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MICROVESICLES: MESSENGERS AND MEDIATORS OF NEUROINFLAMMATION

BIO/14

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1.Introduction

1.1 MVs as mediator of cellular communication

Cell-to-cell communication is required to guarantee proper coordination among different cell types. Communication between cells is mediated mainly by extracellular molecules such as growth factor, cytokines, chemokines, hormones. Some of these molecules operate over long distances, signaling to cells far away; others signal only to immediate neighbors (Alberts, 2002). Cell can also communicate and exchange information through cell-to-cell contact, mediated by specialized adhesion molecules or through the release of membrane vesicle (Camussi et al, 2010). Cell–cell communication mediated by membrane vesicles emerged very early during evolution and served as a template for the further development of intercellular interaction mechanisms involving soluble bioactive mediators and fine-tuned ligand–receptor interactions (Ratajczak et al, 2011). Two classes of membrane vesicles have been described in literature: exosomes and shed vesicles (MVs).

1.1.1 Exosomes

Exosomes are a population of small membrane vesicles (50-90 nm) released by an endocytic pathway (Fig. 1). Exsosomes generation take place inside the lumen of multivesicular bodies (MVBs) by budding, fission ad segregation of their membrane. MVBs are ubiquitous membrane-bound organelle that works as intermediates in endolysosomal transport. Based on their biochemical

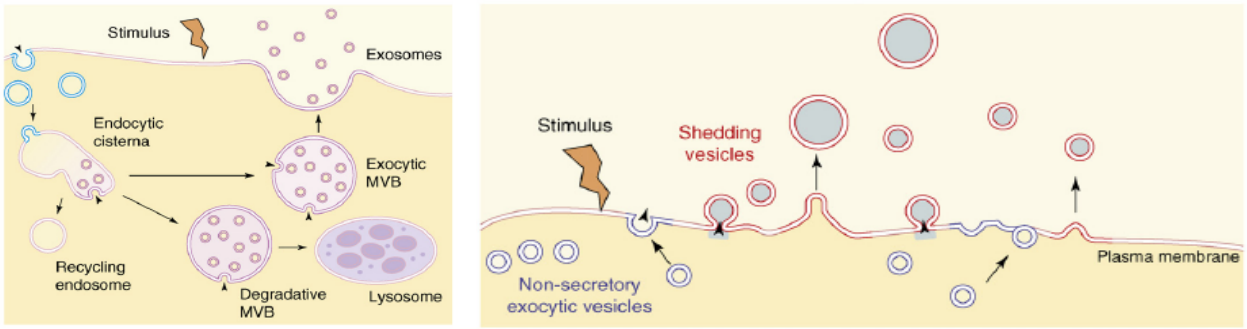

Fig. 1:Generation of exosomes and shed vesicles. (Cocucci et al, 2009)

properties, intracellular MVBs can either traffic to lysosomes, where they are subjected to

proteosomal degradation (degradative MVBs), or to the plasma membrane, where release exosomes in the extracellular space (exocytic MVBs) upon fusion with the plasma membrane (Mathivanan et al, 2010). Sorting of vesicles inside MVBs is a regulated pathway, generally involving the ESCRT (endosomal sorting complex required for transport) machinery, which regulates membrane scission. Recent studies indicate that fission of exosome membrane is catalyzed by components of the ESCRT-III complex, called charged multivesicular body proteins (CHMPs); (Hanson et al, 2009; Wollert & Hurley, 2010; Wollert et al, 2009) and by the AAA-ATPase vacuolar protein sorting-associated 4, VPS4 (Babst, 2005). Several other factors have been identified that promote exosome biogenesis, including the sphingolipid ceramide produced by neutral sphingomyelinase (N-SMase) (Kosaka et al, 2010; Trajkovic et al, 2008). The fusion of MVBs and consequently the release of exosomes in the extracellular space can occur either in a constitutive way or in response to specific stimuli.

Exosomes are enriched in several proteins and lipids of the MVBs membrane, while depleted of many other. Indeed for these constituents, exosome biogenesis serves as a mechanism of regulated assembly of MVB components. As consequence of their endosomal origin exosomes contain numerous protein involved in membrane transport and fusion (i.e. annexin, flotillin, Rab GTPases), in MVBs biogenesis (Alix), besides integrins and tetraspanins (CD63, CD9, CD81, CD82). They are also characterized by the presence of high levels of cholesterol, sphingolipids, ceramide and glycerophospolipids in their membrane (Simons & Raposo, 2009).

1.1.2 Microvescicles

Microvesicles (MVs), also referred to as shed vesicles or ectosomes (Sadallah et al, 2011), are a population of quite large membrane vesicles that are more heterogeneous in size (100nm-1μm) and shape as compared to exosomes. They bud directly from the plasma membrane and are released into the extracellular environment upon cell activation (Fig. 1). Shedding of MVs typically involves a

budding process, in which surface blebs selectively accumulates cellular constituents that are then packaged into MVs. MVs contain a variety of cell surface receptors, intracellular signalling proteins and genetic materials derived from the cell of origin. In terms of composition, MVs shed from distinct cells are molecularly different from each other, reflecting the differential expression of proteins of various donor cells. Composition and biological activity of MVs also vary depending on the state (e.g. resting, stimulated) of donor cells and depending on the agent employed for stimulation (Bernimoulin et al, 2008). However, shed vesicles are generally characterized by the presence of high levels of phosphatidylserine (PS) on their surface.

1.1.3 Shedding of MVs induced by P2X7 receptor activation

A specialized type of MV release exists for cells that express the ATP receptor $P2X_7$, which shed MVs from the cell surface when exposed to ATP. $P2X_7$ receptor is an ATP-gated ion channel that is highly expressed in immune cells, in particular macrophages (Steinberg & Silverstein, 1987) mast cells (Cockcroft & Gomperts, 1979) and microglia (Visentin & Levi, 1997), where it controls the release of inflammatory cytokines. Activation of $P2X_7$ receptor can induce efficient inflammosome assembly and maturation of the inflammatory cytokines IL-18 and IL-1β, which is then followed by rapid cytokine secretion (Qu et al, 2007). $P2X_7$ receptor differs from other members of the P2X family in its relatively low affinity for ATP and the presence of a long cytoplasmic C-terminus that contains several protein–protein interaction motifs. Depending on the concentration of ATP and the duration of stimulation, $P2X_7$ receptor can function as an ion channel or a non selective pore, the latter generally leading to cytoxicity and apoptotic cell death. Many studies have shown that dramatic morphological changes occur in cells endogenously or heterologously expressing $P2X_7$ receptors during and subsequent to receptor activation (Hogquist et al, 1991). These changes consist in rapid formation of cell membrane blebs and are associated to cell death upon sustained $P2X_7$ receptor activation. Membrane blebbing requires several intra-signalling events, which are activated

by occupancy of the receptor such as the action of protein kinases and other effector enzymes (Duan & Neary, 2006). In particular, several lines of evidence indicated that blebbing induced by $P2X_7$ receptor is dependent on P38 and requires ROCK kinase activation, which causes local disassembly of the cytoskeletal elements, associated to the $P2X_7$ receptor C-terminus, (Budagian et al, 2003) (Morelli et al, 2003; Verhoef et al, 2003). Notably, surface blebbing is preceded by loss of plasma membrane asymmetry and exposure of phosphatidylserine (PS) at the outer leaflet of the plasma membrane, a process controlled by specific enzymes, named flippase, floppase and lipid scramblase, which control PS segregation in the inner leaflet of the plasma membrane. Externalized PS is commonly accepted as a marker of a cell undergoing apoptosis. However, a pioneer study by Surprenant and colleagues (MacKenzie et al, 2001) reported that $P2X_7$-induced PS externalization and bleb formation occur within the first few minutes of receptor activation and may be reversible after brief stimulation, thus dissotiating $P2X_7$-induced bleb formation from cell apoptosis in monocytes. MacKenzye and colleagues also showed that during blebbing, MVs with externalized PS may form and be released into the extracellular space as a result of bleb detachment from the cell surface. Notably, during blebbing the pro-inflammatory cytokine IL-1β is packaged into plasma membrane blebs and subsequently released into the extracellular space from reactive monocytes as MVs. Almost ten year ago these results provided the first evidence that $P2X_7$-induced MV shedding acts as a secretory pathway for rapid release of IL-1β and may represent a general mechanism for secretion of leaderless secretory proteins from $P2X_7$-expressing myeloid cells.

Few years ago data obtained in the laboratory demonstrated that a MVs-dependent mechanism IL-1b release, very similar to that first described in monocytes(MacKenzie et al, 2001), occurs in microglial cells (Bianco et al, 2005) and contributed to clarify the mechanism mediating MVs shedding in glial cells that express P2X7 receptors. We found that biogenesis of MVs storing IL-1β is controlled by acid sphingomyelinase (A-SMase), the enzyme which hydrolyzes sphingomyelin (SM) to the sphingolipid ceramide. Following P2X7 receptor activation and

subsequent P38 MAP kinase phosphorilation, A-SMase becomes promptly activated and moves to plasma membrane outer leaflet, where generates ceramide, thereby inducing budding of MVs (Fig. 2) (Bianco et al, 2009a).

Formation of blebs is likely caused by redistribution of extracellularly synthetized ceramide within

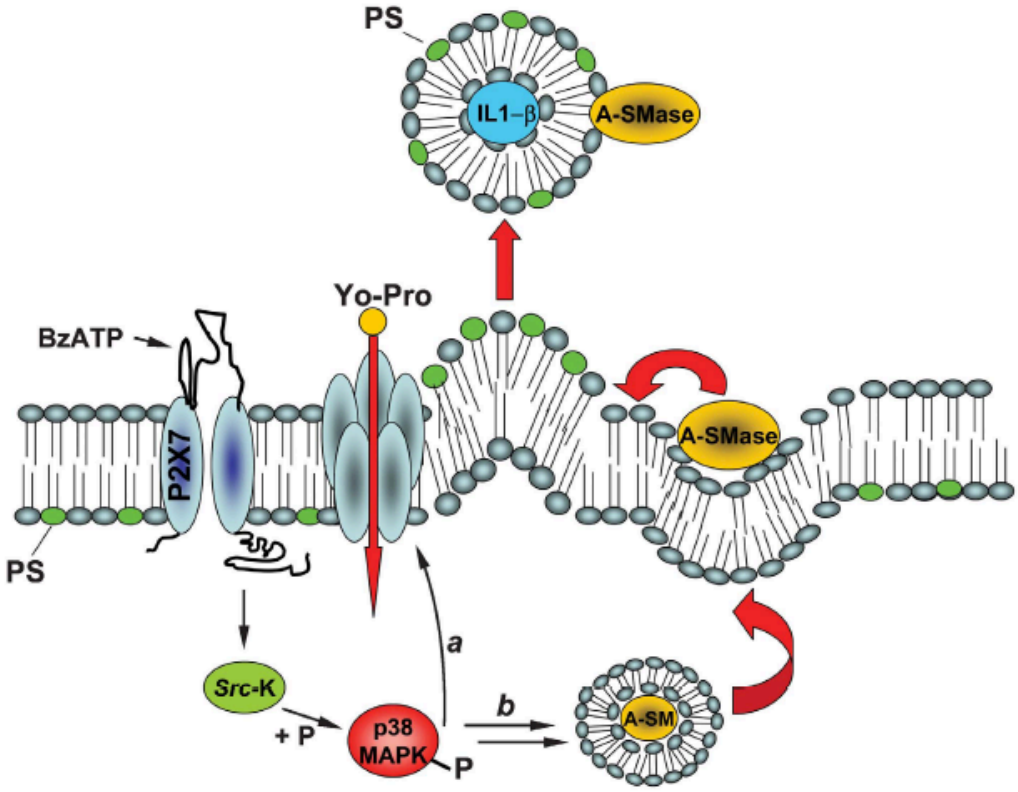

Fig. 2: Model for P2X7R-induced MVs shedding in glial cells (Bianco et al, 2009a)

the bilayer and by local enrichment of the cone-shape sphingolipid into the inner leaflet of the membrane, where it may induce, due to its spontaneous negative curvature, membrane subdomains with curvature different from the adjacent planar membrane (Subra et al, 2007).

The key role of A-SMase in MV formation was demonstrated using pharmacological inhibition and genetic inactivation of the enzyme. Both approaches strongly abolished release of MVs and of IL-1β from reactive glial cells (Bianco et al, 2009a). These results were consistent with the involvement of neutral- (N-) rather than A-SMase and ceramide in the budding of exosomes in oligodendrocytes (Trajkovic et al, 2008) and further indicated that the budding of MVs may share

features with exosome biogenesis. The role of N- and A-SMase in exosome and MV formation, respectively, suggest that different members of the SMase family may control the release of distinct types of extracellular vesicles from brain cells, independently of the ESCRT complex.

Other pathways besides shed MVs have been proposed to mediate IL-1β release from myeloid cells, including exosomes and exocytosis of secretory lysosomes in monocytes (Qu et al, 2007). However, enrichment of IL-1β in larger MVs, derived from the plasmamembrane, and complete blockade of MV shedding and IL-1β release from A-SMase knock-out cells indicated that MV shedding represents the major mechanism mediating secretion of the inflammatory cytokine from reactive microglial cells.

MVs shed from glial cells store the pro-inflammatory cytokine IL-1β (Bianco et al, 2005), angiogenic factors and their respective mRNAs, and matrix metalloproteinases (Al-Nedawi et al, 2009; Proia et al, 2008; Sbai et al, 2010).

1.1.4 MVs role in communication

In spite of the fact that MVs and exosomes contain numerous proteins, lipids ad RNAs and may be released from almost all the cell types both constitutively and upon stimulation; they are characterized by different composition depending on the cell origin ad activation state of parental cells (Distler et al, 2005).

The content of MVs and exosomes do not reflect the exact composition of the cytoplasm of the donor cells. The gene profile array, carry out on MC/9 (a mast-cell line) exosomes displayed essential difference in the levels of mRNA transcripts in the exosomes as compared to donor cells.

Since membrane vesicles are released in the extracellular space they can interact with target cells; some evidence suggest that MVs can interact only with specific target cells and not with any cell present in the microenviroment (Camussi et al, 2010; Losche et al, 2004).

Different ways by which MVs can stimulate target cells are described shown in Fig.3.

