

# Myeloid Microvesicles Are a Marker and Therapeutic Target for Neuroinflammation

Claudia Verderio, PhD,<sup>1</sup> Luca Muzio, PhD,<sup>2</sup> Elena Turola, BSci,<sup>1</sup>  
 Alessandra Bergami, MLT,<sup>2</sup> Luisa Novellino, PhD,<sup>1</sup> Francesca Ruffini, PhD,<sup>2</sup>  
 Loredana Riganti, PhD,<sup>1</sup> Irene Corradini, PhD,<sup>1</sup> Maura Francolini, PhD,<sup>1</sup>  
 Livia Garzetti, BSci,<sup>2</sup> Chiara Maiorino, BSci,<sup>2</sup> Federica Servida, PhD,<sup>3</sup>  
 Alessandro Vercelli, MD,<sup>4</sup> Mara Rocca, MD,<sup>2</sup> Dacia Dalla Libera, MD,<sup>2</sup>  
 Vittorio Martinelli, MD,<sup>2</sup> Giancarlo Comi, MD,<sup>2</sup> Gianvito Martino, MD,<sup>2</sup>  
 Michela Matteoli, PhD,<sup>1,5</sup> and Roberto Furlan, MD<sup>2</sup>

**Objective:** Microvesicles (MVs) have been indicated as important mediators of intercellular communication and are emerging as new biomarkers of tissue damage. Our previous data indicate that reactive microglia/macrophages release MVs in vitro. The aim of the study was to evaluate whether MVs are released by microglia/macrophages in vivo and whether their number varies in brain inflammatory conditions, such as multiple sclerosis (MS).

**Methods:** Electron and fluorescence microscopy and flow cytometry were used to detect myeloid MVs in the cerebrospinal fluid (CSF) of healthy controls, MS patients, and rodents affected by experimental autoimmune encephalomyelitis (EAE), the animal model of MS.

**Results:** Myeloid MVs were detected in CSF of healthy controls. In relapsing and remitting EAE mice, the concentration of myeloid MVs in the CSF was significantly increased and closely associated with disease course. Analysis of MVs in the CSF of 28 relapsing patients and 28 patients with clinical isolated syndrome from 2 independent cohorts revealed higher levels of myeloid MVs than in 13 age-matched controls, indicating a clinical value of MVs as a companion tool to capture disease activity. Myeloid MVs were found to spread inflammatory signals both in vitro and in vivo at the site of administration; mice impaired in MV shedding were protected from EAE, suggesting a pathogenic role for MVs in the disease. Finally, FTY720, the first approved oral MS drug, significantly reduced the amount of MVs in the CSF of EAE-treated mice.

**Interpretation:** These findings identify myeloid MVs as a marker and therapeutic target of brain inflammation.

ANN NEUROL 2012;72:610–624

Small membrane vesicles, released by most cell types, include shed microvesicles (MVs), also called ectosomes, which bud directly from the plasma membrane, and exosomes, which result from exocytosis of multivesicular bodies.<sup>1–4</sup> Shed MVs are characterized by high levels of externalized phosphatidyl serine (PS),<sup>5</sup> whereas exosomes typically express tetraspan proteins such as CD63 or CD9. Membrane vesicles produced from distinct cells are molecularly different from each other,

reflecting the differential expression of proteins of various donor cells. Constituents of MVs may also vary depending on the state (eg, resting, stimulated) of donor cells.<sup>6</sup> MV cargo includes nucleic acids (mRNA, microRNA, DNA), soluble and integral cellular proteins, and lipids. For all these constituents, vesicle biogenesis serves as a mechanism of regulated assembly, protection from dispersal and degradation, efficient extracellular release, or transfer to target cells. Because they serve as units of

View this article online at [wileyonlinelibrary.com](http://wileyonlinelibrary.com). DOI: 10.1002/ana.23627

Received Sep 17, 2011, and in revised form Mar 26, 2012. Accepted for publication Apr 13, 2012.

Address correspondence to Dr Verderio, CNR Institute of Neuroscience and Department of Medical Pharmacology, Università di Milano, Via Vanvitelli 32, 20129 Milano, Italy. E-mail: [c.verderio@in.cnr.it](mailto:c.verderio@in.cnr.it) or Dr Furlan, INSPE, Division of Neuroscience, San Raffaele Scientific Institute, via Olgettina 60, 20132 Milano, Italy. E-mail: [furlan.roberto@hsr.it](mailto:furlan.roberto@hsr.it)

From the <sup>1</sup>Italian National Research Council Institute of Neuroscience and Department of Medical Pharmacology, University of Milan, Milan; <sup>2</sup>INSPE, Division of Neuroscience, San Raffaele Scientific Institute, Milan; <sup>3</sup>Fondazione Matarelli and Department of Medical Pharmacology, University of Milan, Milan; <sup>4</sup>Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Turin; and <sup>5</sup>IRCCS Humanitas Clinical Institute, Rozzano, Italy.

Additional supporting information can be found in the online version of this article.

multiple biochemical information,<sup>1</sup> the efficacy of intercellular signaling allowed by MVs is far superior to that of direct cell to cell contacts or secreted factors. Accordingly, MVs potently influence the phenotype of surrounding cells.<sup>7</sup> Because of their small size, some extracellular vesicles can move from the site of discharge by diffusion and be retrieved from several biological fluids, such as blood, urine, and synovial fluid, where they are emerging as clinically valuable markers of disease states.<sup>8–10</sup> Shed MVs are quite large in size (200–1,000nm in diameter) and can be directly quantified in biological fluids by flow cytometry, in contrast to exosomes (50–100nm), which are below the detection limit of the methodology.

Recent studies have shown that exosomes and shed MVs are released by brain cells. Although little is known about their molecular composition, accumulating evidence indicates that MVs produced by distinct types of brain cells play important functions in the central nervous system (CNS), both in health and after damage.<sup>7</sup> For example, exosomes derived from oligodendrocytes can control myelination,<sup>11</sup> and those produced from Schwann cells may support local axonal protein synthesis by delivering ribosomes to injured axons.<sup>12</sup> Conversely, exosomes released by neurons may exert detrimental function, spreading pathogenic agents or degenerative proteins like beta-amyloid and alpha-synuclein.<sup>8,9,13,14</sup> Finally, MVs released from glioma can transfer oncogenic proteins.<sup>15</sup> A specialized type of MV release has been described for microglia. Microglial cells, like other myeloid cells that express the P2X<sub>7</sub> adenosine triphosphate (ATP) receptor, shed MVs from the cell surface when exposed to P2X<sub>7</sub> agonists.<sup>5,16</sup> Although it is known that MVs derived from reactive microglia contain inflammatory cytokines, such as interleukin (IL)1 $\beta$ , no information is available about their existence and biological activity within the brain. Although microglia are the immune cells of the CNS, which provide the first line of defense in brain pathologies,<sup>17</sup> all reports that exploited the diagnostic value of MVs in CNS diseases have focused so far on MVs derived from platelets, endothelial cells, or oligodendrocytes.<sup>8,9,18</sup> The possibility that microglia-derived MVs exist *in vivo* and may represent biomarkers of inflamed CNS has never been explored. We addressed this hypothesis in the prototypic inflammatory disease of the CNS, multiple sclerosis (MS), and in experimental autoimmune encephalomyelitis (EAE), the widely characterized model of the human disease, in which microglia activation and infiltration of peripheral macrophages are strictly associated with disease severity, contributing to clinical symptoms.<sup>19</sup> Here we report that MVs of myeloid origin are detectable in the cerebrospinal fluid (CSF) of humans and rodents and that their concentration

increases upon brain inflammation. In addition, we show that myeloid MVs, by spreading inflammation, may contribute to the disease pathogenesis.

## Materials and Methods

### Human Subjects

Human CSF samples were obtained for diagnostic purpose from subjects with clinically isolated syndrome (CIS; n = 28) or definitive MS (n = 72) according to revised McDonald criteria,<sup>20</sup> attending the MS Center of the San Raffaele Hospital. Five patients had primary progressive MS (PPMS); among relapsing–remitting MS (RRMS) patients, 39 were clinically stable and 28 were sampled during an acute attack before intravenous steroid pulse. Clinical data are summarized in the Supplementary Table. None of the MS patients was under immunomodulatory or immunosuppressive treatment. Six subjects with neuromyelitis optica (NMO), positive for NMO-Ig, 18 patients with other inflammatory neurological disorders (including aseptic encephalitis, recurrent myelitis, recurrent optic neuritis, Behçet disease, Guillain–Barré syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, inflammatory radiculitis), 18 patients with other noninflammatory neurological disorders (including normal pressure hydrocephalus, parkinsonism, Parkinson disease, motor neuron disease, noninflammatory peripheral neuropathies related to diabetes, vitamin deficiency, alcohol, isolated ischemic nerve palsy, leukodystrophy, spinocerebellar ataxia, pseudobulbar palsy), 5 patients with CNS infectious diseases, and 5 patients with tumors were also included in the analysis. CSF from age- and sex-matched healthy donors (n = 13) 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 written informed consent.

