomotion improvement in mice treated with H-BMCs with respect to stroke-only and N-BMCs-treated rats, suggesting that hypoxic pre-treatment represent an effective stem cell therapy, probably exerting therapeutic effects by promoting neurogenesis by enhancing the trophic and the anti-inflammatory actions of MSCs. Similar results were obtained by Lan and colleagues, who demonstrated for the first time that hypoxic preconditioned MSCs rendered better therapeutic effects than untreated MSCs in a bleomycin induced-pulmonary fibrosis animal model. In particular, hypoxic treatment strongly down-regulated the expression of inflammatory and fibrotic factors in MSCs compared to untreated cells (Lan et al. 2015).

Several studies also reported the effects of cytokine-preconditioned MSCs on innate immunity. Noone and colleagues show that hMSCs suppress NK activation, through PGE-2 secretion in a contact-independent manner and that cell pre-stimulation with interferon gamma (IFN-γ) induced expression of the tryptophan degrading enzyme IDO, thus facilitating enhanced suppression (Noone et al. 2013b). IDO is an IFN-γ-inducible intracellular enzyme that catalyses the first step in tryptophan degradation along the kynurenine pathway. The activity of this enzyme was first identified as a defence mechanism against microbial invasions because it induces the depletion of tryptophan, which is required for the proliferation of bacteria, parasites and viruses (Zelante et al. 2009), producing kynurenine that can be further catabolized into kynurenic acid, anthranilic acid, or 3-hydroxykynurenine, all possessing immunomodulatory properties. PGE-2, is a lipid mediator that is synthesized from arachidonic acid via the actions of COX enzymes, either constitutively or in response to cell-specific trauma, stimuli, or signaling molecules (Smith 1989b). Depending upon the context, PGE-2 has been reported to exert homeostatic (Smith 1989), inflammatory (P. Davies et al. 1984) and anti-inflammatory effects (Takayama et al. 2002). Regarding PGE-2 anti-inflammatory effect, studies have demonstrated its involvement in polarizing M1 macrophages toward M2 anti-inflammatory phenotype. Cytokines, like IFN-β, IFNγ, IL-1β and TLR-agonists, such as LPS have been described as treatment able to

significantly increase the production and release of MSC PGE-2 (Gray et al. 2015), with IL-1β and LPS being the more potent inducers and having the greatest impact on suppression of TNF-*α* production by human macrophages after treatment with MSC-conditioned medium. However, although the combined pretreatment of MSCs with IL-1β and LPS generated a synergistic increase in PGE2 secretion, this double stimulation did not further reduce macrophage TNF-*α* release. This may indicate a lower limit in attenuation of macrophage TNF-*α* production in that system (cultured macrophages treated with MSC-conditioned medium), where further increases in PGE2 will not correspond to a further reduction in TNF-*α* levels. This may indicate that, at least upon a certain level, other molecules need to be modulated to further reduce the production of this pro-inflammatory cytokine. Interestingly, although the synergistic effect in inducing PGE-2 secretion was observed also for interferon β (IFN-β) IL-1β, their combined action failed to antagonize macrophage TNF-α secretion, suggesting potential conflicts in regulatory signals when two potent inducers are used simultaneously.

The important role IDO and PGE-2 in mediating MSC immunosuppressive capabilities was also demonstrated by their ability to enhance the secretion of factor H (Yan Li and Lin 2012), a factor that has been reported to inhibit complement activation, which plays pivotal role in innate immunity (Whaley and Ruddy 1976). The authors found that MSC production of factor H was upregulated by TNF-α and IFN-γ in both dose- and time-dependent manners, while the treatment with the pro-inflammatory cytokine IL-6 did not have a significant impact on MSC factor H production. Moreover, both IDO and PGE-2 inhibitors significantly reduced MSC factor H production, suggesting that factor H produced locally by MSCs may contribute to their broad immunosuppressive activities (Tu et al. 2010). Similar results were obtained by François and colleagues who found that the combined IFN-γ and TNF-γ stimulation of MSCs, as the combination that further increases T cell suppression, with respect to only IFN-γ treatment. The upregulation of IDO following exposure to TNF-α and IFN-γ varied between donors and stronger producers of IDO were more potent inhibitors of T cell proliferation *in vitro*, establishing a correlation between the amount of IDO produced and the level of T cell inhibition. Noteworthy, the authors demonstrated that the enzymatic activity of IDO can mediate a direct and indirect inhibition of T cell proliferation. IFN-γ produced by activated T cells in an inflammatory milieu prompts the expression of IDO in MSCs and thus the conversion of tryptophan into kynurenine, which leads to a direct inhibition of T cell proliferation. Indirectly, they demonstrated that enzymatic activity of IDO and possibly other factors secreted by MSCs, induced the polarization of monocytes into IL-10-secreting $CD206^{+}$ M2-like macrophages, which possess T-cell immunosuppressive properties on their own. These observations were confirmed by both depletion of monocyte and additional of supplementary monocytes to the PBMC proliferation assay (François et al. 2012).

These results indicate that both by low oxygen concentration and pro-inflammatory cytokines represent important tools for enhancing MSC ability to regulate innate immune response, underlining the plasticity of

MSCs that release immunomodulant factors to restore the equilibrium in conditions of disrupted homeostasis.

3.3.6 Mesenchymal Stem Cells in diseases

Among stem cell types available for transplantation in patients, MSCs are often considered best candidates because of their paracrine effects and, given their presence in adult tissues, the chance to be exploited for autologous transplantation, thus avoiding concerns related to allogenic organ rejection and ethics.

In fact, preclinical and clinical studies suggest that MSCs are not APCs, showing low expression of human leukocyte antigen (HLA), MHCI and negligible levels of HLA class II (Le Blanc et al. 2003).

MHCI and MHCII complexes are peptide-binding proteins, well known to activate adaptive immune system: the formers stimulate the cytotoxic T cells to recognize antigen fragments, cleaved by APCs and presented on their membrane; the latter activate helper T-cells by exposing extracellular antigens after they enter the endocytic pathway of the APCs. The low expression of these complexes, as well as of the co-stimulatory molecules B7- 1/B7-2 (CD80/86), CD40 or its ligand CD40L (Pittenger et al. 1999b), prevent lymphocytes from becoming fully activated. These aspects paved the way for allogenic MSC-based therapies.

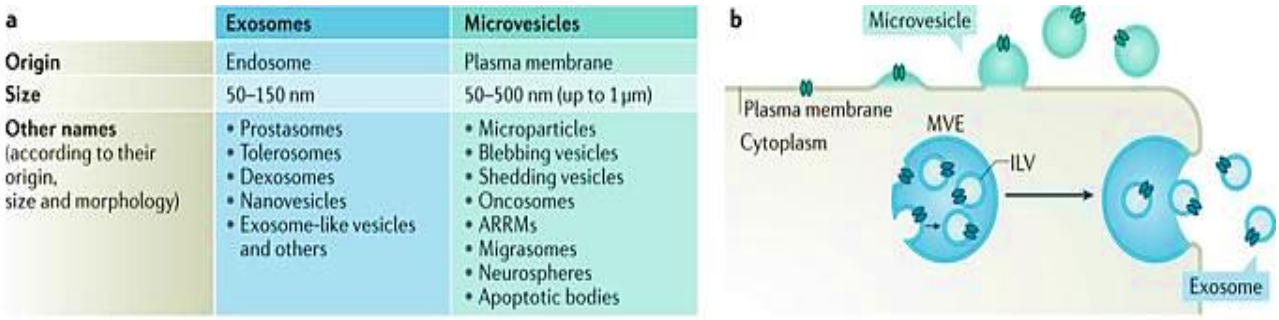
Several studies have demonstrated that MSC neuroprotective effects are associated to functional improvement, following their transplantation in immune-mediated diseases and degenerative diseases. Specifically, the therapeutic immunoregulatory effects mediated by these cells have been observed in different preclinical disease models, such as skin wound, skin-graft rejection, acute kidney injury (AKI), myocardial infarction (MI), acute lung injury (ALI), rheumatoid arthritis, systemic lupus erythematosus, type-I-diabetes, inflammatory bowel disease (Gnecchi et al. 2005; Bruno et al. 2012; Glenn 2014).

Differentiation potential, immunomodulation and production of trophic factors represent the MSC properties that have driven several clinical trials in humans, in order to test MSC efficacy in genetic bone diseases, osteogenesis imperfecta, osteoarthritis, AKI and MI, as well as to foster hematopoietic recovery after BM transplantation and to treat GvHD (Le Blanc et al. 2008). As of date (17.09.2018), in the last three years, a total number of 242 clinical studies involved MSCs for different clinical phases registered on the NIH Clinical Trial Database (https://clinicaltrials.gov/).

Finally, MSCs are extensively considered also as a therapeutic tool against neurodegenerative diseases, such as Alzheimer's Disease (AD), Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Multiple Sclerosis (MS) (Volkman and Offen 2017). In the context of these pathologies, MSCs have been evaluated as a promising therapeutic strategy, reflecting either their ability to transdifferentiate into neural stem cells, whenever specific conditions are guaranteed, or their immunomodulatory actions, which, have been demonstrated to largely be mediated by extracellular vesicles (EVs).

4. Extracellular Vesicles (EVs)

Extracellular Vesicles (EVs) are a heterogeneous group of cell-derived membranous structures released by the majority of the cells and different from secretory vesicles containing hormones or neurotransmitter that are released only by specialized cells. The secretion of EVs was initially described as means of eliminating useless materials from the cells. However, a large amount of published reports has demonstrated that they are more than just waste carriers. Indeed, EVs play important roles in biological communication through their capacity to exchange different molecules between cells (a process defined "horizontal communication"), such as nucleic acids, lipids and proteins. EVs can be found in a variety of physiological fluids such as normal urine, blood, bronchial lavage fluid, breast milk, saliva, cerebrospinal fluid, amniotic fluid, synovial fluid, and malignant ascites. They have been implicated in important processes such as immune responses, homeostasis maintenance, coagulation, inflammation, cancer progression, angiogenesis and antigen presentation, thus acting as signalling vehicles in normal cell homeostatic processes or because of pathological developments. Among the different types of EVs, the most characterized are exosomes and microvesicles (MVs) (Raposo and Stoorvogel 2013). Figure 22 shows a classification of the principal exosomes and MVs.



a	Exosomes	Microvesicles
Origin	Endosome	Plasma membrane
Size	50–150 nm	50–500 nm (up to 1 µm)
Other names (according to their origin, size and morphology)	• Prostasomes • Tolerosomes • Dexosomes • Nanovesicles • Exosome-like vesicles and others	• Microparticles • Blebbing vesicles • Shedding vesicles • Oncosomes • ARRMs • Migrasomes • Neurospheres • Apoptotic bodies

Figure 22. EV classification. a. Over the past two decades, EVs have been named based on their origin (from endosome or plasma membrane), size (typically between 50 nm and 500 nm, but they measure up to 1–10 µm), and morphology. Now, they can be classified into two main distinct classes: exosomes and microvesicles (MVs). **b**. Schematic representation of EV biogenesis: intraluminal vesicles (ILVs) are released in the extracellular environment as exosomes after fusion of multivesicular endosome (MVE) with plasma membrane (PM), while MVs directly bud from PM (Van Niel et al. 2018).

4.1 EV biogenesis and release

Exosomes and MVs have different biogenesis (both involving membrane-trafficking processes): exosomes are generated within the endosomal system as intraluminal vesicles (ILVs) and secreted during the fusion of multivesicular endosomes (MVEs) with the cell surface, whereas MVs originate by an outward budding at the plasma membrane (PM) followed by the shedding of the vesicles. Even though the generation of MVs and exosomes occurs at distinct sites in the cell, common intracellular mechanisms and sorting machineries are involved in the biogenesis of both these types of EVs, thus hindering the possibility of distinguishing between the different vesicle subpopulations. Cargoes are important regulators of EV formation. In particular, cargoes

planned for the secretion within EVs must be targeted to the site of production, either at the PM (for MVs) or at the membrane of the MVE (for exosomes). For example, the ectopic expression of MHC class II cargo has been shown to promote MVE and ILV generation (Ostrowski et al. 2009), probably by recruiting the necessary machineries for their generation.

Exosomal cargoes reach endosomes from the Golgi apparatus or are internalized from the PM before being sorted to ILVs during endosome maturation (Fig. 23). Therefore, cargoes that are preferentially recycled to the PM are not likely to be sorted in exosomes. Consequently, any impairment of regulators of endosomal recycling, retrograde transport from endosomes to the Golgi or internalization from PM might influence the targeting of some cargoes to different types of EVs (Van Niel et al. 2018).

Exosomes are generally thought to derive from the endosomal compartment. They form within the intraluminal vesicles (ILVs) that derive from an unconventional inward budding of MVEs, which are intermediates within the endosomal system. The formation of MVEs is coordinated by a complex of proteins called endosomal sorting complex required for transport (ESCRT) (Raposo and Stoorvogel 2013). ESCRT-0 and ESCRT-I cluster ubiquitylated transmembrane cargoes on microdomains of MVEs and recruit, via ESCRT-II and kinases which phosphorylate key subunits on ESCRT-0 recruiting at the endosomal membrane other subcomplexes such as ESCRT-III, that perform budding and fission of that microdomain (Adell et al. 2014). Therefore, ESCRT exert a main role in channelling molecules into ILVs.

Exosomes can also be formed in a ESCRT-independent process, as revealed by studies showing that MVEs, containing ILVs loaded with CD63, were still formed upon depletion of the ESCRT complexes (Stuffers et al. 2009). The first ESCRT-independent mechanism of exosome biogenesis was shown to be dependent on the activity of neutral type II sphingomyelinase enzyme, that hydrolyses sphingomyelin to ceramide. This was supported by the high levels of ceramide found within purified exosomes and the reduction of EV release after inhibition of the enzyme (Trajkovic et al. 2008). In addition, proteins of tetraspanin family have been shown to regulate ESCRT-independent endosomal sorting, such as CD63, CD81, CD82 and CD9 (Theos et al. 2006; Buschow et al. 2009). These proteins form dynamic membrane platform with other tetraspanins as well as transmembrane and cytosolic proteins contributing to the formation of the microdomains that will bud (Charrin et al. 2014). Other proteins, such as Alix and tumor susceptibility gene derived protein (Tsg101) are directly involved in ILVs formation (Kowal et al. 2014).

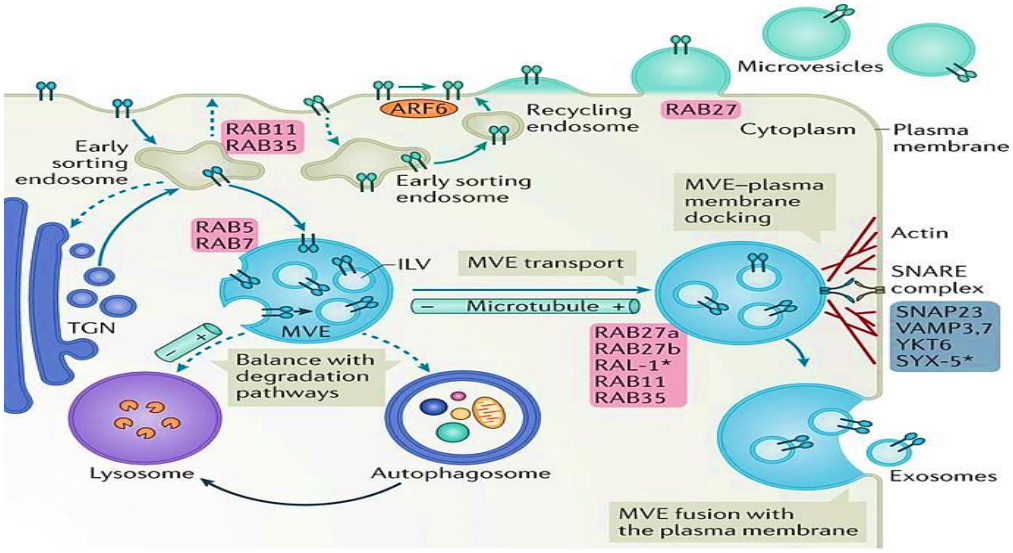


Figure 23. EV biogenesis. EV generation and the targeting of cargoes within these vesicles require tuned regulation of multiple intracellular pathways (blue arrows for exosomes, *green* arrows for MVs) that involve various RAS-related protein (RAB) GTPases. Cargoes directed to MVEs originate from endocytosis at the plasma membrane (PM) or are directly targeted to MVEs or to early sorting endosomes via the biosynthetic pathway (from the *trans*-Golgi network, TGN). Retrograde transport towards the TGN or recycling back to the PM will divert cargoes from their targeting to the MVE (*dashed arrows*) and therefore their incorporation into intraluminal vesicles (ILVs). Once matured, MVEs can target lysosomes or autophagosomes for degradation or - along microtubules - dock to the PM for fusion and release of exosomes. In the case of MV biogenesis, endocytic uptake (*dashed arrow*) and recycling will decrease and increase, respectively, the targeting of membrane-bound cargoes to MVs. ARF6, ADP-ribosylation factor 6; RAL-1, RAL (Ras-related GTPase) homolog; SNAP23, synaptosomal-associated protein 23; SYX-5, syntaxin 5; VAMP3, vesicle-associated membrane protein 3. *Denotes *Caenorhabditis elegans* proteins (Van Niel et al. 2018).

The sequestration of cytosolic proteins into ILVs has been described to depend on co-sorting with other proteins, such as chaperon heat shock 70 kDa protein (HSP70) (Théry et al. 2001). Nucleic acids, including mRNAs, non-coding RNAs, microRNAs (miRNA) and DNA sequences have also been found in exosomes (Valadi et al. 2007; Thakur et al. 2014). Interestingly miRNAs seem to be differently sorted on the basis of specific motifs in their sequence that are bound by heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1)(Villarroya-Beltri et al. 2013). This suggests that the incorporation of nucleic acids is an active regulated process. However, the mechanisms involved in targeting nucleic acids to exosomes are so far elusive. These MVEs can either fuse with autophagosomes and lysosomes for degradation or travel back and fuse with the plasma membrane, a process that is likely to be mediated by SNARE proteins and synaptotagmin family members (Jahn and Scheller 2006). Various Rab-GTPases coordinate the transport of MVBs to the plasma membrane and cytoskeletal rearrangements eventually leads to ILVs secretion in the extracellular environment, as exosomes, upon fusion with plasma membrane (Cocucci et al. 2009).

The biogenesis of **microvesicles** (MVs) requires several molecular rearrangements within the plasma membrane, including changes in lipid and protein composition, and modifications in Ca^{2+} levels (Minciacchi et al. 2015). Ca^{2+}-dependent enzymes such as aminophospholipid translocases (flippases and floppases), scramblases and calpain drive rearrangements in the composition of membrane phospholipids (exposition of phosphatidylserine from the inner leaflet of cell surface to the outer one), which causes physical bending

of the membrane and restructuring of the underlying actin cytoskeleton, followed by fission process and subsequent release of the vesicle in the extracellular space (Tricarico et al. 2017). In particular this fission requires an interaction of actin and myosin with subsequent ATP-dependent contraction (McConnell et al. 2009). The activation of small GTP-binding proteins including ARF6 and ARF1 leads to the phosphorylation of myosin light chain (MLC) and actomyosin contraction, which allows the vesicles to bud off from the membrane (Muralidharan-Chari et al. 2009). In addition to increased levels of Ca^{2+} concentration, robust release of MVs has been reported through the activation of protein kinase C by phorbol esters (Cocucci et al. 2009) and after ATP-mediated activation of $P2X_7$ receptors, which leads to rearrangements of the cell membrane by recruiting acidic rather than neutral sphingomyelinase (Bianco et al. 2005). This suggests that different members of the sphingomyelinase family control the biogenesis of exosomes (Trajkovic et al. 2008) and the release of MVs, but in both cases, through ESCRT-independent mechanisms.

As for cargo targeting to exosomes, lipids and other membrane-associated molecules are incorporated into MVs through their affinity for lipids raft, while cytosolic components are targeted to MVs through their binding to the inner leaflet of the PM through specific anchors (palmitoylation, prenylation, myristoylation) that concentrates them to specific membrane microdomains from which forming MVs will bud (Shen et al. 2011). The mechanism by which nucleic acids are incorporated into MVs is still unclear.

Despite the different biogenesis pathways, exosomes and MVs display a similar appearance, overlapping size and often common composition that make it difficult to ascertain their origin once isolated from the extracellular medium or from biological fluids. Of note, the tightly regulated steps of transport, tethering and fusion of MVE to the plasma membrane for the release of exosomes, could account for the time difference between the generation and release of the two types of EVs.

4.2 Binding to and internalization by target cells

Once released in the extracellular space, EVs can interact and be uptaken by recipient cells. Target cell specifity is likely to be determined by proteins enriched at the surface of EVs and receptors expressed on the PM of recipient cells. Several mediators have been described to perform these interactions, including tetraspanins, integrins, lipids, lectins, heparan sulfate proteoglycans and extracellular matrix (ECM) components (Fig. 24 inset). Integrins on EVs interact with intercellular adhesion molecules (ICAMs) at the surface of recipient cells, as well as extracellular matrix proteins, mostly fibronectin and laminin, that play important roles in exosome (Sung et al. 2015) and MV (Leiss et al. 2008) binding to the target cells. In EV binding, the ECM can act as a ‘zipper’ between integrins present on EVs and target cells. Moreover, EV lipid composition has been reported to mediate interaction with target cells by recruiting specific lipid-binding proteins (Morelli et al. 2004). EVs can be internalized by cells either by fusion with PM or via endocytosis (Mulcahy et al. 2014). Different mechanisms have been described for the uptake by endocytosis including

clathrin-mediated endocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, macropinocytosis and phagocytosis (Fig. 24). The uptake mode of EVs may be dependent on the type of the cell and its physiological state. For example, clathrin-dependent endocytosis or phagocytosis has been described in neurons, macropinocytosis in microglia, phagocytosis or receptor-mediated endocytosis in dendritic cells, caveolin-mediated endocytosis in epithelial cells, and cholesterol- and lipid raft-dependent endocytosis in tumor cells (Abels et al. 2017). The presence of specific structures - such as lipids rafts - at the PM of the target cell might influence the fate of EVs (Escrevente et al. 2011). In a study the authors investigated the temporal and spatial dynamics of vesicle-cell interactions by using optical manipulation and live-cell microscopy to directly drive EVs produced by microglial cells onto the surface of astrocytes or microglia in primary culture. They found that MVs derived from microglia show largely different dynamics of interaction with membranes of microglia and astrocytes. MVs promptly adhered to both glial cell types; however, after adhesion, MVs remained stuck to the plasma membranes of astrocytes, whereas they moved slowly along the surface of microglia. Phosphatidylserine residues on MVs were found to critically regulate this interaction as their cloaking on MVs with annexin V inhibited MV–cell contact and reduced MV movement on the microglial surface (Prada et al. 2016). Importantly, this study validated the optical tweezers as a powerful tool to explore the dynamics of MV interaction with cells and to understand the contribution of surface molecules to MV uptake by cells. Importantly, given that microglial MVs have been described as the vehicles of pathogenic cargo proteins in neurodegenerative diseases (Joshi et al. 2014), exploitation of optical manipulation will be crucial for a better understanding of how microglial MVs interact with neurons and oligodendrocytes and may contribute to their damage.

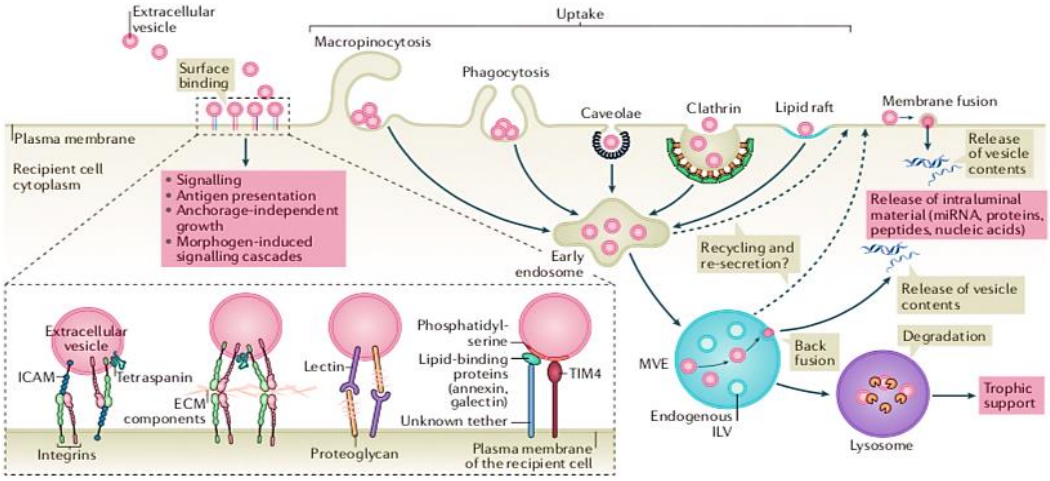


Figure 24. EV Interaction with target cells. On the target cell, exogenous EVs will bind to the cell surface (see inset) and following various fates. Depending on the cell type, they can remain bound to the surface (e.g., to integrins), activating specific intracellular signalling pathways (e.g., antigen presentation) or can be internalized by multiple pathways. Internalization will target exogenous EVs into the canonical endosomal pathway, whereby they reach MVEs, in which the internalized vesicles are likely to mix with endogenous intraluminal vesicles (ILVs). Fusion of MVEs with the lysosome will lead to the degradation of EVs and the recycling of their contents to provide trophic support to the recipient cell. EVs, docked either at the plasma membrane or at the limiting membrane of MVEs, can release their intraluminal contents (e.g., miRNA) into the cytoplasm of the recipient cell by fusion. Uptaken vesicles could also be recycled to the PM (*dashed arrows*). ECM, extracellular matrix; ICAM, intercellular adhesion molecule; TIM4, T cell immunoglobulin mucin receptor 4 (Van Niel et al. 2018).

4.3 Biological content of EVs

After interaction with the target cells, EVs can elicit a variety of functional responses by means of their cargo. The biologically active molecules of EVs comprise:

- *Lipids,* such as sphingomyelin, gangliosides and disaturated lipids, are increased in exosomes while phosphatidylcholine and diacylglycerol proportion are decreased relative to the membranes of their cells of origin. Some studies have also described increased fraction of cholesterol (Laulagnier et al. 2004), as well as an higher proportion of phosphatidylserine in the outer leaflet (Llorente et al. 2013), that may facilitate their internalization by the recipient cells. The increased enrichment of sphingomyelin and disaturated lipids compared to cell membranes implies a higher rigidity of the exosome lipid bilayer. This rigidity has been suggested to be pH dependent and it may be linked to the origin of exosomes, being the pH in MVEs lower than in the cytoplasm (Laulagnier et al. 2004). Finally, the greater rigidity of EVs may contribute to their resistance to the degradation and therefore their stability as carriers of various biomolecules. In addition to these lipids, EVs contain enzymes involved in their metabolism, including phospholipases D and A2;

-*Nucleic acids*, such as RNA and DNA molecules. The total amount of RNA in EVs varies depending on the cell type of origin and the stimulus to which they have been subjected. RNA found in EVs is predominantly short in size [200 nucleotides less than the average of cellular fraction (Eirin et al. 2015)], while in the pool of long transcripts (more than 200 nucleotides), both coding and noncoding RNAs were found (Huang et al. 2013).

The sequencing of total RNA from serum-derived EVs suggested that microRNAs (miRNA) and transfer RNA (tRNAs) constitute about 15% of EV-RNA (Bellingham et al. 2012). Interestingly, some profiles of EV-RNA do not mirror those of cellular RNA (Skog et al. 2008). Some cancer-derived EVs contain more total RNA than those derived from non-cancer cells (Balaj et al. 2011) and some RNAs were found to be enriched in the EVs released from adipose MSCs (Eirin et al. 2015). In particular, EVs preferentially contain mRNA for transcription factors (e.g. MDFIC, POU3F1, NRIP1) and genes involved in angiogenesis (e.g. HGF, HES1, TCF4) and adipogenesis (e.g. CEBPA, KLF7). EVs also transport Golgi apparatus genes (ARRB1, GOLGA4) and genes involved in TGF-β signaling. In contrast, mitochondrial, calcium signaling and cytoskeleton genes are selectively excluded from EVs. This suggests that specific mechanisms exist for selective packaging of RNA molecules within EVs. As described before, miRNA packaging into EVs has been reported to depend on recognition of specific motifs in nucleotide sequence by the ribonucleoprotein hnRNPA2B1 (Villarroya-Beltri et al. 2013). In addition, the sorting of miRNAs to exosomes may be driven by 3’ end post-transcriptional modifications (Koppers-Lalic et al. 2014). Importantly, RNAs have been found to be functionally transferred to recipient cells by EVs (Zaborowski

et al. 2015). Finally, DNA molecules have been found in EVs, but their functional significance remain unknown (Thakur et al. 2014).