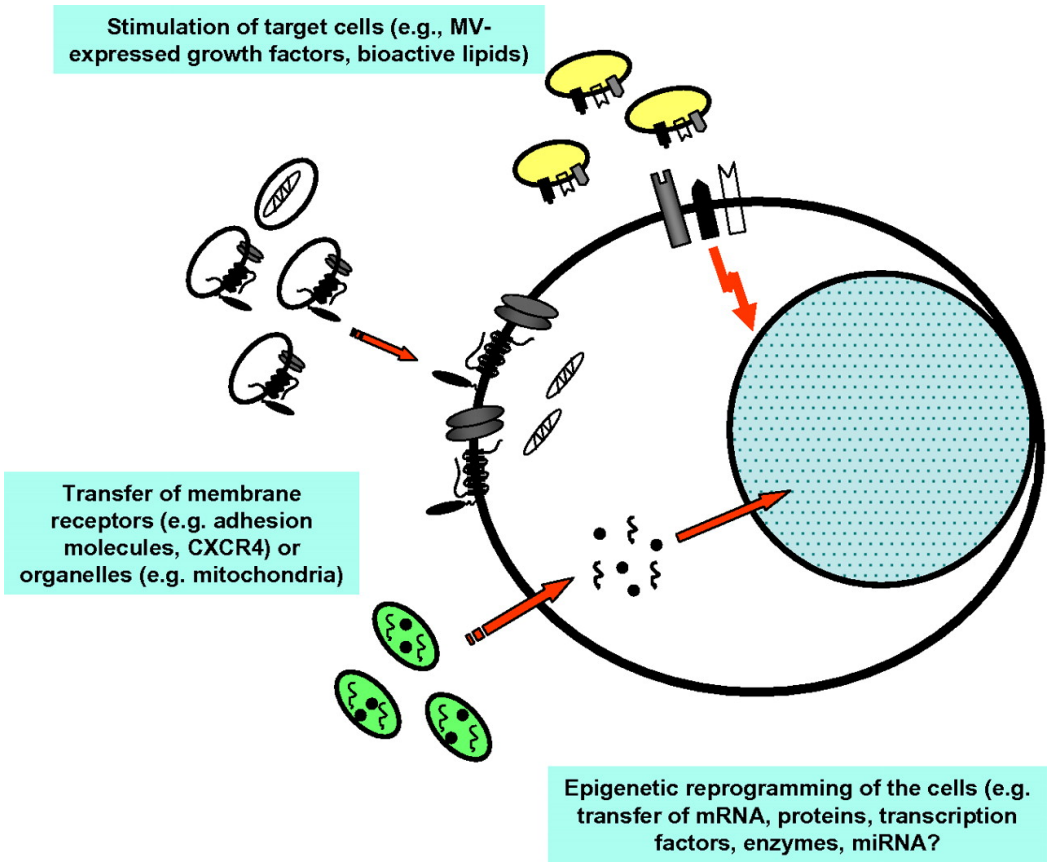

Fig. 3: Model for P2X7R-induced MVs shedding in glial cells (Bianco et al, 2009a)

MVs can interact with other cells directly by surface expressed proteins like growth factors or by bioactive lipids. For example, platelet derived MVs have an important role in coagulation because their membrane, that is enrich in externalized phosphatydilserine, favours the assembly of clotting factors. Membrane vesicles can also transfer surface receptors between cells; it has been reported that MVs derived from tumor can transfer FAS ligand to T cells inducing apoptosis and promoting tumor immune escape. Furthermore MVs can deliver proteins to other cells and mediate horizontal transfer of genetic material such as mRNA and miRNA. Among other evidence, Deregibus et al. (Deregibus et al, 2007) reported that MVs derived from endothelial progenitors cells could transfer mRNA to endothelial cells both *in vitro* and *in vivo*. Interestingly, Valadi and coworkers

demonstrated that mRNAs taken up by recipient cells can be translated within the cells (Valadi et al, 2007). Indeed they identified three distinct mouse proteins in human mast cells after exposure to murine exosomes Notably, these proteins were not present in exosomes secreted from murine mast-cells (Valadi et al, 2007). The presence of several miRNA has been reported in many microvesicles, including those derived from primary human glioblastoma, lung cancer, gastric cancer and ovarian cancer (Skog et al, 2008).

miRNA are small non coding RNA that negatively regulate their mRNA target inducing degradation or translational repression. It has been estimated that a single miRNAs could interact with more than 200 different mRNA and that minor changes in the expression of a miRNA could have big impact on the expression of their target. So the MVs mediated transfer of miRNA can have important effect on the phenotype of the recipient cell (van der Vos et al, 2011).

1.1.5 Physological and pathological role of MVs

Despite MVs were originally described as inert debris, MV shedding from the plasma membrane is now a recognized mode of intercellular communication and the role of MVs has been reported in several physiological and pathological process such as cell proliferation, coagulation, vascular function, apoptosis, inflammation and tumor progression. For example platelet derived MVs are important mediators of both coagulation and thrombosis. These MVs are released upon collagen stimulation and are characterized by the presence of tissue factor (TF) on their membrane. In physiological conditions platelet-derived MVs, by acting on macrophages, neutrophils and other platelets work as trigger of coagulation. However, p vesicles are also present in the lipid core of atherosclerotic plaque where promote thrombotic signals(Cocucci et al, 2009).

Moreover, vesicles secreted from different cells can influence the immune response; for example MVs can present antigen to T cells, or transfer the antigen-MHCII complex to dendritic cells, or directly activate natural killer cells and macrophages (Thery et al, 2009). During inflammation MVs

can act both as anti-inflammatory or pro-inflammatory mediators; neutrophil derived microvescicles stimulate the production of anti-inflammatory cytokines and MVs released from fibroblasts promote the synthesis of pro-inflammatory cytokines, such as inteleukin-6 (IL-6), the monocyte chemotactic protein 1 and metalloproteinase.

The most well characterized membrane vesicles are those released from blood cells, i.e. platelets, leukocytes, erythrocytes, and endothelial cells. However accumulating evidence demonstrate that MVs and exosomes can also be released by brain cells and that these particles play an important function in the central nervous system (CNS) both in pathologic or healthy conditions.

For example, exosomes derived from oligodendrocytes control myelination, and those produced from Schwann cells support local axonal protein synthesis by delivering ribosomes to injured neuron. Moreover exosomes released by neuron may contribute to the spreading of pathogenic agents or degenerative proteins like beta-amyloid and alpha-synuclein (Emmanouilidou et al, 2010). Also MVs derived from glioma contribute to transfer oncogenic proteins.

1.1.6 Clinical prospective

Besides their important function in cell-to-cell communication and their role in physiological and pathological process MVs are emerging as important diagnostic tool and therapeutic target (Cocucci et al, 2009). Because of their small size, released MVs can move from the site of discharge and enter into biological fluids; MVs has been described in almost all body fluids, including plasma, urine, milk, cerebrospinal fluid (CSF) (Camussi et al, 2010).

MVs derived from activated blood platelets, leucocytes and endothelial cells continuously circulate in peripheral blood under steady-state conditions, with their number increasing during stress situations (e.g. infection, organ tissue damage or neoplasia) (George et al, 1982; Quesenberry et al, 2010; Ratajczak, 2011). The presence of MVs in body fluid make them easy accessible and analysis of their concentration and molecular composition can open a window on the damaged tissue

(Camussi et al, 2010).

As a consequence of cell activation elevated levels of MVs mainly derived from platelets, endothelial cells and tumor were reported in several pathologies such as cardiovascular disease, rheumatoid arthritis, sepsis, cancer and also in CNS diseases as summarized in table 1. Their levels may be a measure of inflammation, or predictive of metastasis in case of tumor derived MVs (ratajczak). Therefore extracellular MVs released from blood or tumor cells are emerging as new biomarkers from a specific tissue undergoing activation or damage *(Doeuvre et al, 2009)*.

Pathology	Compartment	MP pattern	Technique used	Reference
Stroke	Blood	Elevated platelets-derived MPs	Flow cytometry	Cherian *et al.* 2003
Stroke	Blood	Elevated platelets-derived MPs	Flow cytometry	Pawelczyk *et al.* 2009
Stroke	Blood	Association of endothelial MPs with lesion	Flow cytometry	Simak *et al.* 2006
Transient ischemic stroke	Blood	Elevated platelets-derived MPs	Flow cytometry	Lee *et al.* 1993
Cerebral malaria	Blood	Elevated endothelial-derived MPs	Flow cytometry	Combes *et al.* 2004
Multiple sclerosis	Plasma	High level of endothelial MPs	Flow cytometry	Jy *et al.* 2004a,b
Multiple sclerosis	CSF	Presence of oligodendrocyte-derived MPs	Electron microscopy	Scolding *et al.* 1989
Traumatic brain injury	Plasma and CSF	Presence of platelet and endothelial MPs	Prothrombinase assay	Morel *et al.* 2008a,b
Glioblastoma	CSF	Presence of platelet and endothelial MPs	Immunoblot	Huttner *et al.* 2008

Table 1: Cell-derived microvesicles in CNS disease(Doeuvre et al, 2009)

Clinical interest on MVs is not only related to their potential role as biomarkers of pathology. Also the possible therapeutic role of MVs has been recently considered.

MVs derived from mesenchimal stem cells (MSC) have been reported to protect the kidney against ischemia–reperfusion-induced acute and chronic kidney injury. A single administration of MVs immediately after ischemia–reperfusion injury protected experimental animals from acute kidney injury by inhibiting apoptosis and stimulating tubular epithelial cell proliferation. This phenomenon was strongly dependent on mRNA present in MVs. Considering that MVs have specific targets and that do not interact with all cell types they can also be used as vehicle to delivery drugs or other molecules to specific cells in order to regulate the immune response such as in tumor or in organ transplantation (Camussi et al, 2010; Ratajczak, 2011).

1.2 Microglia in inflammation

Microglial cells constitute 20% of the total glial cell population within the brain and are the resident macrophages of the central nervous system (CNS). These cells are derived from myeloid precursor that migrate into the CNS during embryogenesis (Kettenmann et al, 2011).

After invading the brain parenchyma microglial cells precursors mature into a ramified cells characterized by a small soma and long thin process (Fig.4) and display the so-called "resting" or"surveillant" microglia phenotype. (Hanisch & Kettenmann, 2007)

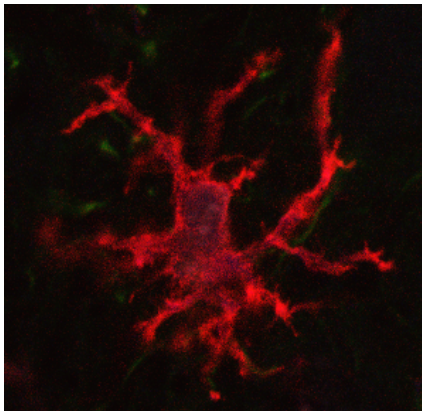

Fig. 4: Resting microglial cell

Although they are called resting, ramified microglial cells are far from inactive. Studies based on *in vivo* two-photon microscopy on transgenic mice that express EGFP in the locus of CX3CR1 chemokine receptor, demonstrates that the process of microglial cells are highly mobile and continuously survey their surrounding parenchyma without disturbing the fragile neuronal structure. It was estimate that the complete brain parenchyma could be monitored every few hours by surveillant microglia ss. The rapid scanning process rapidly changes to a targeted movement toward the site of injury when microlesion are induced. (Hanisch & Kettenmann, 2007) (Kettenmann et al, 2011)

One of the most remarkable properties of microglial cells is their ability to respond both to internal

signal such as damaged or stressed cells and to external signal like pathogens. Activation can be considered a stepwise transformation of resting cells that occurs upon disturbance of tissue homeostasis or upon experimental stimulation.

Infection, trauma, ischemia, neurodegenerative disease, loss of brain homeostasis can evoke rapid and profound changes in the microglial cell shape, gene expression and functional behavior which summarily are defined as "activated" microglia (Hanisch & Kettenmann, 2007).

During activation microglial cells change their appearance and acquire an amoeboid phenotype. Microglial cells also become motile, proliferate, unfold their phagocytotic activities, release chemoattractive factors and present antigen to T cells (Hanisch & Kettenmann, 2007).

Regarding tissue resident macrophages, which represent the peripheral counterpart of microglial cells, several studies have shown that they can undergo two different forms of polarized activation. The first is the classic (M1) activation, characterized by high capacity to present antigen, high production of NO and ROS and of pro-inflammatory cytokines. M1 cells act as potent effectors that kill microorganisms and tumor cells, drive the inflammatory response and may mediate detrimental effects on neural cells.

The second phenotype (M2) is an alternative apparently beneficial activation more related to a fine tuning of inflammation, scavenging of debris, promotion of angiogenesis, tissue remodeling and repair. Specific environmental signals are able to induce these different polarization states (Porta et al, 2009).A bipolar polarization has been also recently suggested for microglia, by showing that these cells, under certain conditions, can indeed be pushed to both extremes of the M1 and M2 differentiation spectrum (Michelucci et al, 2009). Therefore, classically activated microglia (M1) refers to cytotoxic microglial cells which secrete ROS and proinflammatory cytokines. In contrast, alternatively activated microglia (M2) indicate myeloid brain cells that block proinflammatory responses and produce high levels of anti-inflammatory cytokines and neurotrophic factors (Buechler et al, 2000).

1.3 Multiple sclerosis

Multiple sclerosis (MS) is a leading cause of disability in young adults in the western world. (Compston & Coles, 2002).It is considered an autoimmune inflammatory demyelinating disease of the CNS. The disease is due to myelin autoreactive T cells, activated at the periphery, which cross the blood–brain barrier and drive an inflammatory process in the white matter. Clinically the most common form of the disease is partitioned into an early phase characterized by relapses an remission of neurological disability and a later chronic phase which shows a more progressive, non remitting feature (Compston & Coles, 2002). In MS patients, acute lesions in the nervous tissue are observed. These lesions are normally localized in the white matter and are characterized by breaking of the blood brain barrier, edema and demyelination, typical signals of an ongoing inflammatory process. In the last years, most of the research has focused on the inflammatory response of MS patients. The pathological hallmark of MS is, in fact, the presence within the CNS of inflammatory infiltrates containing few autoreactive T cells, a multitude of pathogenic nonspecific lymphocytes, macrophages and microglia (Prineas et al, 2001; Sriram & Rodriguez, 1997). A significant microglia activation is present in all the major steps of MS, including the secondary progressive form. This is why it's believed that microglial activation underlies the insidious and persistent inflammation which mediate demyelination and progressive axonal loss in MS (Hemmer et al, 2002; Takahashi et al, 2003). The MS brain is also characterized by elevated levels of microglia-derived cytokines, including the pro-inflammatory cytokine IL1 beta, which is thought to have a major role in neuroinflammation. Microglia and peripheral macrophages, together with the products of inflammation (cytokines, nitroxide, etc.) and the variable participation of an antibody response, are currently considered the major sources of tissue lesion.

A central point in understanding the pathogenesis of the MS is the role of inflammation and the relationship between inflammation, demyelination and axonal degeneration. There is still a consensus among most researchers that immunological process play a pivotal role in the

pathogenesis and progression of MS (Fig. 5) but there is also an alternative hypothesis that propose that activation of autoimmune cells is a consequence of toxic insults to CNS cells (Hauser & Oksenberg, 2006).

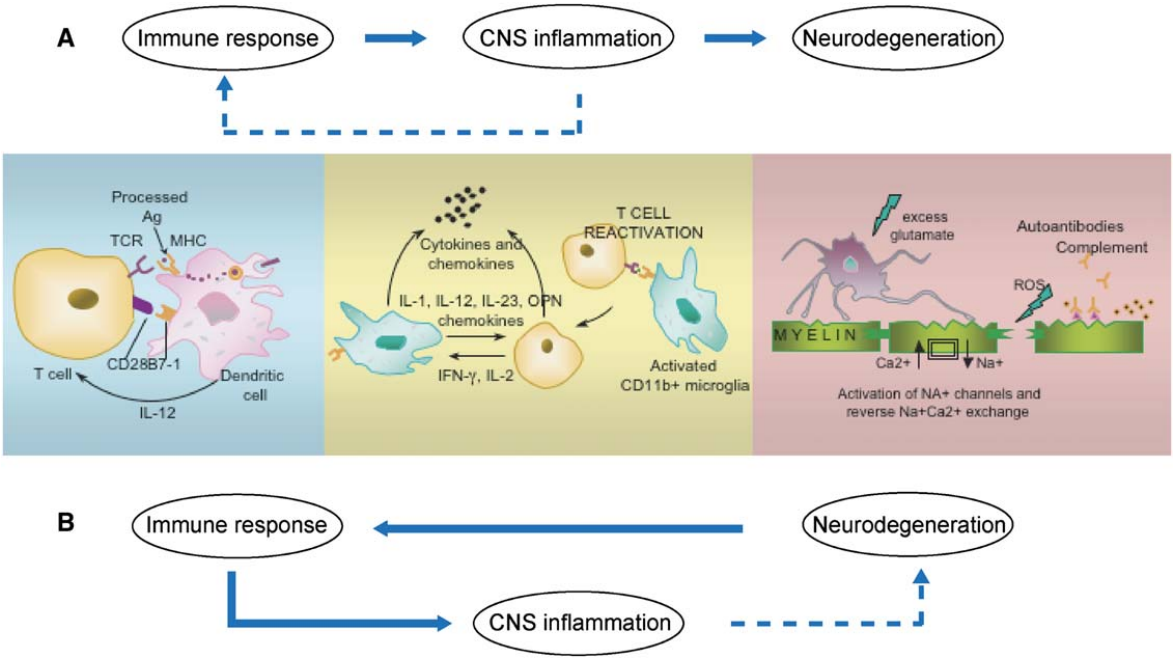

Fig. 5: Models of disease pathogenesis in MS. (Hauser & Oksenberg, 2006)

1.4 Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is the most widely used model of multiple sclerosis. Different types of EAE have been developed in order to investigate this heterogenic human disease (Mix et al, 2010).