### Animals

Sprague Dawley and Lewis rats, and C57BL/6 and SJL/j mice were purchased from Charles River (Wilmington, MA). Enzyme acid sphingomyelinase (A-SMase) knockout (KO) mice<sup>21</sup> were a kind gift of Dr Edward H. Schuchman to A.V. CX3CR1-EGFP<sup>22</sup> mice were kindly provided by F. Kirchhoff. All efforts were made to minimize animal suffering and to reduce the number of animals used, in accordance with the European Communities Council Directive of September 20, 2010 (2010/63/UE). All procedures involving animals were performed according to the guidelines of the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute.

### Isolation of MVs from Rodent CSF

Rats or mice were anaesthetized by intraperitoneal injection of 4% clorinium hydrate; CSF was sampled from the cisterna magna using a glass capillary and checked for the absence of blood contamination. CSF pooled from 2 to 5 rats was diluted with 0.5 to 1ml ice-cold phosphate-buffered saline (PBS)

containing protease inhibitors and either subjected to differential centrifugation to obtain 3 vesicles pellets—P2, P3, and P4—as previously described,<sup>5</sup> or pelleted at  $110,000 \times g$  for 1 hour to obtain the whole MV fraction. The resulting pellets were either resuspended in sodium dodecyl sulfate (SDS) sample buffer for Western blotting, or resuspended (and fixed when needed) for electron microscopy (EM) or fluorescence microscopy.

### EM

P2, P3, and P4 CSF vesicles or apoptotic bodies from ultraviolet-irradiated N9 microglial cells (Supplementary Fig 1) were fixed with 4% paraformaldehyde and adsorbed to 400-mesh Formvar/carbon-coated grids. Grids were contrasted with 1% uranyl acetate and analyzed with a Philips (Best, the Netherlands) CM10 transmission electron microscope. Immunogold labeling for CD11b/c (OX42) was performed on MVs adsorbed to grids and incubated for 1 hour with antirat primary antibody (Ab), followed by 12nm gold-coupled secondary Ab. MVs were then treated with 1% glutaraldehyde for 20 minutes and finally subjected to negative staining. MVs from primary rat microglia were used as positive controls, whereas synaptic vesicles from rat crude synaptome preparation were used as negative controls.

### Fluorescence Microscopy

MVs from rat or mouse CSF were resuspended in about 20 $\mu$ l of PBS buffer, stained with Annexin-V-FITC, CD11b-PE, NBD-C6HPC, or IB4-FITC, spotted on glass slides, and observed with an inverted Zeiss (Thornwood, NY) Axiovert 200M microscope. Staining of fixed MVs with Abs directed against intracellular epitopes, that is, IBA-1, glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), CD63, and SNAP-25, was performed as follows. Primary Ab was added in a 1:1 volume of PBS buffer containing goat serum and 0.3% TritonX-100, and incubations were allowed for 1 hour at room temperature (RT). Primary Ab-conjugated MVs were then washed with PBS and pelleted before incubation with fluorochrome-conjugated secondary Abs for 2 hours at RT and further washing in PBS. Repelleted labeled MVs were then spotted on glass slides and observed under the microscope. CSF collected from CX3CR1-EGFP mice was directly stained for Cd11b, spotted on a glass microscope, sealed, and analyzed.

### Western Blotting

P2, P3, and P4 MVs of rat CSF were resuspended in SDS sample buffer, loaded on a single lane of a 12% polyacrylamide gel, and blotted onto nitrocellulose filters (see Supplementary Fig 1); 0.2 $\mu$ g of corpus callosum homogenate, 10 $\mu$ g of rat brain, and 1 $\mu$ g of cortical astrocyte lysate were run in the same gel, as positive controls. Selected proteins were detected with specific Abs followed by horseradish peroxidase-conjugated secondary Abs and revealed using an ECL system. Samples from MV-stimulated cultured astrocytes (10 $\mu$ g) were processed similarly. Optical density of immunolabeled bands was measured with version 1.35 ImageJ software (National Institutes of Health, Be-

thesda, MD), and average values and standard error (SE) were calculated over 3 independent experiments.

### Flow Cytometry Analysis of Rodent and Human CSF

Human or mice CSF was directly stained with fluorescein isothiocyanate (FITC)-conjugate isolectin B4 from *Bandeiraea simplicifolia* (IB4-FITC; Sigma, St Louis, MO) and/or annexin-V-APC in 1% bovine serum albumin. Specificity of IB4 labeling 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 minutes (Supplementary Fig 4) as previously described.<sup>23</sup> CSF was then diluted in PBS buffer, and labeled MVs were acquired within a fixed time interval on a Canto II HTS flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using FCS 3 software (Becton Dickinson). Unstained and single-color controls were used to properly set photomultiplier tube voltages and compensations. Forward scatter (FSc) height and width were used to discard doublets or aggregates. Using side scatter and FSc, a vesicle gate was determined over the instrument noise (set by running PBS filtered through a 100nm filter). Within this gate, IB4-positive events (number of events/ $\mu$ l) were evaluated as a parameter of myeloid MV concentration in the CSF. In the first set of experiments, the vesicular nature of detected events was confirmed by using the lipophilic membrane styryl dye FM1-43. In addition, in vitro generated MVs were analyzed and sorted using similar flow-activated cell sorting (FACS) parameters on a FACSAria followed by fluorescence microscopy analysis.

### Isolation and Quantification of MVs Shed In Vitro

A Micro BCA protein assay kit (Thermo Fischer Scientific, Waltham, MA) was used to determine the protein concentration of MVs shed upon exposure to 3'-O-(4-benzoyl) benzoyl ATP (BzATP; 100 $\mu$ M) or constitutively released from microglial cells or mesenchymal stem cells.

Spectrophotometric quantification of MVs shed from primary microglia was performed in Kreb's Ringer solution as described previously.<sup>5</sup> Briefly, cells were incubated with 50 $\mu$ M NBD-C<sub>6</sub>-HPC, washed, and stimulated with 100 $\mu$ M BzATP for 20 minutes. Supernatant was collected and centrifuged for 10 minutes at  $300 \times g$  and 4°C to remove cells and debris, and the total fluorescence was assayed at 485/535nm with the spectrophotometric system Tecan Infinite500 (Tecan Group, Männedorf, Switzerland).

### Glial Cells Cultures and In Vitro Stimulation

Primary microglia and purified cultures of astrocytes were isolated from mixed cultures of cortical astrocytes, established from E21 embryonic rat pups, and maintained as previously described.<sup>16</sup> Murine BV2 microglial cells were provided by Dr F. Aloisi and grown in RPMI, supplemented with 5% fetal bovine serum. Murine N9 microglia cells were grown as previously described.<sup>16</sup> Glial cells were exposed to either 0.4 $\mu$ g/ml lipopolysaccharide (LPS) or Th1 cytokines (100U/ml IL1 $\beta$ ,

200U/ml tumor necrosis factor [TNF] $\alpha$  and 500U/ml interferon [IFN] $\gamma$  for 24 hours to shed MVs for different time points (24, 48, and 72 hours). Shed MVs, used as stimulus (P2 and P3 fractions), were freshly isolated from the supernatants of primary microglia, conditioned by cells for 30 minutes upon 100 $\mu$ M BzATP stimulation. 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; 2 $\mu$ g/ml) unless otherwise specified. To reduce the level of activation, recipient astrocytes were prestarved overnight in serum-free medium and kept in low (1%) serum medium during exposure to MVs. To minimize the activation of donor and recipient microglia, half of the medium in which microglia were kept after shaking from mixed glial cultures was replaced with fresh low (1%) serum medium. At the end of activation, recipient glia were washed and either lysed in SDS sample buffer for Western blotting, harvested with TRIzol for reverse transcriptase polymerase chain reaction (RT-PCR) analysis, fixed with 4% paraformaldehyde for immunocytochemistry, loaded with the calcium dye FURA-2/AM for calcium imaging, or analyzed by flow cytometry after surface staining with CD86-PE for 20 minutes (at least  $5 \times 10^4$  events/sample were analyzed).

### RT-PCR

Total RNA was isolated from rat primary astrocytes/microglia using an miRNeasy Qiagen (Valencia, CA) kit following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. cDNA synthesis was performed using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) and random hexamers as primer. The resulting cDNAs were amplified using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA). The mRNA expression was normalized to the label of glyceraldehyde-3-phosphate dehydrogenase mRNA.