- *proteins*: the protein composition of EVs is related to the cell type and mode of biogenesis. Exosomes that originate from the endolysosomal compartment tend to be more enriched in major histocompatibility complex class II (MHC class II) and tetraspanins CD37, CD53, CD63, CD81, and CD82 (Tauro et al. 2012). Since ESCRT pathway play a critical role in MVE formation, proteins belonging to this complex, including Alix, tumor susceptibility gene protein 101 (TSG101) and chaperones, such as Hcs70 and Hsp90, are generally enriched in exosomes, irrespective of the cell type (Théry et al. 2001). Some studies have also reported that, compared to the whole cells, exosomes are enriched in glycoproteins and transmembrane proteins (Sinha et al. 2014). On the contrary, owing to their origin via direct budding from the plasma membrane, MVs tend to be enriched in a different repertoire of proteins as compared to those of exosomes, including integrins, glycoprotein Ib (GPIb), and P-selectin (Heijnen et al. 1999). Compared to exosomes, MVs carry more proteins with post-translational modifications, such as glycoproteins or phosphoproteins (Palmisano et al. 2012). Noteworthy, all these proteins, especially tetraspanins, ESCT proteins, Alix, TSG101,and HSPs, are commonly used as general EV markers (Théry et al. 2001). In contrast, proteins contained within mitochondria (e.g., aconitase), the Golgi apparatus (e.g., GM130), the endoplasmic reticulum (e.g., calreticulin) and some cytoplasmic proteins (e.g., α-tubulin) have been reported to be depleted in EVs isolated by differential centrifugation (Sinha et al. 2014). Therefore, the negativity of EV samples for these markers confirm the purity of EV preparations as long as no cell stress and/or death was induced. The protein composition of exosomes and MVs shows a substantial overlap, although some proteins are more enriched in one than in other EV subtypes (Palmisano et al. 2012). Although it is unclear whether this overlap may be due to the applied isolation techniques - which currently do not allow the complete separation of EV subtypes - the separation of EV fractions by sucrose gradient centrifugation indicates a reduced content - but not the complete absence - of glycoprotein 1b alpha in exosomes when compared with MVs (Heijnen et al. 1999). Up to date there is no marker that permits to univocally distinguish one subtype of vesicle from the another. Proteins transferred by EVs are mostly represented by nucleic acid binding proteins (Sinha et al. 2014) - given the abundant RNA and DNA present in these vesicles - transcription factors, receptors, enzymes. Moreover, EVs were found to elicit functional responses by activating receptors on recipient cells. This was observed for the first time *in vitro* with the discovery that human and murine B cell released EVs containing MHC-II, thus acting as antigen-presenting vesicles able to induce antigen-specific MHC class II-restricted T cell responses (Raposo et al. 1996). Finally, since the profile of EV proteins reflect the status of the parental cells, they may also assume a relevant significance as biomarkers for a wide range of diseases (Colombo et al. 2012; Im et al. 2014).

5. Therapeutic perspectives of MSCs and their derived EVs in Alzheimer's Disease

As described before, recent studies have indicated a role for immune system in AD pathogenesis (Heneka et al. 2015; Villegas-Llerena et al. 2016). The recent identification - by genome wide association studies (GWAS) - of new variants of microglial genes associated with the increased risk of late-onset AD (LOAD) had shed the light on neuroinflammation as a factor driving pathogenetic cascade in AD (Karch and Goate 2015)(par. 2.5) Given the well-known plasticity, migration ability toward damaged tissues and immunomodulatory abilities, MSCs are remarkable cells in that could be exploited to target inflammatory processes in AD.

Kim and colleagues investigated the role of human adipose-derived MSCs (ASCs) in a Tg2576 AD model to elucidate the preventive and therapeutic potential of stem cells in AD (Kim et al. 2012), by evaluating the cognitive ability (behavioural test) and the neuropathology (molecular study). Cells were intravenously injected in 3-month-old mice biweekly for 7 months to evaluate the ability of hASCs to prevent or delay the onset of disease, or bilaterally transplanted (one injection) into the dentate gyrus of the hippocampus of older mice (11-month-old) in order to examine the therapeutic potential of stem cell treatment when disease has already progressed. The authors found that in both the experimental paradigms, hASCs showed therapeutic or preventive potentials rescuing cognitive impairments, reducing protein levels of Aβ peptides and upregulating IL-10 and neurotrophic factors in the brain of treated mice. Interestingly, intravenously injected MSCs were able to pass the BBB and migrate into the brain. Moreover, animals of the treated group evidenced increased neurogenesis and dendritic stability in the hippocampus. The ability of stem cells to pass the BBB was also confirmed by Naaldijk and colleagues who evaluated the effects of intravenous infusion of mouse BM-MSCs in 12-15-month APP/PS1 transgenic mice (Naaldijk et al. 2016). MSCs were found around Aβ plaques 28 days after infusion, suggesting the important propensity of MSCs to home inflamed tissue. Although no transplant-related changes in total amyloid-β were observed, the MSC-treated group displayed an increase in the number of hippocampal small size plaques, but decrease in medium size plaques, compared to control group. MSC treatment reduced microglial number and size in cortex as well as decreased levels of pro-inflammatory mediators TNF-α and MCP-1 in cortex and hippocampus, and IL-6 only in hippocampus. These results suggest that MSCs may exert their therapeutic action in AD through the modulation of neuroinflammation, mainly altering microglial cell functionality. Importantly, in contrast with experimental paradigm of Kim and colleagues, the beneficial effects in this model were obtained by a single administration of MSCs in mice tail vein.

MSCs have been also described to prevent accumulation of Aβ plaques, by inducing the rapid clearance of amyloid aggregates in an acutely induced AD model obtained by injecting Aβ into the dentate gyrus (DG) of the hippocampus of C57BL/6 mice (Lee et al. 2009). The authors found that bilaterally transplantation of MSCs in the DG of mice after Aβ injection was able to promote microglia activation favouring the clearance of Aβ deposits. The ability of MSCs to polarize microglia toward a phagocytic phenotype has important

implications, since increased levels of Aβ plaques have been demonstrated to result in ROS generation, as a consequence of impairment in mitochondrial function, ultimately leading to cellular damage (Sheng et al. 2009). In a study conducted by Yan and colleagues, ADSCs were found to exert profound anti-oxidative effects after transplantation into the hippocampi of 8 months APP/PS1 AD mice (Yan et al. 2014). Moreover, MSC treatment was able to increase hippocampal neurogenesis in the subgranular zone of the hippocampus - as evidenced by the higher number of $BrdU^+$ newborn cells in the dentate gyrus of ADSC-transplanted mice compared to controls - and alleviated cognitive impairments. These studies suggest that a therapeutic approach able to reduce inflammation as well as the oxidative stress may have relevant neuroprotective effect in AD.

Since the beneficial effects of MSCs in several disease models have been mainly attributable to their paracrine actions (par. 3.3.2), considerable attention has been paid to investigate the role of **EVs** as active therapeutic factors secreted by these cells. The administration of stem cell-derived EVs offers several advantages over their cellular counterparts. First, EVs appear to be safer than cells: as they do not contain nuclei, they cannot self-replicate avoiding any risk connected to tumorigenic growth. Moreover, due to their smaller size, they are easier to handle and should rise fewer administration troubles, such as obstruction of small vessels or accumulation into lung or liver. In addition, the small size makes EVs ideal candidates for therapeutic applications against disease of CNS, as they can easily pass the BBB. MSC-EVs have shown encouraging therapeutic effects in a wide spectrum of tissue injuries, such as those in the liver, lungs, kidneys, heart and brain (Rani et al. 2015). In the brain systemic administration of MSC-EVs has been reported to significantly promote neurovascular remodeling and functional recovery in a rat model of stroke (Xin et al. 2013). Cultured neurons treated with exosomes from MSCs exposed to 72 hours post middle cerebral artery occlusion (MCAo), significantly increased the number neurite branches and total neurite length, probably through miR-133b shuttled by EVs to neurons (Xin et al. 2012). Altogether, the evidence suggests that MSC-EVs may represent a potential cell-free therapy for AD.

Katsuda and colleagues showed that human ADSCs secreted exosomes carrying enzymatically active neprilysin (neutral endopeptidase: NEP), a type II membrane-associated metalloendopeptidase, that appears to be the most important protease involved in the proteolysis of Aβ (Iwata et al. 2001). When ADSC-derived exosomes were added to N2a (mouse neuroblastoma cell line) culture, they were incorporated into cells, decreasing both secreted and intracellular Aβ levels (Katsuda et al. 2013). The therapeutic effects of intracerebroventricular injected MSC-EVs in APP/PS1 model of AD has been investigated by Wang and colleagues (Wang et al. 2018). The authors found that injection of EVs into the right ventricle of the brain (once per two days for two weeks) were able to rescue impairment of CA1 synaptic transmission and LTP. The EV treatment also improved cognitive behaviour in APP/PS1 mice while no recovery was observed in

control mice treated with PBS. Then the authors investigated the potential mechanisms underlying the beneficial effects of MSC-derived EVs in mice cognitive function. To do this primary cortical neuron cultures cells prepared from APP/PS1 pups were treated with different concentrations (0.5, 2.0, 5.0 μM) of $A\beta_{1-42}$ and 100 μg/ml EVs for 12 h. The authors found that in cultured primary neurons, iNOS mRNA and protein levels were significantly reduced when treated with MSC-derived EVs, suggesting that amelioration in cognitive behaviour and hippocampal synaptic plasticity of APP/PS1 mice was probably achieved through suppressing iNOS expression.

In another recent study it was assessed and compared for the first time the role of exosomes from normoxic and hypoxic MSCs on learning and memory improvement of 11 month APP/PS1 mice (Cui et al. 2018). Since hypoxia has been demonstrated to increase survival, anti-apoptotic and anti-inflammatory properties of MSCs, the authors hypothesized that this strategy may also improve the therapeutic effects of their derived EVs. Although mice treated with exosomes from normoxic MSCs showed improved learning and memory performance compared to control AD mice, the treatment with exosomes derived from hypoxia preconditioned MSCs exerted a more robust effect. Moreover, the treatment with exosomes from hypoxia-preconditioned MSCs was able to inhibit astrocyte and microglia activation, decrease the level of pro-inflammatory cytokines TNF-α and IL-1β and increase those of the anti-inflammatory cytokines IL-4 and IL-10 in the cortex and hippocampus of AD mice. Importantly, hypoxia preconditioned MSCs enhanced the anti-inflammatory properties of their derived EVs compared to non-preconditioned cells. In addition, the preconditioning revealed to alleviate more efficiently plaque deposition and enhanced synaptic protein expression. To further explore the possible molecular mechanisms of inflammatory responses regulated by exosome injection, the authors focused on the contribution miR-21 that has been shown to control the balance between pro- and anti-inflammatory responses (Garo and Murugaiyan 2016). They found that miR-21 was upregulated in the exosomes derived from preconditioned MSCs and the expression of miR21 in the brain of exosome-injected mice was also higher than that in the control AD mice, suggesting that this miRNA was transferred to the brain tissues after injection. Of note, the injection of miR-21 by lentiviral vector rescued memory deficits in APP/PS1 mice, suggesting the important contribution of this miRNA enriched in EVs derived from hypoxia preconditioned MSCs in dampening inflammation and preventing pathological features.

In a recent study it was evaluated the effects of long-term (10 days) and short-term (48h) hypoxia under different oxygen tension (2%, 5% O_2) on human and porcine bone marrow MSC functional characteristics (Antebi et al. 2018). The authors found that preconditioning with short term 2% hypoxia provided the greatest benefits, as evidenced by the increased cell proliferation and self-renewing capacity, and enhanced MSC anti-inflammatory and neurotrophic potential compared to control MSCs grown in normoxia conditions.

Preconditioning of MSCs with pro-inflammatory cytokines has been also reported to attenuate neuroinflammation by polarizing microglia towards protective M2-phenotype and foster functional recovery in a model of spinal cord injury (SCI) (Ruppert et al. 2018).

In a study evaluating - through miRNA microarray and bioinformatics analysis - how preconditioning may alter miRNA composition of EVs (Zhao et al. 2017), the authors found 62 miRNA (of which 44 were up-regulated and 18 were down-regulated) were significantly differentially expressed miRNAs (DEMs) in EVs derived from IFN-γ preconditioned MSCs compared with non-preconditioned MSC-EVs. Interestingly, many of these miRNA were critical regulators of immunological processes, such as miR-222, that showed the most significant increase and that, according to recent publications, is reported to negatively regulate the release of pro-inflammatory cytokines (Ueda et al. 2009).

In conclusion all these studies provide clear evidence that preconditioning may represent a potent tool to enhance the immunomodulatory properties of MSCs by regulating the release of highly immunoregulatory EVs. In the context of AD, MSC-EVs could be exploited as useful cell-free, natural, non-invasive vehicles for therapeutic delivery of a wide variety of potential disease-modifying molecules, owing to their ability to cross the BBB for horizontal transferring of biologically active molecules.

AIM OF THE STUDY

The aim of this study is to analyse the role of p-MSC-EVs as an appealing therapeutic approach to modulate the neuroinflammatory response in AD.

MSCs were *ex vivo* preconditioned in order to increase their immunomodulatory abilities, that were evaluated through the analysis of the expression of specific immunomodulatory markers. After isolation of an immunocompetent phenotype, we tested the ability of MSC-derived EVs to immunoregulate microglia functions following the next steps:

- In an *in vitro* model of inflammation, we evaluated the ability of MSC-EVs to affect the polarization of microglia - previously switched towards a M1 neurotoxic phenotype - by evaluating the expression of different microglia phenotypic markers and cytokine release;
- In a *in vivo* AD model, we investigated, upon intranasally injection of EVs, their efficacy in counteracting microglia activation (analysis of cell number, morphological changes, expression of phenotypic markers) in specific brain regions and - given the corroborated role of microglia in interacting with synaptic structures - preventing dendritic spine degeneration;

- Finally, in a basic *in vivo* model, we exploited the leech (*Hirudo verbana*) as a paradigm of inflammation (preliminary study) to investigate the effect of MSC-EVs on inflammatory processes specifically driven by microglia cells.

Our preclinical results designate MSC-EVs as a promising therapeutic tool, given their anti-inflammatory and neuroprotective effects. We hope that these data could support the use of MSC-EVs in AD, with the aim to develop a safer, less immunogenic and more specific cell-free therapy.

MATERIALS AND METHODS

1. *In vitro* studies

1.1 Isolation and culture of human Bone Marrow-derived Mesenchymal Stem Cells -

Human Bone Marrow-derived Mesenchymal Stem Cells (hBM-MSCs) were provided by the research group of prof. G. D'Amico from Fondazione Tettamanti [Monza (MB), IT)].

Bone marrow mononuclear cells (BM-MNCs) were isolated from the washouts of sealed healthy donors bone marrow collection bags and filters by Ficoll gradient separation, as previously described by P. Vinci and colleagues (Vinci et al. 2017). BM-MNCs were then seeded (160.000/cm^2) in low glucose (1 g/L) Dulbecco's Modified Eagle Medium (DMEM; *Lonza, Basel, CH*), 10% heat-inactivated fetal bovine serum (FBS; *Biosera, Al Barsha South, Dubai UAE*), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM), all from *Lonza*. Cells were incubated at 37°C with 95% humidity and 5% CO_2. After 48 hours, non-adherent cells were removed from cell culture by washing with phosphate buffered saline [PBS1x, *Euroclone, Pero (MI), IT*] and fresh medium was replaced. When 70-80% confluence was achieved, adherent cells were harvested using 0.05% trypsin-EDTA [372 mg/L ethylenediaminetetraacetic acid (EDTA), 500 mg/L Trypsin; *Euroclone*]. Then, the complete medium was added to stop the trypsin reaction and the cell suspension centrifuged at 800x g for 10 min. Finally, cells were resuspended in complete medium and cultured [passage 1(P1)]. Human MSC (hMSC) culture was amplified by plating cells at a density of $1x10^3$ cells on vented cap T-182 culture flask (*Euroclone*). For the experiments, cells were used between P4 and P9.

Isolated MSCs have been characterized on the basis of the three minimal criteria established in 2006 by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al. 2006, par.3.3.1) . First, MSCs must be plastic-adherent when maintained in standard culture conditions and incapable of growing without anchorage in a semisolid fluid. Second, MSCs must highly express typical stem cell markers (>80% of CD73, CD90, CD105) while lacking the expression of hematopoietic/endothelial molecules lack (<10% of CD14, CD34, CD45). Third, MSCs must have multipotent potential *in vitro*.

In addition, the cells have been subjected to all quality controls required for clinical use as defined in 2006 by the ISCT (Galipeau et al. 2016). Release criteria include: absence of karyotype alterations, lack of detectable microbial contamination (aerobic or anaerobic bacteria, fungi, and mycoplasma) according to European Pharmacopoeia, cell viability >90%, endotoxin levels in the final product <5 EU/kg. Cell lots were cryopreserved and thawed right before use.

1.2 Determination of MSC surface antigen profile

After four culture passages, BM-MSCs were characterized for the expression of markers, following the ISCT guidelines, by Fluorescence-Activated Cell Sorting (FACS). After trypsinization, cells were centrifuged at 800 rpm for 10 min and resuspended in FACS buffer (PBS1x supplemented with 1% human FBS) for aspecific binding sites saturation. Cell suspension was then collected in FACS tubes (20.000 cells/tube) and centrifuged at 1800 rpm for 5 min, the surnatant was removed and cell pellet was disaggregated and resuspended in 50 μl FACS buffer for staining procedure.

Then MSCs were checked for positive expression of CD105, CD73, CD90, CD54, MHC-I and the lack of expression of CD45, CD34, CD14 and MHC-II using the following antibodies according to the product datasheet:

- R-phycoerythrin (PE)-conjugated mouse anti-human CD14 (*eBioscience, San Diego, CA, USA*), for labeling of monocytes/macrophages and neutrophils;
- PE-conjugated mouse anti-human CD90 (*eBioscience*), MSC marker, but also expressed on thymocytes and neurons;
- PE-conjugated mouse anti-human CD105 (*eBioscience*), MSC marker, also labeling vascular endothelial cells and activated macrophages;
- PE-conjugated mouse anti-human CD45 (*BD Pharmingen, San Jose, CA, USA*), for labeling of lymphocytes, monocytes, granulocytes, eosinophils;
- Allophycocyanin (APC)-conjugated mouse anti-human CD54 (*BD Pharmingen*), for labeling of endothelial cells and staining of resting (weak) and activated (moderate) lymphocytes and monocytes.
- PE-conjugated mouse anti-human CD73 (*BD Pharmingen*), MSC marker, but also expressed on subsets of T and B lymphocytes.
- Fluorescein isothiocyanate (FITC)-conjugated mouse anti-human MHC-I (*BD Pharmingen*), for labeling of dendritic cells;
- PE-conjugated mouse anti-human MHC-II (*BD Pharmingen*), for labeling of B cells, dendritic cells, monocytes, macrophages, and thymic epithelial cells.
- PE-conjugated mouse anti-human CD34 (*BD Biosciences, San Jose, CA, USA*), for labeling of hematopoietic progenitor cells.

Samples were acquired using a BD FACS Canto flow cytometer (*BD Biosciences*), and data were analysed with FlowJo 7.5.5 (*Tree Star, Inc., Ashland, OR, USA*) and BD FACSDIVA™ (BD Biosciences). Quadrant markers were set accordingly to unstained controls.

1.3 *Ex vivo* preconditioning and characterization of MSCs

In order to isolate/select a highly immunocompetent MSC phenotype for *in vitro* and *in vivo* experiments, cells were plated in 60 mm petri dishes (*Falcon, New York, USA*) at a density of 1000 cells/cm^2. After reaching 90% confluence, MSCs were rinsed two times with sterilized PBS1x, to remove any serum contaminants, especially bovine serum-derived EVs.

The cells underwent two *ex vivo* experimental preconditioning paradigms:

- Oxygen Deprivation (OD) + Re-Oxygenation (or recovery; OD+R) preconditioning.
- Pro-inflammatory cytokine (PC) preconditioning.

For exposure to OD MSCs were incubated in serum free low glucose Dulbecco's Modified Eagle Medium (SF-DMEM) in a hypoxia chamber (*Billups–Rothenberg, Del Mar, CA*) saturated for 10 min with 5% CO_2: 95% N2 and sealed at 37°C for 3 hours (3h OD) or 16 hours (O/N OD). After OD, cells were supplemented with fresh serum-free (SF) medium and maintained in normal culture conditions for the reestablishment of normoxia (37°C in a 5% CO_2 atmosphere) for different times of re-oxygenation: 1 hour (R 1h) or 24 hours (R 24h).

For preconditioning with pro-inflammatory cytokines MSCs were cultured in SF DMEM for 24 or 48 hours with or without the addition of TNF-α (20 ng/ml) and IFN-γ (25ng/ml) (all from *Peprotech, Rocky Hill, New Jersey, USA*).

For both types of preconditioning protocols control MSCs were maintained in complete DMEM in normal culture conditions (37°C in a 5% CO_2 atmosphere) for the same period. Differentiation potential of preconditioned MSC (pMSC) was assessed by evaluating their ability to be committed towards osteogenic and adipogenic lineages, after administration of a specific differentiation medium. Stemness of pMSCs was investigated by Western Blot (WB) by evaluating the expression of CD73 and CD90 markers.

1.4 Multilineage differentiation of cytokine-preconditioned MSCs

Preconditioned MSCs were plated in 6-multiwell plates either in control or differentiation medium. Control medium consisted of complete medium as described above (par. 1.1).

Osteogenic differentiation medium consisted of control medium supplemented with 100 nM dexamethasone, 10 mM sodium β-glycerophosphate, 0.05 mM ascorbic acid (all *from Sigma-Aldrich, St. Louis, MO, USA*).

Adipogenic differentiation medium consisted of control medium [high glucose (4.5 g/L) instead of low glucose (1g/L) DMEM] supplemented with 1 µM dexamethasone, 0.5 mM indomethacin, 10 µg/ml insulin and 100 mM 3-isobutyl-1-methylxanthine (IBMX) (all from *Sigma-Aldrich*).

Cells were cultured in osteogenic differentiation medium for 31 days and in adipogenic differentiation medium for 21 days, with the medium being changed twice a week.

The occurred adipogenic and osteogenic differentiation were analyzed by visualization of fat aggregates (lipid droplets) and mineralized materials (calcium deposits) in cultured cells by staining with Oil Red and Alizarin Red (all from *Sigma-Aldrich*), respectively.

1.5 Isolation of preconditioned MSC-EVs

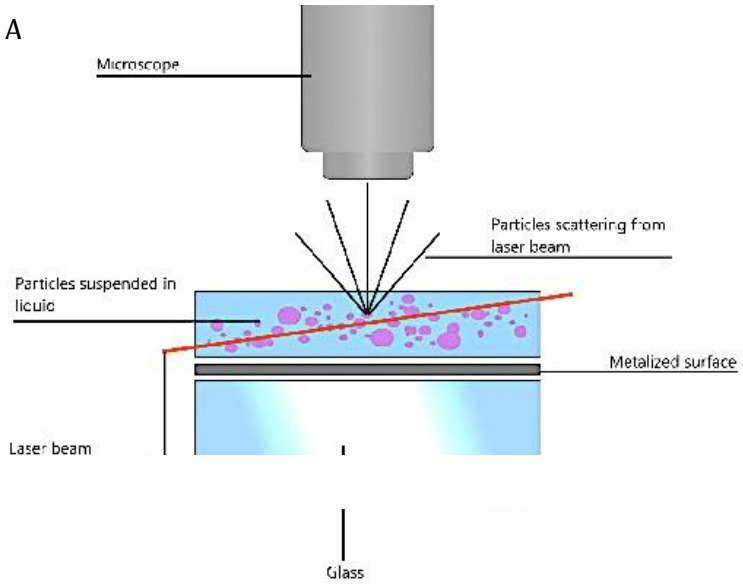
MSC conditioned medium was collected and subjected to a series of centrifugations at 4°C according to a modification of the widely used protocol by Théry and colleagues (Théry et al. 2006), in order to isolate EVs (exosomes and microvesicles). Briefly, the medium was subjected to the following steps :

- 1000x *g* for 10 minutes, to remove death cells;
- 2000x *g* for 20 minutes to discard cell debris;
- 110.000x *g* for 70 minutes to separate EVs (pellet) from supernatant (soluble factors).

In order to better discard any soluble factor contaminant and reach a high purity of EV sample, the pellet was resuspended in PBS1x (Lonza) and recentrifuged at 110,000x *g* for 70 minutes (wash passage).
Finally EVs were resuspended in Minimal Essential Medium (MEM)1x (*Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA*) and PBS1x for *in vitro* and *in vivo* experiments, respectively.

1.6 Quantification of preconditioned MSC-EVs

Quantification of EVs was performed by determination of their protein contents by means of bicinchoninic acid assay (microBCA assay) (*Thermo Fisher Scientific*).
In order to further characterize the vesicles, isolated EVs were diluted in 1 ml of sterile PBS1x for the analysis of size distribution and concentration by NanoSight NS300 instrument (*Malvern, Worcestershire, UK*), which exploits nanoparticle-tracking analysis (NTA) as method for the visualization and analysis of EVs. Briefly, this method takes advantage of two different principles of physics. First, the Brownian motion - which is the random motion of particles present in a liquid suspension - according to which the diffusion of different particles in a fluid is inversely proportional to their size. Moreover, temperature and viscosity of the liquid influence the motion of the particles. Second, the light scattering, a phenomenon by which light (photons) changes direction after incidence on an object. In Nanosight instrument (Fig. 1A), a laser beam is passed through the sample chamber and hits EVs in suspension along its path. Scattered light is visualized by a 20x magnification microscope onto which is mounted a video camera which captures a video file of vesicles moving in stochastical way.



B

$$D = \frac{RT}{N_A} \frac{1}{6\pi\eta a}$$

Figure 1. **A**. Schematic visualization of Nanoparticle Tracking Analysis (NTA) (*Malvern*); **B**. Stokes-Einstein equation for the derivation of the diffusion coefficient (D) of a spherical particle in a liquid suspension (D: Diffusion coefficient; T: Absolute temperature; N: Avogadro's number; R: Gas constant; η: viscosity of diffusion medium; a: radius of the particle).

The Nanoparticle Tracking Analysis (NTA) software tracks many EVs individually and generates digital images of scattered light from single EV (even if aggregation events cannot be ruled out), providing data regarding their speed. Therefore, by using Stokes-Einstein equation (Fig. 1B), the software calculates EV hydrodynamic diameters, determining size distribution.

Total EV count is derived from the analysis of tracked particles captured in each video file.

In addition, NTA measures the peak intensity (i.e. sum of all pixel values within a particle image) exhibited by the particles during the time they are being tracked. This allows the relative light scattering intensity of the particle to be plotted against the estimated particle size. These independent but simultaneous measurements allow to differentiate each particle from the other based on the different refractive index. However, two particles scattering light in a similar manner can also be discriminated because of different diffusion coefficients (e.g. diameters).

In our study EVs were tracked using the following parameters:

- camera type: sCMOS;
- camera level: 15; FPS: 25;
- detect threshold: 5;
- temperature: 23.9-24.1 °C;
- dilution factor: 1:100;
- viscosity (water): 0.908-0.912 cP (centipoise);
- 3 videos per each analyzed sample.