The history of EAE started in the first half of the 20th century; experiment were performed first in guinea pig and monkeys, and later in rat and mouse, enabling more extensive immuno-genetic, histopathological and therapeutic studies.

Currently, the most common mode of EAE induction is based on the injection of an encephalitogenic peptide, mostly MOG $_{35-55}$ or PLP $_{139-151}$, which is emulsified in CFA containing

mineral oil and Mycobacterium tuberculosis, followed by intraperitoneal injection of pertussis toxin(Mix et al, 2010).

The phenotype of immunized animals depends mainly on the antigen source and the genetic background of the species and the strain of animal used. For example PLP induces relapsing remitting EAE in SJL mice, while MOG induces chronic-progressive EAE in C57BL mice (Gold et al, 2006; Mix et al, 2010).

1.5 mir-146a in reactive microglia

MicroRNAs represent an evolutionary conserved class of endogenous ~ 22 nucleotide non coding-RNA that act as small regulatory molecules involved in post-transcriptional gene repression(Cao et al, 2006). Several miRNA have been described in the CNS and they are found to play a crucial role in several biological process. Among miRNAs present in the brain, Mir-146a is known to be an important regulator of the immune response. miRNA-146a is induced by different pro-inflammatory stimuli, such as IL1-β and TNFα and its up-regulation is reported in various human pathologies associated with activation of inflammatory responses. (Aronica et al, 2010). Studies in recent years have shown that miRNAs have a unique expression profile in cells. For this reason they are emerging as potential biomarkers. Large miRNA expression studies have shown that miRNA profiles may reflect the differential status of a tumor and have supported the role of miRNA as either prognostic and/or diagnostic markers in various types of cancer (Calin & Croce, 2006; Rusca & Monticelli, 2011).

In this context, a very interesting study by Ponomarev and colleagues recently demonstrated that expression of Mir-146a reflects the M1 pro-inflammatory phenotype displayed by microglia and peripheral macrophages during the active phase of EAE (Ponomarev et al, 2011).

2. Aim of the study

Extracellular microvesicles (MVs) are important mediators of cell-to-cell communication and are emerging as new biomarkers of tissue damage.

Results of the laboratory have recently shown that the typical danger signal ATP, which accumulates in the brain during inflammation, induces shedding of microvesicles (MVs) from microglia, which store and release pro-inflammatory cytokines. However no information is available about existence of microglia-derived MVs within the brain and of their possible biological activity. Although microglia are the immune cells of the CNS, which provide the first line of defense *in brain pathologies* (Graeber et al, 2011), all reports which exploited the diagnostic value of MVs in CNS diseases have focused so far on platelet-, endothelial cell- or oligodendrocyte-derived MVs (Doeuvre et al, 2009; Emmanouilidou et al, 2010; Scolding et al, 1989). The possibility that microglia-derived MVs exist *in vivo* and may represent biomarkers of inflamed CNS has never been explored.

Aim of the present study was to address this hypothesis in the prototypic inflammatory disease of the CNS, multiple sclerosis (MS).

Cerebrospinal fluid represents an easily accessible source of material for diagnosis and monitoring of neurological diseases. It is a potential indicator of abnormal CNS states such as inflammation, infection, neurodegenerative processes and tumor growth (Zougman et al, 2008). Therefore, to investigate the presence of microglia-derived MVs in vivo we analyzed by fluorescence microscopy, western blotting and flow cytometry the CSF collected from healthy rodents. We found that rodent CSF contains microvesicles (MVs) of microglial origin, similar in size and content to MVs released in vitro from microglia. We also showed that microglia-derived MVs deliver a pro-inflammatory signal to glial cells, both to astrocytes and microglia, and that contribute to neuroinflammation in the course of Experimental Autoimmune Encephalomyelitis (EAE). All together these findings demonstrated a previously unappreciated role for microglia-derived MVs in

neuro-inflammation and identified microglial MVs as a novel biomarker of brain diseases characterized by microglia activation.

3.Material and methods

3.1 Animals

Sprague Dawley and Lewis rats, C57BL/6 and SJL/j mice were purchased from Charles River. A-SMase KO mice (Horinouchi et al, 1995a) are a gift of Prof. Edward H. Schuchman. CX3CR1-EGFP (Jung et al, 2000) mice were provided by F. Kirchhoff.

Animals were housed at constant temperature (22-18°C) and relative humidity (50%) under a regular light–dark schedule (lights on 7 a.m. to 7 p.m.). Food and water were freely available. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

3.2 Human patients

Human CSF samples were obtained for diagnostic purpose from subjects with CIS (n=18) or definitive MS (n=40) according to revised McDonald's criteria (Polman et al, 2011), attending the MS Center of the San Raffaele Hospital. Two patients had a primary progressive course (PPMS), while among relapsing remitting MS patients (RRMS), 23 were clinically stable and 15 were sampled during an acute attack. Two subjects with neuromyelitis optica (NMO), positive for NMO-Ig, were also included in the analysis. CSF from age and sex matched healthy donors (n=7) without known neurological disease was collected from subjects undergoing local anesthesia for orthopedic surgery. This research project was approved by the ethical Committee of the San Raffaele Scientific Institute and all subjects signed a written informed consent.

3.3 Astrocytes and Microglia primary culture

Primary mixed glial cultures from embryonic rat pups (embryonic days 20–21) were obtained as previously described (Bianco et al, 2009). Dissociated cells were plated on poly-L-lysine-treated (10 μg/ml, Sigma-Aldrich) T75 flasks at density $1x10^{6}$ cells/flask, and grown in MEM (Invitrogen Life Technologies) supplemented with 20% FCS (Gibco) and 100 IU/ml penicillin, 10 mg/ml streptomycin, and 5.5 g/L glucose (glial medium). Purified microglial cultures were harvested by shaking 2-weeks old mixed glial cultures. $1x10^{6}$ microglia cells were plated onto poly-D,L-ornithine-coated (final concentration 50 μg/ml; Sigma-Aldrich) 60mm tissue culture dish and maintained in the same medium.

3.4 Hippocampal neuron

Primary neuronal cultures were prepared from the hippocampi of 18-day-old fetal rats, as described by Bartlett and Banker (1984). Dissociated cells were plated on poly-L-lysine-treated (1mg/ml, Sigma Chemical Co., St Louis, MO, USA) glass coverslips in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen).

3.5 Isolation of MVs from cells

MVs shedding is induced by treatment of microglial cells with ATP (1 mM) for 30 min in Krebs-Ringer solution (125mM NaCl, 5mMKCl, 1.2mM MgSO4, 1.2mM KH2PO, 2mM CaCl2, 6mM D-glucose, and 25mM HEPES/NaOH, pH 7.4). MVs were then isolated by differential centrifugation as previously described(bianco) The shedding vesicles were pelletted at 10.000 g for 30 min while exosomes were pelleted at 110.000 g., (Bianco et al, 2009). Micro BCA protein assay kit (Thermo Fischer Scientific) was used to determine the protein concentration, according to manufacturer's specifications. We obtained 4,2± 0,15 μg of MVs (n=5, ±SE) from $1X10^{6}$ primary microglia.

3.6 Isolation of MVs from rodent CSF

Rats or mice have been anaesthetized by intraperitoneal injection of 4% Cloralium Hydrate; CSF has been sampled from the *cisterna magna* using a glass capillary and checked for the absence of blood contamination. CSF pooled from 2-5 rats has been diluted in ice-cold PBS containing protease inhibitors and subjected to differential centrifugation to obtain three vesicles pellets corresponding to larger shed vesicles (P2), smaller shed vesicles (P3) and exosomes (P4) (Bianco), or pelletted at 110,000g for 1h to obtain the whole MV fraction.

3.7 Fluorescence microscopy

MVs from rat or mouse CSF were re-suspended in 20 µl of PBS buffer and stained with Annexin-V-FITC, CD11b-PE or IB4-FITC, spotted on glass slides and observed with an inverted Zeiss Axiovert 200M microscope. Staining of fixed MVs with Abs directed against intracellular epitopes, like Iba-1, GFAP, MBP and SNAP-25 was performed adding primary Ab was in a 1:1 volume of PBS buffer containing goat serum and 0.3% TritonX-100 and incubations were allowed for 1h at RT. Primary Ab-conjugated MVs were then washed with PBS and pelletted before incubation with fluorochrome-conjugated secondary Abs for 2h at RT and further washing in PBS. Re-pelletted labelled MVs were then spotted on glass slides and observed at the microscope. CSF collected from CX3CR1-EGFP mice was directly stained for Cd11b, spotted on glass microscope, sealed and analyzed.

3.8 Mir-146a real time PCR

MVs and exosomes were collected from 24x106 primary cortical astrocytes after exposure to ATP for 30 minutes. P2, P3 (shed vesicles) and P4 (exosomes) fractions were isolated by differential centrifugation as reported above. Total RNA was extracted from MVs pellet using MirVana

miRNA isolation Kit (ambion) following the manufacturer's protocol.

TaqMan miRNA assays (Applied Biosystem) used the stem-loop method to detect the expression level of mature microRNA. As a first step total RNA was retrotrascribed using RT primer specific for the mir of interest; cDNA was then used for PCR reaction along with TaqMan primers. The RT primer, the TaqMan primer and probe for mir-146a and RNU6B, which was used for normalization, were from Applied Biosystem

3.9 Mir-146a functional assay

To evaluate the levels intracellular mir-146a in neuronal culture a functional analysis was done using a luciferase-based assay.

Primary culture of hippocampal neuron at DIV 6 were transiently transfected with PM146a/PsiCheck-2. In this the mature sequence of hsa-miR-146a in the antisense orientation was cloned downstream to the *Renilla Luciferase* coding region (Rom et al, 2010).

The day after transfection cells were exposed to MVs released from microglial cells, approximately 2,8 mg/ml. As positive control neuron were co-transfected with PM146a/PsiCheck-2 and mir146a/block-it a plasmid that induces the overexpression of mir-146a in the cells (Rom et al, 2010).

Twenty-four hours after the exposure to MVs, Firefly and *Renilla* luciferase activities were measured using the dual-luciferase assays (Promega) following the manufactures instruction. Briefly cells were washed in PBS and then scraped using the passive lysis buffer that is furnished with the kit. *Renilla luciferase* activity was normalized to firefly luciferase activity.

Plasmids were a gift from Peruzzi F. (LSU Health Sciences Center, New Orleans,).

3.10 Western blotting

MVs fraction collected from rat CSF as previously described were re-suspended in SDS sample

buffer, loaded on a single lane of a 12% polyacrylamide gel and blotted onto nitrocellulose filters. 0,2μg of corpus callosum homogenate, 10 μg of rat brain and 1μg of cortical astrocyte lysate were run in the same gel, as positive controls. Selected proteins were detected with specific Abs followed by HRP-conjugated secondary Abs and revealed using an ECL system. Samples from MVs-stimulated cultured astrocytes (10 μg) were processed similarly.

3.11 Flow cytometry analysis of rodent and human CSF

Human or mice CSF was directly stained with FITC-conjugate Isolectin B4 from *Bandeiraea Simplicifolia,* (IB4-FITC, SIGMA), and/or annexin-V-APC in 1% BSA. Specificity of IB4 labelling was evaluated on MVs produced by cultured microglia, by pre-treating IB4-FITC with 1M melibiose (6-O-a-D-galactopyranosyl-D-glucose) for 30 min as previously described (Ayoub & Salm, 2003). CSF was then diluted in PBS buffer and labelled MVs were acquired within a fixed time interval on a Canto II HTS flow cytometer (Becton Dickinson). Data were analyzed using FCS 3 software (Becton Dickinson, Franklin Lakes, NJ, USA). Unstained and single-color controls were used to properly set PMT voltages and compensations. Forward scatter (FSc) height and width were used to discard doublets or aggregates. Using side-scatter (SSc) and FSc a vesicle gate was determined over the instrument noise (set by running PBS filtered through a 100 nm filter). Within this gate, IB4 positive events (number of events/μl) were evaluated as a parameter of myeloid MVs concentration in the CSF.

In a first set of experiments, the vesicular nature of detected events was confirmed by using the lipophylic membrane styril-dye FM1-43. In addition, *in vitro* generated MVs were analyzed and sorted using similar FACS parameters on a FACSAria followed by fluorescence microscopy analysis.

3.12 Spectrophotometric quantification of shed MVs

Spectrophotometric quantification of MVs shed from primary microglia was performed in Kreb's Ringer. Cells were incubated with 50μM NBD-C_6-HPC (Molecular Probes, Invitrogen), washed and stimulated with 100μM BzATP for 20 min. Supernatant was collected, centrifuged 10 min 300*g* at 4°C to remove cells and debris, and the total fluorescence was assayed at 485/535 nm with the spectrophotometric system Tecan Infinite500, (TecanGroup Ltd , Switzerland).

3.13 Glial cells cultures and in vitro stimulation

Glial cells were exposed to either 0.4μg/ml LPS for 6 hours or Th1 cytokines (100 U/ml IL1-β, 200 U/ml TNFα and 500 U/ml IFNγ) for 48 hours. Recipient glial cells were exposed to an amount of MVs produced by twice as many donor microglia (1:2 receiving cells to donor cells relative ratio). At the end of activation, recipient glia were washed and either lysed in SDS sample buffer for western blotting or harvested with TRIZOL (invitrogen) for RT-PCR analysis or fixed with 4% paraformaldehyde for immunocytochemistry or loaded with the calcium dye FURA-2/AM (Invitrogen) for calcium imaging or analyzed by flow cytometry after surface staining with CD86-PE for 20 min (at least 5×10^4 events/sample were analyzed).

3.14 Semiquantitative reverse transcriptase-coupled PCR

Total RNA was isolated from rat primary astrocytes/microglia using miRNeasy Qiagen kit following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. Reverse transcriptase was performed using SuperScript® III First-Strand Synthesis System (Invitrogen) and oligo (dT)20 as primer in a final volume of 20 μl. The resulting cDNA was amplified using FastStart Taq DNA Polymerase (Roche) and the following primers for rat proinflammatory genes IL-1β (339 bp), sense, 5'- CAG GAA GGC AGT GTC ACT CA -3';

antisense, 5'- GGG ATT TTG TCG TTG CTT GT -3'; IL-6 (473 bp), sense, 5'- CCG GAG AGG AGA CTT CAC AG -3'; antisense, 5'-TGG TCC TTA GCC ACT CCT TC -3'; iNOS (595 bp), sense, 5'- AAG TCC AGC CGC ACC ACC CT -3'; antisense, 5'- TGC AGA CGC CAT GGT GCA GG -3'; CD206 (493 pb) sense, 5'- ACC TGG CAA GTA TCC ACA GC -3'; antisense, 5'-TTT TCA GGC CTC AAT CCA AC -3'; COX-2 (334), sense, 5'-GAG CAC CTG CGG TTC GCT GT -3'; antisense, 5'-GCA GCA GCG GAT GCC AGT GA -3';. Amplified products were electrophoresed on a 2% agarose gels and visualized by SYBR® safe staining. Actin (sense, 5'-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG -3'; antisense, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA -3') was used to ascertain that an equivalent amount of cDNA was synthesized from different samples.