### Cell Fluorescence Analysis of Recipient Glia

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

### [Ca<sup>2+</sup>]<sub>i</sub> Determination

Astrocytes, after exposure to shed MVs for 72 hours, were loaded with 10 $\mu$ M Fura-2/AM for 45 minutes at 37°C in culture medium. Polychrome IV (TILL Photonics, Leuven, Belgium) 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 and 1 to 4Hz. 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.

### Lentivirus Injections

Mice (n = 5 per group) were anesthetized with 2,2,2-tribromoethanol (10mg/ml; 1/27 of body weight), and the head was placed in a stereotactic injection apparatus (David Kopf Instruments, Tujunga, CA). Vesicular stomatitis virus-pseudotyped lentivirus (LV) LV-PGK-IFN $\gamma$  and LV-PGK-TNF $\alpha$ , previously described in Muzio et al,<sup>24</sup> 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.

### Relapsing–Remitting and Nonrelapsing Rodent EAE

Nonrelapsing EAE (nr-EAE) was induced in female C57Bl/6 mice by immunization with 200 $\mu$ g/mouse of MOG<sub>35–55</sub> (Espikem, Florence, Italy) and 2 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-week-old female A-SMase<sup>-/-</sup> mice with a SV129 background 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.<sup>21,25</sup> To obtain subclinical EAE for focal MV injections, mice were immunized with 50 $\mu$ g/mouse of MOG<sub>35–55</sub> and 250ng/mouse of pertussis toxin. Relapsing–remitting EAE (r-EAE) was induced in female SJL/j mice by 2 immunizations, 7 days apart, with 200 $\mu$ g/mouse of PLP<sub>139–151</sub> (Espikem) and 4 injections of 500ng of pertussis toxin the day of immunization and 48 hours later. CSF was collected at 10, 20, and 60 days postinjection (dpi), in nr-EAE and at 29, 35, and 48 dpi in r-EAE mice. Female Lewis rats weighing about 150g were immunized under the skin of the flanks using 1mg lyophilized spinal cord homogenate emulsified in a total of 200 $\mu$ l of complete Freund adjuvant. Weight and clinical score were assigned according to a standard and validated on a 0 to 5 scale, described in Furlan et al.<sup>26</sup> Cumulative disease score was calculated by summing the neurological scores recorded daily for each mouse along the whole period of observation.

### MV Injections in EAE Mice

Naive mice and mice with subclinical EAE (see above), 20 days postimmunization, were stereotactically injected in the corpus callosum (coordinates: 0mm anterior, 1.0mm lateral to the bregma, and 2.2mm in depth) with MVs (P2–P4 fractions) derived from primary microglia dissolved in 1 $\mu$ l of sterile saline (2 $\mu$ g/ $\mu$ l). MVs from bone marrow–derived mesenchymal cells (2 $\mu$ g/ $\mu$ l) and liposomes, mimicking the phospholipid composition of the plasma membrane (8nmol/ $\mu$ l), were used as controls. Liposomes were prepared as follows. Bovine brain phosphatidylcholine (PC), PS, sphingomyelin (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 resuspended in PBS at 40°C to obtain multilamellar vesicles. Small unilamellar vesicles were obtained by sonicating multilamellar vesicles. Mice were killed 72 hours after MV/liposome injection to analyze formation of focal lesions at the site of administration.

### Neuropathological and Flow Cytometry Analysis of Spinal Cords

Brain tissue sections were fixed, embedded in paraffin, and stained with hematoxylin and eosin, Luxol fast blue, and Bielschowsky to reveal perivascular inflammatory infiltrates, demyelinated areas, and axonal loss, respectively. Infiltrating microglia and T cells were stained using IB4, anti-CD3, and anti-CD4, revealed using a biotin-labeled or fluorescent (Alexa Fluor Dyes; Molecular Probes, Eugene, OR) secondary antibody. Inflammatory infiltrates, demyelinated areas, and axonal loss were quantified on an average of 10 complete cross sections of spinal cord per animal, representative of whole spinal cord levels. Perivascular inflammatory infiltrates, T cells, and macrophages were evaluated as the number per square millimeter, whereas demyelinated areas and axonal loss were expressed as the percentage per square millimeter.

To analyze by flow cytometry infiltrating immune cells, spinal cords were dissected out, and homogenized through a 70 $\mu$ m cell strainer. Mononuclear cells were isolated by 30/40/80/100% Percoll gradient, and 40 to 80 fractions were washed twice before FACS staining with allophycocyanin (APC)-conjugated anti CD45, APC-Cy7-conjugated anti-CD11b, and PE-Cy7-conjugated anti-CD4 Abs (all Abs from BD Biosciences, San Jose, CA) to detect microglia/macrophages or T cells, respectively. Cells were collected on Canto II HTS flow cytometer and analyzed using FCSexpress software (Tree Star, Ashland, OR).

### Chemicals and Antibodies

ATP, BzATP, LPS, Fura-2/AM, fluorescein isothiocyanate (FITC)- and APC-annexin V, IB4-FITC, IB4-Texas red, phalloidin-Texas red, and melibiose were from Sigma-Aldrich (St Louis, MO). CellTracker green CMFDA was from Molecular Probes, propidium iodide (PI) was from BD Biosciences, NBD-C<sub>6</sub>-HPC was from Invitrogen, IL1 $\beta$  was from Euroclone (Devon, UK), TNF $\alpha$  and IFN $\gamma$  from R&D (Minneapolis, MN). The following antibodies were used: antimouse CD11b-PE (BD Biosciences), antihuman CD63-PE (BD Biosciences) or antimouse CD63 (M13; Santa Cruz Biotechnology, Santa Cruz, CA), antirat CD86 (eBioscience, San Diego, CA), antimouse GFAP (Sigma-Aldrich), antihuman calnexin (Sigma-Aldrich), antimouse 2',3'-cyclic nucleotide 3'-phosphodiesterase (Chemicon, Temecula, CA), antimouse MBP (Chemicon), antimouse SNAP-25 SMI 81 (Sternberger Monoclonals, Lutherville, MD), antirabbit Iba-1 (Wako Chemicals, Richmond, VA), IB4-FITC (Sigma), anti-CD3 (Serotec, Oxford, UK), anti-CD4 (BD Biosciences), anti-OX42 (Harlan Sera-Lab, Loughborough, UK). PC, PS, cholesterol, and SM were from Sigma-Aldrich. Mesenchymal stem cells were purchased from Neuro-Zone (Milan, Italy).

### Statistical Analysis

All data are presented as mean or median  $\pm$  standard deviation or SE from the indicated number of experiments. Data were compared using the Student *t* test for parametric data or the Mann-Whitney *U* test for nonparametric data or non-normally distributed data. Differences were considered to be significant if *p* < 0.05.