1.7 Mixed glial culture preparation and microglia isolation

Primary cultures of microglial cells were prepared from cortices and hippocampi of postnatal 1/3 day old C57BL/6 mice, which were provided from Dr. C. Elia from the group of Prof. M. Matteoli [Humanitas University, Rozzano (MI)]. After the decapitation of newborn mouse pups, the heads were placed into a 60 mm Petri dish containing cold and sterile dissection media [Hank's Balanced Salt Solution (HBSS) (*Gibco, Thermo Fisher Scientific*), supplemented with Hepes 0.3 M (*Sigma-Aldrich*), penicillin (100 U/mL), streptomycin (100 μg/mL), both from *Euroclone*]. The scalp was opened by using fine scissors to cut along the midline starting posteriorly and ending near the snout. By putting one sharp tip of the scissors beneath the skull at the posterior end of the brain, the skull was cut proceeding toward the anterior part of the brain. Therefore, the brain was scooped out and immersed in cold dissection media in a new Petri dish.

Cortices and hippocampi were isolated from brainstem, striatum and substantia nigra and meninges were eliminated. Then brain tissues were crumbled with a scalpel for mechanical digestion. Fine mechanical digestion was also performed by means of a pipette tip after collecting brain tissue pieces in a conical centrifuge tube. Both tissue isolation and mechanical digestion were performed at 4°C in order to minimize tissue protease activation. The obtained cell suspension was filtered with a 70 μm nytex membrane (*Falcon*) and harvested in a new centrifuge tube to be centrifuged 10 min at 800x g at RT. Then the supernatant was removed and a second centrifugation (10 min at 800x g at RT) was performed after resuspension of cell pellet in complete glial medium [(Eagle's minimal essential medium (MEM, *Gibco, Thermo Fisher Scientific*) supplemented with 20% inactivated FBS (*Sigma-Aldrich*), 33 mM Glucose (*Sigma-Aldrich*), 2mM L-ultra glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin, all from *Lonza*].

Finally, cells were seeded into poly-L-lysine (0.02 mg/ml, *Sigma-Aldrich*) pre-coated (2 hours at 37 °C) vented cap T-75 culture flask (*Euroclone*) in complete glial medium and incubated at 37°C with 95% humidity and 5% CO_2. For cell plating, we considered the ratio 1 brain: 1 T-75 flask.

After seeding, cells were left grow without changing the medium to let microglial cells to lay on the top of a confluent astrocyte monolayer. After 14 days microglial cells were harvested after shaking the flasks at 230 rpm for 50 min at RT. Then, the medium from each flask was collected in a tube and centrifuged at 800x g for 10 min. In order to promote microglia survival and the development of a ramified phenotype, astrocyte factors coming from the microglial culture medium are required (Butovsky et al. 2014; Bohlen et al. 2017). Based on our observations regarding the conditions that favoured a ramified/spindle-shaped (resting) cellular morphology *in vitro*, we decided to resuspend microglial pellet obtained by centrifugation in a medium consisting of 5/6 glial medium without serum (1/6) and 0.22 μm-filtered astrocyte conditioned medium (1/6). Finally, cells were seeded at about 200.000 cells/well on 24-multiwell plate (*Euroclone*), pre-coated (2 hours at 37 °C) with 0.05 mg/ml poly-Ornithine (*Sigma-Aldrich*).

1.8 Microglia cultures and *in vitro* experimental design

After isolation, microglia were cultured on 24-multiwell plate. In order to drive microglia polarization, cells were plated for 24 hours before being exposed to the proinflammatory stimuli [TNF-α (20 ηg/ml) and IFN-γ (25 ηg/ml), both from *Peprotech*] that have been reported to polarize cells toward the M1 classical activated phenotype (Verderio et al. 2012). 2 and 24 hours after the inflammatory challenge or in control conditions, EVs (4.5 μg/ml) derived from cytokine preconditioned MSCs were administered to microglial cells. Control culture cells were administered with cytokines and/or EVs, were treated with buffer used for cytokine reconstitution and/or MEM used for EV resuspension, respectively. 48 hours after stimulation, culture medium was collected for cytokine release evaluation while cells were lysed with denaturation buffer for western blot (WB) analysis (Fig. 2).

For the analysis of vesicle uptake by primary microglial cells, EVs were stained with the lipophilic fluorescent dye PKH26 (*Sigma-Aldrich*) following the protocol described by van der Vlist and colleagues (van der Vlist et al. 2012). Then labeled EVs were added to microglia, previously stained with Hoechst 33342 dye (*Thermo Fisher Scientific*), according to the experimental paradigm described above. Images were acquired on a Zeiss LSM710 (*Zeiss, Oberkochen, DE*) with a 40x objective.

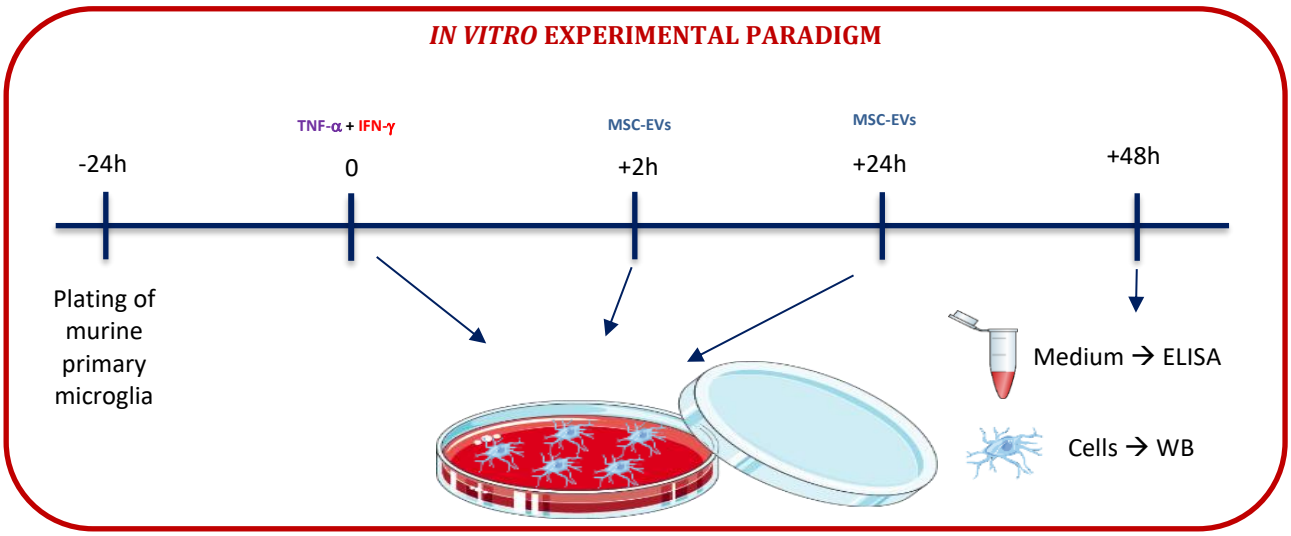


Figure 2. ***In vitro* experimental paradigm**. Primary cultures of microglial cells were prepared from cortices and hippocampi of postnatal 1-3 days old C57BL/6 mice. Microglial cells were plated for 24h before being exposed to pro-inflammatory stimuli [TNF-α (20 ηg/ml) + IFN-γ (25 ηg/ml)], to induce M1 classical activation. EVs (4.5 μg/ml) derived from cytokine-preconditioned MSCs were added twice, after 2h and 24h from the inflammatory challenge. After 48 hours cell medium was collected to perform ELISA on cytokine release and cells were lysed for WB analysis of microglia phenotypic markers.

1.9 Enzyme-Linked Immunosorbent Assay (ELISA)

The pattern of cytokines secretion by microglial cells after the inflammatory challenge with or without EVs (CYT, CYT+EVs) and in control conditions (CTRL, EVs) was estimated 48 hours after the stimulation with pro-inflammatory cytokines. Supernatants were collected and the levels of interleukins IL-1β, IL-6, IL-10 and IL-4 were measured by a commercially available kit of enzyme-linked immunosorbent assay (ELISA, *Peprotech*),

following manufacturer's instructions. In particular, ELISA was performed according to the "sandwich" immunoassay format. This capture assay requires two antibodies specific for different epitopes of the same cytokine. Briefly, one of the antibodies is coated on the surface of the multi-well plate and used as a capture antibody to allow for antigen immobilization. The second, biotinylated, antibody facilitates the detection of the cytokine by interacting with both the antigen and streptavidin, that is conjugated to an enzyme (horseradish peroxidase, HRP). The addition of the appropriate substrate [ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid) diammonium salt] results in an end product which gives rise to a colorimetric reaction (read as absorbance), whose intensity is proportional to the cytokine concentration.

Optical density (absorbance) values were acquired by Fluostar Omega microplate reader (*BMG LABTECH, Offenburg, DE*) and data analysis was performed by interpolating absorbance values on the standard curve (calibration curve) of each cytokine, in order to derive the corresponding concentration value.

1.10 Western blotting

MSCs

After preconditioning with OD+R and CYTO in 60 mm Petri dishes (Falcon), MSCs were rinsed with PBS1x and lysed by adding 100°C previously heated denaturation buffer [(DB: 2% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl (pH 6.8) and protease inhibitor cocktail (all from *Sigma*)]. After 5 minutes adherent cells were scraped off the dish using a plastic cell scraper, collected in a microcentrifuge tube (*Eppendorf, Hamburg, DE*) and heated at 100 °C for 5 min to guarantee protein denaturation. In order to perform SDS-polyacrylamide gel electrophoresis, samples were added with a 4x loading buffer (8% SDS, 4 % 2-mercaptoethanol, 40% glycerol, 0.004% bromophenol blue, 0.2 M Tris-HCl, pH 6.8).

For the analysis of MSC markers we used a 12% Bis-Acrylamide (*Euroclone*) gel (0.375 M Tris-HCl, 0.01% SDS, 0.1% Ammonium persulfate (APS), 0.05% Tetramethylethylenediamine (TEMED), all from *Sigma-Aldrich*). Gel electrophoresis was performed at RT, at 100 V for 1.30h in a running buffer composed by 0.1% SDS in Tris-Glycine 1x. After electrophoresis, proteins were transferred to a nitrocellulose membrane (*GE Healthcare Life Sciences, Buckinghamshire, UK*) in a transfer buffer (0.013% SDS, 20% methanol in Tris-Glycine 1X). Protein transfer was performed at 4°C, at 250 mA for 2.30h.

For the immunostaining, membranes were blocked in TBS-Tween (TBS-T) 0.1% buffer containing 5% bovine serum albumin (BSA, *Sigma-Aldrich*) for CD90 and CD73 phenotypic markers or 5% non-fat milk for COX2 and IDO immunomodulatory markers for 1 hour at RT. After 4°C o/n incubation in agitation with the primary antibodies (all 1:1000 from *Cell Signaling, Danvers, Massachussets, USA*), the nitrocellulose membranes were incubated for 1h at RT, with HRP-conjugated goat anti-rabbit antibody (1:20000, *Thermo Fisher Scientific*). Proteins detection by Enhanced Chemiluminescence (ECL) was performed with long lasting

chemiluminescent substrate LiteAblot Extend (*Euroclone*) and immunoblot bands were analysed and semi-quantified by ImageQuant™ LAS 4000 (*GE Healthcare Life Sciences*).

Microglia

Microglia markers were investigated by the following primary antibodies: rabbit monoclonal anti-Iba-1 (1:1000, *Wako, Mountain View, CA, USA*), rabbit polyclonal anti-iNOS (1:200, *Santa Cruz Biotechnology, Dallas, Texas, USA*), rat monoclonal anti-CD68 (1:400, *Biorad, Hercules, CA, USA*), rat monoclonal anti- CD206 (1:400, *Biorad, Hercules, CA, USA*).

Cell lysis, SDS-polyacrylamide gel electrophoresis - carried on by using an 8% Bis-Acrylamide gel - protein membrane transfer and ECL were conducted, as described for MSCs.

CD68 and CD206 microglia markers were investigated in non-reducing conditions, as specified by manufacturer's instructions. At this aim, protein samples were prepared in loading buffer (see above), without 2-mercaptoethanol.

Blocking and antibody probing occurred in TBS-T 0.1% buffer containing 5% non-fat milk, as for MSC's. Secondary anti-rat antibodies (1:5000, *Genetex, Irvine, CA, USA*) were used for detection of rat anti-mouse CD68 and CD206 antibodies.

Extracellular vesicles

After isolation by ultracentrifugation, EVs were lysed in 20 μl DB and prepared according to NuPage™ Bis-Tris Protein Gel (*Thermo Fisher Scientific*) guidelines for reduced sample preparation. Specifically, protein samples were prepared with a loading buffer (1x NuPAGE LDS sample buffer, 1x NuPAGE Reducing Agent in deionized water).

For gel electrophoresis (RT, 200 V, 50 min), NuPAGE MOPS 1x in deionized water was used and proteins were then transferred to a nitrocellulose membrane (4°C, 250 mA, 2.30h).

Therefore, nitrocellulose membranes were blocked in TBS-T 0.1% buffer containing 5% BSA (*Sigma-Aldrich*). Then, CD63, CD9, CD81 rabbit anti-human primary antibodies (1:1000, *System Biosciences, Palo Alto, CA, USA*) and secondary anti-rabbit antibodies were probed in the same buffer. Finally, membranes were subjected to ECL using the sensitive chemiluminescent substrate LiteAblot Turbo (*Euroclone*).

Total protein amount was evaluated by means of microBCA (*Sigma-Aldrich*). 30 μg (for MSCs), 20 μg (for microglia and EVs) protein of each sample was subjected to SDS-polyacrylamide gel electrophoresis. Nitrocellulose membrane transferred proteins were revealed by Ponceau staining (*Sigma-Aldrich*).

All the data were normalized to β-actin (1:1000, *Sigma-Aldrich*), except for CD68 and CD206 microglial markers whose expression normalization was carried out with respect to the total amount of proteins detected by Ponceau staining, allowing a straightforward correction for lane-to-lane variation (Li and Shen 2013; Moritz 2017).

2. *In vivo* studies

2.1 Transgenic Mice

In vivo experiments were performed in collaboration with the research group of Prof. M. Buffelli from the University of Verona (VR, IT).

Procedures involving animals and their care were conducted in conformity with the EU guidelines (2010/63/UE) and Italian law (DL 26/14) and were approved by University of Verona ethical committee and local authority veterinary service. For our experiments eight (four for each group) 7-month female AD triple-transgenic (3xTg-AD) mice expressing three mutant human transgenes - $PS1_{M146V}$, APP_{Swe} and Tau_{P301L} - were purchased from The Jackson Laboratory (*Sacramento, CA, USA*). Although the 3xTg-AD mice were originally derived from a 129/C57BL6 background, genetic analysis showed that our 3xTg-AD mouse colony matched ~80% of the allelic profiles of C57BL/6 mice after ten generations of random mating.

All efforts to minimize animal suffering, as well as to reduce the number of animal involved in this study were made. Animal use was approved by the Italian Ministry of Health, in agreement with the EU Recommendation 2007/526/CE (http://eur-lex.europa.eu/legal).

2.2 *In vivo* experimental design

EVs were resuspended in sterile PBS1x solution at a concentration of 300 μg/mL (approximately $15x10^9$ vesicles). 7-month 3xTg female mice were anaesthetized with isoflurane before being carefully intranasally administered with PBS1x solution or EVs in ~5-μL spurts per nostril. Anaesthesia was performed before every IN administration and each mouse received two injections of 50 μl vehicle (PBS1x) or EVs separated by 18 hours.

After 21 days each mouse was anesthetized using Tribromoethanol drug [(TBE), *Sigma-Aldrich*], and perfused transcardially with 0.1 M PBS1x followed by paraformaldehyde (PFA) 10% V/V, buffered 4% W/V (*Titolchimica, RO, IT*). Finally, brains were extracted and left in fixative solution o/n in order to be processed for both immunofluorescence (evaluation of microglia activation by ImageJ software) and Golgi-Cox staining (dendritic spine analysis by Imaris software)(Fig.3). For EV tracking studies, mice, injected with PKH26-labeled EVs, were perfused 6 hours following the second intranasal administration.

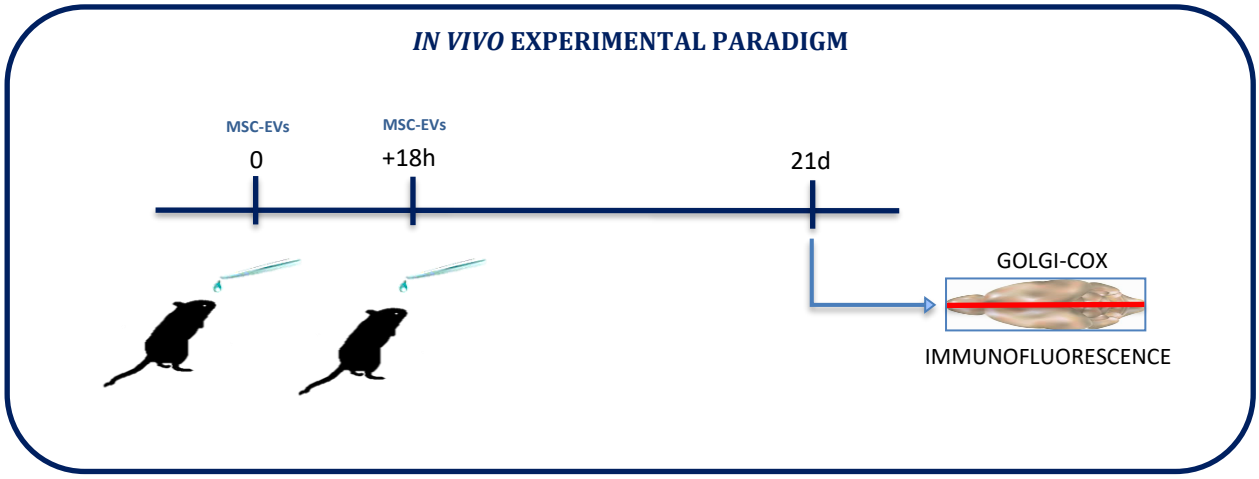


Figure 3. ***In vivo*** **experimental paradigm**. EVs were prepared using sterile PBS1x solution at a concentration of 300 μg/mL (~15x10^9 vesicles). 7-month 3xTg female mice were intranasally (IN) administered with PBS1x solution or PBS1x resuspended EVs in ~5μl- spurts separeted by 5 min via a 10-ul micropipette. Each mouse received two IN injections of 50 μl of vehicle (PBS1x) or EVs separated by 18h. After 21 days mice were transcardially perfused with saline and 4% PFA. Then brains were removed and processed half for immunofluorescence (microglia activation) and half for Golgi-Cox (dendritic spine).

2.3 EV labeling for tracking studies

For EV *in vivo* tracking in the brain, MSCs were labeled with PKH26 (MINI26; *Sigma-Aldrich*) according to the procedures for general cell membrane labeling, with some modifications.

Briefly, 7 x 10^6 single cells were trypsinized and placed into a conical polypropylene centrifuge tube. Cells were pelleted at 800 rpm for 10 min at RT and resuspended in 1 ml of Diluent C (*Sigma-Aldrich*) by gently pipetting to ensure complete dispersion. Immediately prior to staining, a 2x dye solution (4x10^{-6} M) was prepared by adding 4 μl of the PKH26 ethanolic dye solution to 1 ml of Diluent C in a new polypropylene centrifuge tube. Then, the 2x dye solution was added to the 2x Diluent-C cell suspension and rapidly mixed by pipetting for 5 minutes. In order to stop the staining reaction, 2 ml of FBS were added for 1 minute to cell/dye suspension, allowing the aspecific binding of excess dye. Finally, the cells were pelleted at 800 rpm for 10 minutes and subjected to three wash passages in complete MSC medium, changing the polypropylene centrifuge tube at each passage to remove any further excess of the unbound dye. Therefore, MSCs were plated and let grow for three days until they were subjected for 48 hours to cytokine preconditioning protocol in order to isolate PKH26-labeled EV for the *in vivo* tracking studies.

2.4 Immunofluorescence

Immunofluorescence was performed on 40 μm coronal sections of prefrontal cortex (from + 2.40 mm to 2.80 mm Bregma), CA1 region of medial hippocampus (from -1.955 mm to - 2.355 mm Bregma) and entorhinal cortex (from -2.80 to -3.30 Bregma). Slices were treated for 30 min with a blocking solution [3% BSA (*Sigma-Aldrich*) and 0.3% Triton X-100 (*Sigma-Aldrich*) in PBS1x]. Then, the slices were incubated o/n at 4°C with the

following primary antibodies: rabbit monoclonal anti-mouse Iba-1 (1:500, *Wako*), rat monoclonal anti-mouse CD68 (1:200, *Biorad)*, rat monoclonal anti-mouse CD206 (1:200, *Biorad*).

For EV tracking studies monoclonal anti-mouse GFAP (1:1000, *Invitrogen*), anti-mouse NeuN [neuronal nuclear antigen, 1:500, *Chemicon*, *Temecula, CA, USA*)] and anti-mouse Iba-1 primary antibodies were used to investigate the internalization of PKH26-labelled EVs in astrocytes, neurons and microglia. Appropriate negative controls were run without the primary antibodies and none non-specific fluorescent signal was observed after immunofluorescence reactions. Then, after 3 washes in PBS1x, slices were incubated at RT for 2 h with the following fluorophore-conjugated secondary antibodies: Alexa Fluor 594 anti-rat and Alexa Fluor 488 anti-rabbit secondary antibodies (1:1000, all from *Invitrogen, Carlsbad, California, USA*). Finally, after incubation for 5 min with 4',6-Diamidino-2-Phenylindole, Dihydrochloride [(DAPI), 1:2000, *Sigma-Aldrich*] PBS1x solution, slices were mounted on Xtra slides (*Leica Biosystems, WetzLar, DE)* using Para-Phenylenediamine (PPD) and covered with cover glass.

Samples were acquired using a confocal laser scan microscope (*Sp5, Leica*). Fluorescent images were derived by Z-stack projections (maximum intensity) of sections obtained with the open source software for image processing ImageJ [(http://rsbweb.nih.gov/ij/), *ImageJ, National Institute of Health, NIH, Bethesda, Maryland, USA*]. Microglia activation was assayed in the hippocampus, entorhinal and prefrontal cortices of control and EV-treated mice, using a 20x or a 63x magnification. 1.4 NA objective immersion oil was used for the analysis of the internalization of PKH26-labelled EVs, in different cell types and to study neuron-microglia interaction.

All the acquisition settings (laser power, photomultiplier gain offsets and pixel dwell time) were kept constant for each single experiment.

2.4.1 Analysis of microglia activation

Microglia activation was investigated by evaluating cell density and the size of cell soma of Iba-1$^+$cells, signal fluorescence intensity of the marker of activation Iba-1, CD68 (M1 marker) and CD206 (M2 marker).

Microglia density was calculated as the total number of Iba-1$^+$ /DAPI positive cells, within the Z-projection acquired for each slice. Specifically, we collected stacks of 50 μm for a total volume of 0.0144 mm^3.

Further, semi-quantitative analysis, aiming at determining microglia cell soma size and expression of M1 and M2 microglia markers, was performed using a specifically designed macro with ImageJ software.

Briefly, cell soma size of Iba-1$^+$ cells was identified by applying an intensity threshold (histogram-based manual intensity selection) that better fitted the total morphology of cells, depending on the overall intensity of the staining. Moreover, since Iba-1 staining gave a higher signal intensity in the cell bodies than in most of the dendritic processes, the application of an intensity threshold, filtered out most of the microglial processes from the analysis, as it has been described by Tynan and colleagues (Tynan et al. 2010).

With this method, only pixels with a staining intensity above a certain value in the intensity range scale (0-255) were counted. After the conversion of each thresholded images into a binary mask, soma was defined by automatic removal (*ERODE COMMAND*) and addition (*DILATE COMAND*) of pixels from the edges in order to exclude all the signals deriving from the dendritic processes. Therefore, the obtained mask was used to calculate the area of Iba-1$^+$ soma using the custom plugin of ImageJ named "*Analysed Particles*" with the following settings: "*size=10-Infinity show=Outlines display summarize stack*".

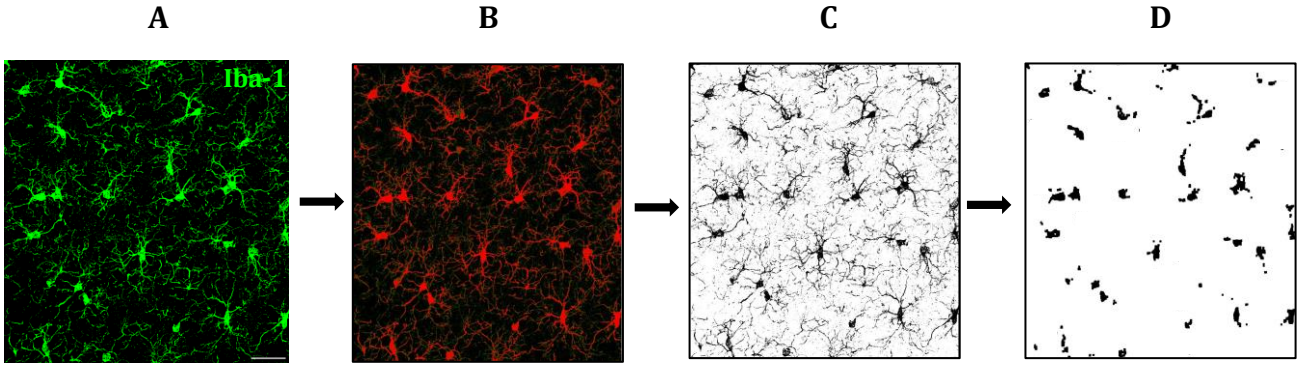


Figure 4. Illustration of semi-automatic digital image processing procedure used to measure the soma size of microglia by Image J software. **A.** A 63x image of Iba-1-positive cells underwent processing through thresholding method in order to tell microglia apart from the background. **B**. The pixels within the manually selected threshold value that better fit the morphology of the cells are displayed in red. **C**. All pixels in the image whose values lie above the threshold are converted to black while all pixel values under the chosen threshold are converted to white, or vice-versa. Hence, a binary mask of the cells is generated. **D**. This binary mask is required for automatic cell soma size analysis. Microglial processes are filtered out from the analysis in virtue of their lower signal intensity compared to the soma and the area of the cell body is derived (Scale bar: 30 μm).

Expression levels of Iba-1, CD68 and CD206 was performed by quantifying their signal derived from the integrated density (ID) in Iba-1$^+$-cells. Since almost no signal was observed for CD68 and CD206 in the processes of microglial cells, pixel intensity analysis was carried only in cell soma, semi-automatically drawn by applying an intensity threshold as described above. The average intensity of each slide was obtained as the ratio of the total integrated density of the signal over the total number of cells.

2.5 Golgi-Cox staining

The day after mouse sacrifice, each half brain was removed from the fixative solution, transferred into an individual small bottle containing 200 ml of Golgi-Cox solution and stored at RT in the dark for 2 weeks.

The Golgi-Cox solution contains (Das et al. 2013; Zaqout and Kaindl 2016):

- 1% Mercury Chloride ($HgCl_2$): Solution A
- 1% Potassium Dichromate ($K_2Cr_2O_7$): Solution B
- 1% Potassium Chromate (K_2CrO_4): Solution C

Briefly, for the preparation of 1 liter of Golgi-Cox solution the impregnation stock solutions (A, B, C) were prepared by dissolving 10 g from of the above-mentioned powders in MilliQ double distilled water (dd-H_2O mq) following these passages:

1. Dissolving 10 g $HgCl_2$ in 200 ml H_2Omq (A);
2. dissolving 10 g $K_2Cr_2O_7$ in 200 ml H_2Omq (B);
3. heating A Solution at 80°C and let it cool;
4. gently pouring B solution into A solution;
5. dissolving 10 g K_2CrO_4 in 600 ml H_2Omq (C);
6. pouring C solution into A-B mixed solution;
7. Filtering the final solution to avoid deposits.

After mixing the three impregnation solutions, the bottle was covered with aluminium foil and kept to settling at RT for at least 24 hours before use to allow precipitate formation.