Primer specific for the mouse and rat isoforms of IL-1β were also used: IL-1βrat (600 pb), sense 5'-CCA AAT TCA ATT CAT CCC ATA-3' antisense 5'-TGC CCG TGG AGC TTC CAG GA-3' and IL-1βmouse (500pb), sense 5'- GGG ATT TTG TCG TTG CTT GT-3' antisense 5'- CCC TGG AGA TTG AGC TGT CTG-3'. All the primers were designed using Primer3 (REF).

3.15 Quantitative real time PCR

RNA was extracted from primari microglial cells and astrocytes as desribed above. cDNA synthesis from total RNA was performed using ThermoScript™ RT-PCR system (Invitrogen) and Random Hexamers as primer. IL1-β, iNOS, COX-2, CD206, TGFβ and IL-10 mRNA levels were measured by real time PCR using Taqman® Gene Expression Assays on the ABI-Prism7000 sequence detection system (Applied Biosystems). 50 ng of starting RNA were used as template. The mRNA expression was normalized to the levels of GAPDH mRNA.

3.16 Cell fluorescence analysis of recipient glia

Surface stainings for CD11b-PE and IB4-Texas red were carried out for 20 min at 4 °c or 3 min at RT, before fixing the cells. Iba-1, GFAP and phalloidin staining was performed on cells fixed with 4% paraphormaldeyde. Nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). Cells were mounted and observed with a Leica SP5 confocal microscope.

3.17 [Ca2+]i determination

Astrocytes, after exposure to shed MVs for 72h, were loaded with 10 μm Fura-2/AM for 45 min at 37°C in culture medium. Polychrome IV (TILL Photonics) was used as a light source. Fura-2 fluorescence images were collected after excitation at 340 and 380nm wavelengths and the emitted light was acquired at 505nm at 1–4 Hz. The ratio values in discrete areas of interest were calculated from sequences of images to obtain temporal analyses. Calcium concentrations were expressed as F340/380 fluorescence ratios.

3.18 Lentivirus injections

Mice were anesthetized with 2,2,2-tribromoethanol (10 mg/ml; 1/27 of body weight) and the head was placed in a stereotactic injection apparatus (David Kopf Instruments, Tujunga, CA, USA). Vesicular stomatitis virus-pseudotyped lentivirus (LV) LV-PGK-IFNγ and LV-PGK-TNFα, previously described in (Muzio et al, 2010), were injected within the right lateral ventricle at the following coordinates: A. +0; L. + 0.8 and D. −2.4. CSF was collected 10 days after lentivirus injection.

3.19 Relapsing-remitting and chronic rodent EAE

Chronic progressive EAE (c-EAE) was induced in female C57Bl/6 mice by immunization with 200μg/mouse of MOG_{35-55} (Espikem, Florence, Italy) and two injections of pertussis toxin (500ng/mouse) the day of immunization and 48 hours later. A reduced concentration of pertussis toxin (250ng/mouse) was used to induce EAE in 6 weeks old female A-SMase$^{-/-}$ mice and their littermates, in consideration of the young animal age, necessary to avoid interference with the inherent phenotype of these mice, consisting of ataxia and mild tremors which appears after about 10 weeks of age (Otterbach & Stoffel, 1995). To obtain sub-clinical EAE for focal MV injections, mice were immunized with 50μg/mouse of MOG35-55 and 250ng/mouse of pertussis toxin. Relapsing-remitting EAE (r-EAE) was induced in female SJL/j mice by two immunizations, seven days apart, with 200 μg/mouse of $PLP_{139-151}$ (Espikem, Florence, Italy) and four injections of 500ng of pertussis toxin the day of immunization and 48 hours later. CSF was collected at 10, 20, and 60 days post injection (dpi), in c-EAE and at 29, 35, and 48 dpi, in r-EAE. Female Lewis rats weighing about 150gr were immunized under the skin of the flanks using 1mg lyophilized spinal cord homogenate emulsified in a total of 200μl of complete Freund adjuvant. Weight and clinical score were assigned according to a standard and validated 0 to 5 scale, described in g (Furlan et al, 2009) .

3.20 MVs injections in EAE mice

Mice with sub-clinical EAE, 20 days post immunization, were stereotactically injected in the corpus callosum (coordinates: 0 mm anterior, 1.0 mm lateral to the bregma and 2.2 mm in depth**)** with the whole fraction of vesicles (MVs and exosomes) derived from primary microglia dissolved in 1 μl of sterile saline (2μg/μl) or with liposomes, mimicking the phospholipid composition of the plasma membrane, which were prepared as follows: bovine brain PC, PS, SM and cholesterol (60:10:10:20, molar ratio), were dissolved in chloroform. The lipid mixtures were evaporated under a nitrogen stream, dried for 1 hour at 50°C and re-suspended in PBS at 40°C in order to obtain multilamellar

vesicles. Small unilamellar vesicles were obtained by sonicating multilamellar vesicles. Mice were sacrificated 72 hours after MV/liposome injection to analyze formation of focal lesions at site of administration.

3.21 Neuropathological analysis

Brain tissue sections were fixed, embedded in paraffin and stained with H&E and Luxol fast Blue to reveal perivascular inflammatory infiltrates and demyelinated areas respectively. Infiltrating microglia and T cells were stained using IB4 and anti-CD3, revealed using a biotin-labeled secondary anti-rat antibody. Inflammatory infiltrates, demyelinated areas and axonal loss were quantified on an average of 10 complete cross-sections of spinal cord per animal. Perivascular inflammatory infiltrates, T cells, and macrophages were evaluated as the number per mm^2, while demyelinated areas and axonal loss were expressed as the percentage per mm^2.

3.22 miRNA profiling

To map miRNAs MVs and exosomes were isolated from $15x10^6$ microglial cells exposed for 24 hours to inflammatory Th1 cytokines and to the anti-inflammatory cytokines IL4. Total RNA has been isolated from MVs collected using miRNeasy mini Kit; cDNA has been prepared by a single in vitro transcription amplification step, using Universal cDNA synthesis kit (Exiqon) starting from 22 ng of total RNA. The obtained cDNA was used as template for microRNA quantitative real-time PCR using microRNA Ready-to-Use PCR, Mouse&Rat panel I, V2.M (Exiqon). The experiment was run on Roche LightCycler 480. Analysis of data has been carried out with GenEx software (Exiqon) using UniSp6 (Spike in RNA) as calibrator and mir-499c as refefence gene.

3.23 Chemicals and antibodies

ATP, BzATP, LPS, FICT- and APC-annexin V, IB4-FITC, IB4-Texas red, phalloidin-Texas red, melibiose were from Sigma-Aldrich. CellTracker green CMFDA, were from Molecular Probes, PI was BD Biosciences, NBD-C_6-HPC was from Invitrogen, IL1-β was from Euroclone, TNFα and IFNγ from Peprotech. The following antibodies were used: anti mouse CD11b-PE (BD Biosciences), anti human CD63-PE (BD Biosciences) anti-rat CD86 (eBioscience), anti-mouse GFAP (Sigma-Aldrich), anti-human calnexin (Sigma-Aldrich), anti-mouse CNPase (Chemicon), anti-mouse MBP (Chemical), anti-mouse SNAP-25 SMI 81 (Sternberger Monoclonals) anti-mouse Iba-1 (Wako), anti-CD3 (Serotec Ltd), anti-OX42 (Harlan Sera-Lab, UK). PC, PS, cholesterol and SM were from Sigma-Aldrich.

3.24 Statistical Analysis

All data are presented as mean or median $\pm$ SD or SE from the indicated number of experiments. Data were compared using the Student's *t*-test for parametric data or the Mann–Whitney U-test for non-parametric data or non normally distributed data. Differences were considered to be significant if $p<0.05$ and are indicated by an asterisk; those at $p<0.01$ are indicated by double asterisks, and $p<0.0001$ by triple asterisk.

4. Results

4.1 MVs shedding is increased upon inflammation

The release of shedding vesicles takes place in resting cells, but it is increased upon stimulation such as during inflammation and in tumor cells.

To verify whether the activation state of microglia may influence MV production, cultured microglia were exposed for 48 hours to Th1 cytokines (IL-1β- TNFα- IFNγ). Cells were incubated in vivo with antibodies directed against CD11b, then were fixed with PFA 4% and counter stained for Iba1. Fluorescence microscopy analysis showed the presence of several blebs at the cell surface, characterized by Iba1 accumulation in Th1 treated cells not detectable in unstimulated cells. (Fig. 6) This finding suggests that exposure to a pro-inflammatory enviroment may strongly induce the process of shedding in microglial cells.

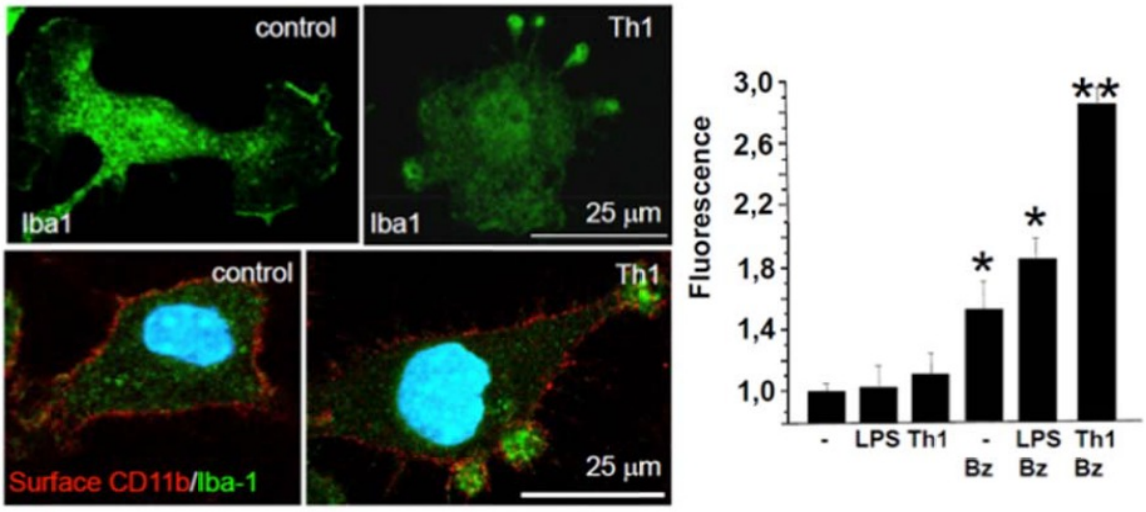

Fig. 6: MVs shedding in activated cells. Primary microglia exposed to Th1 cytokines for 24h, showing numerous blebs at the plasma membrane enriched in Iba-1 (upper panels). Double staining for surface CD11b and Iba-1 in control and Th1-treated BV2 microglial cells (lower panels).The histogram shows the spectrophotometric quantification of MVs shed from resting or reactive microglial cells, prelabelled with NBD-C6-HPC and exposed to the P2X7 agonist BzATP (100 µM) for 20 min

Consistent with the ability to induce massive formation of blebs at the cell surface, spectrometric quantification. of ATP-induced MVs shedding from resting microglia and cells pre-

activated with Th1 cytokines or LPS indicated that Th1 cytokines are the best priming stimuli for the process(Fig.6)

4.2 MVs interaction with glial cells: astrocytes and microglia

To evaluate whether microglia-derived MVs may have a pathological role in inflammation we analyzed the effect of MVs on primary glial cells both astrocytes and microglia.

$1x10^6$ astrocytes were exposed for 48 hours to MVs released from $2x10^6$ microglial cells (ratio 1:2) at concentration of 2 μg/ml. After exposure to MVs astrocytes appeared e more reactive, they looked hypertrophic, displaying more thicker process, and showed higher levels of GFAP (Fig. 7).

72 hours after exposure to MVs we also evaluate basal calcium concentration of astrocytes exposed to MVs. produced either from LPS or TH1 primed microglial cells. To this aim, cells were loaded with the fluorescent calcium dye FURA-2, and calcium levels were analyzed evaluating F340/F380 fluorescence ratio. We found that that astrocytes exposed to MVs have higher basal calcium

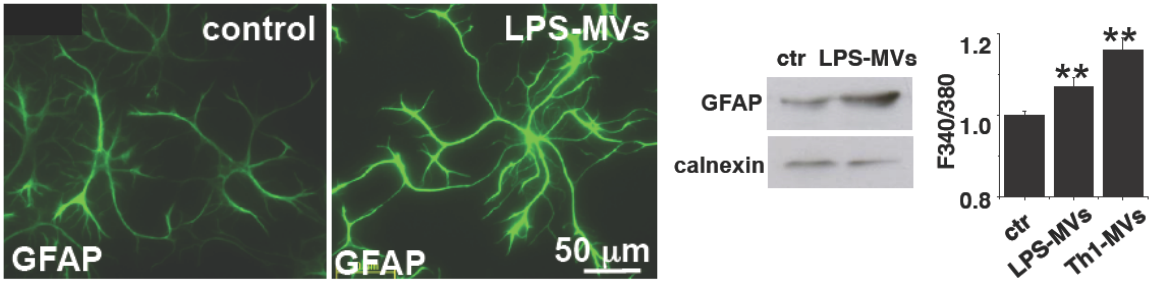

Fig. 7: Effect of MVs on astrocytes. 1-Fuorescent staining for the activation marker GFAP of naïve cortical astrocytes and astrocytes exposed to MVs shed from LPS-treated microglia. 2-Western blotting for GFAP of control astrocytes and astrocytes activated by MVs ; the endoplasmic reticulum protein calnexin was used as loading control. 3-Cytoplasmic calcium concentration, expressed as F340/380 fluorescence ratio, of astrocytes, loaded with the fluorescent calcium dye FURA-2, 72 hours after exposure to MVs produced from LPS- or Th1-treated microglia.

concentration as compeared to control cells, confirming that they are activated.

To further analyze the phenotype acquired by astrocytes after exposure to MVs we used semi-quantitative PCR and Real Time PCR.

Exposure of astrocytes to MVs produced by LPS-treated microglia induced up regulation of the

pro-inflammatory markers iNOS, COX-2, IL1β, IL6 and COX-2 as indicated by both by semi-quantitative PCR and real time PCR without changing the level of expression of the anti-inflammatory molecules TGF-β and IL-10. Inflammatory markers were induced in a dose-dependent manner, as indicated by linear correlation between the amount of MVs, used as a stimulus, and level of expression of markers of astrogliosis in recipient astrocytes. Of note, a less robust response was evoked by MVs produced by resting microglia (Fig.8).