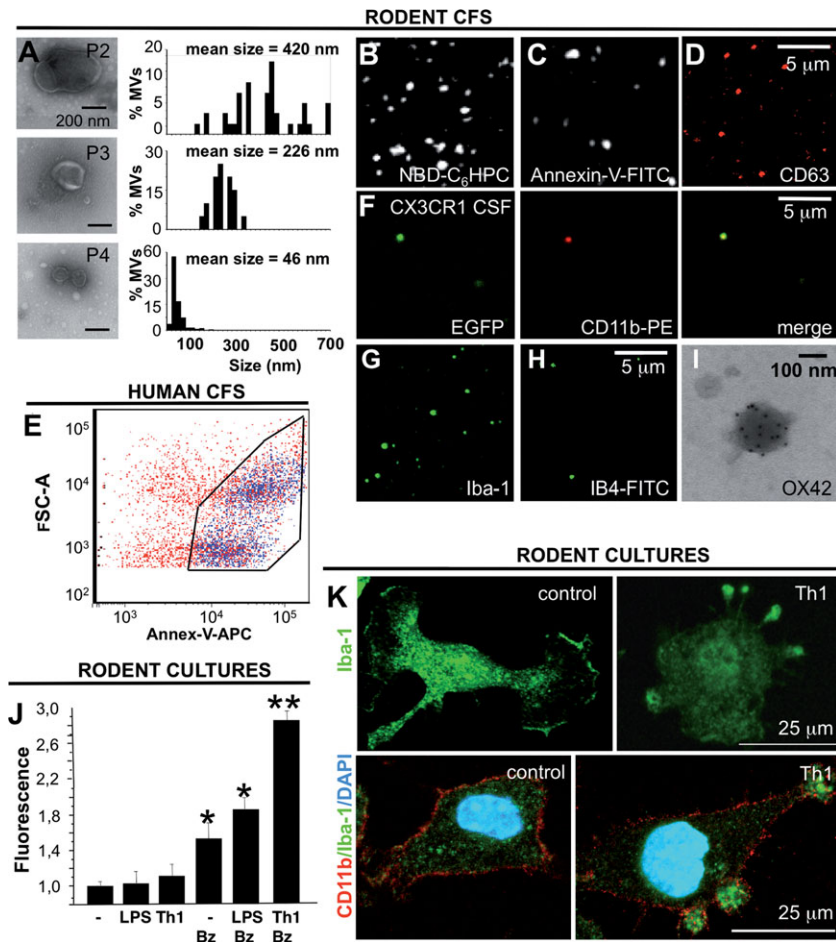
## Results

### Microglia/Macrophage-Derived MVs Can Be Detected in Healthy CSF

Small membrane MVs, similar in size to both shed MVs and exosomes released from cultured glial cells, were detected by negative staining EM in the CSF collected from healthy rodents (Fig 1). The presence of shed MVs, characterized by externalized PS, was assessed by fluorescence microscopy and by direct flow cytometry analysis with annexin-V, which binds externalized PS. We excluded the presence of apoptotic bodies, which are much larger and denser than the vesicles we found in the CSF (see Supplementary Fig 1C–E). Fluorescence microscopy and Western blotting analysis revealed that MVs display neuronal, astrocytic, oligodendroglial (see Supplementary Fig 1A, B), or microglia/macrophage markers, thus indicating that they originate from all these brain cells. The microglia/macrophage origin of MVs was definitely confirmed by immunogold EM with antibodies to CD11b/c (OX42; Supplementary Fig 2A, B) and by the recovery of enhanced green fluorescent protein (EGFP)-positive MVs in the CSF collected from CX3CR1-EGFP transgenic mice,<sup>22</sup> in which microglia express EGFP. Given the fact that peripheral macrophages under normal CNS conditions do not penetrate into the brain,<sup>27</sup> the presence of EGFP-labeled MVs in the CSF suggests that they derive from resident microglia. Thus, the healthy CSF drains MVs from all the neural cell lineages tested, including those, like neurons and oligodendrocytes, that are strictly parenchymal and have no contact with liquor spaces.

### Neuroinflammation Enhances the Levels of Microglia/Macrophage-Derived MVs in the CSF

We and others have shown that both the typical danger signal ATP and the bacterial component LPS enhance the release of MVs from microglia/macrophages.<sup>16,28,29</sup> Here we show that Th1 cytokines (TNF- $\alpha$ , INF- $\gamma$ , and IL1 $\beta$ ) are the best priming stimuli for MV shedding (see Fig 1J), consistent with their ability to induce massive formation of blebs at the cell surface, characterized by accumulation of Iba-1 (see Fig 1K). Notably, a linear correlation exists between production of MVs and levels of in vitro cell activation (Supplementary Fig 3). To validate these results in vivo and verify whether production of MVs from microglia/macrophages increases during brain inflammation, we stereotactically injected a group of mice into the ventricular cavity with lentiviral vectors codifying for INF- $\gamma$  or TNF- $\alpha$ . This treatment induces infiltration of macrophages and expansion of microglia in the periventricular area and in the choroid plexus,

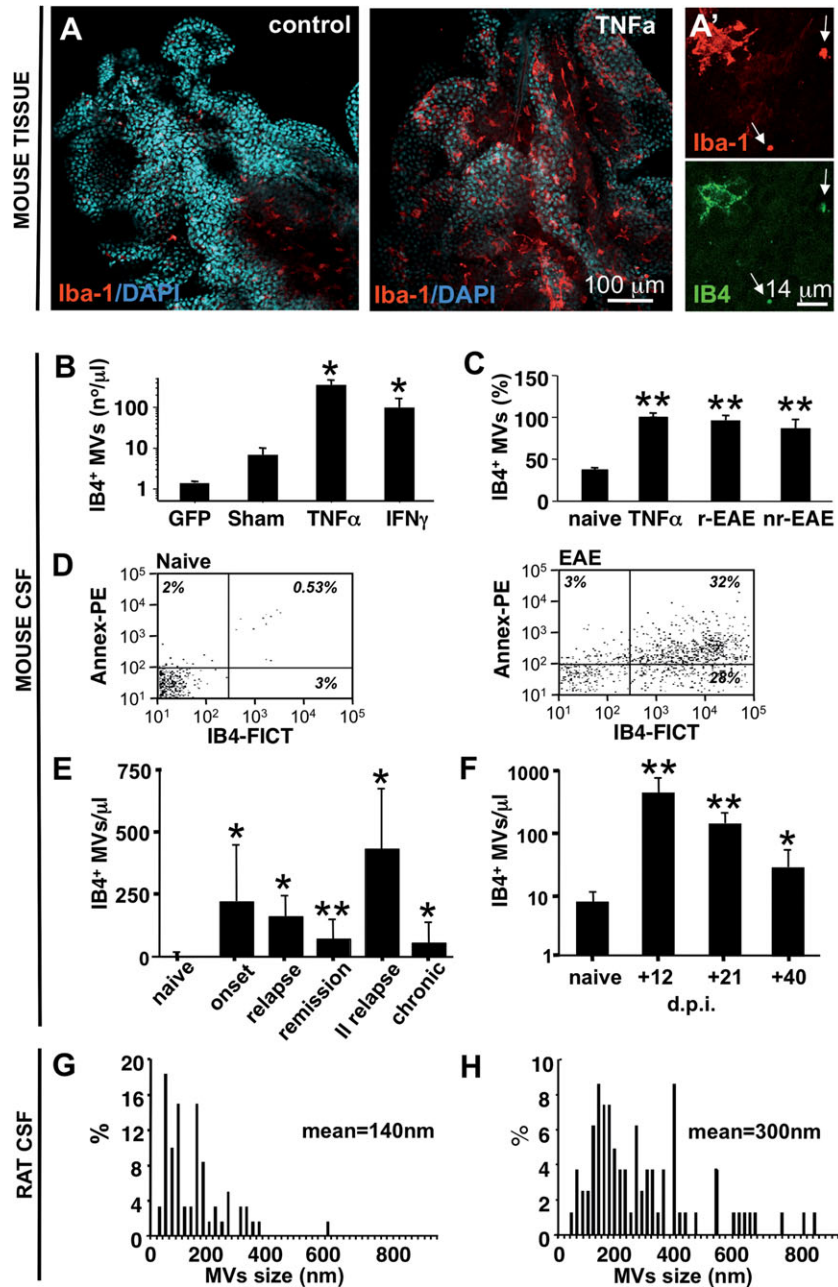


**FIGURE 1:** Microvesicles (MVs) of microglia/macrophage origin are present in the healthy cerebrospinal fluid (CSF). (A) MVs of decreasing size isolated from the CSF of healthy rats by differential centrifugation (P2, P3, P4 fractions) and analyzed by negative staining electron microscopy. Corresponding frequency histograms, indicating the size distribution of MVs pelleted at increased centrifugal force, are shown on the right (P2,  $n = 51$ ; P3,  $n = 73$ ; P4,  $n = 237$ ). (B–D) Examples of CSF MVs analyzed by fluorescence microscopy using the fluorescent phosphocholine analog NBD-C<sub>6</sub>-HPC to label the lipid bilayer of MVs (B), the shed MVs marker annexin-V-FITC (C), and the exosomal marker CD63 (D). (E) Flow cytometry analysis of MVs present in human CSF, directly stained for annexin-V-APC. (F) Fluorescence micrographs of enhanced green fluorescent protein (EGFP)<sup>+</sup> MVs present in the CSF of CXCR3-EGFP transgenic mice, stained for the microglia/macrophage marker CD11b-PE before microscope observation. (G, H) CSF MVs positive for the microglia/macrophage markers Iba-1 (G) or IB4-FITC (H). (I) Immunogold labeling of a CSF MV for the myeloid marker OX42. (J) The histogram shows the spectrophotometric quantification of MVs shed from resting or reactive microglial cells, prelabeled with NBD-C<sub>6</sub>-HPC and exposed to the P2X<sub>7</sub> agonist 3'-O-(4-benzoyl) benzoyl adenosine triphosphate (Bz; 100 μM) for 20 minutes. Note the significant increase in MV production in microglia preactivated with Th1 cytokines or lipopolysaccharide (LPS). (K) Primary microglia exposed to Th1 cytokines for 24 hours, 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). \* $p < 0.05$ , \*\* $p < 0.01$ .

mimicking the expansion of myeloid cells, which occurs during EAE (Fig 2).<sup>24,30,31</sup> Flow cytometry analysis of IB4<sup>+</sup> MVs revealed a dramatic increase of myeloid cell-derived MVs in the CSF of mice injected with cytokine-expressing vectors as compared to mice who received control vector and sham-treated animals (see Supplementary Fig 4 for MV characterization). This increase leads myeloid MVs to become the vast majority (>90%) of the total population of CSF MVs in both TNF- $\alpha$ - and IFN- $\gamma$ -injected mice (not shown). These results represent the proof of principle that the amount of IB4<sup>+</sup> micro-

glia/macrophage-derived MVs in the CSF reflects the activation state of myeloid cells in vivo.