After two weeks, the brains were removed from Golgi-Cox solution and put in 30% sucrose solution (in PBS1x) for 24 h in order to reduce the tissue fragility during the sectioning procedure (Gibb and Kolb 1998). Then, 100 μm thick slices of hippocampus, prefrontal and entorhinal cortices were collected using vibratome (Leica VT1200, *Wetzlar, DE*) in 6% sucrose solution (in PBS1x) and transferred onto Xtra gelatin-coated slides for the developing step. Therefore, the slices were passed in Kodak Developer and Fixer (*GBX Carestream Dental, Congers, New York, USA*) for 5 and 15 minutes, respectively, and washed in distilled water for 5 min after each step. Finally, slices were dehydrated using increasing concentrations of ethanol (50%-60%-75%-90%). Although it is being reported in several published protocols, we did not perform 100% dehydration, as it induced, at least in some cases, slice fragmentation. Finally, the slices were mounted on Xtra slides (*Leica Biosystems*) using Eukitt (*Sigma-Aldrich*) and covered with cover glass.

2.5.1 Brightfield microscopy and dendritic spine analysis

Images were collected using Olympus BX63 microscope (*Olympus Corporation, Tokyo, J*) and acquired by Neurolucida 64-Bit software (*MBF Bioscience, Williston, North Dakota, USA*). Acquisition of dendritic spines in CA1 region of medial hippocampus, prefrontal and entorhinal cortices occurred at 100x. Images were collected of 117 x 88 μm and analyzed three slices per mice at the same Bregma points, as for immunofluorescence staining. Every stack was acquired using a Z-stack unit of 35 μm. Images were deconvolved through *Autoquan*t software, converted in 8bit images and, then, black signal was inverted to allow the analysis with Imaris image processing software (*Bitplane Software, Belfast, UK*). Dendritic length and the number of neuronal spines were reconstructed by using using *Autopath* system of Imaris (*FILAMENT*

COMMAND) with each single spine detected by the Software being manually checked to avoid false positive signals. To reduce the bias related to different dendrite lengths, the medium spine density for each animal was calculated by dividing the total number of spines with the total length of every dendrite.

2.6 Combined Golgi-Cox and Immunofluorescence technique

In order to better study the spatial relationship between neurons and microglia in the brain of mice treated or not with EVs, we combined Golgi-Cox and immunofluorescence stainings.

This simultaneous Golgi-Cox and immunofluorescence technique has been set-up by M. Pedrazzoli (PhD student in M. Buffelli's Lab from the University of Verona), by modifying a previous published protocol (Spiga et al. 2011). After the perfusion and the o/n fixation in PFA solution, each mouse brain was transferred in Golgi-Cox solution for 2 weeks, following the procedures described in paragraph 2.5.

In this case, we collected 60 µm thick slices of the different brain areas, being this thickness the best compromise for the slice processing with the combined techniques. After the cut, slices were immersed in PBS1x into a 24-multiwell plate in order to be processed with Kodak Developer and Fixer (developing step).

Then, the Golgi-Cox staining was stopped and slices were proceeded with the immunofluorescence procedure (modified by Spiga et al. 2011). Slices were treated for 30 minutes with the blocking solution, composed of 3% BSA (*Sigma-Aldrich*) and 0.3% triton in PBS1x. In order to label microglia cells, the slices were then treated with Iba-1 primary antibody (1:500, *Wako*) for 36 hours at 4°C, in the dark, followed by Alexa 488-conjugated secondary anti-rabbit antibody incubation for 2 hours at RT. Finally, slices were stained with DAPI solution (1:2000, in PBS1x), mounted on Xtra slides (*Leica Biosystems*) using PPD and covered with cover glass.

For image analysis, brightfield signal of Golgi-Cox stained neurons was using Reflectance Confocal Microscopy (RCM) that detects backscattered light from illuminated tissue (Gambichler et al. 2015), allowing the visualization of metallic impregnated tissues. In particular, to detect Golgi-Cox material, the microscope was automatically set up in reflection mode as previously described by Spiga and colleagues (Spiga et al. 2011). Iba-1 microglial signal was captured in canonical fluorescent mode. Imaris software (*Bitplane Software*) was, then, used to 3D rebuilt the cells, through the "*Cell*" function, taking advantage of the automatic threshold in order to reconstruct the whole visible cells.

Both the neuron and the microglial cells were reconstructed using 2 "surface" protocols: one, with higher sensibility, to build the body of the cells; the other with higher threshold for filaments reconstruction. Finally, all the surfaces were enclosed to obtain the 3D image. Figure 5 shows an example of the 3D reconstruction of a neuron (purple) and a microglial cell (green).

By this combined technique, we will investigate the dynamics of neuron-microglia interaction in order to understand the mechanisms underlining the neuroprotective effects of EVs (see *Discussion*).

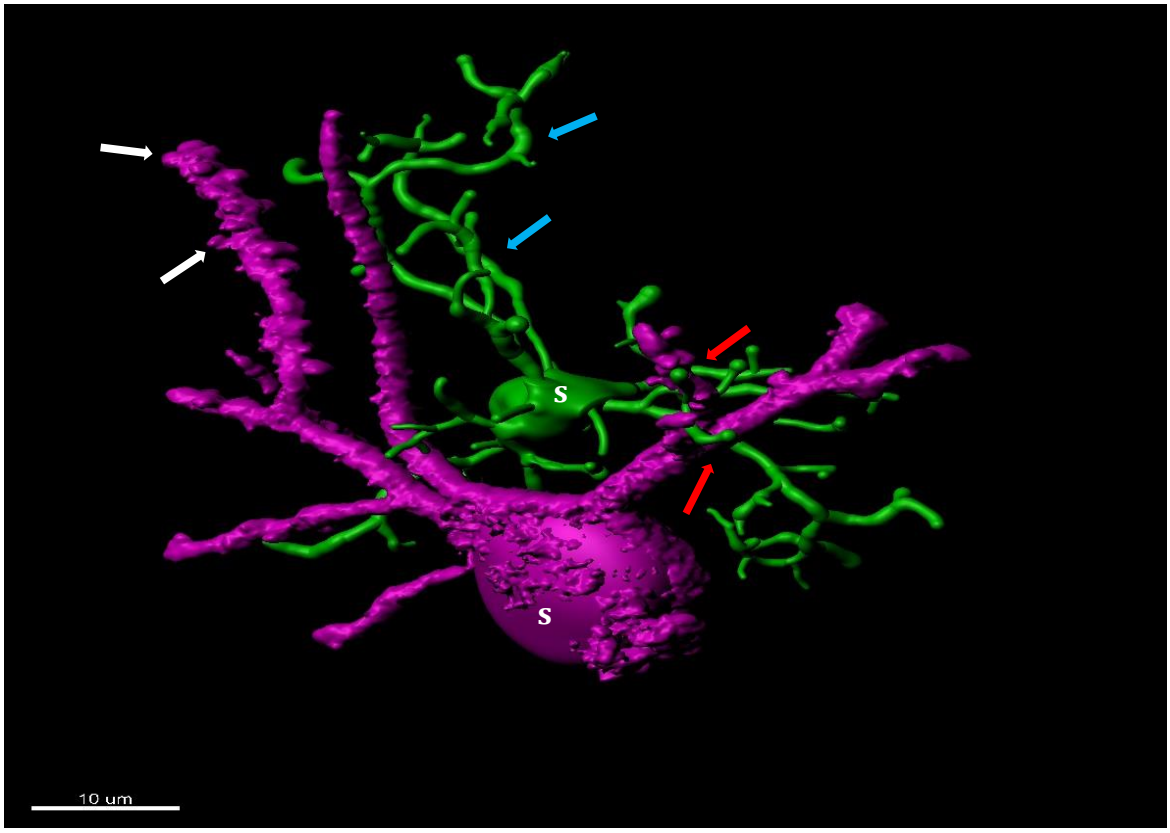


Figure 5. 3D reconstruction performed by Imaris software of a neuron (*purple*, Golgi-Cox staining) and a microglial cell (*green*, stained for Iba-1 by IF) in a hippocampal (CA1) slice processed with the combined Golgi Cox and Immunofluorescence technique. Neuronal dendrites and microglial processes were reconstructed with *FILAMENT TRACER* semi-automatic tool by Imaris-Bitplane. *White arrows*: neuronal dendritic spines; *light blue arrows*: microglial processes; *red arrows*: putative contact points; *s*: cell somata.

2.7 Experimental paradigm in leech (*Hirudo Verbana*)

Medicinal leeches [*Hirudo Verbana* (Annelida, Hirudinea) from Ricarimpex, Eysines, France] measuring 10 cm were kept in tap water at 19–20 °C in aerated tanks and fed weekly with calf blood. Before each experiment, performed in collaboration with the group of Prof. A. Grimaldi from University of Insubria, leeches were anesthetized with a 10% ethanol solution. Animals were divided into 3 separate experimental groups (3 animals for each group):

1) unstimulated leeches, to verify the normal and correct morphological information about the CNS;
2) LPS (100 ng/ml from *Escherichia coli*, serotype O55:B5, *Sigma-Aldrich*) + PBS1x, as control;
3) LPS + preconditioned MSC-EVs;

Given that the leech segmental nerve cord is formed by 21 segmentally homologous ganglia localized in the ventral region, to stimulate the CNS, anesthetized leeches were injected with LPS (100 ng/ml in PBS1x) in the

ventral side at the level of 20th, 40th, 50th, 60th and 80th segmental annuli, starting from the oral sucker (Fig.6). All the injections were performed in the connective tissue surrounding the nerve cord. Since in a study evaluating axon regeneration after nerve lesion, microglia was shown to move within the first minutes after the injury and accumulate at the site of lesion within two hours (McGlade-McCulloh et al. 1989), for our experiments we decided to use the same temporal interval between LPS challenge and EV injection.

Therefore, after 2 hours, leeches were anaesthetized (as described before) and administrated in the same metameric ventral segments with 10 μl of EV-resuspended PBS1x (2 μg of corresponding vesicular protein) derived from 48 h cytokine preconditioned MSCs. Control leeches, were injected with 10 μl PBS1x in each aforementioned point of the chain ganglia. After 24 hours, the treatment with EVs and PBS1x was repeated, to recapitulate the same experimental design utilized for the *in vitro* experiments performed on microglia cells (*Materials and Methods* par. 1.8). In total, each leech (control and EV treated animals) received a volume of 100 μl (50 μl/day) PBS1x. In particular, EV treated leeches received a total amount of 20 μg (10 μg/day) corresponding vesicular protein. After 48 hours from the LPS injection animals of each group were anaesthetized with a 10% ethanol solution and decapitated. Then the ventral segmental nerve cords were dissected out from each leech and fixed for 1 hour at RT in 4% PFA. Each nerve cord was then cut into different pieces, in order to isolate the portions of the connectives subjected to the different treatments. All protocols regarding the use of leeches were carried out in strict accordance with the Italian legislation and European Treaty and were in compliance with the Helsinki Declaration.

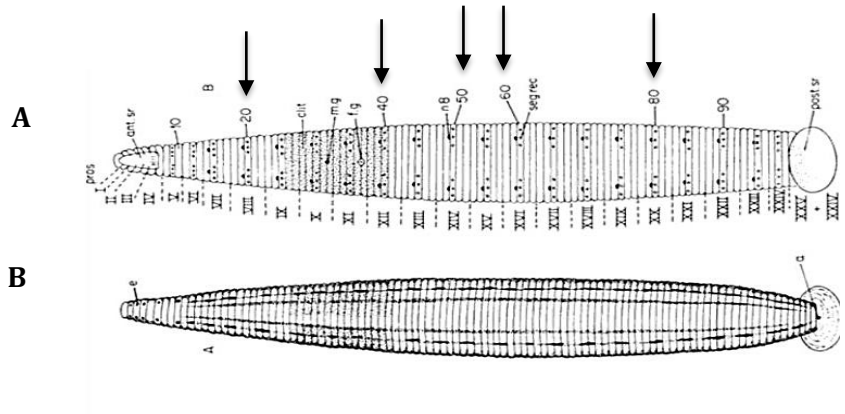


Figure 6. Leech metameric structure. A. Dorsal View. **B**. Ventral view. Segments are numbered in roman numerals, the segmental annuli in arabic. Arrows indicate the points where LPS, PBS and EV injections were performed according to the experimental paradigm described above (Mann 1962).

2.7.1 Whole mount immunochemistry

After fixation in 4% PFA, CNS (chain ganglia) were rinsed several times in PBS1x and permeabilized for 24 hours at 4°C in 0.1M glycine, 1% Triton X-100 in PBS1x. Non-specific background staining was blocked with

saturation buffer (1% Triton X-100, 3% normal donkey serum, 1% BSA and 1% ovalbumin in PBS1X-glycine 0.1M) for 5 hours at 4°C. Samples were then incubated o/n at 4°C with the following primary rabbit polyclonal anti-hirudo antibodies: anti-IL-6 (*Sigma-Aldrich*), anti-IL-10 (*Santa Cruz Biotechnology*), anti-TNF-□ (*Abcam, Cambridge, UK*), anti IL-18 (*Abnova, Taipei, Taiwan*), all diluted 1:250 in saturation buffer. After rinsing with permeabilization solution, samples were incubated for 1 h at 37°C with the secondary anti-rabbit FITC-conjugated antibody (*Abcam*). Nuclei were counterstained with Propidium iodide (PI, 4% in PBS1X) for 3 minutes and slices were mounted with Citifluor (*Citifluor Ltd, London, UK*). In negative control experiments primary antibodies were omitted and sections were incubated only with the secondary antibodies. Immunofluorescence was analyzed using an inverted laser-scanning confocal microscope (*TCS SP5, Leica*) equipped with a HCX PL APO lambda blue 63x 1.40 NA OIL UV objective. Fluorescent images were acquired using the Leica TCS software (emission windows fixed in the 488/525 or 551–626 range), after performing Z-stack projection of sections with the open source image software Fiji (average intensity) (Schindelin et al. 2012).

3. Statistical analysis

Comparison between groups of *in vitro* studies used paired, one-tailed Student's *t* test. Data are presented as mean ± SEM from at least three independent experiments. Data from *in vivo* studies are expressed as mean ± SD and groups were compared using the unpaired, two-tailed Student's *t* test. Differences were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

1. *In vitro* experiments

1.1 Characterization of hBM-derived MSCs

hMSCs derived from bone marrow (BM) of healthy donors were cultured and expanded *in vitro:* MSCs have been selected *in vitro* after isolation of mononuclear cells from the washouts of sealed bone marrow collection bags and filters (Vinci et al. 2017), and cells plated without further separation in culture growth medium (containing 10% serum); *in vitro* characterization occurred according to ISCT minimal definition criteria (Dominici et al. 2006).

Early after isolation, cultures appear morphologically heterogeneous, containing narrow spindle-shaped to large polygonal cells and some slightly cuboidal cells (Javazon et al. 2004). However, after few days after plating, cultured MSCs display a typical spindle-shaped fibroblastoid morphology (Fig. 1). Cells were characterized by adherent growth to plastic culture plates, as suggested by the guidelines for MSC characterization (Dominici et al. 2006).

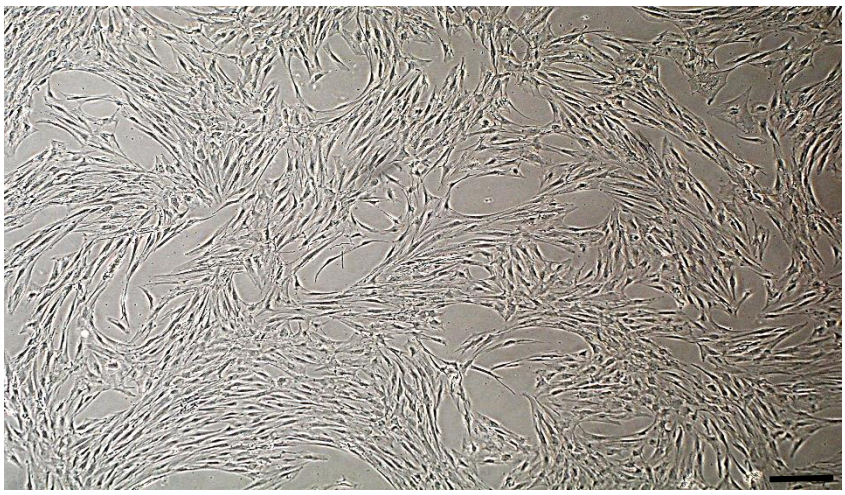


Figure 1. Culture of hMSCs. The phase contrast microscopy image shows plastic-adherent human BM-derived human MSCs (hBM-MSCs) after 5 passages (P5) *in vitro*, cultured in standard conditions. Note the cell typical spindle-shaped morphology. Scale Bar: 100 μm.

Although MSCs phenotypically express a number of markers, none of them, unfortunately, selectively identifies these stem cells. Moreover, any differences in cell surface expression of antigens may be influenced by the donor age, gender, physiological status (healthy vs. patient), tissue source as well as the isolation protocol and culture conditions (Siegel et al. 2013; Mushahary et al. 2017).

However, following the ISCT guidelines, it has been generally agreed that adult human MSCs must express and should lack the expression of specific markers. In order to confirm cell phenotype, the expression of membrane molecules or antigens was assessed by Fluorescence-Activated Cell Sorting (FACS).

Flow cytometric analysis (Fig. 2), performed by the group of Prof. G. D'Amico (Fondazione Tettamanti), evidenced that most of the cells were negative for MHC-II, a complex typically found on the membrane of dendritic cells, monocytes and macrophages, playing a key role in activating adaptive immune response. The absence of MHC-II and co-stimulatory molecules is associated to MSC hypoimmunogenicity (Ryan et al. 2005). Moreover, MSCs did not express hematopoietic-specific antigens CD34, CD14, CD45 (Pittenger et al. 1999) which labels a variety of cells such as hematopoietic progenitor cells, lymphocytes, monocytes, granulocytes and eosinophils.

As expected, MSCs were positive for the typical stem cell markers, such as CD73, CD90, the ultimate stemness marker, and CD105. In particular, as indicated by Dominici and colleagues (Dominici et al. 2006), ≥95% of the cell population expressed CD105, CD73 and CD90 markers. In addition, MSCs were positive for MHC-I and CD54, two markers that have been also used to further characterize these cells (Hass et al. 2011). Interestingly, CD54, also known as intercellular adhesion molecule 1 (ICAM-1), plays an important role in the formation of functional immune synapses mediating an immunosuppressive function on pro-inflammatory macrophages (Espagnolle et al. 2017).

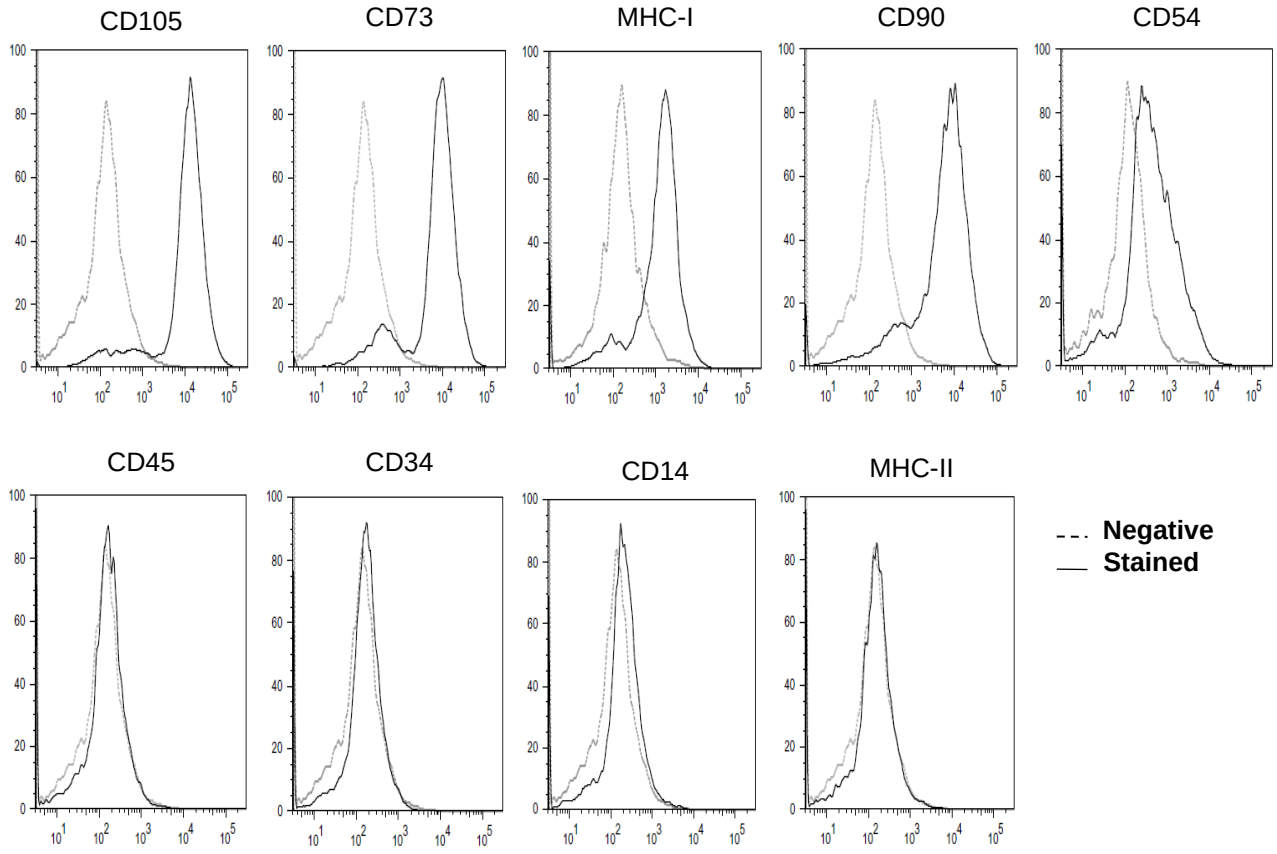


Figure 2. **MSC characterization by flow cytometry (representative histograms)**. MSCs expanded *in vitro* did not express the hematopoietic markers CD45, CD34, CD14 or MHC-II but were positive for CD105, CD73, MHC-I, CD90 and CD54. FACS data were analysed with FlowJo 7.5.5 (*Tree Star, Inc., Ashland, OR*) and BD FACSDIVA™ (*BD Biosciences*).

Therefore, the high expression of CD90, CD73, CD105, essential requisite for staminal ability identification, and the absence of hematopoietic cell antigens provided by the immunological profile through flow cytometry, indicate a high level of purity of our cell culture. In particular, such expression pattern was kept by cells throughout the passages (P4 to P9) used for all the experiments.

1.2 Cytokine preconditioning up-regulates MSC immunomodulatory markers and preserves cell stemness ability

Based on the assumption that preconditioning protocols are key strategies to improve MSC immunomodulatory functions *in vitro* and *in vivo* [(Lan et al. 2015; Noone et al. 2013), see par. 3.3.5 of the *Introduction*], we assessed two different protocols:

1) serum deprivation (SF), in the presence or the absence of a cocktail of cytokines [TNF-α (20 ng/ml) +IFN-γ (25ng/ml)] for 24h or 48h;
2) 3h or 16h (O/N) oxygen deprivation (OD) followed by 1h or 24h of reoxygenation (OD+R);

We monitored the expression of indoleamine 2,3-dioxygenease (IDO) and ciclooxygenase-2 (COX2), as strongly associated to immunocompetence of MSCs (Tu et al. 2010).

Both the *ex vivo* adopted preconditioning protocols did not alter the expression of typical p6 MSC stemness markers (Fig. 3A), thereby fulfilling the ISCT requirements for MSC definition.

Figures 3A1 and 3B1 show the quantification of the immunoblotting bands (shown in Fig. 3A, B) relative to IDO and COX2 expression, after MSC underwent the two preconditioning protocols. In OD experiments, we observed a variable modulation of both COX2 and IDO. COX2 was mostly induced after 16 hours of oxygen deprivation [(O/N OD+1hR, protein expression relative to β-actin ± SEM: COX2: 0.60 ± 0.24; IDO: 1.24 ± 0.25; Fig. 3A1), while IDO exhibited the greatest increment in 3h OD+24hR condition (IDO: 1.44 ± 0.45; COX2: 0.31 ± 0.08; Fig. 3A1). Considering the modulation of both markers in OD, we concluded that the highest overall mean increment was observed in O/N OD+1hR (Fig. 3A1).

With respect to O/N OD+1hR, cytokine stimulation (CYTO) performed for 24h or 48h, induced a more consistent upregulation of both markers (compare the scale of histograms of OD (Fig. 3A1) and CYTO (Fig. 3B1), referring to protein expression relative to β-actin. In particular, the immunomodulatory markers seem to be regulated in a time dependent manner, with COX2 being mainly induced after 24h of cytokine stimulation (24h, COX2: 5.18 ± 2.66; 48h, COX2: 3.97 ± 1.76; Fig. 3B1) and IDO at 48 hours (24h, IDO: 4.23 ± 0.56; 48h, IDO: 11.35 ± 2.10; Fig. 3B1). However, the overall highest increment of the two markers was observed at 48 hours. Therefore, on the basis of the quantification results, we have chosen to use the preconditioning induced by cytokines for 48h, since among those analysed it was found to be the experimental paradigm that induced the highest expression of both markers.

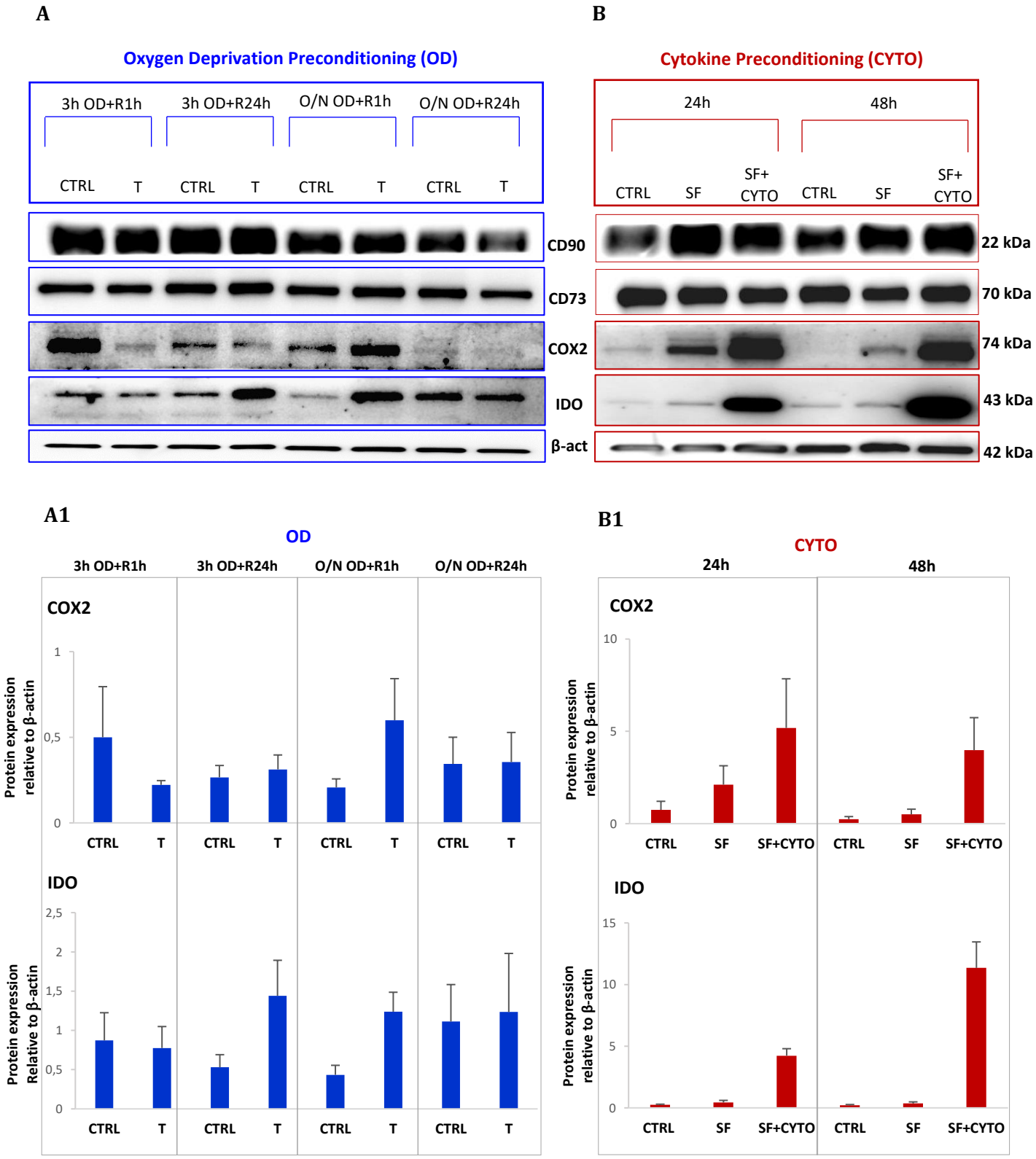


Figure 3. Cytokine preconditioning (CYTO) for 48 hours in a SF-medium causes the highest upregulation of immunomodulatory markers and preserves MSC stemness proteins. A, **B**. Representative WB immunoblotting bands of the typical stemness (CD90 and CD73) and immunosuppressive (COX2, IDO) markers in MSCs (P6) subjected to OD (A) and CYTO (B) preconditionings. **A1, B1.** Immunoblotting histograms relative to the quantification of the immunomodulatory markers in cells subjected to OD (A1) and CYTO (B1) protocols (CTRL: control; SF: serum free; CYTO: cytokines, TNFα and IFNγ; OD: oxygen deprivation; O/N: overnight; R: reperfusion; T: treated). The data are expressed as mean ± SEM (n=3). In some cases, the absence of the correspondence between the bands obtained by WB and their relative quantification (histograms) may be attributable to the high variability of the protein expression observed in primary MSCs underwent to each preconditioning protocol during three independent experiments.