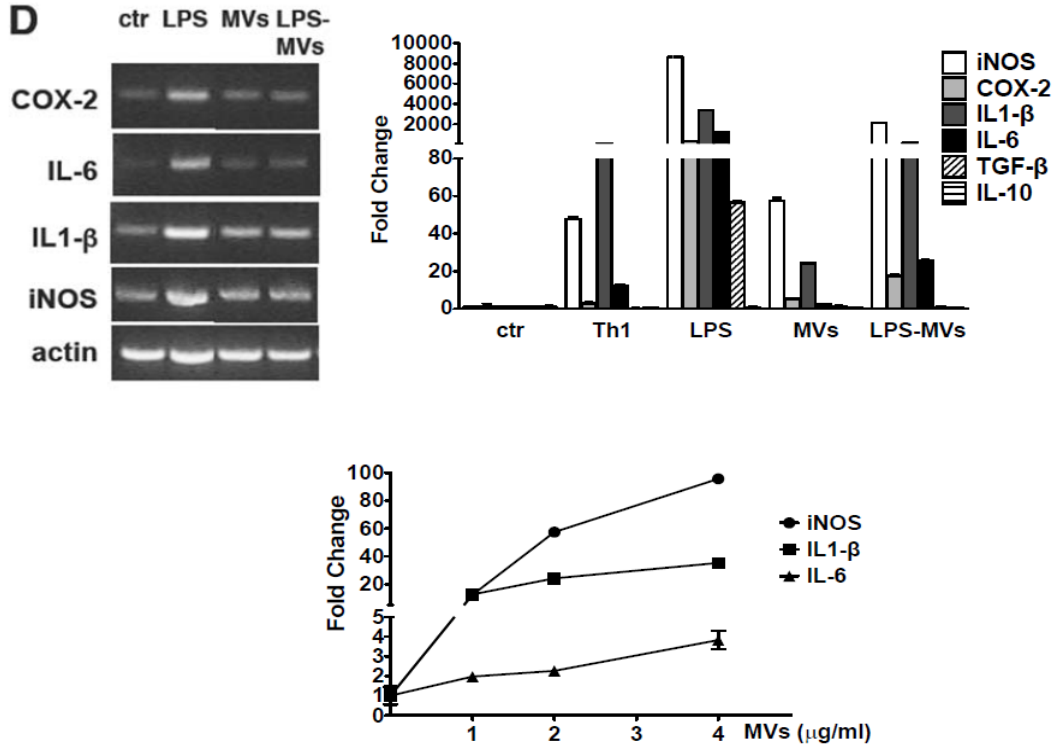

Fig. 8: MVs shed from microglia deliver a pro-inflammatory signal to astrocytes. 1-2 RT-PCR for COX-2, IL-6, IL-1β, and iNOS mRNA in control cells, astrocytes activated overnight with LPS or exposed for 48 hours to MVs produced by resting or LPS--treated microglia. Both amplicons run on agarose and real time quantitative evaluation are shown. 3- dose-response correlation between concentration of MVs and induction of inflammatory markers

Similar results were obtained on microglial cells exposed to MVs.

Microglia cells responded to MVs by up-regulating the T-cell co-receptor ligand CD86 at the cell surface, mimicking the effects induced by exposure to the inflammatory stimulus LPS. This was indicated by flow cytometry analysis of surface expression of CD86 in control microglia and cells exposed to MVs (Fig. 9). Microglia-derived MVs were also able to stimulate expression of inflammatory markers (iNOS, COX-2, IL6, IL1beta) and to reduce the level of expression of the

M2 marker CD206, the mannose-6 phosphate receptor, as indicated by quantitative real time and semi-quantitative PCR analysis (Fig. 9). A treatment of 48 hours with MVs was necessary to induce

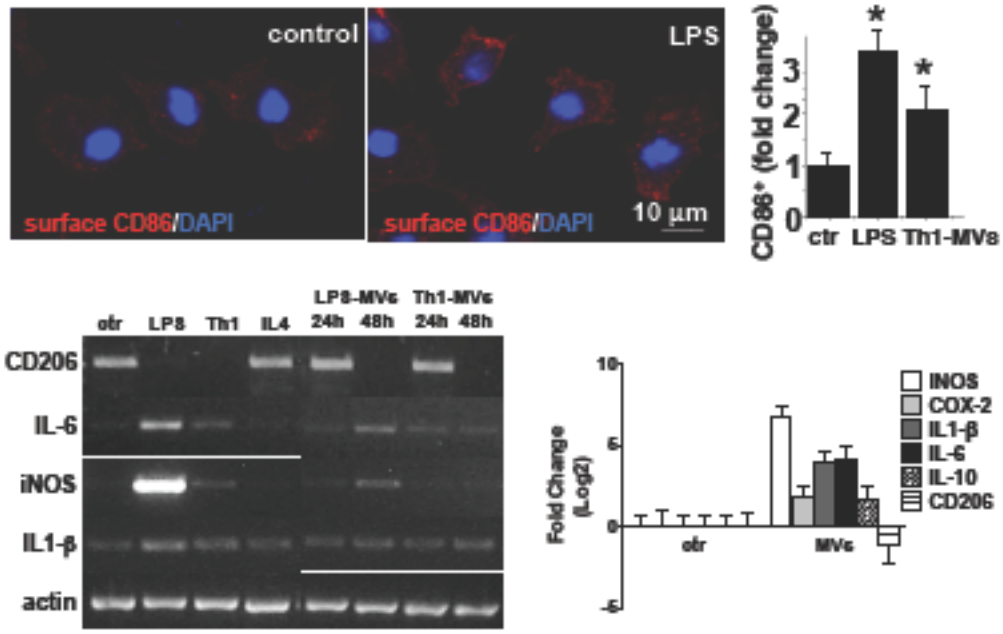

Fig. 9: *MVs shed from microglia deliver a pro-inflammatory signal to microglial cells* 1- Fluorescent images revealing increased surface expression of the activation marker CD86 (red) in primary microglia exposed overnight to LPS, as compared to control. Nuclei are shown in blue with DAPI staining. **2-** Quantitative flow cytometry analysis of CD86 surface expression in microglia maintained in control condition, treated for 3 hours with LPS or incubated overnight with MVs derived from Th1-primed microglial cells.3- RT-PCR for inflammatory genes and for the proregenerative marker CD206 in control microglia and cells stimulated with Th1 cytokines or LPS, or exposed to MVs derived from either resting or LPS/Th1-primed donor microglia. Amplicons run on agarose gels, compared to actin, and real time quantitative analysis.

these variations in the expression of mRNAs.

Activation of glial cells was likely mediated by interaction of MVs with recipient cells and subsequent MV internalization into their cytoplasm. This was indicated by confocal analysis of N9 microglial cells exposed for 1h to GFP labeled MVs ,released from N9 cells, stably expressing GFP. As shown in Fig. 10, staining with the f-actin ligand phalloidin clearly revealed the presence of GFP-labelled MVs inside the cytoplasm of a recipient N9 cell.

To further study the interaction of MVs with target gial cells we also did a surface staining for IB4-Texas red (red) in primary microglia exposed to MVs derived from CMFDA-loaded microglia (green). CMFDA-storing MVs were incubated with recipient microglia for 1h and microglia were briefly exposed to IB4-PE and gently washed before being fixed. This protocol allows staining with

IB4 of only MVs attached to the cell surface of receiving microglia but not of those already internalized into the cells. As shown in Fig. 10, some CMFDA-loaded MVs were IB4 negative (arrows), indicating that they were already internalized into the cytoplasm

To evaluate whether MV internalization into recipient cells may be associated to transfer of cytoplasmic components I settled up a PCR based assay based on the use of primers specific for murine IL1β mRNA.

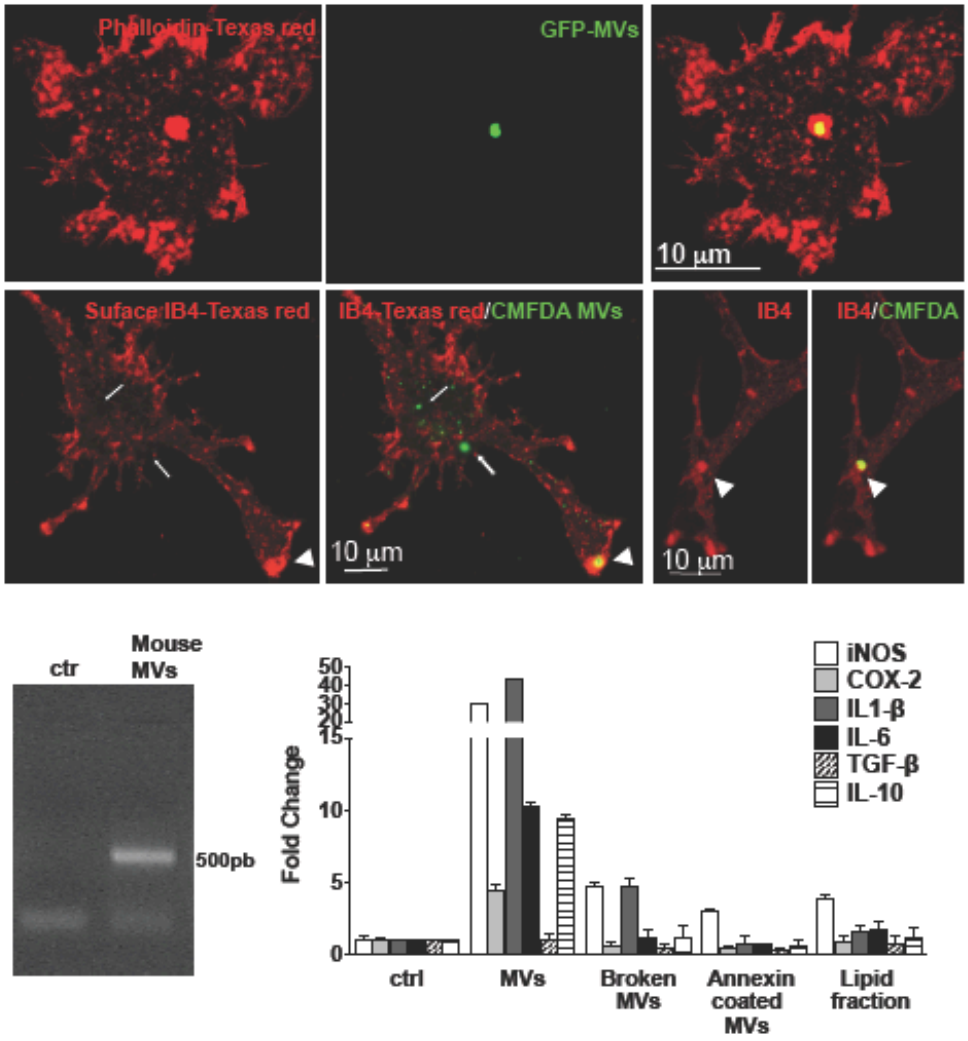

Fig. 10: *MVs interact with by recipient glial cells*. 1- Confocal images of N9 cells exposed for 1 h to GFP-labelled MVs, isolated from the medium of GFP-expressing N9 cells, fixed and stained with the f-actin ligand phalloidin. 2- Surface staining for IB4-Texas red (red) of primary microglia exposed to CMFDA-labelled MVs (green), derived from CMFDA-loaded microglia. CMFDA-storing MVs were incubated with recipient microglia for 1h and microglia were then briefly exposed to IB4 and gently washed before being fixed. 3- PCR amplification of mouse IL-1b mRNA in rat microglia exposed to MVs derived from mouse microglia. 4- Expression of inflammatory marker induced from MVs, MVs depletet of the content, Annexin V coated MVs and to lipid fraction extracted from MVS revealed by Real Time PCR

$1x10^6$ rat primary microglia cells were incubated for 5 hours with MVs released from mouse microglial cells. Cells were then extensively washed before extraction of RNA and amplification with mouse specific primers. Because the low amount of mouse mRNA a nested PCR was necessary. Amplicons were then run on agarose gel. As shown in figure 10 mRNA for the mouse isoform of IL1 beta was present in rat microglia cells incubated with mouse MVs, thus indicating that MVs transfer cytoplasmic components to recipient microglia. We next evaluated whether interaction of MVs with the surface of target cells and MV internalization may contribute to the pro-inflammatory response of receiving cells. To this aim astocytes were exposed to artificially broken MVs, depleted of their content, to the lipid fraction of MVs or to MVs in which externalized PS, that is determinant for recognition on recipient cells, was masked by annexin V. Our preliminary data suggested that inhibition of MV interaction by annexin-V stongly reduce the capability of MVs to activate an inflammatory response. Furthermore these data suggested that that the active components of MVs are located in the MVs lumen rather than in the MV surface. However, further experiments are necessary to confirm that propagation of the inflammatory signal is mediated by MV cargo and to identify the components responsible for the inflammatory activity of MVs

4.3 Mir-146a in MVs

Since mir146a is expressed in primary human fetal microglia and a large amount of small RNAs, including microRNA (miRNA) has been described in extracellular vesicles (Valadi et al, 2007) we evaluate the possibility that mir146a was present in microvesicles.

To assess the presence of mir146a, RNA was extracted from MVs and exosomes produced from the N9 microglia cell line, either unstimulated or exposed or to LPS, and analysed by Real Time PCR. Graph in figure 11 shows that mir146a is present in both shed vesicles (P2-P3 population) and exosomes (P4) and that its expression is increased in vesicles released from LPS primed cells.

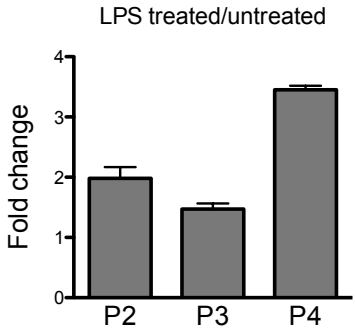

Fig. 11: mir-146a in glial MVs. LPS induced upregulation of mir-146a is in glial MVs and exosomes revealed by Real Time PCR

4.4 MVs interaction with neuron

Recent data of the laboratory have shown that microglia-derived MVs can also interact with the surface of recipients neurons, maintained in primary culture. Confocal analysis of hippocampal neurons exposed for 1 hour to GFP-labelled MVs, extensively washed and processed for immunocytochemistry, revealed the presence of MVs anchored to the surface of neuronal processes as showed in figure 12.

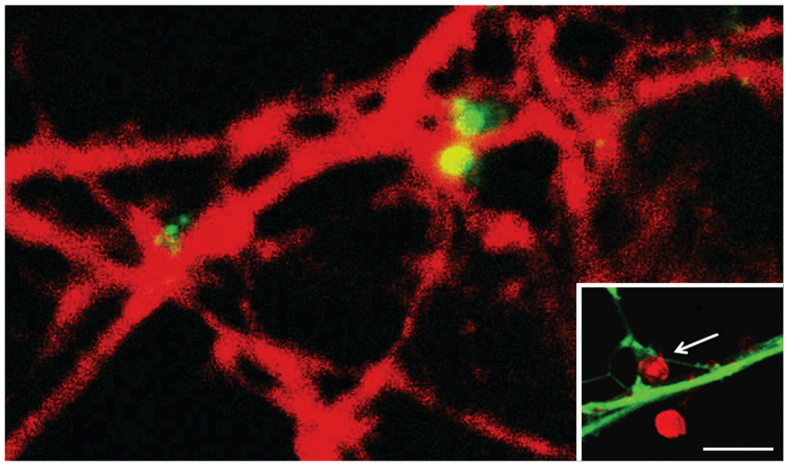

Fig. 12: MVs shed from microglia interact with neuronal surface. MVs shed from N9 microglial cells, stably expressing GFP were incubated for 1 h whit hippocampal neurons. Culture were then fixed and stained for the plasma membrane protein SNAP-25.