To address the possibility that MV production from reactive microglia/macrophages increases also during a chronic neuroinflammatory disease, we quantified myeloid MVs in the CSF of mice affected by EAE. We found that IB4<sup>+</sup> MVs increased significantly, reaching 90% of total CSF MVs in mice with either nr-EAE or r-EAE, mimicking the 2 most common clinical forms of human MS (see Fig 2).<sup>32</sup> The absolute amount of microglia/macrophage MVs was closely associated with the



**FIGURE 2:** The amount of microglia/macrophage-derived microvesicles (MVs) in the cerebrospinal fluid (CSF) increases upon inflammation and reflects experimental autoimmune encephalomyelitis (EAE) activity. (A) Representative choroid plexus from mice injected with lentiviral vectors encoding for green fluorescent protein (GFP; control) or tumor necrosis factor (TNF) $\alpha$  10 days after injection. Staining for Iba-1 (red) and DAPI, to label nuclei (light blue), indicates a massive increase of Iba-1<sup>+</sup> cells in the plexus from a TNF $\alpha$ -injected mouse. (A') Confocal maximum projection image showing 2 MVs (arrows) double positive for IB4 (green) and Iba-1 (red) in the choroid plexus from a TNF $\alpha$ -injected mouse. Flow cytometry analysis of IB4<sup>+</sup> MVs in the CSF collected from naive mice, sham mice, and mice injected with the lentiviral vectors as above, 10 days after treatment (n = 5 mice for each condition; shown is 1 of 2 independent experiments). Histogram in (C) shows the percentage of IB4<sup>+</sup> MVs in the CSF of naive mice, mice injected with lentivirus coding for TNF $\alpha$ , and mice affected by nonremitting (nr-) or relapsing-remitting (r-) EAE. (D) Representative flow cytometry scatter plots of IB4<sup>+</sup> and annexin-V<sup>+</sup> MVs collected from CSF of a naive mouse and an EAE mouse, in the chronic phase of the disease. (E, F) Flow cytometry analysis of IB4<sup>+</sup> CSF MVs at different disease stages during relapsing-remitting (E) and nonremitting EAE (F; n = 5 mice at each disease stage; 1 of 3 independent experiments is shown). (G, H) Frequency histograms indicating the size distribution of MVs in the CSF of healthy rats or EAE rats, at peak of neuroinflammation. The size of MVs (not exceeding 1 $\mu$ m) rules out the apoptotic body nature of the vesicles (number of analyzed MVs: 361 naive CSF; 396 EAE CSF). IFN = interferon. \*p < 0.05, \*\*p < 0.01.

disease course, peaking at onset and during clinical relapses, and decreasing in the chronic phase of the disease. EM analysis of the CSF of EAE rats excluded that the increase of MVs during neuroinflammation was due to apoptotic bodies, and confirmed instead an increase in MVs of larger size, likely plasma membrane-derived MVs.

### **Microglia-Derived MVs Deliver a Proinflammatory Signal**

To define whether microglia/macrophage-derived MVs represent causative and/or amplifying agents of inflammation, primary glial cells were exposed *in vitro* to MVs shed from either Th1/LPS-primed or unstimulated microglia. A dose-dependent activation of astrocytes was induced by MVs produced by LPS-preactivated microglia, as indicated by the upregulation of mRNAs for the inflammatory markers IL1 $\beta$ , IL6, inducible nitric oxide synthase (iNOs), and cyclooxygenase-2 (COX-2) 48 hours after addition of MVs. Of note, a less robust response was evoked by MVs produced by resting microglia (Fig 3). Seventy-two hours after exposure to MVs, astrocytes appeared hypertrophic and displayed numerous thicker processes, showed increased GFAP expression, and increased cytoplasmic calcium concentration. Primary microglia responded *in vitro* to MVs by upregulating the T-cell coreceptor ligand CD86 on the cell surface, suppressing the expression of the tissue repair marker mannose receptor CD206,<sup>33–35</sup> and increasing the expression of the inflammatory markers iNOs, IL6, IL1 $\beta$ , and COX-2. Activation of glial cells was likely mediated by direct interaction of MVs with recipient cells (Supplementary Fig 5A–C) and was associated with the delivery of cytoplasmic components from donor to recipient microglia. By using PCR primers specific for mouse IL1 $\beta$ , we detected murine IL1 $\beta$  transcript in the RNA extracted from rat microglia previously exposed to murine MVs for 5 hours (Supplementary Fig 6). Interestingly, the transfer of MVs between glial cells spontaneously occurred in cultures, as indicated by the association of green fluorescent protein (GFP)<sup>+</sup> MVs, derived from GFP-P2X<sub>7</sub>-expressing microglia, with adjacent astrocytes (see Supplementary Fig 5D).

To validate these findings *in vivo*, we stereotactically injected MVs derived from cultured microglia into the brain of mice affected by subclinical EAE.<sup>36</sup> Injections were performed into the corpus callosum, a site usually devoid of inflammation during EAE.<sup>37</sup> Seventy-two hours after injection, we found infiltrating CD45<sup>+</sup> inflammatory cells close to the site of injection and along the needle track in all MVs-injected EAE mice (10 of 10; Fig 4A). In the same areas, we found several ameboid

Iba-1<sup>+</sup> cells, not detectable in control mice injected with saline (0 of 3), liposomes (0 of 3; not shown), or MVs derived from nonrelated cultures, that is, mesenchymal stem cells (0 of 3; see Fig 4B). Liposomes were injected as MV controls based on the recent finding that lipids from MVs but not synthetic liposomes mediate the stimulatory activity of microglial MVs toward neurons.<sup>38</sup> In all controls Iba-1<sup>+</sup> cells, although recruited along the needle track, were fewer and maintained their ramified morphology. The same occurred in naive mice injected with microglia-derived MVs (not shown). These data suggest that MVs may contribute to the formation of focal inflammatory lesions and propagate microglia activation in the presence of permissive conditions such as the EAE proinflammatory environment.

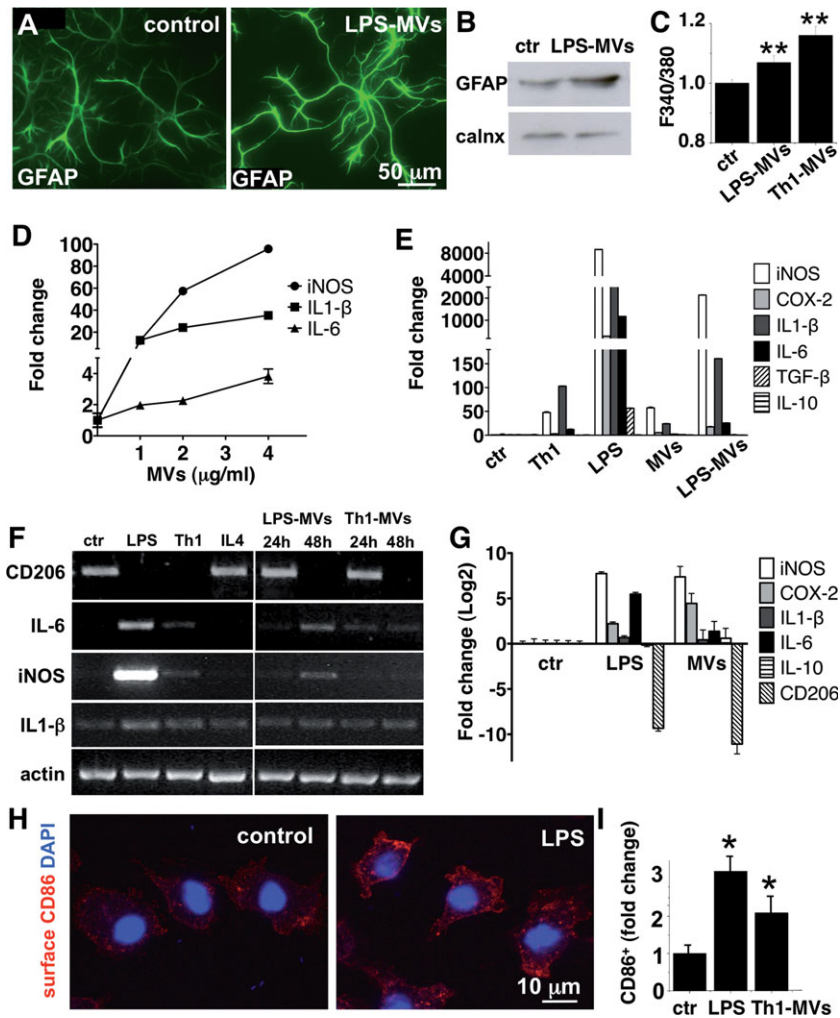
### **A-SMase KO Mice Are Highly Resistant to EAE**

We have previously shown in A-SMase controls the budding of MVs from the plasma membrane and that MV shedding is abolished in A-SMase KO glial cells.<sup>5</sup> Therefore, to further explore the pathogenic role of MVs *in vivo*, we induced EAE in A-SMase KO mice.<sup>21</sup> Because these mutants develop neurological signs starting from 10 to 12 weeks of age, EAE was induced in 6-week-old animals, and the disease was monitored until 8 weeks of age. Notably, A-SMase KO mice were highly resistant to the development of EAE as compared to wild-type (WT) littermates (see Fig 4E; Supplementary Fig 7). The lower score of A-SMase KO mice was associated with an expected lower amount of myeloid MVs in the CSF, absence of tissue damage, and reduced perivascular infiltrates in the spinal cord (see Fig 4C, D). Consistently, a decreased number of CD45<sup>low</sup>CD11b<sup>+</sup> microglia and CD45<sup>+</sup>CD4<sup>+</sup> T cells was detected by flow cytometry among mononuclear cells isolated from the spinal cord of A-SMase KO mice. Overall, these data confirm that shed MVs may have pathogenic functions during EAE.