In order to further investigate if CYTO preconditioning may negatively influence the stemness abilities, we evaluated the capability of MSCs to be committed towards adipogenic and osteogenic lineages, when grown a in a medium enriched with specific differentiation inducing factors. As shown in figure 4, cytokine preconditioned MSC cultures showed calcium deposits visualized by Alizarin red, after 31 days of osteogenic induction (Fig.4, O) and intra-cytoplasmic lipid droplets revealed by Oil-Red staining, after 21 days in adipogenic culture medium (Fig. 4, A) - with the first lipid vacuoles becoming evident after 7 days (not shown). As expected, no differentiation was observed in respective MSC control cultures (Fig.4, CTRL-O, CTRL-A), grown in medium without the addition of any differentiation inducing factors.

Altogether these results suggest that cytokine preconditioning for 48 hours drives the highest upregulation of immunomodulatory markers without altering MSC stemness potential.

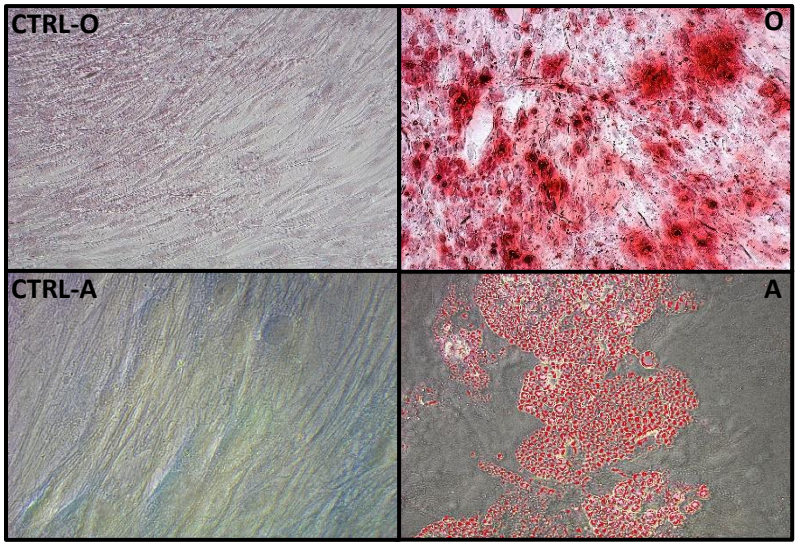


Figure 4. *In vitro* differentiation of cytokine-preconditioned MSCs. MSCs (p6) were committed towards osteogenic (O) and adipogenic (A) lineages. CTRL-O and CTRL-A: controls of MSCs grown in osteogenic (O) and adipogenic (A) inducing differentiation medium. Calcium deposits (O, *in red*) are visualized by Alizarin Red, while fat droplets (A, *in red*) stained by Oil Red, indicating osteocytic and adipocytic differentiation, respectively. Images were acquired by phase contrast microscopy. Magnification 20x.

1.3 Isolation and characterization of preconditioned MSC-derived EVs

As we selected cytokine preconditioning for 48h for *in vitro* and *in vivo* experiments, we then characterized the EVs derived from MSCs subjected to this protocol. After isolation from culture medium of 48-cyto-preconditioned MSCs, EVs were analysed for protein concentration by microBCA assay. The total amount of EVs isolated from ≅ $7*10^6$ cells ranged between 30 and 45 μg corresponding vesicular protein. Additionally, in order to better characterize EVs, nanoparticle tracking analysis (NTA) was performed (Fig. 5A). This analysis allowed us to quantify EV size distribution and particle concentration: the mean size of isolated EVs was 201.1 ± 82.1 nm ±SD; the concentration was $1.01\times10^9 \pm 4.28\times10^7$ particles/ml (Fig. 5B).

The intensity/size distribution (Fig. 5C), derived from three video recordings of the tracked particles for each experiment, shows a major range size (estimated on the basis of light scattered by EVs) from less than 50 nm up to 300 nm. Moreover, the EVs within this range size scattered light predominantly under 1.0 value (a.u.), suggesting the presence of a pure population of EV, without contaminants of different nature, that would exhibit (if present) a distinct pattern of light scattering in virtue of a different refractive index.

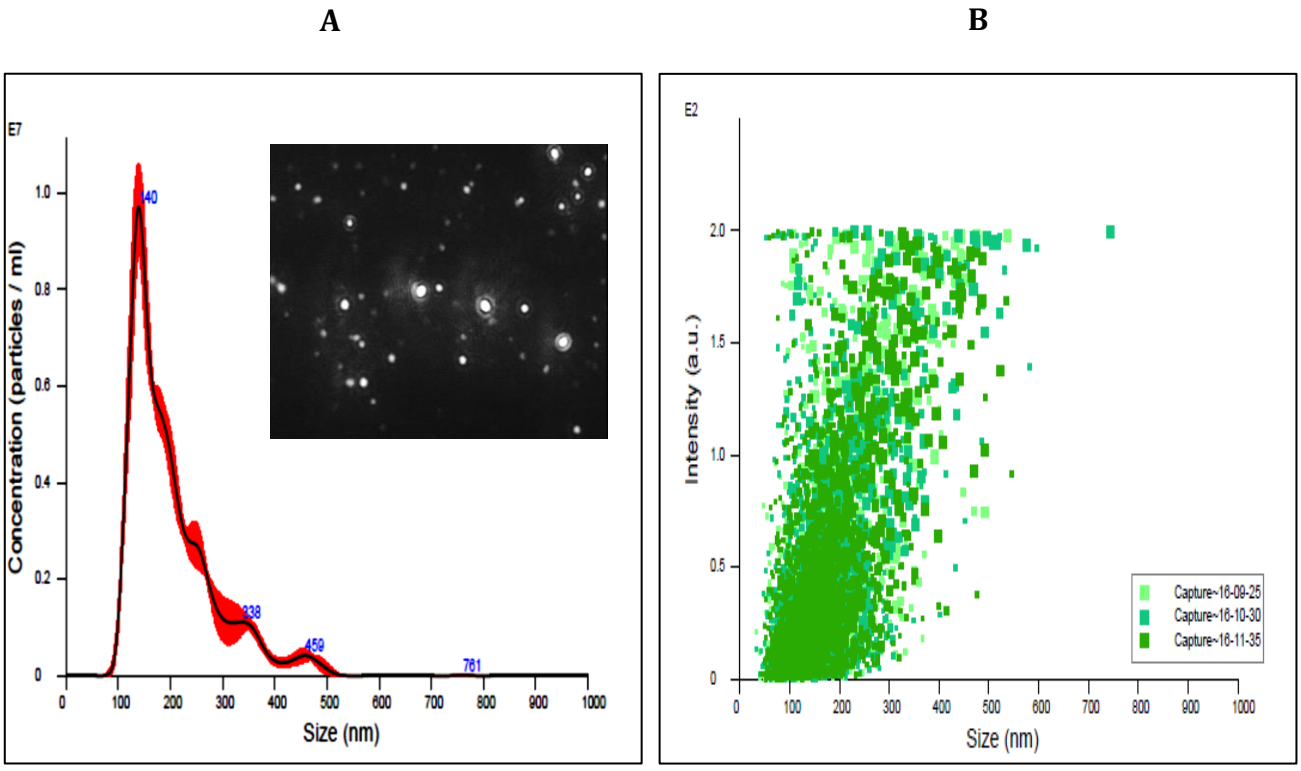


Figure 5. EV nanoparticle tracking analysis (NTA). A. The NTA (NS300 NanoSight instrument) diameter-distribution curve (data are obtained by mean of three tracking video files, for each experiment) shows a range size of 100-500 nm (mean size: 201.1 nm). The picture shows a frame from NTA video, visualizing light scattering EVs derived from cytokine preconditioned MSCs**. B**. 2D intensity/size graph. The graph plot shows the relative intensity of light scattered by EVs plotted against the estimate of EV size, captured during the three video files, each one identified by a different colour green intensity.

EV biochemical features were investigated by Western immunoblot. In particular, we compared CYTO-EVs and SF-EVs (control), respectively derived from 48h serum free (SF) cytokine preconditioned MSCs and MSCs preconditioned in a SF condition only, to evaluate if the inflammatory challenge could alter the expression pattern of their derived typical EV proteins. As shown in the figure 6, CYTO-EVs maintain the positivity for CD9, CD63 and HSP70 markers. Moreover, no signal for the three investigated EV markers was found in the supernatants of the two EV pools, suggesting a high grade of isolation performed by the ultracentrifugation method.

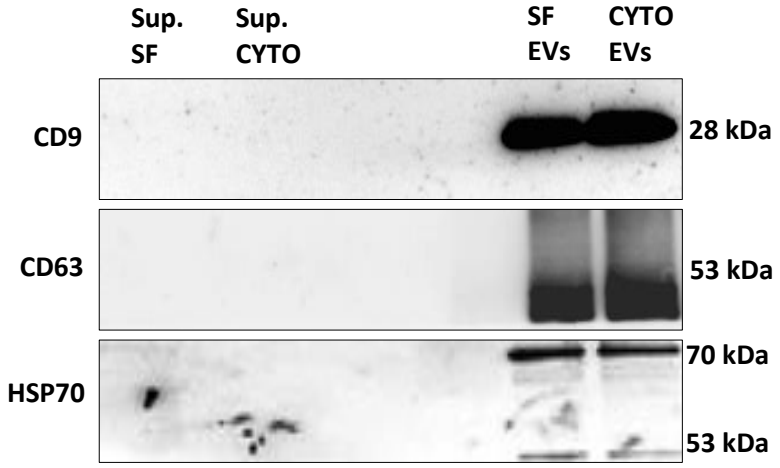


Figure 6. EV biochemical characterization. Expression of EV markers by immunoblotting after serum deprivation (SF) in the presence of cytokines or SF medium alone for 48 hours. EVs were positive for all three typical EVs markers analysed. 20 µg of proteins derived from the EV pellet (EVs SF, EVs CYTO) or respective supernatants (Sup. SF, Sup. CYTO), derived from the last ultracentrifugation before the wash passage (see *Material and Methods* par. 1.5), were loaded into each lane.

Finally, we labeled EVs derived from preconditioned MSCs with PKH26 lipophilic dye to monitor their internalization by primary cultures of microglial cells. A double administration of labeled-EVs for 48h to primary microglia, according to a protocol previously described (van der Vlist et al. 2012), shows a robust uptake of EVs by cells (Fig. 7A), while no signal was detected in control cells treated with PKH26 resuspended in PBS1x (Fig. 7B).

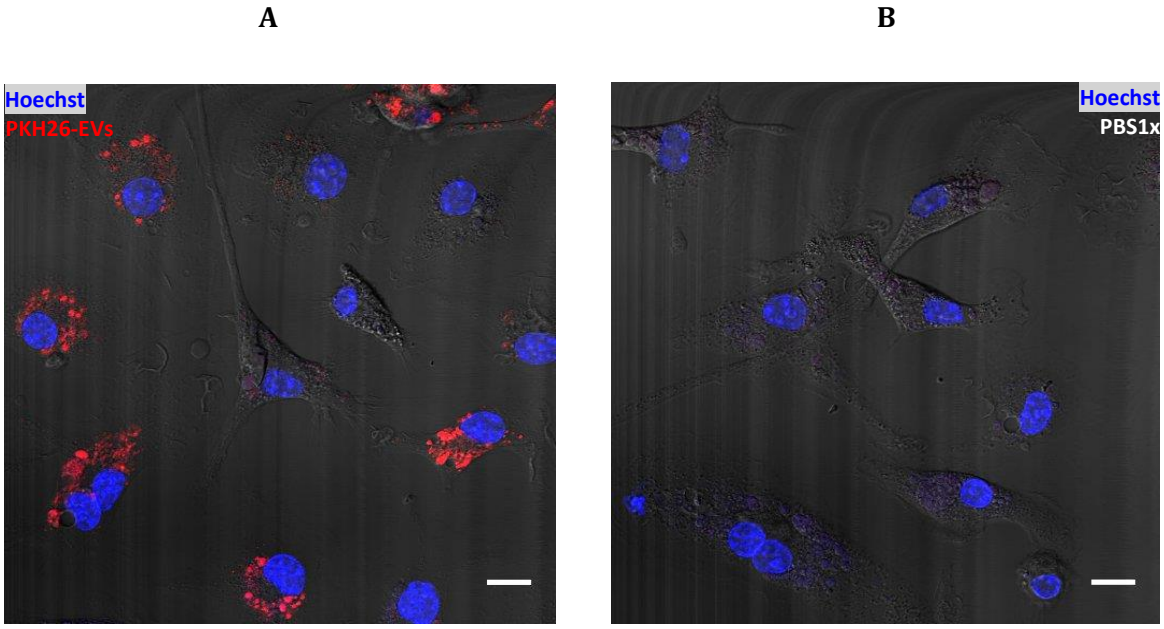


Figure 7. EVs are internalized into primary microglia *in vitro*. **A**. PKH26-labeled EVs (4.5μg/ml) are found within microglial cells 48 hours after the administration. EVs (in red) were labeled by directly adding the dye (final concentration: $5X10^{-6}$ M) to the EV pellet, previously resuspended in 0.2% BSA PBS1x, according to a protocol modified from van der Vlist and colleagues (van der Vlist et al. 2012). After staining EVs were subjected twice to 70 min 110x g ultracentrifugation to remove unbound dye. **B**. Control microglia treated with equal concentration of PKH26 dye, after being resuspended in the same buffer and subjected to the same ultracentrifugation protocol used for EVs. Nuclei in both images are stained by Hoechst 33342 dye (in blue). Images were acquired on a Zeiss LSM710 with a 40x objective. Scale bars: 10 μm.

Altogether, these results show that EVs derived from 48h-SF cytokine preconditioned MSC show a major range size corresponding to that of exosomes and microvesicles, preserve typical biochemical markers and are internalized by microglial cells *in vitro* within 48 hours.

1.4 MSC-EVs do not significantly modulate M1 and M2 microglia markers *in vitro*

To assess the ability of MSC-derived EVs to modulate microglia functionality, EVs were administered to primary microglial culture subjected or not to an inflammatory insult, according to the experimental paradigm described in *Material and Methods* (par. 1.8). We challenged the cells with TNFα and IFNγ, (same concentration used for MSC stimulation) to strongly drive microglia towards M1 phenotype (Verderio et al. 2012). After treatment with cytokines, microglial cells switched their morphology from a resting phenotype defined by long, thin processes to an activated reactive phenotype, characterized by a bigger soma and less branched processes (Fig. 8, CYT). Moreover, microglia number increases, a typical feature of activation process. The presence of EVs did not seem to influence microglial morphology neither in control (Fig. 8, EVs vs CTRL) nor in inflammatory conditions (Fig. 8, CYT vs CYT+EVs).

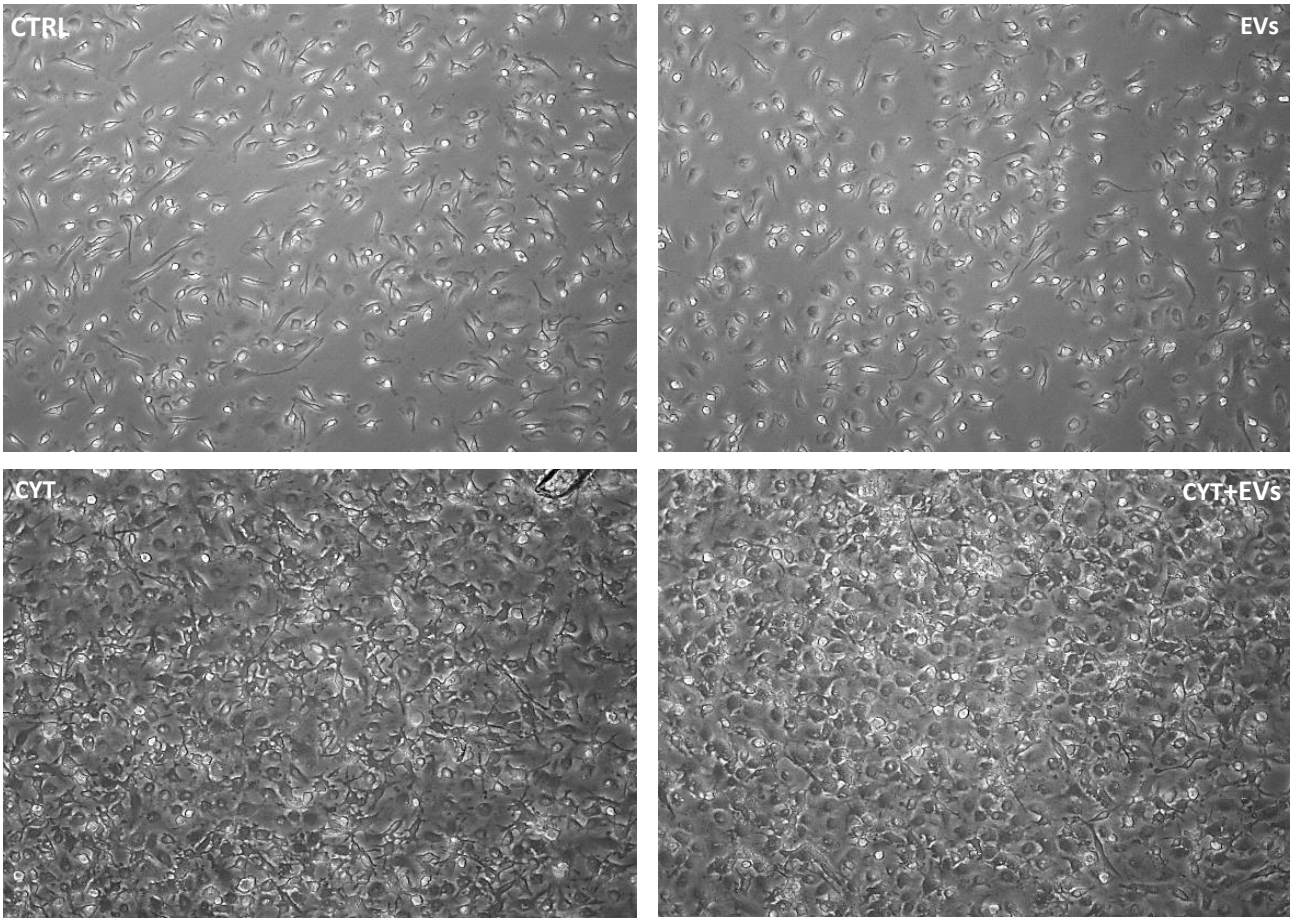


Figure 8. **Microglia morphological evaluation in *in* vitro experimental paradigm.** Cells in control conditions exhibited a spindle-shaped morphology with thin and long processes (CTRL, EVs). After cytokine treatment microglia proliferate and acquire a reactive phenotype characterized by a bigger soma and less branched processes (CYT). The presence of EVs did not modulate microglial morphology neither in control (EVs) nor in inflammatory conditions (CYT+EVs) (CTRL: control; EVs: extracellular vesicles; CYT: cytokines). Images were acquired by phase contrast microscopy. Magnification 10x.

Then we investigated if the treatment with EVs for 48 hours could modulate the expression of M1 and M2 markers that commonly characterize microglial phenotype. As expected, exposure to inflammatory cytokines significantly upregulated the expression of Iba-1 (+545.1% vs. control, $p<0.05$, Fig. 9A), an actin-binding protein that is normally increased after cell activation (Imai et al. 1996). Moreover, activated microglia underwent upregulation of common M1 markers such as the inducible nitric oxide synthase (iNOS) (+975.3% vs. control, $p<0.05$; Fig. 9B) and the lysosomal phagocytic protein CD68 (+37.3% vs. control, $p<0.05$; Fig. 9C), while the typical M2 marker CD206, the mannose receptor with a repair/reparatory function, is significantly downregulated (-33.7% vs control, $p<0.01$; Fig. 9D). However, double EV treatment for 48 hours was unable to revert the expression of M1 or M2 microglial markers in this *in vitro* inflammatory environment.

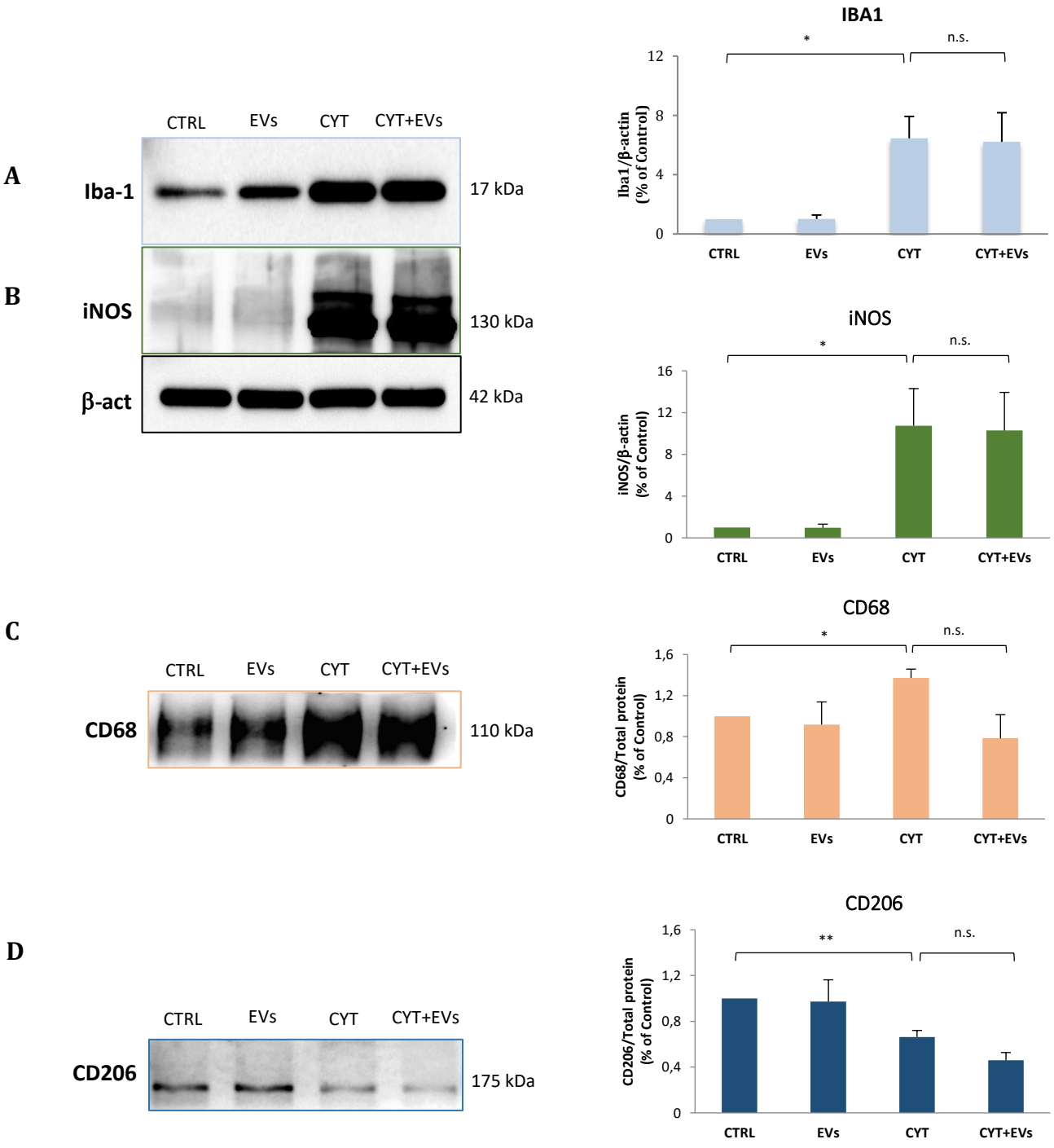


Figure 9. EV treatment did not modulate microglial M1 and M2 markers. **A, B, C, D.** Immunoblotting bands (left column) and representative quantification histograms (right column) of microglial markers. Cytokine treatment caused a significative upregulation of the activation marker Iba-1 (A), as well as iNOS (B) and CD68 (C) M1 markers, while downregulated the M2 marker CD206 (D). EV treatment did not significantly affect the expression of these markers after the inflammatory challenge (Iba-1, CD68: n=3; iNOS: n=4, CD206: n=4). β-actin was used as loading control for Iba-1 and iNOS analysis, while CD68 and CD206 expression was normalized on total protein (Ponceau staining). Comparison between groups (e.g., CYT vs CYT+EVs, CTRL vs CYT) used paired, one-tailed Student's *t* test; *p < 0.05, **p < 0.01 and ***p < 0.001. Data are expressed as mean ± SEM.

1.5 MSC-EVs modulate cytokine release by microglia in an anti-inflammatory manner *in vitro*

Although microglia phenotype markers did not seem to be modulated by EV treatment, we decided to investigate if the vesicles might influence microglial function by evaluating the release pattern of the typical pro- and anti-inflammatory mediators. The treatment of primary microglia with TNFα and IFNγ significantly increased, as expected, the release of pro-inflammatory mediators IL-6 [(CTRL: 22.54 ± 6.97 pg/ml (± SEM); CYT: 45.29 ± 7.96 pg/ml, $p<0.05$; Fig. 10A] and IL-1β (CTRL: 784 ± 148.7 pg/ml; CYT: 970 ± 194.31 pg/ml, $p<0.05$; Fig. 10B), and downregulated the production of the anti-inflammatory cytokine IL-10 (CTRL: 133.72 ± 29.83 pg/ml; CYT: 80.9 ± 6.05 pg/ml, $p<0.05$; Fig. 10C). EV administration to microglia resulted in the downregulation of pro-inflammatory cytokines released by the activated cells, since EVs elicited a significative downregulation of IL-6 (CYT: 45.29 ± 7.96 pg/ml; CYT+EVs: 29.83 ± 10.79 pg/ml, $p<0.05$; Fig. 10A) and IL-1β (CYT: 970 ± 194.31 pg/ml; CYT+EVs: 746 ± 179.13 pg/ml, $p<0.05$; Fig. 10B). In addition, EVs reverted the switch-off effect induced by TNFα and IFNγ on IL-10, by restoring the cytokine release almost to control levels (CYT: 80.9 ± 6.05 pg/ml; CYT+EVs: 104.43 ± 7.49 pg/ml, $p<0.05$; Fig. 10C). IL-4, a prototypical anti-inflammatory cytokine typically associated with M2a-repair/regenerative polarization of microglia, was not detectable in our experimental settings.