I therefore evaluated the possibility that MVs could transfer luminal components to neuronal cytoplasm. By the used of the assay described above I cannot observe any IL1β mRNA transfer from glial MVs to neuron. To confirm this result by a different methodology approach I investigated the possible transfer of mir146a from microglia-derived MVs to neurons. The levels of expression of mir-146a were evaluated using a functional assay based on luciferase activity. Neuron were transfected with a reporter construct that contain the *Renilla* Luciferase gene fused to the perfect match sequence of mir-146a; the plasmid also contained the firefly luciferase gene for normalization. Transfected neurons were then incubated in presence of glial MVs for 24 hours and the mir-146a levels were evaluated by dual luciferase assay. As shown in figure 12 the exposure of neuron to MVs or exosomes induced a significant down-regulation of *Renilla* luciferase activity indicating an increase in the levels of mir-146a in the cells as compare to control cells. As positive control we evaluated the reduction of *Renilla* luciferase activity in neuron transfected with mir-146a; in this sample we observe a reduction of around 70% of luciferase activity.

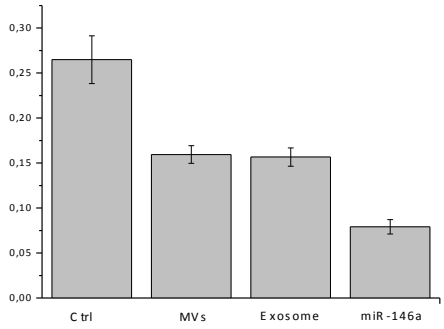

Fig. 13: Dual luciferase assay on mir-146a activity. Levels of mir146a in cells exposed to MVs and exosomes were analyzed using a luciferase reporter sensitive to mir-146a. As positive control mir146a expression was induced in cells. The reduction in luciferase activity indicate an increase in the levels of mir146a.

These data indicate that the levels of mir-146a are increased in neurons exposed to MVs and exsosomes. Data obtained clearly indicated an increase of mir146a in neurons exposed to MVs. However it is not clear at the moment whether this increase results from upregulation of the mirna or transfer..

4.5 MVs in rodent CSF

To demonstrate the existence in vivo of microglia-derived MVs the cerebrospinal fluid collected from healthy rodents was recently analyzed in the laboratory by negative staining electron microscopy. Analysis of three distinct pellets (P2,P3 and P4) obtained by differential centrifugation of rat CSF showed that extracellular vesicles, similar in size to both MVs and exosomes, released *in vitro* from glial cells, are indeed present in the cerebrospinal fluid of healthy rats. (Fig. 13)

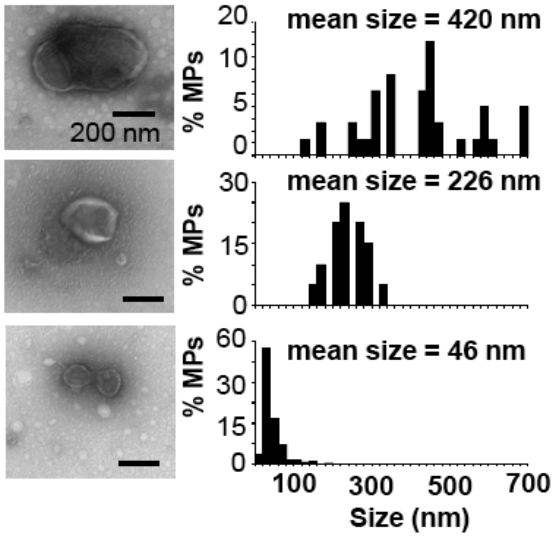

Fig. 14: MVs in rodent CSF. MVs of decreasing size isolated from the CSF of healthy rats by differential centrifugation (P2, P3, P4 fractions) and analyzed by negative staining EM. Corresponding frequency histograms, indicating the size distribution of MVs pelleted at increased centrifugal force, are shown on the right.

To better characterize these vesicles, rodent CSF was also analyzed by fluorescence microscopy (Fig. 15). The membrane vesicles pelleted from the CSF (P2-P4 fraction) were stained with the fluorescent phosphocholine analog NBD-C6-HPC to label the lipid bilayer of the vesicles.

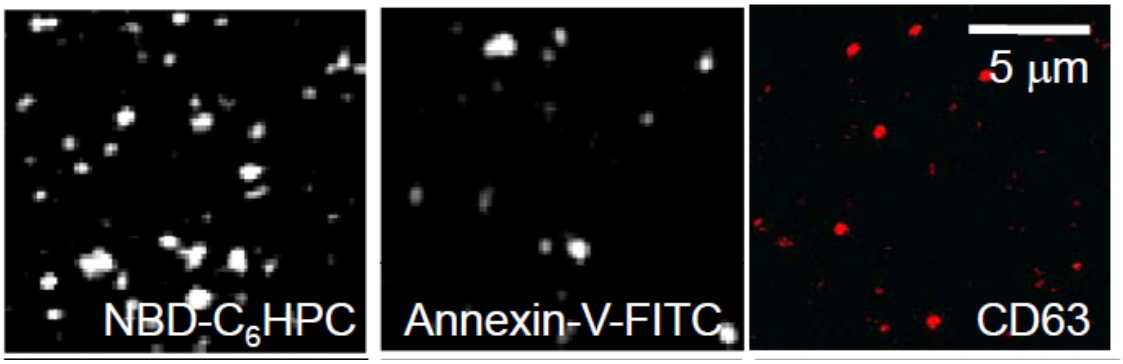

Fig. 15: MVs in the CSF of healthy rodents. Examples of CSF MVs analyzed by fluorescence microscopy using the fluorescent phosphocholine analog NBD-C6-HPC to label the lipid bilayer of MVs, the shed MVs marker annexin-V-FITC, and the exosomal marker CD63

They were also stained with specific markers for shed vesicles or exosomes, such as Annexin V, that binds to phosphatidyl serine on the outer leaflet of the MVs plasma membrane and CD63 that is a typical exosomal marker. This analysis confirmed the exosome nature of part of CSF vesicles and the plasmamembrane origin of larger vesicles.

Rodent CSF was also analyzed by fluorescence microscopy or western blot analysis in order to evaluate the cellular origin of MVs. Vesicle fractions were isolated by differential centrifugation the pellets stained with fluorescent markers for distinct cell types and observed at confocal microscope. Alternatively vesicle fractions were solubilized for WB analysis with the same markers. We observed vesicles immunopositive for markers of distinct brain populations, i.e. SNAP-25 for neurons, GFAP for astrocytes and MBP and CNPase oligodendrocytes. These data indicated that MVs can be released from all these cell types (Fig. 16). To assess the presence of microglial MVs in the CSF we analyzed the CSF of CX3CR1-EGFP transgenic mice, in which microglial cells express the green fluorescent protein GFP. The presence of EGFP+ MVs in the CSF of these mice is a clear demonstration of the microglial origin of some CSF MVs. that are positive also for the microglial marker CD11b stained in red.

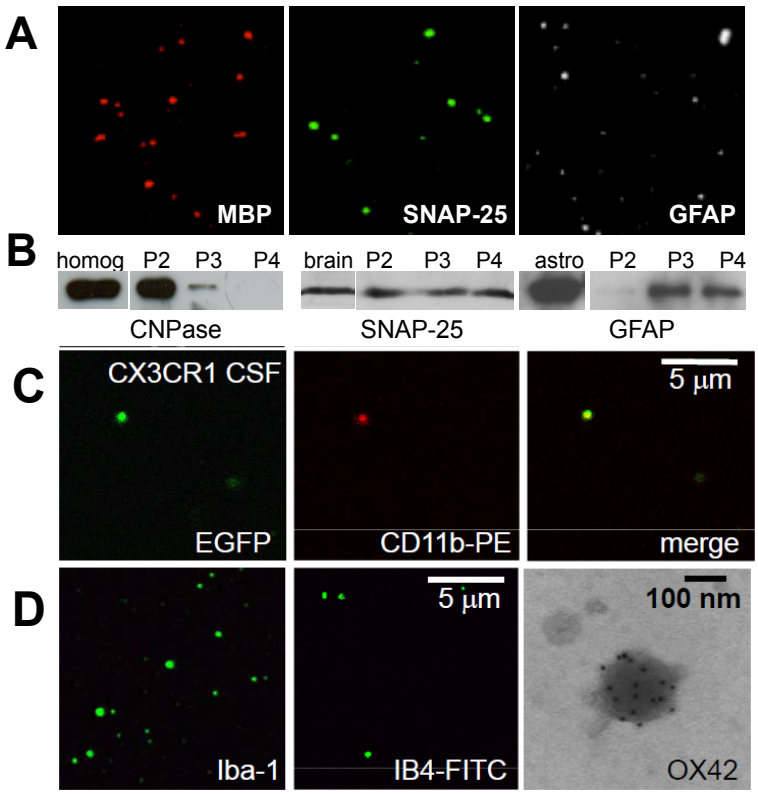

Fig. 16: Origins of MVs in the CSF of healthy rodents. Confocal microscopy and WB analysis of the CSF collected from healthy rodents revealed MVs displaying neuronal (SNAP-25), astrocytic (GFAP), oligodendroglial (MBP-CNPase) and microglia (Iba1, IB4 CD11b) markers. Fluorescence micrographs of EGFP+ MVs present in the CFS of CXCR3-EGFP transgenic mice, stained for the microglia marker CD11b-PE before microscope observation.

The existence of microglia derived microvesicles was also confirmed with fluorescent microscopy and immunogold electro-microscopy staining MVS with specific microglial markers such as Iba1, IB4-FITC and OX-42(CD11b).

4.6 Quantification of MVs in the CSF

Cerebrospinal fluid represents an easily accessible source of material for diagnosis and monitoring of neurological diseases. It is a potential indicator of abnormal CNS states such as inflammation,

infection, neurodegenerative processes and tumor growth (Zougman et al, 2008). CSF is the only body fluid in direct contact with the brain and therefore can act as a recipient of shedding products release by neuronal cells. With the objective of quantify MVs in the CSF of rodent we settled up a cytofluorimetric assay specific for microglia derived MVs.

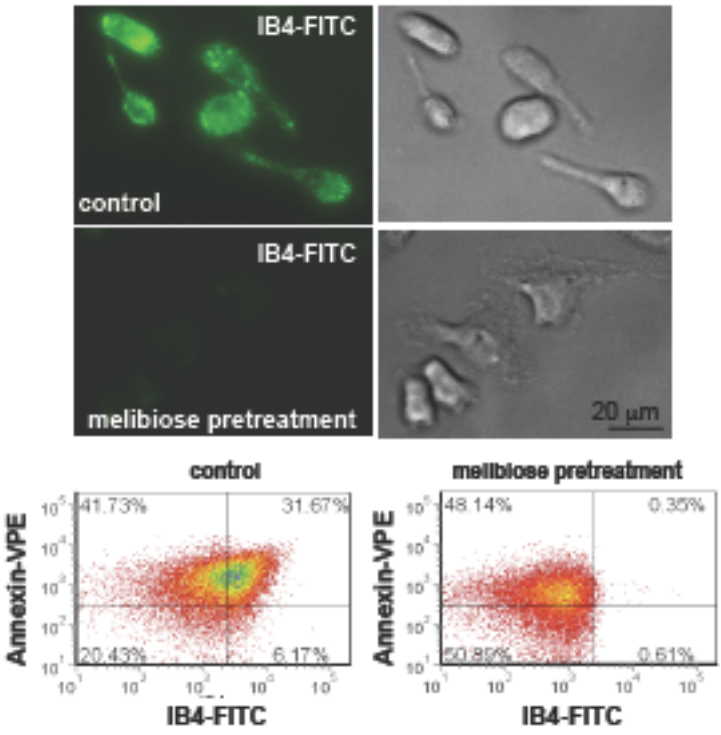

Fig. 17: IB4 labeling of microglia-derived MVs. Differential interference contrast microscopy (right panels) and *in vivo* fluorescent labeling for IB4-FITC (left panels) of rat microglia in primary cultures. Note that IB4-FITC pretreatment with 1M melibiose, a specific ligand for the isolectin IB4, completely prevents its binding to the surface of microglial cells, confirming specificity of the staining. Melibiose pretreatment completely abolished IB4-FITC labeling, confirming the specificity of the FACS assay.

Flow cytometry analysis is a quantitative and reliable methodology to measure MVs, and it has been already used to detect small extracellular vesicles, exosomes, in samples of human plasma. A gate was established on size, using beads of 0.5-2 µm, to analyze particles ranges from 50 to 1000 nm. Within this gate, IB4 events were evaluated as parameter of MVs concentration. CSF sample were directed immunolabeled with IB4-FITC in order to avoid the formation of immuno complexes that can be detectable within the MVs gate. To asses the specificity of IB4 labelling we use

melibiose, a specific ligand for the isolectin IB4. Pretreatment with 1M melibiose completely prevents IB4 binding to the surface of microglial cells confirming the specificity of the staining. (Fig. 17) FACS analysis of microglia MVs pretreated with melibiose completely abolished IB4-FITC staining, confirming the specificity of the FACS assay (Fig. 17).

4.7 CSF MVs during neuroinflammation

We and others have shown that both the typical danger signal ATP and bacterial lipopolysaccharide greatly enhance the release of MVs and MV-dependent secretion of IL1-beta and MHC-II, *in vitro,* from microglia (Fig. 6 Bianco et al., 2005; Qu et al., 2009). To evaluate if a similar phenomenon occurs *in vivo,* healthy C57BL/6 mice were stereotaxically injected into the ventricular cavity with lentiviral vectors codifying for the pro-inflammatory cytokines INF-γ or TNFα. This protocol is known to induce a strong infiltration of macrophage and the expansion of microglia in the periventricular area and in the choroid plexus (Muzio et al, 2010). Consistently the confocal analysis of the *choroid plexus* isolated from mice, ten days after the injection and labeled with microglial marker Iba1 and DAPI, showed a significant increase in microglial cells Iba1 positive in TNFα and IFNγ injected mice. (Fig. 18) Analysis by flow cytometry of MVs of myeloid origin indicated that intrathecal release of pro-inflammatory cytokine dramatically increased microglia-derived MVs of myeloid origin in the CSF. Giving the proof of principle that microglia-MVs were increased in vivo in inflammatory conditions.

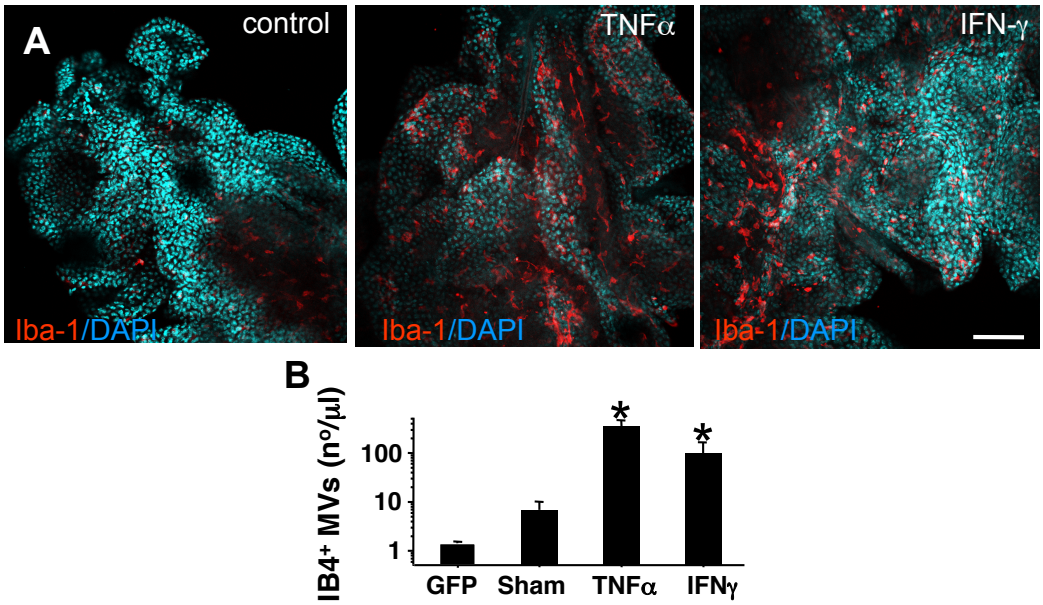

Fig. 18: The amount of microglia-derived MVs in the CSF increases upon inflammation 1-Representative choroid plexus from mice injected with lentiviral vectors encoding for GFP (control), TNFα or IFNγ, ten days after injection. Staining for Iba-1(red) and DAPI, to label nuclei (light blue) 2- Flow cytometry analysis of IB4+ MVs in the CSF collected from naïve mice, sham mice and mice injected with the lentiviral vectors.