### **Microglia/Macrophage MVs Are Elevated in the CSF of MS Patients**

To verify whether the findings obtained in the mouse model can be extended to humans, we collected CSF from 2 independent cohorts of healthy donors, patients with CIS, patients with definite PPMS or RRMS, the latter during a stable phase of the disease (stable RRMS) or during an acute attack (acute RRMS), and patients from other neurologic diseases. Myeloid MVs were significantly increased in the CSF from both CIS and relapsing RRMS patients (Fig 5). Based on receiver operator characteristic curves, we set the cutoff value at 1.6 MVs/ $\mu$ l, obtaining a sensitivity of 85% and specificity of 100% in distinguishing CIS patients from healthy



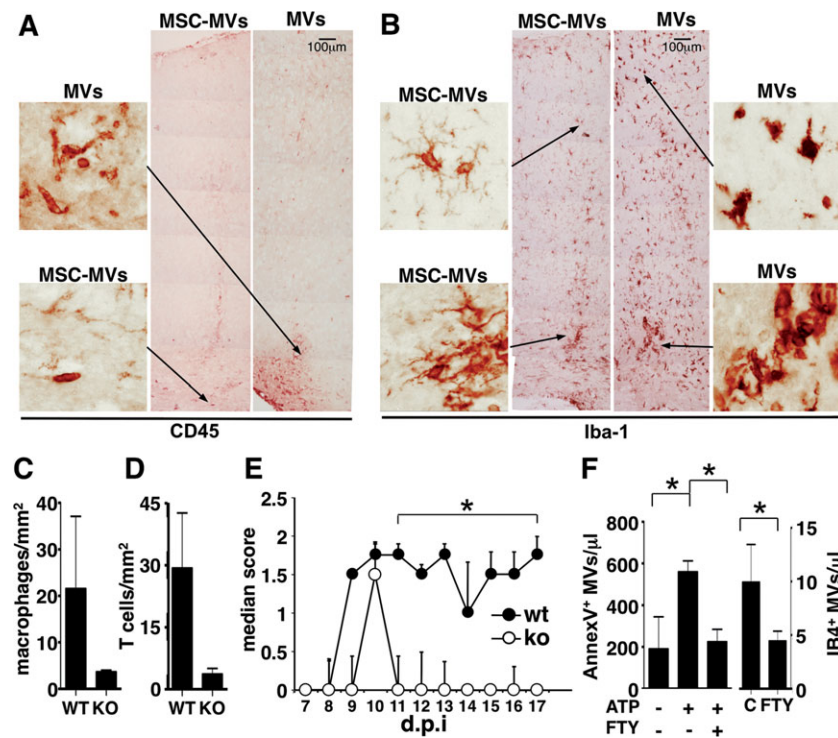


**FIGURE 3:** Microvesicles (MVs) shed from reactive microglia deliver a proinflammatory signal. (A) Fluorescent staining for the activation marker glial fibrillary acidic protein (GFAP) of naive cortical astrocytes and astrocytes exposed to MVs shed from lipopolysaccharide (LPS)-treated microglia (2µg/ml; microglia to astrocyte ratio, 2:1). (B) Western blotting for GFAP of control (ctr) astrocytes and astrocytes activated by MVs as described above. The endoplasmic reticulum protein calnexin (calnx) was used as loading control. (C) 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. Data represent mean values from 2 independent experiments and are normalized to control (number of recorded cells: control, n = 144; LPS-MVs, n = 41; Th1-MVs, n = 139). (D) Quantitative real time polymerase chain reaction (PCR) for interleukin (IL)6, IL1β, and inducible nitric oxide synthase (iNOS) in astrocytes exposed to increasing concentration of MVs derived from LPS-pre-activated microglia. Data are representative of 2 separate experiments. (E) Real time PCR for cyclooxygenase-2 (COX-2), IL6, IL1β, iNOS, IL10, and transforming growth factor (TGF)-β in control astrocytes, and cells activated overnight with LPS or Th1 cytokines or exposed for 48 hours to MVs produced by resting or LPS-treated microglia (microglia to astrocyte ratio, 2:1). (F, G) Real time PCR for inflammatory genes and for the proregenerative marker CD206 and IL10 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, are shown in F, whereas real time quantitative evaluation is shown in G. (H) Data are representative of 3 separate experiments. Fluorescent images reveal 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. (I) 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. \*p < 0.05, \*\*p < 0.01.

controls, and a sensitivity of 82% and specificity of 82% in differentiating stable from relapsing MS patients. Thus, MVs significantly increase when inflammatory processes, as reflected also by magnetic resonance imaging findings, predominate. A linear correlation was found between levels of CSF MVs and gadolinium-positive

lesions in MS patients ( $R^2 = 0.972$ ), but not with other clinical parameters (Supplementary Table). These data indicate CSF MVs as a novel exploratory biomarker of microglia/macrophage activation.

Interestingly, FTY720, a drug recently approved for the treatment of MS, and known to cross the blood–



**FIGURE 4: Microglia/macrophage-derived microvesicles (MVs) amplify brain inflammation.** (A, B) Coronal sections of the brain of experimental autoimmune encephalomyelitis (EAE) mice injected in the corpus callosum with microglia-derived MVs, or MVs produced from mesenchymal stem cells (MSC-MVs). CD45 immunohistochemical staining (A) shows recruitment of peripheral cells at the site of MV delivery and along the needle track, whereas Iba-1 staining (B) reveals an increased number of myeloid amoeboid cells, clearly distinguishable from ramified microglia present in mice injected with control MSC-MVs. Sections shown are representative of 3 controls injected with MSC-MVs, and 10 mice injected with microglial MVs. (C, D) Quantification of IB4<sup>+</sup> macrophages (C) and CD3<sup>+</sup> T cells (D) in the spinal cord of EAE enzyme acid sphingomyelinase (A-SMase) knockout (KO) mice and wild-type (WT) littermates. (E) Median clinical score of A-SMase KO mice and WT littermates affected by nonrelapsing EAE (n = 5 mice per group). (F) Quantitative flow cytometry analysis of annexin-V<sup>+</sup> MVs shed upon 1mM adenosine triphosphate (ATP) into the supernatants from control (C) primary microglia or microglia pretreated with FTY720 (FTY; 10µM) for 30 minutes. Note that FTY720 prevented ATP-induced MV shedding (left side; Mann-Whitney). Flow cytometry quantification of IB4<sup>+</sup> MVs in the cerebrospinal fluid collected at 30 days postinjection (dpi) from nonrelapsing EAE mice, treated daily with FTY720 (1mg/kg) via oral gavage or control saline. The histogram shows a significant reduction in the concentration of MVs in EAE mice treated with the oral drug (right side; Mann-Whitney). \*p < 0.05.

brain barrier, has been identified as a specific inhibitor of A-SMase,<sup>39</sup> the enzyme that controls MV production. This finding opens the possibility that inhibition of MV shedding from reactive microglia or infiltrating macrophages by FTY720 may, at least in part, contribute to its direct CNS therapeutic action.<sup>40</sup> Consistent with this hypothesis, FTY720 completely abolished the ATP-induced release of shed MVs from primary mouse microglia (see Fig 4F) without impairing P2X<sub>7</sub> receptor activation (not shown). Furthermore, clinical amelioration of EAE mice upon treatment with FTY720 (not shown) was associated with decrease of MV to baseline levels in the CSF (see Fig 4F).