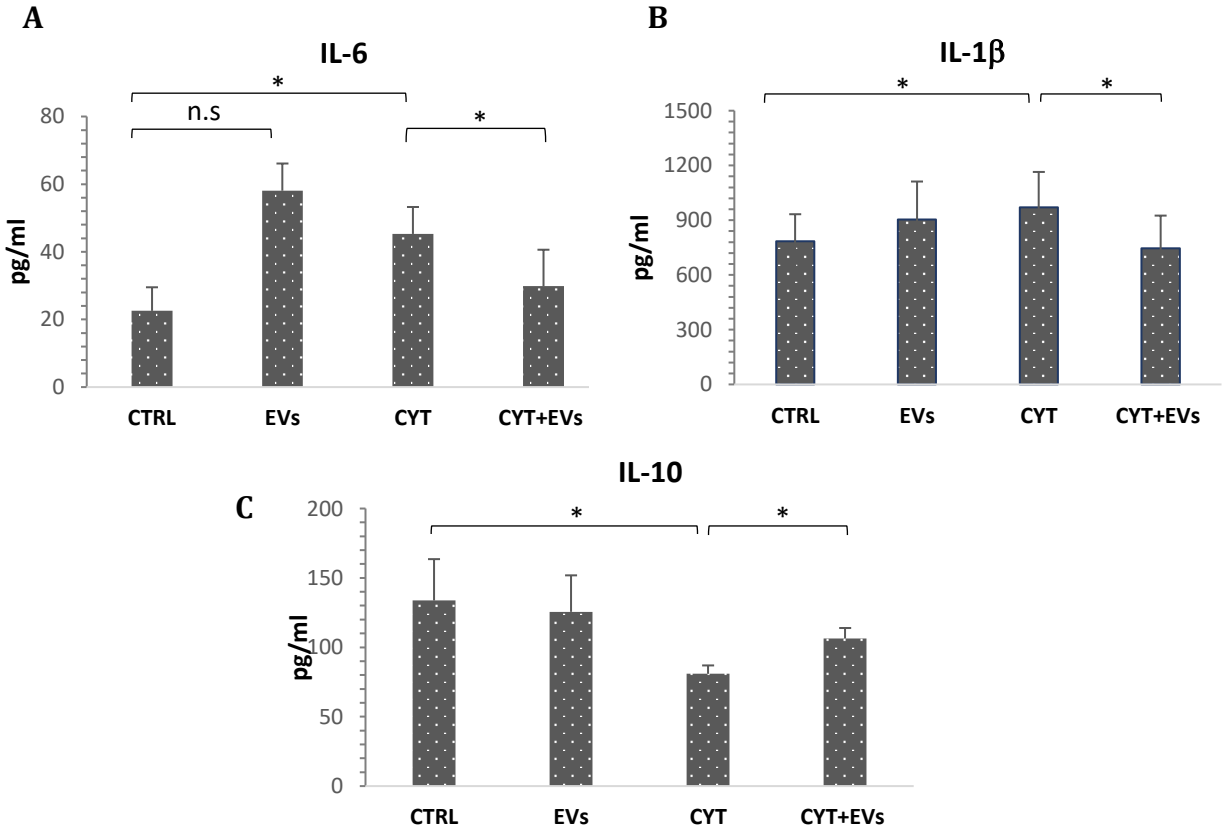


Figure 10. EV treatment polarize microglia *in vitro* toward an anti-inflammatory phenotype. Histograms show the quantification of microglia release of IL-6 (**A**), IL-1β (**B**) and IL-10 (**C**) by ELISA. In activated microglia, EVs were able to induce the release of the anti-inflammatory cytokine IL-10 (n=6) and a significant negative modulation on the pro-inflammatory cytokines IL-6 (n=5) and IL-1β (n=5). For the comparison between groups (e.g., CYT vs CYT+EVs, CTRL vs CYT, CTRL vs EVs) paired, one-tailed Student's *t* test was used; *p < 0.05, **p < 0.01 and ***p < 0.001. Data are expressed as mean ± SEM.

2. *In vivo* experiments

2.1 Intranasal-dispensed MSC-EVs are internalized by microglia and neurons

In the *in vivo* studies we focused our analysis on 3xTg mice hippocampus, entorhinal and prefrontal cortices, since these regions have been reported to be differently affected by the proliferation of microglial cells, with their cell number increasing in early phase (from 6 months of age) in the hippocampus and the entorhinal cortex or remaining unaltered until 15 months in the prefrontal cortex (Mastrangelo and Bowers 2008). Before evaluating the immunoregulatory potential of EVs in 3xTg mice, we qualitatively investigated whether intranasal (IN) administration of PKH26-labelled EVs (~$15x10^9$ vesicles corresponding to 30μg of vesicular protein) could result in the targeting of the vesicles into the aforementioned brain areas (see the *in vivo* experimental paradigm described in *Materials and Methods, par. 2.2*). Six hours after the injection of PKH26-labelled EVs, the animals were sacrificed and the analysis of the EV distribution has been performed. EVs were found in the rostral, medial and caudal hippocampus CA1, in the entorhinal cortex and in the prefrontal cortex. Specifically, PKH26-EVs were frequently seen within the cytoplasm and processes of Iba-1^+ cells in CA1 medial hippocampus (Fig. 11A1); to a lesser extent accumulation of EVs was also observed in pyramidal CA1 neurons (Fig. 11C1), while no internalization was seen in astrocytes (Fig. 11B1).

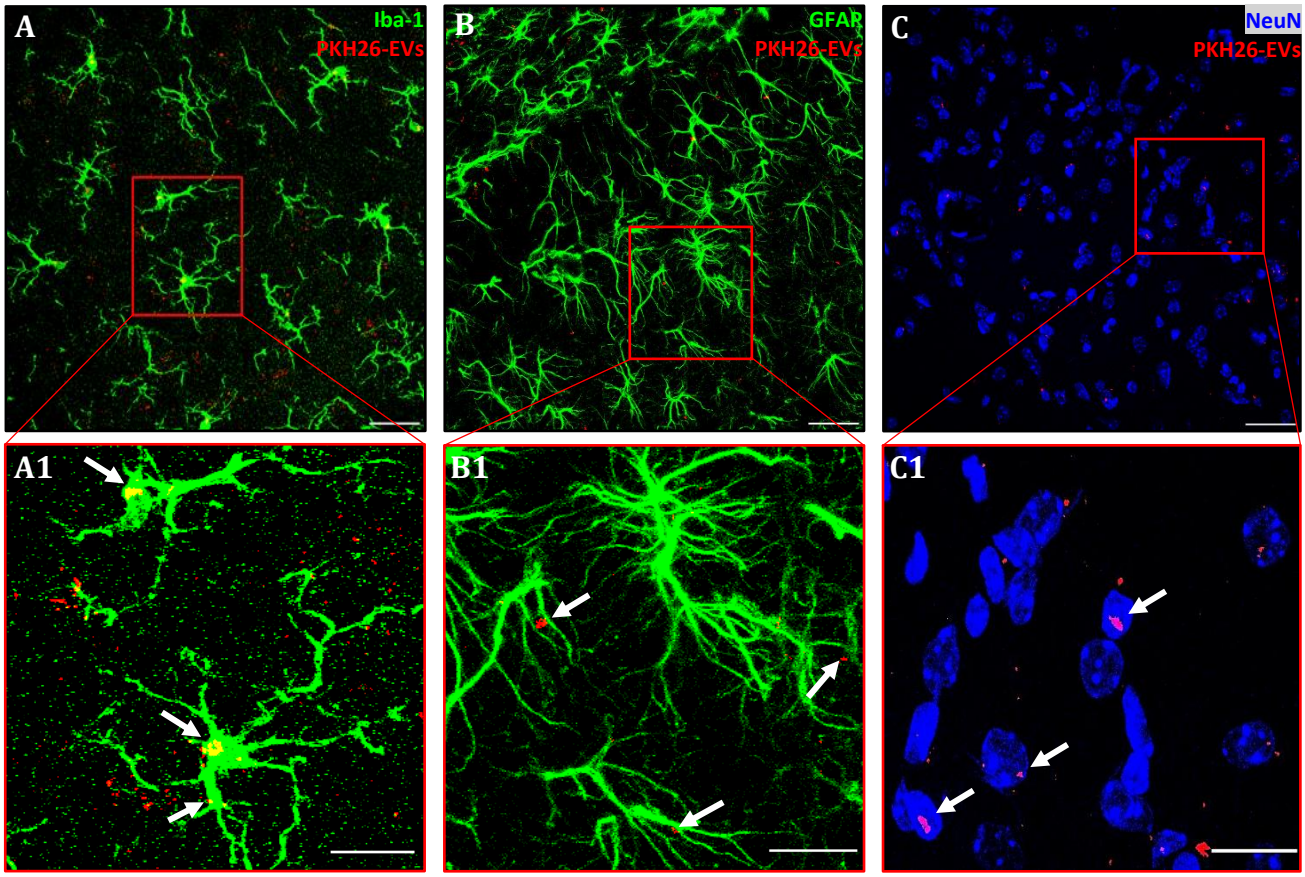


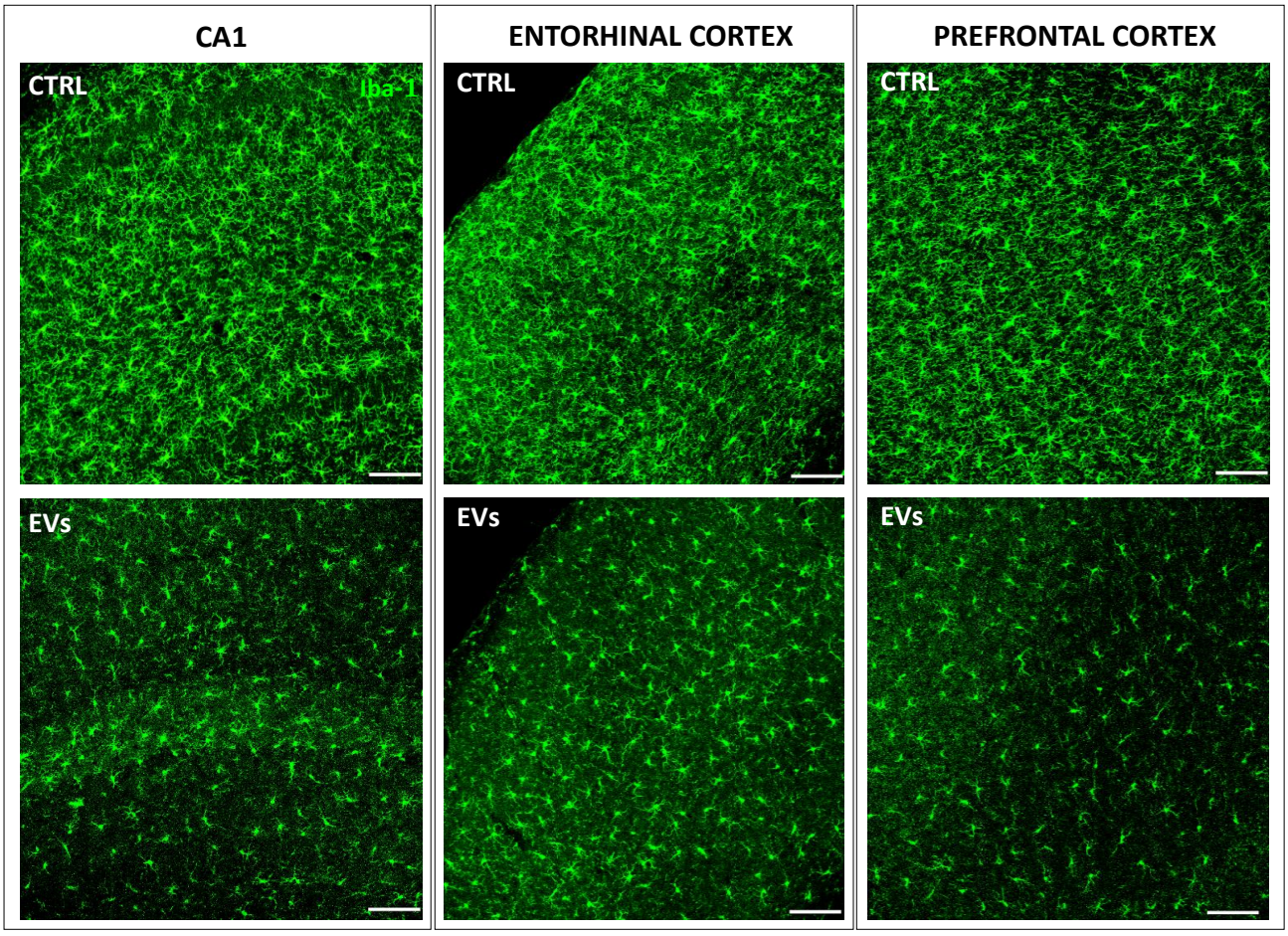
Figure 11. **MSC-derived EVs are internalized by microglia and neurons in medial hippocampus CA1 within 6 h after IN administration**. **A.** The presence of EVs [yellow dots (*white arrows*)] within the soma or processes of some Iba1+ microglia in CA1. **B.** Lack of EVs in the soma of $GFAP^+$ astrocytes. Note the presence of some red spots (EVs) adjacent to astrocyte processes (*white arrows*). **C.** The presence of PKH26-EVs [red and purple dots, (*white arrows*)] within the cytoplasm or in close contact with cell membrane of $NeuN^+$ pyramidal neurons. **A1, B1, C1**: magnifications of boxed regions in A, B, C (Scale bars: A, B, C: 30 μm; A1, B1, C1: 15 μm).

The specific pattern of internalization in these three cell types seems to be conserved among the other regions investigated (data not shown). Thus, within six hours of IN administration, MSC-EVs are robustly incorporated into microglia and, to some extent, into neurons, suggesting a cell specific mechanism that drive vesicle internalization.

2.2 MSC-EVs affect microglia density in 3xTg mice

Microglia activation is characterized by proliferation, morphological changes, such as hypertrophy of the cell soma, increased process branching, upregulation or *de novo* synthesis of cell surface or intracellular molecules (Perry and Teeling 2013). We therefore evaluated the ability of MSC-derived EVs to affect the number of microglia cells, through immunofluorescence staining of coronal sections for Iba-1, in the hippocampus, entorhinal and prefrontal cortices of 7-month-old 3xTg mice receiving vehicle (PBS1x) or EVs (Fig. 12A). Animals of the EV group exhibited a strong decreased density of Iba-1^{+} cells compared to the control group (CTRL) in the hippocampal CA1 area [CTRL: 13807.79 ± 368.26 (mean density ± SD)]; EVs: 11027.06 ± 952.99, **-20.14%** vs. CTRL, p<0.01; Fig. 12B), entorhinal cortex (CTRL: 14830.59 ± 994.44; EVs: 10803.32 ± 1444.36, **-27.16 %** vs. CTRL, p<0.05; Fig. 12B), prefrontal cortex (CTRL: 18026.64 ± 2738.72; EVs: 12769.01 ± 1711.93, **-29.17%** vs. CTRL, p<0.05; Fig. 12B).

A



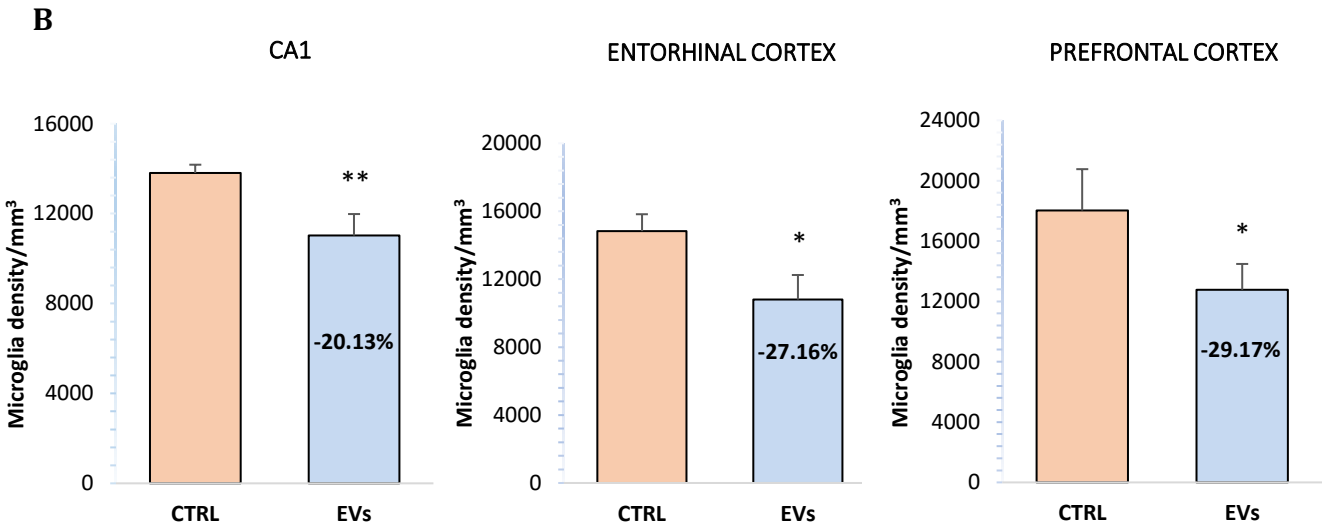


Figure 12. **IN administration of EVs reduces the density of Iba-1$^+$ cells**. **A**. Distribution of Iba-1$^+$microglia in the CA1 medial hippocampus, entorhinal cortex and prefrontal cortex of control (CTRL) and EV treated mice (EVs). **B**. Histograms compare the number of microglial cells in the same areas of A. Note that the animals receiving EVs (*light blue column*) displayed a reduced number of Iba-1$^+$ cells compared to animals in the control group (CTRL, *light red column*) that received PBS. For the comparison between groups (n=4) unpaired, two-tailed Student's t test was used; *p < 0.05, **p < 0.01 and ***p < 0.001. Data are expressed as mean ± SD (Scale bars: 100 μm).

2.3 MSC-EVs decrease microglia cell soma size in 3xTg mice

Although a precise evidence of the correlation between microglia morphology and function still lacks, in the context of neuroinflammation some morphological features that label reactive microglia have been repeatedly reported (Davis et al. 1994).

Figures 13 A1 and B1 show that microglia in CA1 of control mice displayed a more robust body swelling and process thickening, compared to EV treated mice. This morphological trait, typical of activated microglia, was also found in the entorhinal and prefrontal cortices (not shown). In these areas, as for CA1 region, the treatment with EVs, dramatically switched glial cell morphology towards a more "resting" phenotype, characterized by thinner processes and a smaller soma. In order to quantitatively assess changes in microglia morphology, we analysed by ImageJ software the cell body size of microglia in EV-treated (EVs) and non-treated groups (CTRL). When compared to vehicle-treated mice, EVs reduced the average cell body size in all the regions analysed: CA1 hippocampal region [(CTRL: 43712.58 ± 1891.98 (a.u ± SD); EVs: 33765.36± 6249.98, **-22.76%** vs. CTRL, p<0.05; Fig. 13C)], entorhinal cortex (CTRL: 32132.09 ± 2845.62; EVs: 22455.26 ± 3970.50, **-30,12%** vs. CTRL, p<0.05; Fig. 13C) and prefrontal cortex (CTRL: 41662.93 ± 3654.41; EVs: 31924.98 ± 4143.49, **-23.37%** vs. CTRL, p<0.05; Fig. 13C).

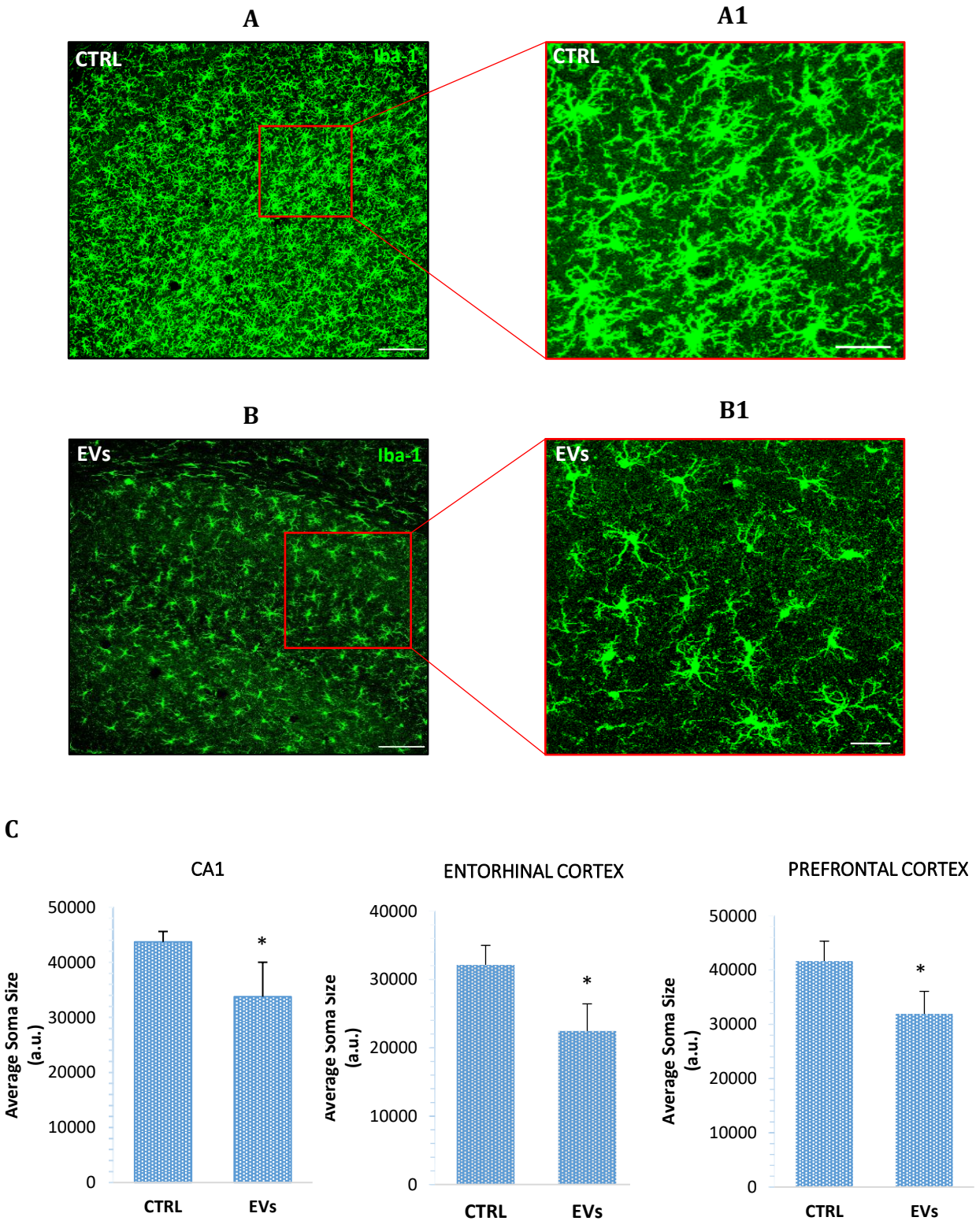


Figure 13. **IN administration of EVs reduces the size of the cell soma of Iba-1$^+$ cells. A, B**. 20x images of microglia cells in hippocampal CA1 of control (CTRL) and treated (EVs) mice. **A1, B1**. Magnified views of boxed regions show that EV administration strongly reduces the size of the soma and dendritic process thickness of Iba-1$^+$ cells (Scale bars: A, B: 100 µm; A1, B1: 30 µm.) **C.** Histograms comparing the reduction [(shown as arbitrary units (a.u.)] of microglial cell body size in CA1 medial hippocampus, entorhinal cortex, prefrontal cortex of control and treated mice. Average cell body size was quantified by ImageJ software within the microglia soma, that was derived by using the thresholding method described in *Material and Methods* (par. 2.4.1). Comparison between groups (n=4) used unpaired, two-tailed Student's *t* test; *p < 0.05, **p < 0.01 and ***p < 0.001. Data are expressed as mean ± SD.

2.4 MSC-EVs decrease Iba-1 and CD68 fluorescence signals in microglia of 3xTg mice

In order to further characterize the activated state of microglia cells, we analysed in the brain of control and EV treated mice the fluorescence intensity of Iba-1, whose expression is prompted by cell activation, and CD68 and CD206, two markers that are typically associated to M1 and M2 phenotypes, respectively.

Figure 14A shows that microglia cells in CA1 hippocampal region of EV-treated mice evidenced a lower intensity of Iba-1 immunofluorescent signal, compared to cells of PBS-treated mice (CTRL). In particular, quantification of the fluorescence integrated density (Fig. 14B) indicates that IN administration of EVs strongly reduced the expression of Iba-1 in CA1 hippocampus [(CTRL: 5415.03 ± 100.05 (a.u ± SD); EVs: 34729.50± 353.6, **-35.86%** vs. CTRL, p<0.001; Fig. 14B)], the entorhinal cortex (CTRL: 4983.64± 541.24; EVs: 1971.41± 1176.96, **-60.44%** vs. CTRL, p<0.05; Fig. 14B) and the prefrontal cortex (CTRL: 4471.21± 402.67; EVs: 2378.76 ± 198.89.7,**-46.79%** vs. CTRL, p<0.001; Fig. 14B), compared to vehicle-treated animals (CTRL).