4.8 CSF MVs in EAE

We then analyzed rodents affected by experimental autoimmune encephalomyelitis (EAE), a neuroinflammatory model for human multiple sclerosis (MS). Quantitative analysis of MVs in mice affected by chronic or relapsing EAE, mimicking the two most common clinical forms of MS, showed that the amount of total MVs, and myeloid MVs was closely associated to disease course, increasing at onset and during clinical relapses, and decreasing in the chronic phase of the disease (Fig 19). During EAE, myeloid cells enter the CNS from the blood stream and accumulate in both parenchimal and meningeal perivascular inflammatory infiltrates. Choroid plexi, which have been identified as the site of first entry of inflammatory cells during EAE (Kivisakk et al, 2009)

are in close contact with the CSF and present therefore the more plausible source of myeloid CSF MVs.

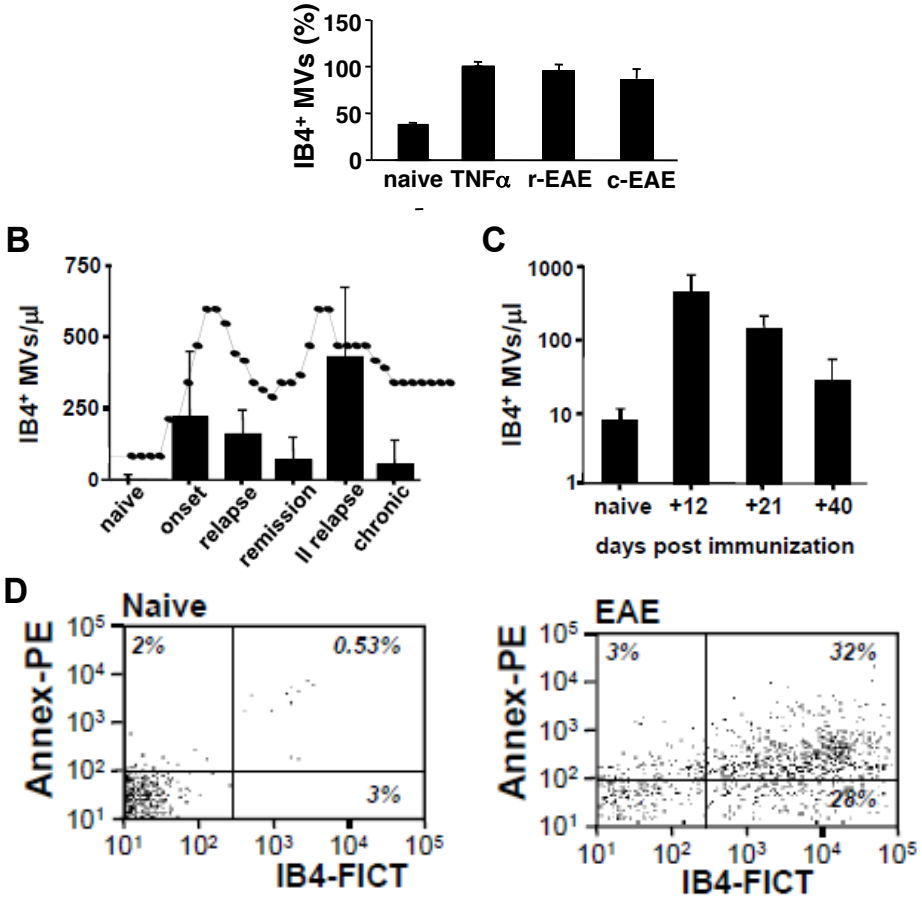

Fig.19: The amount of microglia-derived MVs in the reflects EAE activity. 1-Histogram shows the percentage of IB4+ MVs in the CSF of naïve mice, mice injected with lentivirus codifying for TNFα, and mice affected by chronic (c-) or relapsing-remitting (r-) EAE. 2- Flow cytometry analysis of IB4+ CSF MVs at different disease stages during relapsing remitting (r-EAE) and chronic (c-EAE) 3- Representative flow cytometry scatter plots of IB4+ and annexin-V+ CSF MVs collected from a naïve mouse and a EAE mouse, in the chronic phase of the disease.

In the last panel was reported a representative FACS analysis of MVs in the CSF of EAE mouse compare to a control. Those data open the possibility that MVs can be an useful biomarker to monitor the disease progression.

4.9 Can MVs propagate an inflammatory signal in vivo?

To evaluate whether microglia-MVs can have a role in the propagation of an inflammatory signal *in vivo* during EAE stereotactically injected MVs derived from cultured microglia into the brain of

mice affected by subclinical EAE. Injections were performed into the *corpus callosum,* a site usually devoid of inflammation during EAE. Seventytwo hours after injection we found perivascular inflammatory foci close to the site of injection in all MVs-injected EAE mice and in none of saline- or liposome-injected controls (Fig. 12). Inflammatory perivascular infiltrates were constituted by very few CD4+ T cells and a vast majority of IB4+ microglia. These data suggest that MVs may contribute to formation of focal inflammatory lesion.

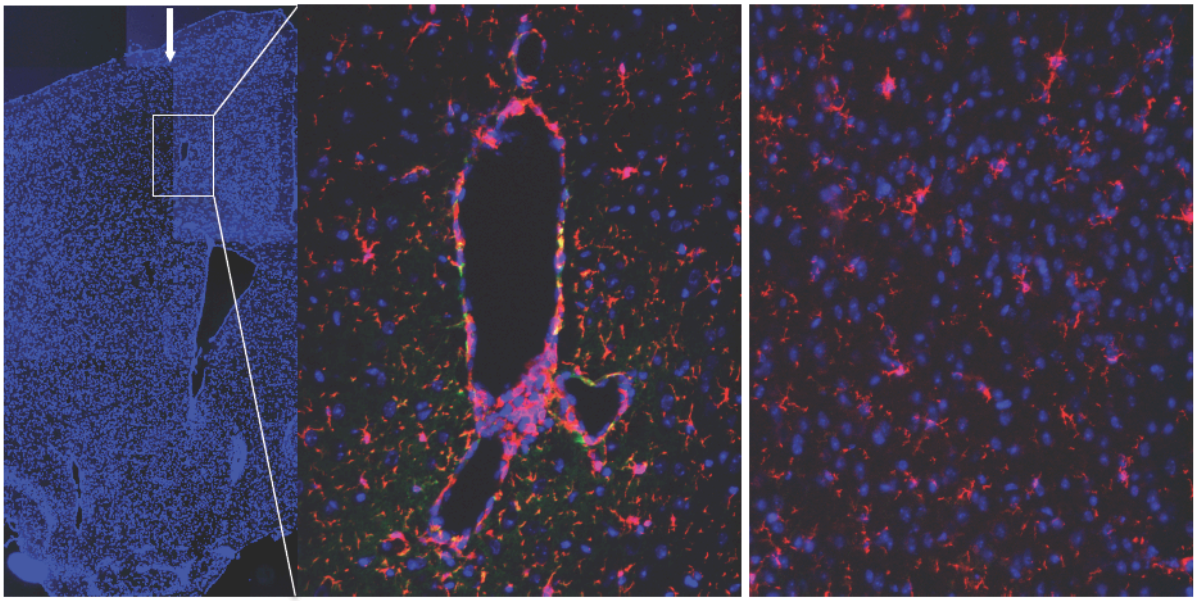

Fig. 20: Microglia-derived MVs amplify brain inflammation. Coronal sections of the brain of EAE mice injected in the corpus callosum with MVs or liposomes mimicking the phospholipid composition of the plasma membrane. Triple staining for DAPI (blue), Iba1 (red) and CD4 (green) shows perivascular inflammatory infiltrates in mice injected with MVs but not with control.

4.10 Pathogenic role of MVs

We have previously shown the enzyme acid sphingomyelinase (A-SMase) controls the budding of MVs from the plasma membrane and that MV shedding is abolished in A-SMase KO glial cells. Therefore, to further explore the pathogenic role of MVs in vivo we induced EAE in A-SMase KO mice. Since these mutants develop neurological signs starting from 10-12 weeks of age, EAE was induced in 6 weeks-old animals and the disease was monitored until 8 weeks of age. Notably, ASMase KO mice were highly resistant to the development of EAE as compared to wild type

littermates (Fig. 21). The lower score of A-SMase KO mice was associated with an expected lower amount of myeloid MVs in the CSF, reduced perivascular infiltrates and absence of tissue damage in the spinal cord, confirming that shed MVs may have pathogenic functions during EAE.

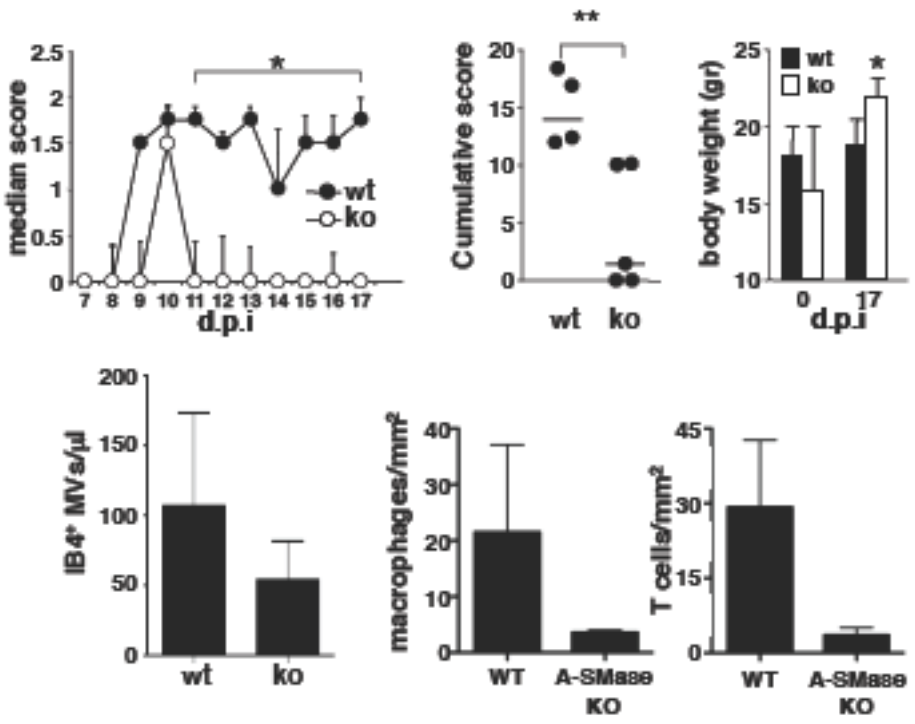

Fig. 21: Pathogenic role of microglia-derived MVs. 1-2-3Body weight and median and cumulative clinical score of A-SMase KO mice and WT littermates affected by c-EAE 4- FACS analysis of IB4+ MVS in CSF of EAE-A-SMase KOmice and EAE WT littermate 5-6 Quantification of IB4+macrophages and CD3+ T cells in the spinal cord of EAE A-SMase KO mice and WT littermates.

4.11 Human sample

In order to verify whether the increase in microglia derived MVs that we demonstrate in the CSF of EAE mice can be extended to humans, we collected CSF from healthy donors, patients with Clinically Isolated Syndrome (CIS), patients with definite primary progressive or relapsing-remitting multiple sclerosis (PPMS and RRMS, respectively), the latter during a stable phase of the disease (stable RRMS), or during an acute attack (acute RRMS). Quantitative flow cytometry analysis of IB4 positive microvesicles was done. Similar to data obtained in the animal model IB4 positive MVs were significantly increased in the CSF from CIS and relapsing RRMS patients (Fig 22), when inflammatory processes are at higher levels.

These data indicate that CSF MVs represent novel *in vivo* biomarker of microglia/macrophage

activation.

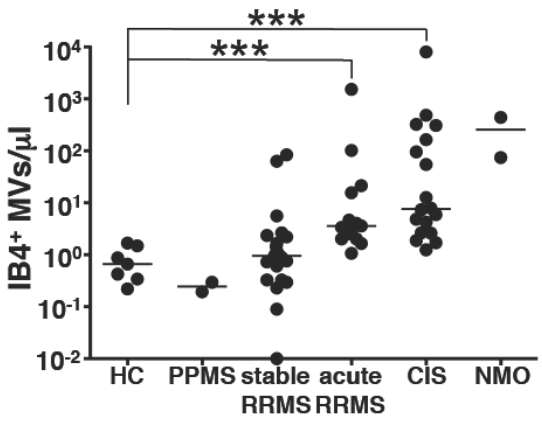

Fig. 22: Microglia derived MVs in Multiple sclerosis patients. Quantitative flow cytometry analysis of IB4+ positive MVs in human CSF collected from CIS patients, relapsing remitting MS patients in a clinical and neuroradiological stable (stable RRMS) or acute (relapsing MS) phase of the disease, primary progressive MS patients, patients with neuromyelitis optica and age-and sexmatchedcontrols .

4.12 MVs miRNA profiling

To address the possibility that CSF MVs contain miRNA signatures that specifically reflect microglial activation occurring in the brain during neuroinflammatory disease we carried out a profiling of miRNAs present in extracellular vesicles.

We analyzed by Real Time PCR the expression of 376 microRNAs in MVs and exosomes released from control cells as well as from cells exposed to Th1 pro-inflammatory cytokines (IL1-b, TNFa, INFg) and to IL4, an anti inflammatory cytokine. We observed a differential expression of several miRNAs (~110) in our samples. As shown in figure 23 some miRNAs were expressed only in TH1 extracellular vesicle; other were expressed only in IL4 vesicles or in control cells. We also compared the expression data on microRNA present in vesicles with those present in parental cells. Interestingly we detected in vesicles miRNA that are not present in cells, indicating that some miRNAs may be uniquely packed into MVs and/or exosomes (Fig. 24). Nevertheless, these data are very preliminary and the presence of the microRNA identified as significantly up-/down-regulated in MVs need to be confirmed by quantitative real-time PCR.