## Discussion

Microglia are myeloid cells of hematopoietic origin, resident in the brain, which show a resting phenotype in

normal CNS and become promptly activated in response to brain alterations. Resident microglia are traditionally distinguished from inflammatory peripheral macrophages, which derive from a common myeloid progenitor, but migrate to the brain only upon CNS damage or inflammation.<sup>41</sup> Previous studies indicated that microglia and other myeloid cells in vitro can shed MVs, which store and release the proinflammatory cytokine IL1β, together with inflammasome components<sup>16,42,43</sup> and MHCII protein, a central player in the adaptive immune response.<sup>28</sup> 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. In addition, our recent results indicated that microglia-derived MVs interact with the plasma membrane of neurons and enhance excitatory transmission,<sup>38</sup> possibly contributing to the excessive potentiation of neurotransmission, which occurs in

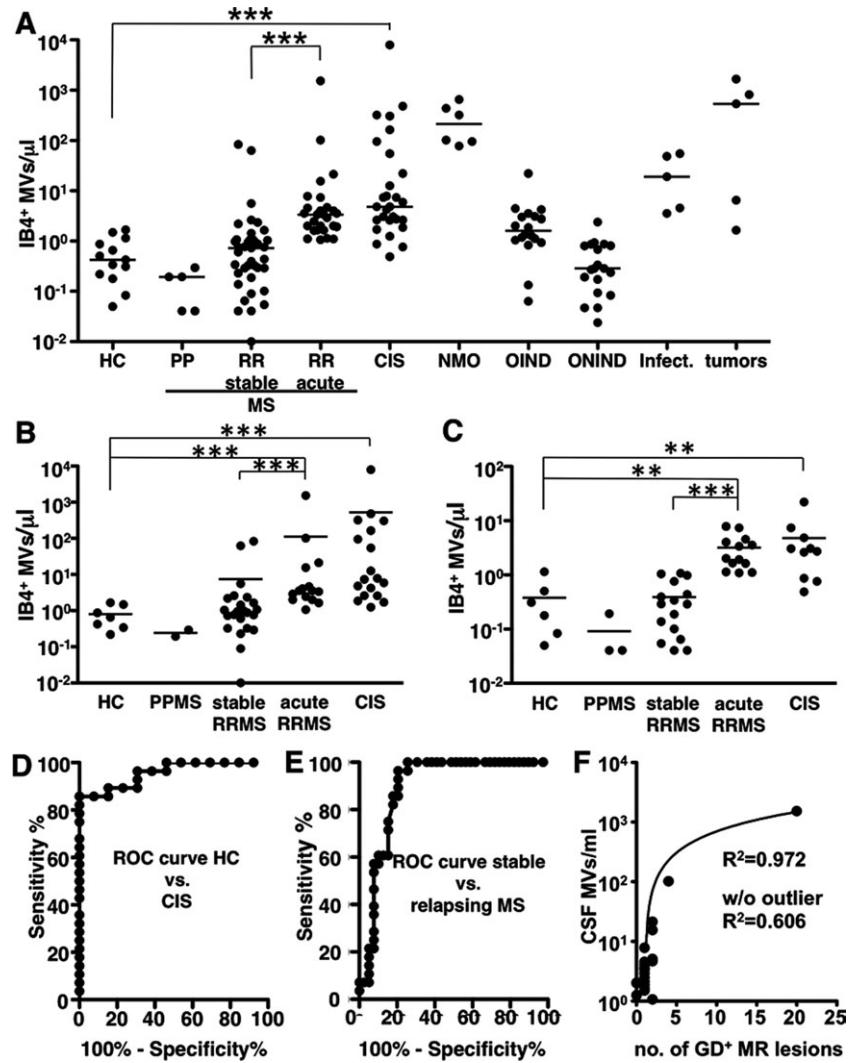


FIGURE 5: Myeloid microvesicles (MVs) are a novel biomarker of neuroinflammation. (A) Quantitative flow cytometry analysis of IB4<sup>+</sup> MVs in all human cerebrospinal fluid (CSF) collected from clinically isolated syndrome (CIS) patients (n = 28), relapsing–remitting multiple sclerosis (MS) patients in a clinically and neuroradiologically stable (stable RR; n = 39) or acute (relapsing MS; n = 28) phase of the disease, primary progressive MS patients (PP; n = 5), patients with neuromyelitis optica (NMO; n = 6), patients with other inflammatory (OIND; n = 18) or noninflammatory neurological disorders (ONIND; n = 18), patients with brain infections (Infect.; n = 5) or tumors (n = 5), and age- and sex-matched controls (HC; n = 13; 2-tailed Mann–Whitney). (B, C) Separate flow cytometry data from the first cohort (A) and the validation cohort (B) of healthy controls, CIS patients and MS patients shown in A. (D, E) Receiver operator characteristic (ROC) curves were used to evaluate the performance of CSF MVs in discriminating among healthy controls and CIS patients (D), or between stable and active MS (E) and determine the ideal cutoff value. (F) Linear regression of CSF MVs and numbers of gadolinium enhancing (GD<sup>+</sup>) lesions observed by magnetic resonance (MR) in 22 MS patients. \*\*p < 0.01, \*\*\*p < 0.0001.

neuroinflammatory diseases.<sup>44</sup> However, whether MVs exist and play a role in vivo is still elusive. The 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 that 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 suggests that MVs originate from resident microglia in the normal brain. However, MVs may also originate from the low number of macrophages that are present at choroid plexus and within leptomeninges in the uninjured brain. The capability of MVs to travel away from parenchymal 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 liquor space. Neurons and astrocytes have been described to

release MVs *in vitro*.<sup>5,45,46</sup> 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/macrophage 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,<sup>30,31</sup> represents the more plausible source of increased amounts of myeloid MVs during neuroinflammation. Consistent with this possibility, myeloid MVs were detected in plexus from mice injected with inflammatory cytokines, and MV shedding could be induced in EGFP-myeloid cells of CX3CR1 transgenic plexus under ATP stimulation (Supplementary Fig 8). Because small volumes of CSF collected from single mice do not allow the use of beads for exosome capture,<sup>10,47</sup> exosomes cannot be accurately analyzed by flow cytometry. We could not therefore distinguish between the 2 populations of extracellular membrane vesicles of myeloid origin. However, the majority of events above the detection limit of the flow cytometer most likely represent 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 exploratory biomarkers of microglia/macrophage activation *in vivo*. In MS, the most common neuroinflammatory disease, CSF MVs may be useful as a companion tool to monitor disease activity and the efficacy of drugs, or to identify very active patients likely to need a prompt shift to second-line treatments or CIS patients needing early treatment. Microglia activation, however, is also associated with several other CNS diseases (eg, neuromyelitis optica, brain tumors) that in fact display increased CSF MVs. Thus, CSF MV monitoring may provide valuable information in several different neurological disorders.

Microglia are considered to be at the borderline between inflammation and neurodegeneration, with both detrimental and protective roles.<sup>41,48</sup> The evidence that different methods of activation result in different functional phenotypes of myeloid cells, ranging from purely

phagocytic and tissue destructive to immunomodulating and promoting tissue remodeling,<sup>49</sup> 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.<sup>50</sup> 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 neuroinflammation. The proinflammatory activity of MVs was demonstrated *in vitro* by the dose-dependent induction of inflammatory markers in recipient glial cells. The glial reaction was associated with interaction with MVs and with MV-mediated transfer of IL1 $\beta$  mRNA. Accordingly, our unpublished observations suggest that MVs depleted of their content greatly lose their capacity to activate astrocytes, thus playing against the possibility that surface lipids of MVs may be responsible for inflammatory activity, as previously suggested.<sup>51</sup> 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 proinflammatory 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,<sup>21,25</sup> an inherited disease characterized by progressive visceral organ abnormalities and neurodegeneration, which leads to growth retardation and death in early childhood. A-SMase KO mice appear normal at birth and develop normally until about 8 to 10 weeks of age, when ataxia and mild tremors become noticeable, as a result of Purkinje cell neurodegeneration.<sup>25</sup> Of note, despite growth defects, incipient neurodegeneration, and slight gliosis in the gray and white matter,<sup>25</sup> we found that 8-week-old EAE KO animals weighed about 20% more than EAE WT littermates. Moreover, EAE KO mice displayed no demyelinating lesions and less perivascular infiltrates in the spinal cords as compared with WT littermates, thus strongly linking A-SMase deficiency and

impairment in MV shedding to EAE amelioration. We cannot exclude, however, that other changes in the CNS and immune system of A-SMase KO animals may contribute to the reduced inflammatory response.

The possibility that MV shedding may represent a contributor to EAE pathogenesis is in agreement with the amelioration of EAE severity in mice treated with antagonists selective for the ATP receptor P2X<sub>7</sub><sup>52</sup> and with the reduced incidence of the disease in P2X<sub>7</sub> KO mice, where P2X<sub>7</sub>-induced MV shedding is necessarily impaired.<sup>53</sup>

The pathogenic contribution of MVs to the disease is finally suggested by our observation that FTY720, a recently approved oral drug for MS<sup>54</sup> that protects mice from EAE,<sup>55</sup> strongly inhibits MV shedding from myeloid cells both in vitro and in EAE mice, possibly blocking A-SMase activity.<sup>39</sup> Although reduction of CSF MVs detected in FTY720-treated EAE mice may reflect multiple therapeutic actions, exerted by FTY720 both inside the CNS and in the periphery,<sup>40</sup> the possibility arises that the therapeutic efficacy of the drug may at least in part derive from reduced MV production and limited propagation of MV-mediated inflammatory signal within brain parenchyma. Given that 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.