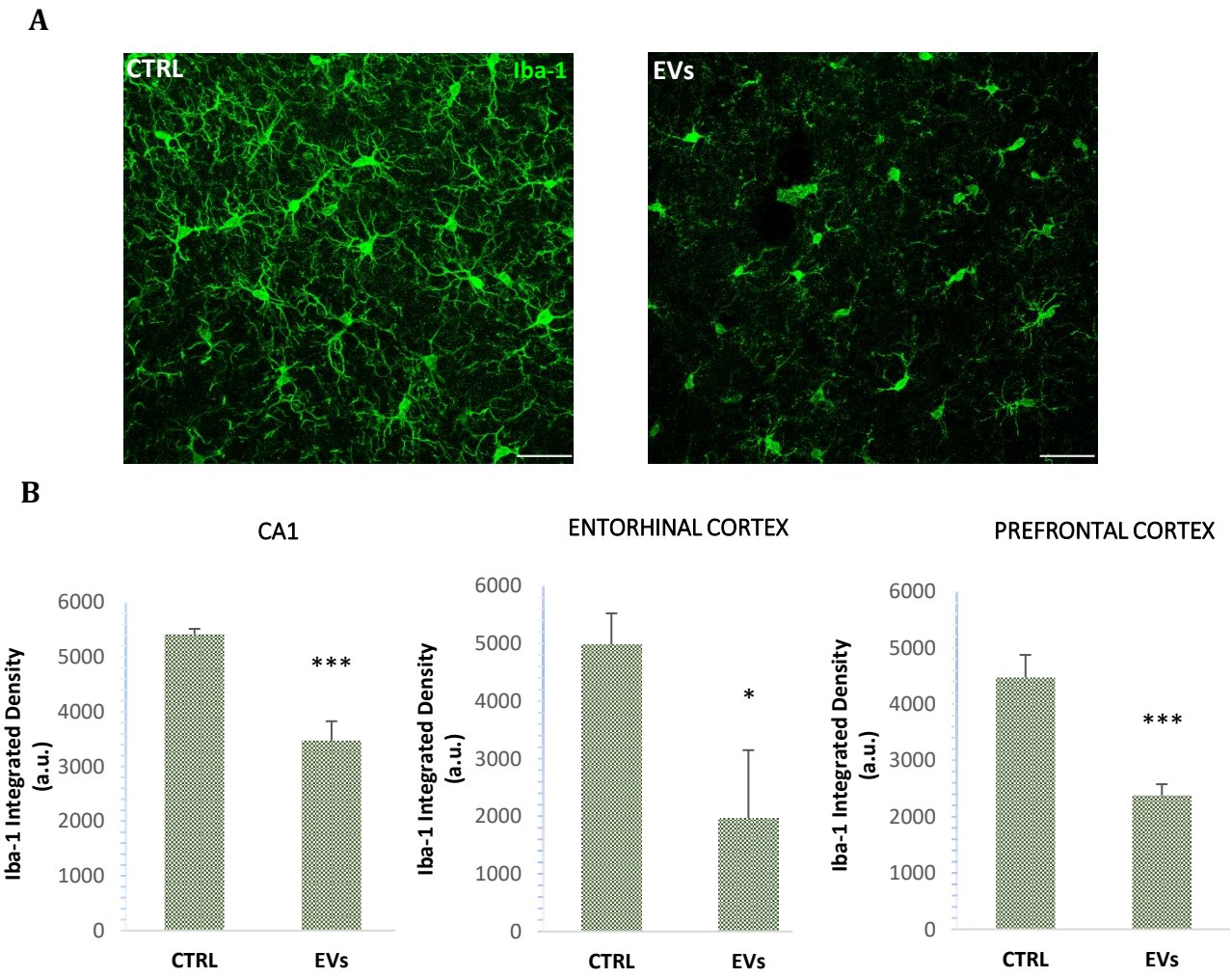
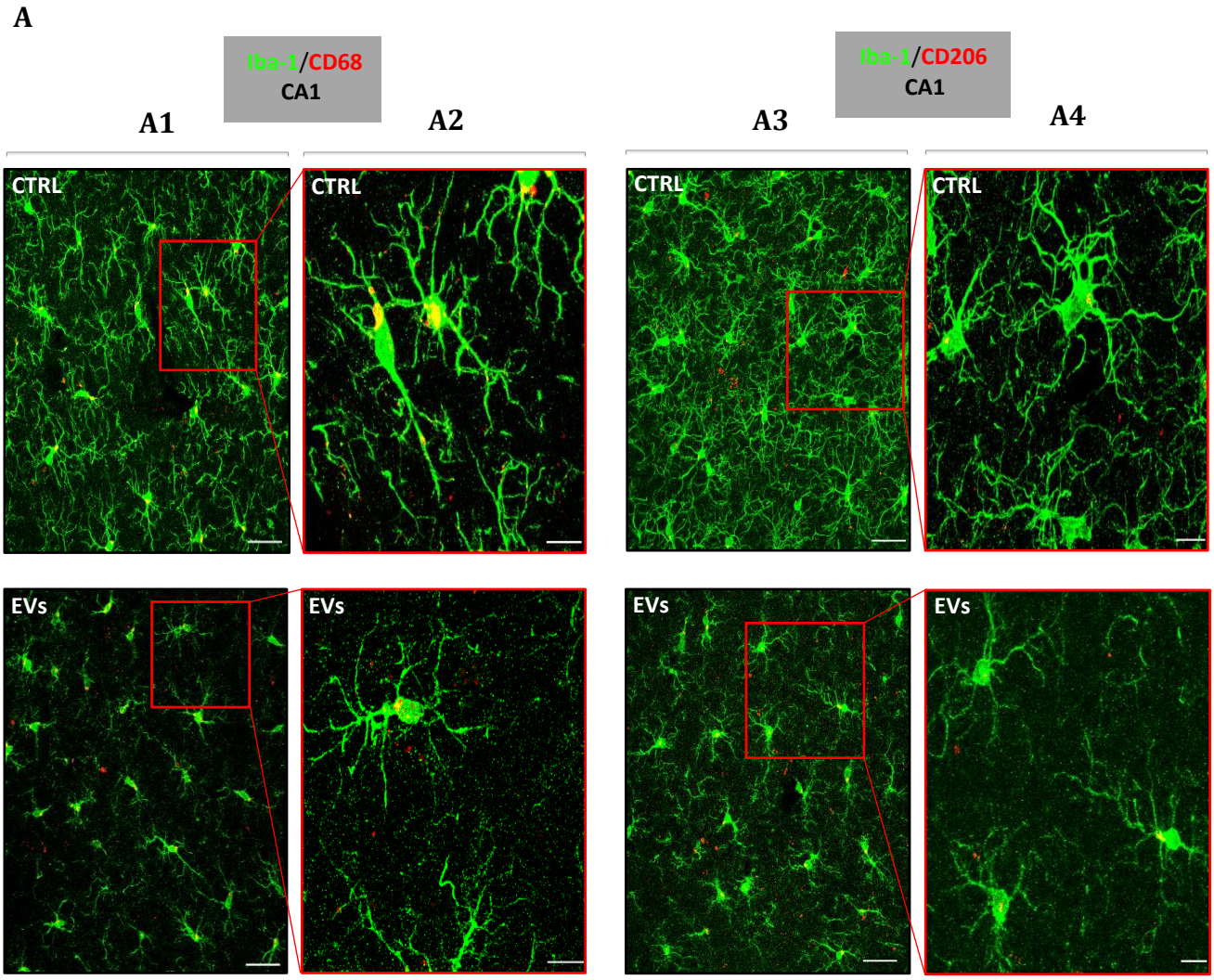


Figure 14. **MSC-derived EVs reduce Iba-1 expression microglia**.**A.** Confocal images of CA1 hippocampus evidencing the lower signal intensity (green) of Iba-1 in microglial cells of EV-mice compared to control mice. Scale bars: 30µm. **B.** Bar charts comparing the quantification of fluorescence intensity of Iba-1 (n=4) in CA1 region of medial hippocampus, entorhinal cortex and prefrontal cortex. Fluorescence intensities (a.u.) were quantified by ImageJ software within the microglia soma, that was derived by using the thresholding method described in *Material and Methods* (par.2.4.1). Comparison between untreated and treated groups (CTRL vs EVs) used unpaired, two-tailed Student's *t* test; *p < 0.05, **p < 0.01 and ***p < 0.001. Data are expressed as mean ± SD.

We then analysed the expression of CD68 and CD206, in order to examine the polarization state of microglia. Figures 15A3, A4 qualitatively evidence no changes of CD206 expression in CA1 Iba-1^{+} cells of control (CTRL, top) and EVs treated mice (EVs, bottom), while a strong decrease in the colocalization (yellow dots) for Iba-1 and CD68 was observed in treated EV-group, compared to the control (Fig. 15A1, A2). Quantitative analysis of fluorescent integrated signal showed that EV treatment did not modulate CD206 expression (Fig. 15B, C,D) compared to the control group, but was able to significantly reduce the fluorescence intensity of CD68 in the CA1 area of hippocampus [(CTRL: 694.58 ± 43.48 (a.u ± SD); EVs: 438.36 ± 92.76, **-36.89%** vs. CTRL, p<0.01; Fig. 15B), the entorhinal cortex (CTRL: 649.33 ± 75.81; EVs: 434.23 ± 36.82,**-33.13%** vs. CTRL, p<0.01; Fig. 15C) and the prefrontal cortex (CTRL: 535.94 ± 44.18; EVs: 376.45 ± 11.8,**-29.76%** vs. CTRL, p<0.01; Fig. 15D). These results suggest a dampening effect mediated by MSC-EVs on M1 polarized activated microglia.



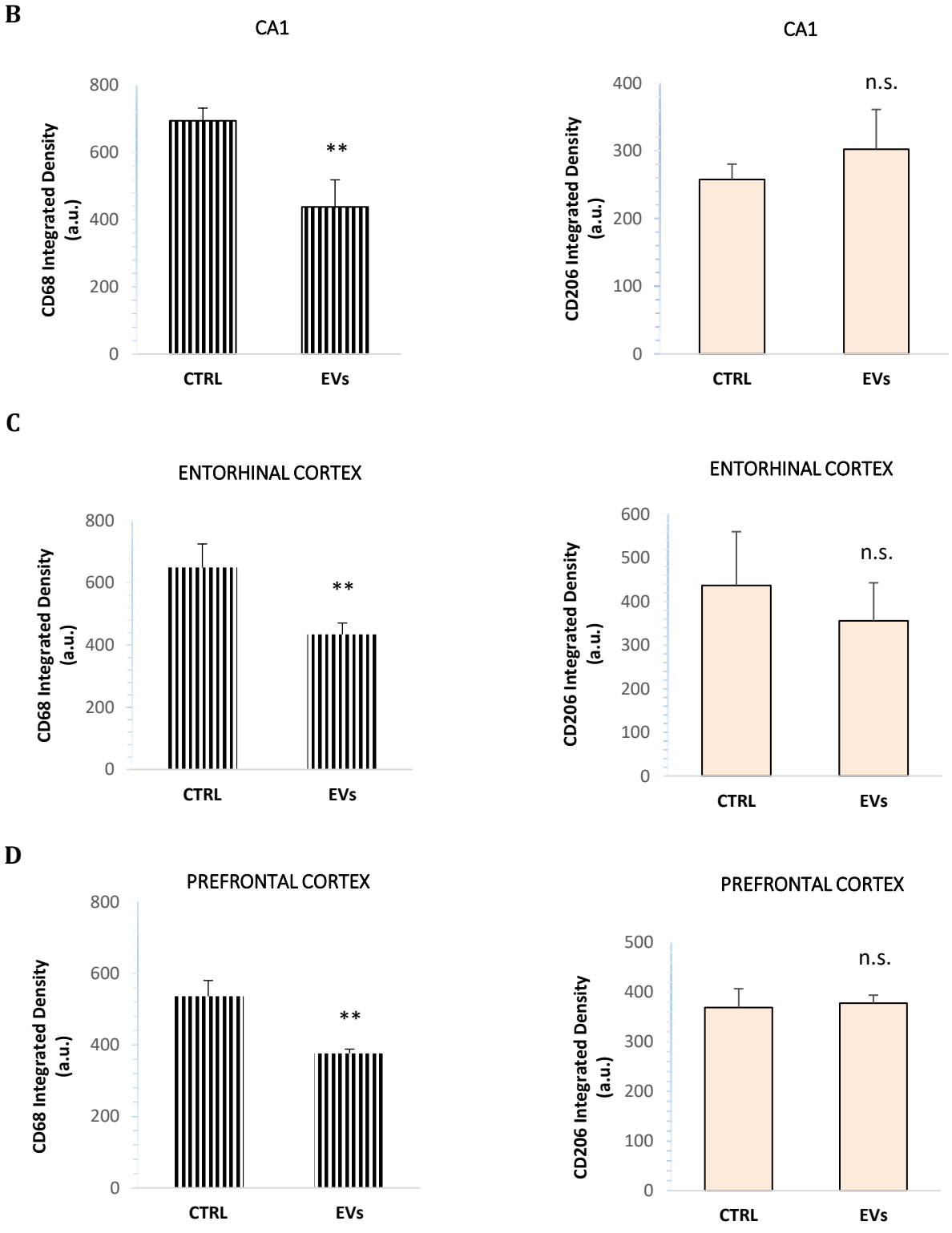
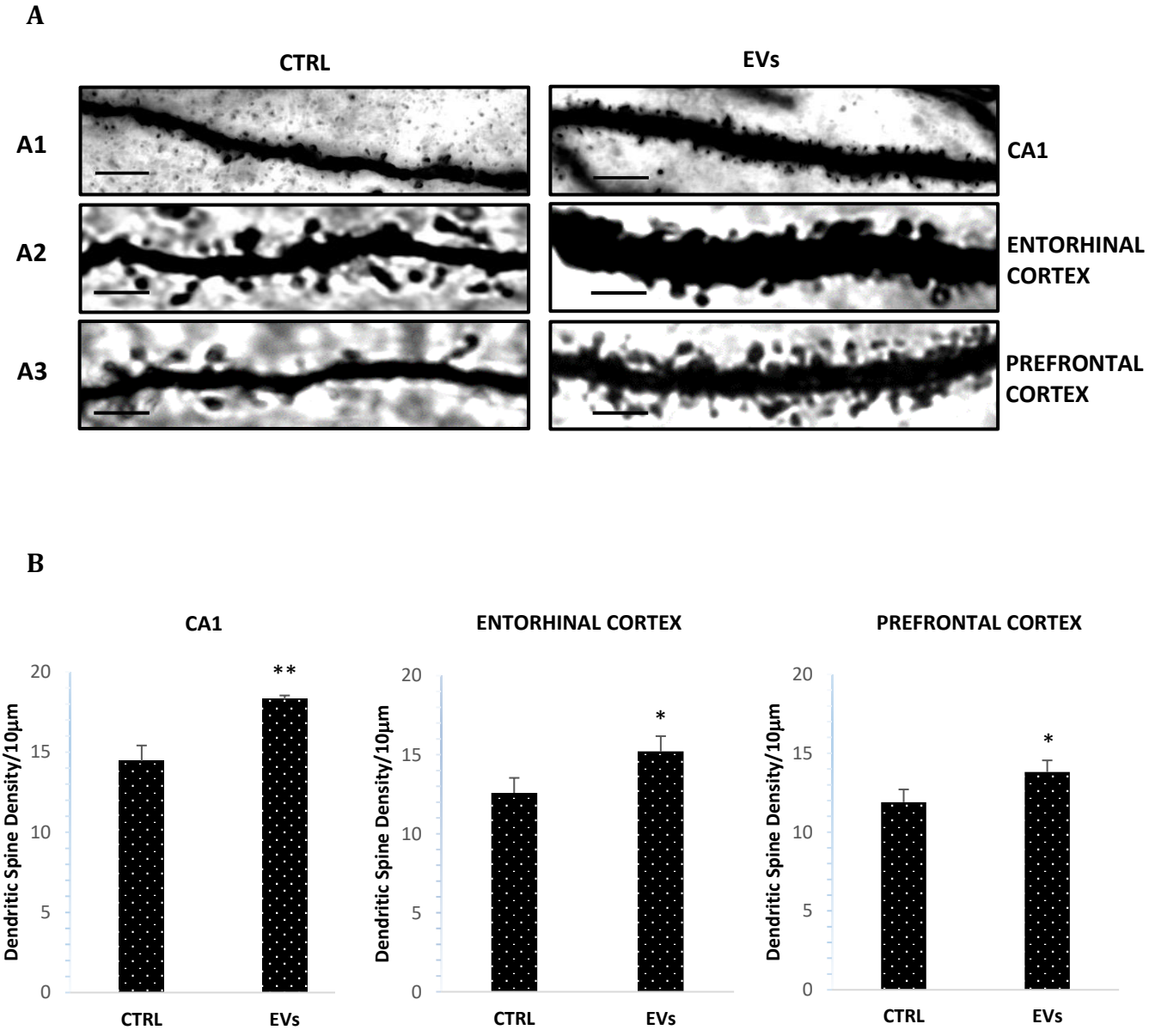


Figure 15. **MSC-derived EVs reduce CD68 expression in Iba-1$^+$ cells, while not affect CD206 expression. A.** Confocal images of CA1 hippocampus of control (CTRL) and EV treated mice (EVs) showing Iba-1$^+$ microglial cells (*green*) stained for CD68 (*yellow*/*orange dots*, A1, A2) or for CD206 (*yellow*/*orange dots*, A3, A4). Scale bars: A1, A3: 30µm; A2, A4 (magnified views of boxed regions in A1, A3): 10µm. **B, C, D.** Histograms comparing the fluorescence intensity quantification of CD68 (n=4, black and white striped charts, *left column*) and CD206 (n=4, orange charts, *right column*) in CA1 region of the medial hippocampus, entorhinal cortex and prefrontal cortex of control (CTRL) and EV treated mice (EVs). Fluorescence intensities (a.u.) were quantified as described in *Material and Methods* (par.2.4.1). Comparison between untreated and treated groups (CTRL vs EVs) used unpaired, two-tailed Student's *t* test; *p < 0.05, **p < 0.01 and ***p < 0.001. Data are expressed as mean ± SD.

2.5 MSC-EVs increase dendritic spine density in 3xTg mice

Since reactive microglia has been demonstrated to actively mediate synapse loss in AD by different mechanisms (Shi et al. 2017; Rajendran and Paolicelli 2018), we investigated if EV-induced modulation of microglia activation might affect dendritic spine density. We performed Colgi-Cox staining in the same brain regions analysed for microglia activation. Our results showed that EV treatment was able to strongly increase dendritic spine density in the hippocampal CA1 region (**+ 26.72%** vs. CTRL, p<0.01; Fig. 16B), the entorhinal cortex (**+20.83%** vs. CTRL, p<0.05; Fig. 16B) and the prefrontal cortex (**+16.08%** vs. CTRL, p<0.05; Fig. 16B).



C

Table	CA1		ENTORHINAL CORTEX		PREFRONTAL CORTEX	
Group	**CTRL**	**EVs**	**CTRL**	**EVs**	**CTRL**	**EVs**
Mice (n°)	**4**	**4**	**4**	**4**	**4**	**3**
Dendritic Length (μm)	**1882.37**	**1698.94**	**1716.38**	**1029.77**	**669.17**	**695.52**
Dendritic Spines (n°)	**2735**	**3099**	**2181**	**1562**	**794**	**956**
Mean density/ 10 μm	**14.44**	**18.30**	**12.58**	**15.20**	**11.89**	**13.80**

Figure 16. MSC-derived EVs increase dendritic spine density in 3xTg mice. **A.** Representative photomicrographs of Golgi-Cox stained dendritic segments from hippocampal CA1 pyramidal neuron (**A1**), entorhinal cortex (**A2)** and prefrontal cortex (**A3)** neurons, of control (CTRL, *left column*) and EV-treated mice (EVs, *right column*). **B.** Histograms show the quantification of dendritic spine density (spines/10μm) in the same areas. Note that animals treated with EVs display a significative higher number of dendritic spines compared to the non-treated group (CTRL). **C**. The table indicates the numeric values corresponding to total number of counted dendritic spines (Dendritic Spines n°) and the total length of dendritic processes (Dendritic Length μm) that were considered for the analysis of dendritic spine density/10μm (Mean density/10μm). Comparison between untreated and treated groups (CTRL vs EVs) used unpaired, two-tailed Student's *t* test; *p < 0.05, **p < 0.01 and ***p < 0.001. Data are expressed as mean ± SD. Scale bars: 5μm.

2.6 MSC-EVs modulate the inflammation in leech (*Hirudo Verbana*) CNS: a preliminary result

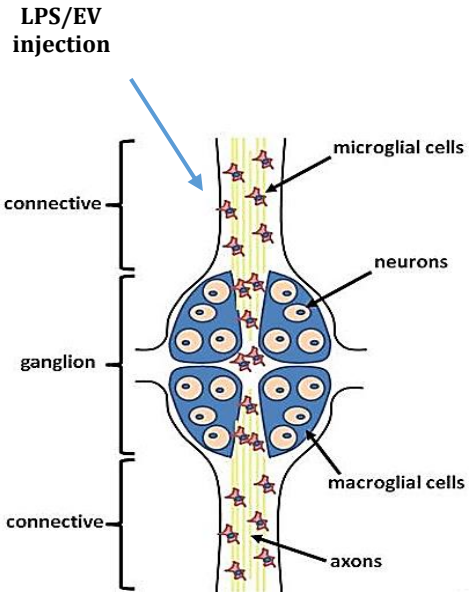
In order to clarify the mechanisms underlying the immunomodulatory action on microglia, in collaboration with the group of Prof. A. Grimaldi from the University of Insubria, we are performing experiments on a leech (*Hirudo Verbana*) model of inflammation induced by LPS.

In our study EVs derived from 48h cytokine preconditioned MSCs were administered in the same points of gangliar ventral chain injected with LPS.

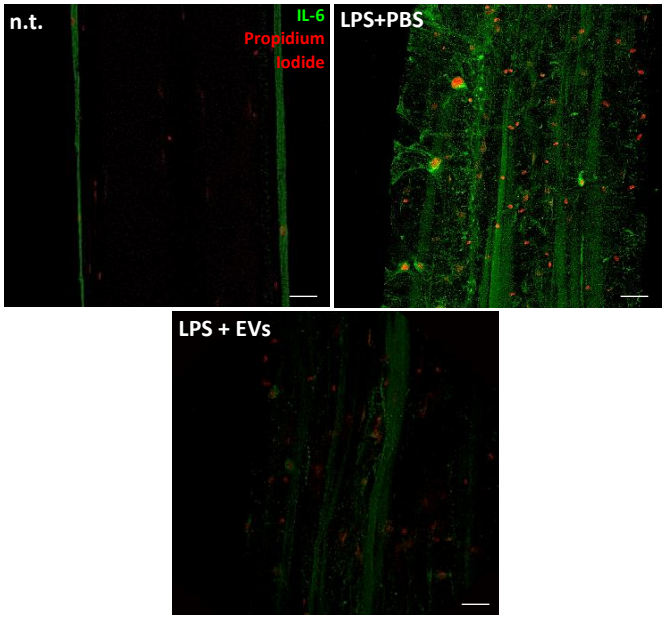
Preliminary immunofluorescence results indicate that the treatment with EVs two hour after the inflammatory challenge reduced microglia cell recruitment at the site of LPS injection, as evidenced by the decreased number of nuclei stained by Propidium Iodide (Fig. 18B, C, D, E). Although this dye cannot discriminate between different cell types, the presence of only two glial cells compared to thousands of microglia that have been described in the leech CNS connectives (Drago et al. 2014), indicated that these nuclei most likely virtually identify only microglial cells.

The treatment with MSC-EVs appeared to downregulate the expression of the typical LPS induced pro-inflammatory cytokines IL-6 (Fig. 18B), TNFα (Fig. 18C), IL-18 (Fig. 18D) and increased the anti-inflammatory cytokine IL-10 (Fig. 18E).

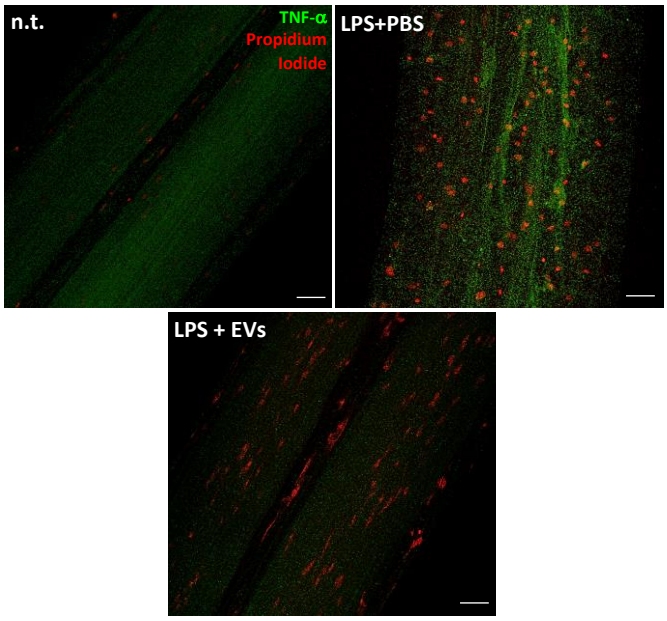
A


LPS/EV
injection
microglial cells
connective
neurons
ganglion
macroglial cells
connective
axons

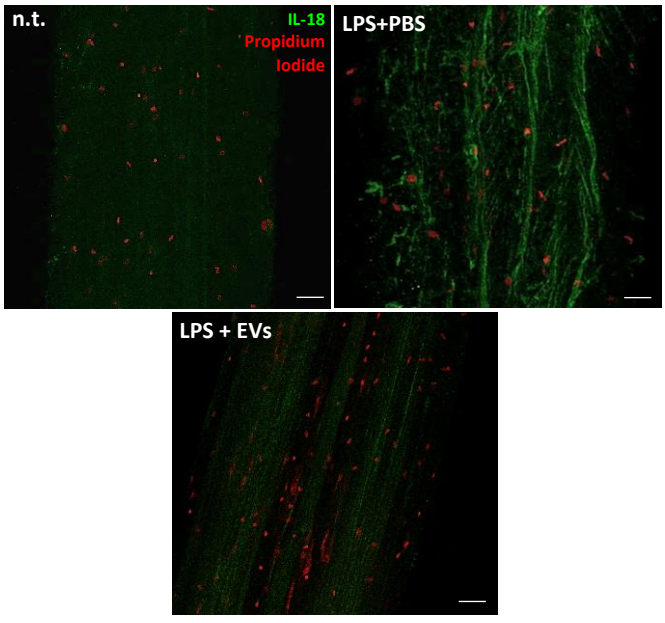
B


IL-6
n.t.
IL-6
Propidium
Iodide
LPS+PBS
LPS + EVs

C


TNF-α
n.t.
TNF-α
Propidium
Iodide
LPS+PBS
LPS + EVs

D


IL-18
n.t.
IL-18
Propidium
Iodide
LPS+PBS
LPS + EVs

E

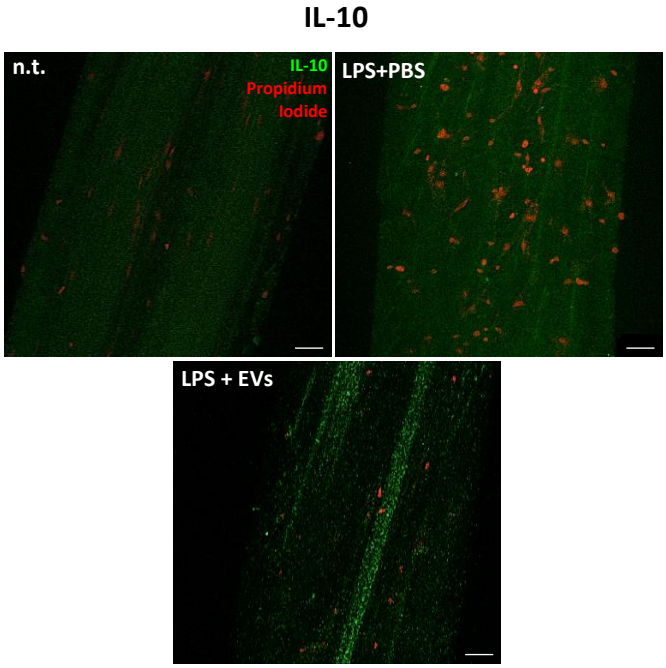


Figure 18. **Immunohistochemistry on whole mounted leech CNS connectives**. **A**. Schematic representation of leech CNS. Note the presence of microglia cells into the connectives (axons). Here, microglia represent the main cell type among with only two connectival glial cells (not shown). **B**, **C**, **D**, **E**. Immunofluorescence images from CNS samples (connectives) of non-treated leech (n.t.), and leeches treated with LPS plus PBS (LPS+PBS) or EVs derived from 48h cytokine preconditioned MSCs (LPS+EVs). Connectives were immunoistained for IL-6 (B), TNF-α (C), IL-18 (D), IL-10 (E) (*green*). Nuclei were counterstained with Propidium Iodide (red). Scale bars: 20 µm.

DISCUSSION

In this study we demonstrated for the first time the ability of intranasal injected Mesenchymal Stem Cell (MSC)-derived Extracellular Vesicles (MSC-EVs) to positively affect microglia activation and dendritic spine density in a triple transgenic mouse model of Alzheimer's disease (3xTg AD).

The success of therapeutic MSC use in many preclinical and clinical trials was fundamentally associated to their trophic influence on damaged tissues and safety as allogenic cell transplant, that is feasible without any substantial risk of immune rejection. Over the past few decades the emerging evidence of their immunomodulatory properties greatly enhanced the possibility to exploit them in tissue and organ repair (Patel et al. 2013). However, the high levels of pro-inflammatory, pro-oxidant factors and death signals, typically found in chronic diseases, may hinder MSCs to function properly, thus compromising their regenerative actions. To avoid this, preconditioning of MSCs by hypoxia, pharmacological and chemical agents, such as trophic factors, cytokines, prior to their application *in vivo* have been pursued as significant tools to make these cells able to counter any rigorous harsh microenvironment. In fact, *in vitro* pre-treated MSCs have shown to better survive, to increase differentiative capacities, to encourage the migration to injured sites and improve their paracrine activities (Saparov et al. 2016; Qazi et al. 2017). In particular, taking into account that MSCs mediate their therapeutic effects in a paracrine manner, these strategies have been pursued to specifically boost MSC immunosuppressive properties (Németh et al. 2009; Liu et al. 2017), that have been largely ascribed to their derived EVs (Bruno et al. 2015). Before investigating the immunomodulatory actions of MSC-EVs, we compared two types of preconditioning in order to select a specific MSC immunoregulatory phenotype. In order to pre-stimulate MSCs, we added a cocktail of two cytokines (CYTO) or we subjected the cells to oxygen deprivation (OD), in virtue of the large number of published papers assessing the effectiveness of these preconditioning protocols in endowing MSCs with increased immunomodulatory abilities (Wei et al. 2012; Noone et al. 2013). Both the preconditioning protocols did not alter the expression of MSC stemness markers *(Results*, Fig. 3A,B). Moreover, WB analyses (data not shown) evidenced no activation of the apoptotic protein caspase-3, revealed by the detection only of the pro-enzyme (uncleaved, 35kDa) of apoptotic executioner caspase-3 (cleaved and active form, 17 kDa). Since the pleiotropic nature of MSC immunoregulation - demonstrated on cells of both innate and adaptive immunity (Contreras et al. 2016) - we decided to focus our evaluation on the expression of COX2 and IDO markers, known to be associated to a MSC polarized phenotype endowed with augmented capacity to regulate the innate immune response (Tu et al. 2010). In particular, these markers have been reported to promote a homeostatic response toward M2 macrophage polarization (Németh et al. 2009; François et al. 2012). In our experiments the preconditioning with TNFα and IFNγ cytokines, especially at 48 hours, drove the highest upregulation of COX2 and IDO with respect to oxygen deprivation and reoxygenation paradigm

(OD+R). This is likely due to the fact that the two cytokines proved to be more potent inducers of the two investigated markers. However, we cannot rule out the induction of other immunoregulatory molecules after OD protocol, since it has been reported that MSCs, that were grown under hypoxia conditions, exhibited a robust anti-inflammatory function (Schive et al. 2017). Notably, MSCs normally reside *in vivo* in a microenvironment characterized by low oxygen tension (e.g. 1–5% O_2), while the oxygen concentration of the standard culture environment (e.g. 20–21% O_2) is much higher than their native physiological conditions (Choi et al. 2014). The changed culture oxygen tension settings *in vitro* has been demonstrated to negatively affect different properties of MSCs, as indicated by the reduced release of trophic, anti-apoptotic and anti-inflammatory factors, in the conditioned medium of normoxia cultured cells compared to those grown under hypoxia conditions (1% O_2) (Schive et al. 2017).