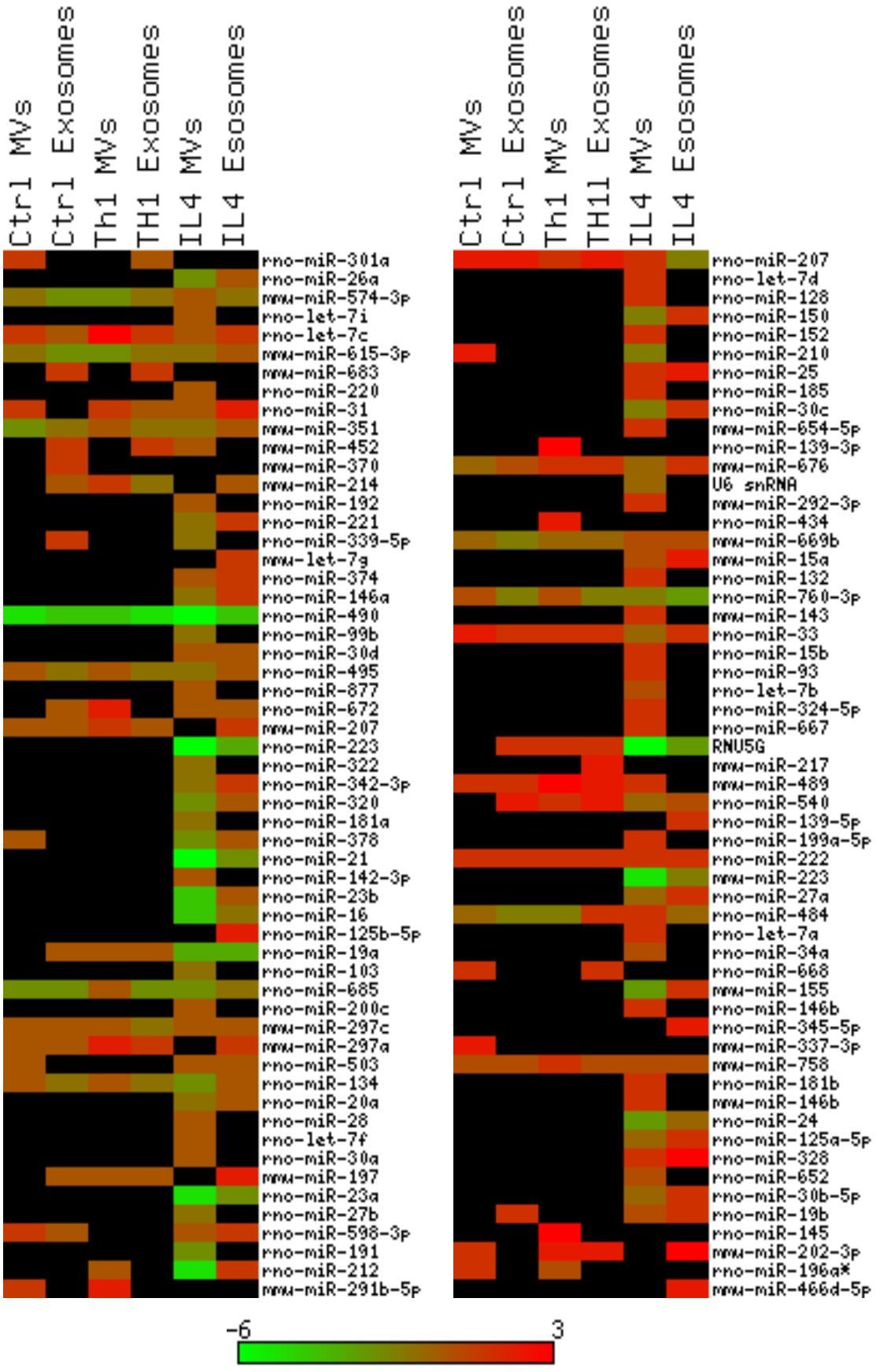

Fig. 23: miRNAs relative expression in extracellular vesicles. Heatmap of the relative expression of miRNA in MVs and exosomes release from differently activated microglial cells. The wap was done at http://www.chibi.ubc.ca/matrix2png/bin/matrix2png.cgi

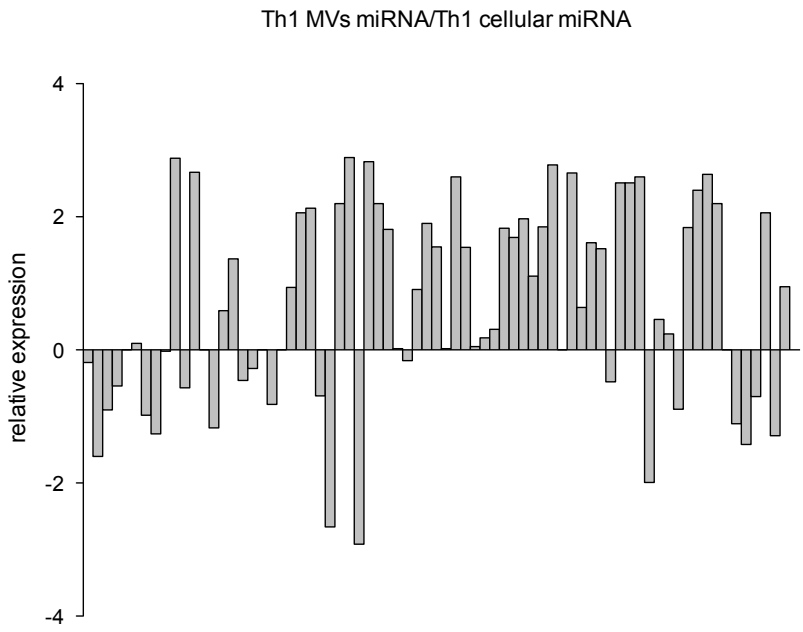

Fig. 24: Relative abundance of miRNAs in MVs released from Th1 primed microglia compared to parental cells .

5 Discussion

5.1 Inflammatory role of Mvs in vitro

Microglial cells constitute the resident macrophage population in the brain. They are generally considered the immune cells of the CNS. They show a resting phenotype in normal CNS where they provide tissue maintenance and immune surveillance (Cameron & Landreth, 2010). Microglia cells become promptly activated in response to alteration of brain homeostasis (Hanisch & Kettenmann, 2007).

We and others have shown that both the typical danger signal ATP and the bacterial membrane component lipopolysaccharide (LPS) enhance the release of MVs (Bianco et al, 2009a; Qu et al, 2007) Here we show that Th1 cytokines (TNF-α, INF-γ and IL1-β) are the best priming stimuli to mediate shedding of MVs and that microvescicles shedding takes place very efficiently in microglia exposed in vitro to a pro-inflammatory environment. Several study indicate that microvesicles contribute to inflammation via their influence on cell-cell communication and cytokine release. In this work we demonstrate that microglia derived microvesicles are able to propagate an inflammatory signal to glial cells. iNOS, IL1beta, IL6, COX2 expression is increased in astrocytes and microglia exposed to MVs as indicated from Real Time PCR analysis. Interestingly MVs produced by microglia exposed to an inflammatory environment, such as those released from LPS primed microglia, induced a stronger up-regulation of the inflammatory markers in recipient glial cells.

Microvesicles may influence the behaviour of target cells in several ways, including the transfer of protein, mRNAs and miRNAs (Ratajczak et al, 2006). Our data indicate that MVs can be internalized from microglial cells and astrocytes in culture and as a consequence of this process, mRNAs that are present inside the MVs are transferred to recipient cells. Nevertheless the role of

MVs internalization and mRNA transfer in the propagation of the inflammatory signal remain to be largely explored.

We also show that MVs can interact with the surface of cultured neurons, thereby causing an increase in the levels of mir-146a in hippocampal cultures. Mir-146a is an inflammatory microRNA, and its expression is induced by NF-kB activation (Aronica et al, 2010). Hence, the increase in Mir-146a suggest that MVs propagate an inflammatory signal to neurons, besides glial cells. However, lack of detectable levels of murine mRNA for Il1 beta in the extract from rat neurons exposed mouse MVs, indicate that MVs fail to transfer components from their lumen to the neuronal cytoplasm. Based of this observation, it is unlikely that the increase in mir-146 levels evoked in neurons by MV exposure is mediated by a direct transfer of the miRNA. Although further study are required to clarify this point, it is likely that MVs induce an up-regulation of neuronal of mir-146a through the activation of inflammatory pathway in neurons.

5.2 Role in vivo of microglia-derived MVs

Previous studies indicated that microglia and other myeloid cells *in vitro* can shed MVs, which store and release the pro-inflammatory cytokine IL-1b, together inflammosome components (Mathivanan et al, 2010) and MHCII protein, a central player in the adaptive immune response (Qu et al, 2007). These data suggest that MVs produced from reactive myeloid cells may propagate inflammation and provide an efficient route for rapid dissemination and presentation of antigens. This possibility has been clearly demonstrated by the in vitro experiments described in this thesis. In addition, recent results of our laboratory indicated that microglia-derived MVs interact with the plasma membrane of neurons and enhance excitatory transmission (Antonucci et al., in press), possibly contributing to the excessive potentiation of neurotransmission, which occurs in neuroinflammatory diseases (Centonze et al, 2009a). However, whether MVs of microglial origin

exist and play a role *in vivo* was still elusive. Aim of this work was to investigate the existence of MVs of microglia origin *in vivo* and to explore their possible role during inflammatory brain diseases. We showed, by electron and fluorescence microscopy, the presence of MVs positive for myeloid markers in the CSF of healthy rodents and humans, indicating that myeloid cells can secrete MVs *in vivo*. Given peripheral macrophages are virtually absent in healthy brain parenchyma, the presence in the CSF of MVs positive for myeloid markers or derived from CX3CR1-EGFP expressing cells suggest that MVs originate from resident microglia in the normal brain. However, MVs may also originate from the low number of macrophages which are present at choroid plexus and within leptomeninges in the uninjured brain. The capability of MVs to travel away from parechymal microglia and to enter the CSF is consistent with the presence in the CSF of MVs of neuroectodermal origin, including neurons and oligodendrocytes, that are strictly parenchymal and have no contact with liquoral space. Neurons and astrocytes have been described to release MVs *in vitro*(Faure et al, 2006)·(Taylor et al, 2007)·(Bianco et al, 2009b). However, to our knowledge, this is the first evidence that rodent and human CSF contain MVs derived from these brain cells.

Quantification of MVs by flow cytometry indicated an increase in the absolute amount of microglia/macrophages MVs in EAE mice. Choroid plexus, which is in direct contact with the CSF and has been identified as the site of first entry of inflammatory cells and peripheral macrophages during EAE(Engelhardt et al, 2001; Kivisakk et al, 2009), represent the more plausible source of increased amounts of myeloid MVs during neuroinflammation. Consistent with this possibility myeloid MVs were detected in plexus from from mice injected with inflammatory cytokines. Due to the fact that small volumes of CSF collected from single mice do not allow the use of beads for exosome capture(Gyorgy et al, 2011)·(Ostrowski et al, 2010), exosomes cannot be accurately analyzed by flow cytometry. We could not therefore distinguish between the two populations of extracellular membrane vesicles of myeloid origin. However, the majority of events above the detection limit of the flow cytometer most likely represents large MVs, originating from the plasma

membrane of microglia/macrophages. Consistent with this possibility, MVs of larger size were detected by EM in the CSF of EAE rats as compared to naive animals.

One of the main accomplishments of this study is the finding that the concentration of microglia/macrophage-derived MVs in mouse CSF reflects the course and severity of EAE. Consistently, the amount of MVs in human CSF is higher in patients presenting with the first clinical symptom of MS or in relapsing patients as compared to patients in a stable phase of the disease or healthy controls. These results link the events of microglia activation and infiltration of peripheral macrophages to the process of MV secretion. They also identify CSF myeloid MVs as novel biomarkers of microglia/macrophage activation *in vivo,* useful as companion tool for disease diagnosis and for monitoring the efficacy of drugs targeting MS. Microglia is considered at the crossroad between inflammation and neurodegeneration with both detrimental and protective roles(Ransohoff & Cardona, 2010; Schwartz & Shechter, 2010). The evidence that different methods of activation result in different functional phenotypes of myeloid cells, ranging from purely phagocytic and tissue destructive, to immuno-modulating and promoting tissue remodeling(Biswas & Mantovani, 2010), has led to the idea that suppressing microglia or modulating/redirecting its activation holds a therapeutic potential for progressive forms of MS and for neurodegenerative disorders(Centonze et al, 2009b). However, information on the quality of the microglial activation *in vivo* in humans is missing as are reliable biomarkers to monitor the efficacy of drugs targeting activated microglial cells. We propose that MVs produced by microglia/macrophages and leaking into the CSF may represent a rich source of information on microglia/macrophage activation in the brain, which may lead to the identification of specific disease cell signature through the analysis of their content.

The second main accomplishment of our study is the demonstration that increased amounts of microglia/macrophage-derived MVs actually promote neuro-inflammation The pro-inflammatory activity of MVs was demonstrated *in vitro* by the dose-dependent induction of inflammatory markers in recipient glial cells. The glial reaction was associated to MV interaction and transfer of

IL-1β mRNA in the cell cytoplasm. Accordingly, our unpublished observation suggested that MVs depleted from their luminal content greatly lose their capacity to activate target astrocytes and the lipid fraction from MVs only weakly stimulates the expression of inflammatory markers in astrocytes, thus ruling out that phospholipids of microglia-derived MV are responsible for the inflammatory activity. However, further studies are required to identify the active component of MVs and to determine whether MV internalization and transfer of genetic information contribute to the pro-inflammatory response target cells. The pathogenic role of MVs in the inflammatory response was demonstrated *in vivo* by showing that injection of microglia-derived MVs induces the formation of inflammatory foci at the site of delivery. This was further corroborated by the finding that A-SMase KO mice, genetically impaired in MV production, are largely protected from EAE. Mice lacking A-SMase develop a phenotype similar to Niemann-Pick type A disorder(Horinouchi et al, 1995b), (Otterbach & Stoffel, 1995), an inherited disease characterized by progressive visceral organ abnormalities and neurodegeneration, that leads to growth retardation and death in early childhood. A-SMase KO mice appear normal at birth and develop normally until about 8-10 weeks of age, when ataxia and mild tremors become noticeable, as a result of Purkinje cell neurodegeneration (Otterbach & Stoffel, 1995). Of note, despite growth defects, incipient neurodegeneration and the slight gliosis in the gray and white matter (Otterbach & Stoffel, 1995) we found that 8 week-old EAE KO animals were about 20% more the weight of EAE WT littermates. Moreover, EAE KO mice displayed no demyelinating lesions and less perivascular infiltrates in the spinal cords as compared WT littermates, thus strongly linking A-SMase deficiency and impairment in MV shedding to EAE amelioration.

Give these data MVs are a unique way for exchanging integrated signals, targeting MVs may represent a therapeutic strategy more advantageous than classical approaches aimed at neutralizing single inflammatory molecules in MS.

From the data exposed in this thesis Microglia derived MVs are emerging as new biomarkers for neuroinflammatory desease. To address the possibility that CSF MVs contain signatures which

specifically reflect microglial activation occurring in neuroinflammatory disease we analyse their miRNAs contents. RNAs profiling of human MVs is hardly feasible, due to the small amount of organelles available for the analysis. Therefore we will identify putative multiple sclerosis markers by mapping miRNAs expressed in MVs shed in vitro upon activation with either Th1 inflammatory cytokines and anti-inflammatory cytokine (IL4).

Data collected from the miRNAs Exiqon's panels revealed several miRNAs that are differently expressed in MVs. Moreover miRNA that are significantly up-/down-regulated in MVs shed from Th1 microglia as compared to resting cells or cells stimulated with IL4, will be confirmed by quantitative real-time PCR and their expression will be evaluated by Real Time PCR also in MVs isolated from the CSF of MS patients or healthy donor

5.3 Conclusions

Overall our data link activation of myeloid cells *in vivo* to propagation of MVs, as cargo structures delivering pro-inflammatory signals, and identify myeloid MVs as a novel therapeutic target and a companion tool for MS diagnosis.

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