Otherwise, the lower induction of COX2 and IDO after OD may be due to the relative short-term exposure (no longer than 16 h) to OD, which was unable to mighty activate the signaling pathways that control their expression. In a recent published work, aiming at elucidating the ideal hypoxia conditions (i.e. best duration of the treatment and oxygen tension) that would endow MSCs with enhanced therapeutic potential, the authors found that 48h of hypoxia (2% O_2) preconditioning of MSCs more efficiently increased cell proliferation, self-renewing and augmented their trophic and anti-inflammatory properties, compared to cell cultured in normoxia conditions (Antebi et al. 2018). However, the OD protocol we used envisaged that cells were placed in complete anoxia (0% O_2) conditions - to more prominently stress cells that normally live in low-oxygen tension conditions - only for 3h or 16h. Therefore, we cannot rule out that OD preconditioning of a longer duration may have higher inducing effects on the expression of the investigated immunomodulatory markers. The use of primary cell cultures may represent a putative explanation for the high variability observed in the COX2 expression of control MSCs underwent to the different OD protocols. Further experiments need to be planned in order to better clarify this unexpected fluctuation in COX2 protein levels. In MSCs subjected to CYTO preconditioning we observed a time-dependent upregulation of both IDO and COX2. However, while the former was induced most at 48h, the latter exhibited the highest upregulation at 24h [see section of the *Results*, Fig. 3 (red box)]. Interestingly, in contrast to IDO, we observed that COX2 was induced by serum deprivation (serum free condition, SF) more at 24h, progressively decreasing at 48h and 72 hours (data not shown). Consequently, the higher expression of COX2 at 24h may be attributable to the more consistent contribution of SF at this time point. The modulation of COX2 expression by SF has been reported in a study that investigated the cell cycle-dependent expression of the enzyme in human fibroblasts. SF has been shown to induce the cells to enter into the G_0 phase, where they remain in a quiescent state and express COX2 at higher levels than at other phases such as G_1, S, and G_2/M (Gilroy et al. 2000). Based on our results, we could speculate that higher expression of MSC COX2 at 24h may be dependent upon the specific cell cycle phase (G_0 phase) at that time point, and decreases when cells - probably after 48h - enter the other phases. However, the overall high expression of both markers at 48h, let us to select this time point for the

cytokine stimulation of MSCs.

In our *in vitro* experiments, the treatment with cytokines switched microglia phenotype from a spindle-shaped morphology with thin and long processes towards an activated phenotype characterized by a bigger soma and less branched processes. Interestingly, these morphological changes were observed right after 10 minutes from TNF-α and IFN-γ administration (not shown). As expected, pro-inflammatory cytokines positively modulated M1 markers (CD68, iNOS) and Iba-1 - a protein constitutively expressed by microglia, but increased after cell activation (Sasaki et al. 2001) - and downregulated CD206, the mannose receptor that is widely recognized as a typical M2 protective microglial marker with important functions in pinocytosis and phagocytosis (Marzolo et al. 1999; Durafourt et al. 2012). The treatment with preconditioned MSC-EVs did not affect either cell morphology or the expression of these markers in cytokine activated primary microglia. However, ELISA *in vitro* results, investigating cell functional properties, showed that EVs counteracted the microglia reaction to this inflammatory challenge by switching the phenotype from the cytotoxic to the beneficial state. Indeed, EVs reduced the release of IL-6 and IL-1β cytokines, known to play an important role in the neuroinflammation linked to AD, and whose expression is typically increased in AD brains (Griffin et al. 1989; Strauss et al. 1992; Paradowski et al. 2008). EVs were also able to enhance the secretion of IL-10, a potent anti-inflammatory cytokine inducing the M2c polarization state, that is associated to the deactivation of microglia phenotype, the inhibition of pro-inflammatory cytokine release and neuronal synapse formation (Szczepanik et al. 2001; Lim et al. 2013). These data are in line with previous *in vitro* evidence showing that MSC-EVs are able to limit the inflammatory response by preventing the production of pro-inflammatory molecules by microglia/macrophages (Jaimes et al. 2016; Harting et al. 2017). The unexpected absence of the correspondence between the expression of phenotype-specific markers (*Results*, Fig. 9) and cytokines release (*Results*, Fig. 10) may be related to the different times of action of specific miRNAs, horizontally transferred to microglial cells via EVs, on the pathways regulating their synthesis. Since MSCs are activated by pro-inflammatory signals to express and secrete, through a homeostatic-like mechanism, anti-inflammatory molecules (Choi et al. 2011), we can hypothesize that a regulated sorting of specific anti-inflammatory miRNAs to the vesicles may occur after cytokine stimulation, thus explaining the modulation of the microglial cytokine pattern profile, compared to unaltered marker expression. In line with this hypothesis is a study investigating the differentially expressed miRNAs (DEMs) within EVs derived from IFN-γ preconditioned compared to non-preconditioned MSCs. This study showed that among all miRNAs that underwent upregulation (44) or downregulation (18) in preconditioned MSC-EVs, the most significant variation (i.e. an increase) was observed for miR-222 (Zhao et al. 2017). This miRNA appeared to negatively regulate the release of pro-inflammatory cytokines (Rao et al. 2015). Furthermore, in addition to non-coding RNAs, EVs contain different functional (e.g. capable of encoding polypeptides) mRNAs that can exploit the expression machinery of targeted cells (Valadi et al. 2007). One could therefore hypothesize that the higher

levels of IL-10 detected in the medium of CYT+EV treated cells, may be derived from the direct translation in microglia of the MSC cytokine mRNA transferred via EVs. However, no significant increment in IL-10 was observed after treating control microglia with EVs alone, suggesting that protein translation may depend on the physiological status of the cell (activated vs. non-activated). Indeed, regardless the specific EV cargo that may drive these effects, a significative immunomodulatory action on microglia cytokine release was observed only after the inflammatory insult *in vitro* [*Results*, Fig. 10 (CYT+EVs vs. CYT groups)], but none variation was recorded in control conditions (EVs vs. CTRL). The unresponsiveness of control microglia - with respect to cells activated by the pro-inflammatory stimuli - to the treatment with EVs can be also observed for IL-6 and IL-1β release, with the former appearing - unexpectedly - to be positively, although not significantly, modulated. Further experiments will be performed in order to unravel the mechanisms regulating the secretion of IL-6 in M1 and control microglia after EV treatment. Nevertheless, this different modulation of cytokine release by microglia could suggest that cytokine activated MSCs are "instructed" to release EVs endowed with anti-inflammatory potential, in order to restore the disrupted (detected) equilibrium. These EVs may act in a homeostatic-like manner by targeting specific inflammatory pathways activated in cytokine M1 polarized, but not in control microglia, thus explaining the observed modulation of cytokine release in "CYT+EV" compared to "EV" microglia. The so-called *feedback loop* by which MSCs can "sense" and dampen inflammatory processes has been described in co-culture systems between MSCs and macrophages. Briefly, bacterial LPS- activated macrophages release TNF-α that interacts with its receptor on MSCs, activating the expression of a variety of genes, including TNF-α stimulated gene/protein 6 (TSG-6). MSC-released TSG-6 interact with CD44 on macrophages, blocking the NFκ-B signaling, which results in decreased secretion of TNF-α.

To summarize our *in vitro* results, we can conclude that the preconditioning with pro-inflammatory cytokines increases the expression of MSC immunomodulatory markers. MSC-EVs are able to functionally switch microglia from M1 phenotype - characterized by the release of pro-inflammatory cytokines (IL-6, IL-1β) which are associated with detrimental actions - to the anti-inflammatory M2 state, characterized by the increased release of IL-10 and associated to cellular debris removal and tissue repair (Walker et al. 2014; Orihuela et al. 2016). Our results are in line with a previous study showing immunomodulatory properties of preconditioned MSC-EVs on microglia (Ruppert et al. 2018). Further studies will be addressed to identify the specific EV factors that are responsible for these effects.

The data regarding the capability of MSC-EVs to positively affect microglia behaviour *in vitro* are coherent with our *in vivo* results that showed an evident polarization of these cells towards the protective phenotype, induced by EV treatment in a mouse transgenic model of AD. Transplantation of MSCs in rodent AD models has been reported to inhibit Aβ and tau-related cell death (Zilka et al. 2011; Lee et al. 2012), with reduced

Aβ deposits and plaque formation (Lee et al. 2012; Yang et al. 2013; Naaldijk et al. 2016), stimulate neurogenesis, synaptogenesis and neuronal differentiation (Zilka et al. 2011; Yang et al. 2013; Oh et al. 2015) and rescue spatial learning and memory deficits (Lee et al. 2012; Yun et al. 2013; Kim et al. 2013). Despite of all these beneficial effects, some risk factors are associated with stem cell therapy. First of all, the potential chance of tumor formation; in fact, stem cell features resemble some of the characteristics of cancer cells, such as relative apoptosis resistance, long life span and ability to proliferate for extended periods of time (Li et al. 2006). In addition, cell rejection, undesired immune response (although MSCs are less immunogenic with respect to other cell types), accumulation in lung, spleen and livers (following intravenous injection), and unwanted biological effects (e.g. *in vivo* differentiation in undesirable cell type), represent important factors that may hinder the use of stem cells in clinics. In this scenario, EVs represent a safer alternative to their cellular counterpart, as they are easier to handle due to their small size and are safer because they lack nuclei, thus preventing self-replication, and likely they do not possess endogenous tumorigenic potential. Moreover, EVs can be used for overcoming tissue barrier, as BBB, for targeting distinct cell populations within the brain (Lener et al. 2015).

For our *in vivo* studies, we decided to assay the immunomodulatory effects of EVs derived from cytokine preconditioned MSCs in 3xTg-AD mice. This represents the first animal model developed to date that facilitates the study of neuroinflammation in the context of both amyloid and tau pathologies, that appear with a regional pattern closely mimicking that observed in AD patients (Mesulam 1999).

In order to more selectively study the effects of EVs on targeting inflammatory processes, 7-month-old 3xTg mice were chosen because they, at that time, virtually do not display Aβ plaques and NFTs (Aβ plaques clearly appearing by 12 months in the frontal cortex and 15 months in the hippocampus, and NFTs by 12 months in the hippocampus and 18 months in the frontal cortex), although some amyloid plaque begins to appear in the frontal cortex already at 6 months of age (Oddo et al. 2003).

Proliferation of microglia in the brain of 3xTg mice has been described before the appearance of Aβ deposits in the hippocampus and entorhinal cortex (Janelsins et al. 2005; Rodríguez et al. 2010). According to a detailed immunohistochemical characterization of temporal and spatial progression of the disease in this model, proliferation of microglial cells has been reported to occur in the CA1 subfield of the hippocampus and the entorhinal cortex of 3xTg mice before the formation of extracellular Aβ plaques (6 months of age), while microglia number unchanged until 18 months in the frontal cortex (Mastrangelo and Bowers 2008). Therefore, we focused on these three areas that differently show microglia proliferation. Noteworthy, a large amount of evidence underlined the critical involvement of the hippocampus and the entorhinal cortex at the earlier stages of the disease (Braak et al. 1993; Pennanen et al. 2004).

We firstly evaluated the biodistribution of intranasally injected (IN) EVs in the brain of the animals. To do this PKH26-labeled EVs were used evidencing a wide biodistribution of the stained EVs throughout the hippocampus, the entorhinal and the prefrontal cortices, 6 hours after the last intranasal injection. Specifically, we observed a strong internalization of EVs into microglial cells present in these areas; to a lesser extent EVs were found also in neurons, while none vesicle was detected in the cell body of astrocytes, but only in the proximity of their processes. These results are in agreement with those of a study that investigated the effects of IN injected MSC-exosomes on inflammation and memory impairments after the induction of a status epilepticus, suggesting a cell-specific mechanism adopted by cells to uptake these vesicles (Long et al. 2017).

Then we evaluated the ability of MSC-EVs to affect microglia density, as microglia activation and proliferation are typical traits in post-mortem AD brains (McGeer et al. 1987) and in the preclinical models (Jimenez et al. 2008; Meyer-Luehmann et al. 2008), and strategies targeting expansion of microglia population have shown beneficial effects in AD mice models (Fyfe 2016; Olmos-Alonso et al. 2016). In our study EVs were found to strongly reduce the number of Iba-1-positive cells in all the analysed regions of treated group after 3 weeks, compared to control mice. In contrast to Mastrangelo and colleagues who showed evidence of microglia proliferation in frontal cortex of 3xTg mice only at later stages (18 months of age) (Mastrangelo and Bowers 2008), in our study, the frontal cortex of 7-month-old control mice was the region that exhibited the highest density of microglia cells (frontal cortex: 18026.64 ± 2738.72; entorhinal cortex: 14830.59 ± 994.44; CA1: 13807.79 ± 368.26) and, at the same time, the most robust decrease after EV treatment (frontal cortex: -29.17%; entorhinal cortex: -27.16%; CA1: - 20.13%). These disparate outcomes may be the result of the different genders of mice used in our study (female mice) compared to theirs (male). In addition to the increased cell number, microglia activation process is characterized by profound changes in cell shape. Specifically, one of the criteria that has been used to morphometrically characterize “primed” and “reactive” microglia is the hypertrophy of the cell soma (Perry and Teeling 2013). Our morphological analyses evidenced that microglia in non-treated mice displayed a significative increased of the size of cell body compared to microglia of EV treated mice. Moreover, with respect to EV-treated mice, control microglia were characterized by thicker processes, suggesting a polarization towards a highly activated state (Davis et al. 1994). Morphological changes have been described as reliable indicators of the cell function, as evidenced by concomitant changes in the expression of specific antigens and the production of mediators that promote inflammatory response or injury resolution and tissue repair (Ransohoff and Cardona 2010). Iba-1 - a widely employed immunohistochemical marker for the study of both ramified and activated cells (Ahmed et al. 2007) - is a protein involved in actin-crosslinking necessary for membrane ruffling of microglia. This process represents an essential step for the morphological changes that guide the transition from quiescent ramified microglia to activated amoeboid microglia. For these reasons, microglial activation is typically associated to the increased Iba-1 expression (Sasaki et al. 2001).

Therefore, in order to define the activation state of microglia, we measured the expression level of Iba-1 in the CA1 subfield of the hippocampus, entorhinal and prefrontal cortices through the quantification of fluorescence intensity. The significative downregulation of the semiquantitative evaluated expression of Iba-1 in EV-treated group compared to control mice could suggest an EV-induced dampening effect on microglia activation.

When we evaluated the fluorescence intensity of CD68 and CD206, respectively M1 and M2 markers, no changes in CD206 expression was observed, while animals treated with EVs showed a strong decrease in the phagocytic marker CD68 expression, suggesting a functional switch of microglia towards a less phagocytic cell population. This switch is consistent with data obtained in a mice model of traumatic brain injury (TBI) in which the authors clearly demonstrated a downregulation of CD68 expression following ICV injection of MSCs (Zanier et al. 2014). However, in our *in vitro* experiments no modulation of CD68 expression could be reported. This discrepancy between *in vitro* and *in vivo* results may be due to the longer time windows (3 weeks) chosen to analyse the effects *in vivo*, possibly allowing EV non-coding RNAs to efficiently modulate protein expression (see also above). In addition, the knowledge that microglia are able to respond to several environmental stimuli may delineate a role of neurons or other glial cells by releasing factors that could take part in modifying the expression of this phagocytic marker. Something that in our experimental *in vitro* study cannot occur.

In addition to evaluate the effect of EVs on microglia, we decided to focus also on their putative role on neurons, whose function is strongly affected by the activation of microglial cells.

Synaptic dysfunction and LTP deficits are the features that better correlate with memory and cognitive alterations characterizing AD (DeKosky and Scheff 1990; Scheff et al.1991). Amyloid-β fibrils, diffusible oligomers or the intracellular accumulation of amyloid-β have been found to alter the function and structure of dendritic spines by distinct mechanisms in AD (Dorostkar et al. 2015).

In 3xTg mice, intraneuronal Aβ - described as the earliest neuropathological manifestation both in the cortex and the hippocampus of this AD model (Oddo et al. 2003), as well as in the brain of AD patients (Gouras et al. 2000) - has been suggested to underlie synaptic dysfunction and LTP deficits at 6 months of age, since at this age the other principal hallmarks have not been clearly displayed yet (Oddo et al. 2003).

Since hyper-activated microglia has also been shown to dramatically contribute to synapse loss in AD (Rajendran and Paolicelli 2018) and early microglia proliferation has been demonstrated in 3xTg mice from 6 months of age (Janelsins et al. 2005), we wondered if the observed effects of EVs on microglia cell activation could somehow result in a protective effect on neurons.

Although dendritic spine density has been reported to decrease in the hippocampus and in the frontal cortex of this model only from 15 months on, compared to non-transgenic mice (Bittner et al. 2010), our data

showed that EV administration produced a significative increment in dendritic spine density in the hippocampus (+ 26.72%), the entorhinal (+20.83%) and the prefrontal (+16.08%) cortices of 7-month-old 3xTg mice, compared to the controls. This suggests a protective effect on a degenerative process, hitting dendritic spines at early stages. Moreover, the fact that the lower increment in dendritic spine density with respect to controls was observed in the prefrontal cortex, a region that, according to Oddo and colleagues, seems to be already affected by plaque deposition at 7 months of age (Oddo et al. 2003), may suggest a neurotoxic action of Aβ deposits, thus leading to a milder protective effect exerted by EVs in this region.

As far as we know, only one study reported early dendritic spine degeneration in this model at 4 months of age (Bittner et al. 2010). However, the role of microglia was not investigated and loss of dendritic spines (observed only in cortical layer III) was suggested to be the consequence of neuronal loss, which, in turn, was caused by oligomeric Aβ that started to accumulate at this age.

Although a direct correlation is still to be proven, in our study we can hypothesize that the preservation of dendritic spines may be correlated to the EV immunomodulatory effects, due to the polarization towards the M2 microglia phenotype observed *in vitro*. This would suggest that EVs counteract the degeneration of dendritic spines by reducing the levels of inflammatory mediators, that, in turn, contribute to neuronal damage (Ransohoff and Cardona 2010). Importantly, our *in vitro* results evidenced that EVs are able to significantly reduce the release of IL-6 and IL-1β and augment the secretion of IL-10 by primary microglia cells. Interestingly, IL-1β has been shown to alter BDNF-induced expression of molecules critical for activity-dependent synaptic plasticity in organotypic hippocampal slices, leading to the inhibition of BDNF-dependent LTP and dendritic spine genesis (Tong et al. 2012). Conversely, application of IL-10 to hippocampal neurons *in vitro* has been reported to induce neuronal synapse formation and increase dendritic spine density (Lim et al. 2013).

In order to further clarify the impact of inflammation (or that of soluble Aβ species, the most neurotoxic ones) on dendritic spines, it would be interesting to compare the levels of brain cytokines and Aβ oligomers in EV treated and control mice.

In addition to the release inflammatory mediators, microglia has been demonstrated to contribute to synapse loss through the direct phagocytosis of synaptic material, through the classical complement cascade (Hong et al. 2016). In particular, microglia have been shown to express higher levels of the lysosomal protein CD68 in the hippocampus of J20 AD-mice compared to WT. Engulfed synaptic proteins have been found to colocalize with CD68, suggesting an internalization into lysosomal compartments. In our study, the treatment with EVs promoted a functional switch of microglia towards a less phagocytic cell population (decreased CD68 expression compared to control mice). Therefore, we may hypothesize that a reduction in microglia phagocytic activity could represent at least one of the mechanisms underlining the higher dendritic spine density observed after EV treatment. In order to explore this possibility, we are performing simultaneous

Golgi-Cox and immunofluorescence techniques to allow the visualization of the spatial relationship between neuron and microglia. This combined staining method (*Materials and Methods*, Fig. 5) has been set-up by M. Pedrazzoli (PhD student in M. Buffelli's Lab from the University of Verona), by modifying a previous published protocol (Spiga et al. 2011). By combining the detailed information about morphological characteristics of neurons visualized by Golgi-Cox impregnation and the biochemical features (by immunofluorescence) of microglia, we will investigate whether the protective effect on dendritic spines observed after EV treatment might underlie a different behaviour of microglia interacting with neuron dendrites. Moreover, by exploiting markers that specifically label microglia M1 or M2 phenotype, we will carry out studies to assess the specific polarization state of microglia that preferentially contacts dendritic spines in control and EV treated mice.

The analysis of astrocyte-mediated actions in preventing/contributing to dendritic spine loss should also not be neglected. In fact, the crosstalk between activated microglia and astrocytes can result in the amplification of inflammatory response, thus contributing to the neurodegeneration and helping to drive the disease progression (Liddelow et al. 2017). In analogy to the M1/M2 macrophage nomenclature, A1 astrocytes (induced by M1 activated microglia) could exert harmful activities by releasing factors destructive to synapses, while A2, the protective astrocytic phenotype, is characterized by the release of many neurotrophic factors. Therefore, one could wonder whether it might be possible that EV neuroprotective effect on dendritic spines may be mediated by A2 polarized astrocytes. Since none internalization of PKH26-EVs was evidenced in these cells in all the analysed brain regions, we can suppose that the beneficial astrocytic polarization may strictly depend on EV-induced microglia M2 phenotype. At the same time, we cannot rule out that the protective effect may derive by a direct action of EVs on neuronal cells, as suggested by some data from the literature that have indicated that MSC-exosomes are able to promote neurite outgrowth in peripheral and central nervous system (Lopez-Verrilli et al. 2016).

Since in 3xTg mice both plaques and tangles develop in an age- and region- specific manner, it would be interesting to examine the animals during the disease progression up to 20 months of age, in order to evaluate if the observed EV immunomodulatory effects may be effective in halting or at least ameliorating the progression of the other pathological hallmarks. In this direction, behavioural studies will be planned to verify if the desirable synaptic recovery could eventually correlate with a reduced severity of cognitive impairments (spatial learning and memory), that has been reported to affect 3xTg mice already at 6 months of age (Stover et al. 2015).

In 3xTg-mice, amelioration of memory deficits have been observed following ICV infusion of hMSCs (Ruzicka et al. 2016) or after pharmacological inhibition of microglia proliferation through PLX5622 [an inhibitor of colony-stimulating factor 1 receptor (CSF1R)] (Dagher et al. 2015). In this latter study, chronic treatment with the low dose of PLX5622 in aged 3xTg-AD mice resulted in the sustained elimination of microglia (30 %). This suggests the potential therapeutic effect of therapies affecting microglia activation in the brain.

Other studies have investigated the therapeutic role of MSC-EVs in other AD models. MSC-EVs have been reported to alleviate memory impairment in APP/PS1 AD-mice probably by reducing Aβ induced iNOS expression (Wang et al. 2018). In another study EVs rescued synaptic function, eased inflammation and ameliorated the cognitive decline in treated AD mice (Cui et al. 2018). However, in these studies EV administration was performed intravenously (IV) or, even by more invasive routes, as the ICV route, for weeks or months.

We believe that the striking aspect of our study is that the observed effects on both microglia activation and dendritic spine protection were obtained by only two IN MSC-EV injections, most likely because the amount of EVs delivered upon this administration route is higher than those delivered through IV or ICV (Haney et al. 2015). Moreover, it affords a rapid onset of the therapeutic effect, as it does not imply any hepatic first-pass metabolism and, consequently, a higher bioavailability of EVs reaching the CNS. This allowed us to avoid repeated injections in order to achieve enough EVs in the brain. Given the low-invasiveness of IN administration route it would be interesting to evaluate the effects of the chronic treatment on the course of the neuropathology.

In conclusion, this study highlights the therapeutic potential of an anti-inflammatory cell-free strategy in the early phases of the disease, considering that synaptic deficits are among the first signs of the neuronal degenerative process and occur in early AD and mild cognitively impaired (MCI) patients before the appearance of the plaques (Mucke and Selkoe 2012).

Preliminary study in leech

In order to better and more specifically characterize the immunomodulatory potential of EVs on microglia and the putative molecules involved in this effect, we are exploring and exploiting a leech model (*Hirudo Verbana*) of inflammation induced by LPS, a prototypical stimulus known to drive microglia M1 polarization also in leech.

Leech has been described as a valuable model to study neuroinflammation, in virtue of its well characterized, although simple, CNS (Le Marrec-Croq et al. 2013). Particularly, the functional similarities of leech microglia cells with those found in vertebrates, the low-level of infiltration of peripheral macrophages in CNS after injury, and the absence of the other principal glial cells (astrocytes and oligodendrocytes) (Boidin-Wichlacz et al. 2012), make leech a very useful model to specifically study the role of microglia in orchestrating neuroinflammatory and regenerative processes, without the contribution of any other cell type.

In collaboration with the group of prof. A. Grimaldi from the University of Insubria, we are investigating the anti-inflammatory effects of EVs derived from 48h cytokine preconditioned cells.

Our preliminary results suggest that the treatment with EVs reduces the recruitment of microglial cells at the site of LPS injection [connectival fibers (i.e. neuronal axons)]. Notably, microglia could be considered virtually

the only cell type found in the connectives, along with only two connectival glial cells that envelop the axons. Typically, in this model their recruitment at the lesion site is observed, as it occurs in vertebrates.

Moreover, the treatment with EVs downregulated the expression of the pro-inflammatory cytokines IL-18, IL-6, TNF-α, while increases the release of the anti-inflammatory cytokine IL-10. In particular, the modulation of IL-6 and IL-10 is in line with what observed *in vitro* on primary microglial cells. Further experiments will be carried out to confirm these preliminary data in order to identify the EV cargoes (and possibly the molecular mechanisms associated) responsible for the immunoregulation on microglia. To do this, we will compare the effects and the molecular content (miRNAs, proteins, lipids) of EVs derived from 48h cytokine preconditioned and non-preconditioned cells (MSCs grown in SF medium only). In fact, although data from the literature have clearly demonstrated that cytokine preconditioning increases the ability of EVs to attenuate inflammation (Harting et al. 2018; Ruppert et al. 2018), the ultimate mechanism by which microglia behaviour can be modulated by MSC-EVs remain to be elucidated. Moreover, given the ability of the leech to repair and restore normal functions in response to nerve lesion and the critical role exerted by microglia in these processes, an evaluation of EV effects on leech CNS regeneration, as well as the molecular mechanisms involved in this process will be carried out.

CONCLUSIONS

Our current study indicates that IN injection of EVs derived from cytokine-preconditioned MSCs is a feasible method to reduce microglia activation and to prevent dendritic spine degeneration in the hippocampus, the entorhinal cortex and the prefrontal cortex of 7-month-old 3xTg mice. However, to ascertain the beneficial effects of EVs, it will be necessary to analyse the progression of the pathological hallmarks as well cognitive performance up to later stages. In this case, given the low invasiveness of the procedure of EV administration, a chronic treatment may be considered in order to potentiate the promising effects observed on microglia and dendritic spines after only two IN injections. Hopefully, the experiments performed in the leech will help us to elucidate some aspects involved in the observed EV neuroprotective effects. Finally, we believe that our results support the therapeutic potential of EVs derived from preconditioned MSCs for the treatment of AD, and potentially for other neurodegenerative diseases, given their efficacy and safety.

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