### Conclusions

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

### Acknowledgments

This study was supported in part by the Italian Ministry of Health 2008XFMEA3 and FISM 2010/R/39 to C.V., by CARIPLO 2008 3184 to M.M., and by INSPE to R.F. The research leading to these results has also received funding from the European Union Seventh Framework Program under grant agreement HEALTH-F2-2009-241498.

We thank C. Perotta, E. Clementi, and S. Pluchino for critical discussion; P. Giussani, E. Tonoli, A. Palini, A. Finardi, and G. Fossati for technical assistance; L. Bellocchio and M. Baronio for providing some CSF samples of healthy controls; and F. Di Virgilio for cDNA coding for P2X<sub>7</sub>-GFP.

### Authorship

C.V. and R.F. designed the studies, shared the supervision of the work, analyzed data, and wrote the manuscript. L.M.

performed injections of lentiviruses and MVs and helped with data interpretation. E.T. and L.R. performed MV isolations, spectrometric assays, and analysis of glial response to MVs, and helped with EAE experiments. A.B. performed flow cytometry assays with the help of L.G. L.N. characterized CSF MVs and cultured apoptotic bodies. F.R. collected CSF from mice, and performed EAE experiments and pathological analysis. I.C. collected rat CSF. M.F. helped with EM microscopy. C.M. helped with lentiviruses experiments. F.S. carried out flow cytometry in some experiments. A.V. provided A-SMase KO mice. M.R., D.D.L., V.M., and G.C. provided human CSF samples. D.D.L. collected clinical data and performed data analysis. G.M. and M.M. discussed the hypothesis and helped with data interpretation.

### Potential Conflicts of Interest

C.V., M.M. and R.F. have deposited a patent application (PCT111346) entitled "Increase of myeloid microvesicles in the cerebrospinal fluid as biomarker of microglia/macrophage activation in neurological disorders." L.M., G.M., G.C., and R.F. have received consulting fees, travel support, or received funding for speaking from several pharmaceutical companies, including FTY720 license holder Novartis, with commercial interest in the field of multiple sclerosis and neuroinflammation, but none has directly or indirectly supported this research. M.R.: consulting, Bayer Schering Pharma; travel expenses, Biogen Dompè; speaking fees, Biogen Dompè. V.M. consultancy Sanofi Aventis, Merck Serono; speaking fees, Genzyme Europe; travel expenses, Sanofi Aventis, Bayer Schering, Teva Pharmaceuticals, Merck Serono, Biogen Dompè, Novartis. G.C.: consultancy, TEVA Pharmaceutical, Merck Serono, Biogen, Novartis, Bayer, Serono Symposia International Foundation, Sanofi-Aventis, Actelion; speaking fees, TEVA Pharmaceutical, Merck Serono, Biogen, Novartis, Bayer, Serono Symposia International Foundation, Sanofi-Aventis. R.F.: grants/grants pending, Merck-Serono; speaking fees, Teva, Biogen Dompè, Merck-Serono.

### References

1. Al-Nedawi K, Meehan B, Rak J. Microvesicles: messengers and mediators of tumor progression. *Cell Cycle* 2009;8:2014–2018.
2. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009;19:43–51.
3. Ratajczak J, Wysoczynski M, Hayek F, et al. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 2006;20:1487–1495.
4. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009;9:581–593.
5. Bianco F, Perotta C, Novellino L, et al. Acid sphingomyelinase activity triggers microparticle release from glial cells. *EMBO J* 2009;28:1043–1054.

6. Bernimoulin M, Waters EK, Foy M, et al. Differential stimulation of monocytic cells results in distinct populations of microparticles. *J Thromb Haemost* 2009;7:1019–1028.
7. van der Vos KE, Balaj L, Skog J, Breakefield XO. Brain tumor microvesicles: insights into intercellular communication in the nervous system. *Cell Mol Neurobiol* 2011;31:949–959.
8. Doeuvre L, Plawinski L, Toti F, Angles-Cano E. Cell-derived microparticles: a new challenge in neuroscience. *J Neurochem* 2009;110:457–468.
9. Emmanouilidou E, Melachroinou K, Roumeliotis T, et al. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J Neurosci* 2010;30:6838–6851.
10. Gyorgy B, Szabo TG, Pasztoi M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;68:2667–2688.
11. Bakhti M, Winter C, Simons M. Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. *J Biol Chem* 2011;286:787–796.
12. Court FA, Midha R, Cisterna BA, et al. Morphological evidence for a transport of ribosomes from Schwann cells to regenerating axons. *Glia* 2011;59:1529–1539.
13. Ardoin SP, Pisetsky DS. The role of cell death in the pathogenesis of autoimmune disease: HMGB1 and microparticles as intercellular mediators of inflammation. *Mod Rheumatol* 2008;18:319–326.
14. Vella LJ, Sharples RA, Lawson VA, et al. Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J Pathol* 2007;211:582–590.
15. Al-Nedawi K, Meehan B, Micallef J, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol* 2008;10:619–624.
16. Bianco F, Pravettoni E, Colombo A, et al. Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. *J Immunol* 2005;174:7268–7277.
17. Graeber MB, Li W, Rodriguez M. Role of microglia in CNS inflammation. *FEBS Lett* 2011;585:3798–3805.
18. Scolding NJ, Morgan BP, Houston WA, et al. Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement. *Nature* 1989;339:620–622.
19. Ponomarev ED, Veremeyko T, Barteneva N, et al. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Nat Med* 2011;17:64–70.
20. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011;69:292–302.
21. Horinouchi K, Erlich S, Perl DP, et al. Acid sphingomyelinase deficient mice: a model of types A and B Niemann-Pick disease. *Nat Genet* 1995;10:288–293.
22. Jung S, Aliberti J, Graemmel P, et al. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol* 2000;20:4106–4114.
23. Ayoub AE, Salm AK. Increased morphological diversity of microglia in the activated hypothalamic supraoptic nucleus. *J Neurosci* 2003;23:7759–7766.
24. Muzio L, Cavasinni F, Marinaro C, et al. Cxcl10 enhances blood cells migration in the sub-ventricular zone of mice affected by experimental autoimmune encephalomyelitis. *Mol Cell Neurosci* 2010;43:268–280.
25. Otterbach B, Stoffel W. Acid sphingomyelinase-deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann-Pick disease). *Cell* 1995;81:1053–1061.
26. Furlan R, Cuomo C, Martino G. Animal models of multiple sclerosis. *Methods Mol Biol* 2009;549:157–173.
27. Prinz M, Mildner A. Microglia in the CNS: immigrants from another world. *Glia* 2011;59:177–187.
28. Qu Y, Ramachandra L, Mohr S, et al. P2X7 receptor-stimulated secretion of MHC class II-containing exosomes requires the ASC/NLRP3 inflammasome but is independent of caspase-1. *J Immunol* 2009;182:5052–5062.
29. Sarkar A, Mitra S, Mehta S, et al. Monocyte derived microvesicles deliver a cell death message via encapsulated caspase-1. *PLoS One* 2009;4:e7140.
30. Engelhardt B, Wolburg-Buchholz K, Wolburg H. Involvement of the choroid plexus in central nervous system inflammation. *Microsc Res Tech* 2001;52:112–129.
31. Kivisakk P, Imitola J, Rasmussen S, et al. Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. *Ann Neurol* 2009;65:457–469.
32. Hemmer B, Archelos JJ, Hartung HP. New concepts in the immunopathogenesis of multiple sclerosis. *Nat Rev Neurosci* 2002;3:291–301.
33. Colton CA, Mott RT, Sharpe H, et al. Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. *J Neuroinflammation* 2006;3:27.
34. Mantovani A, Sica A, Sozzani S, et al. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004;25:677–686.
35. Michelucci A, Heurtaux T, Grandbarbe L, et al. Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: effects of oligomeric and fibrillar amyloid-beta. *J Neuroimmunol* 2009;210:3–12.
36. Pluchino S, Muzio L, Imitola J, et al. Persistent inflammation alters the function of the endogenous brain stem cell compartment. *Brain* 2008;131:2564–2578.
37. Politi LS, Bacigaluppi M, Brambilla E, et al. Magnetic-resonance-based tracking and quantification of intravenously injected neural stem cell accumulation in the brains of mice with experimental multiple sclerosis. *Stem Cells* 2007;25:2583–2592.
38. Antonucci F, Turolo E, Riganti L, et al. Microvesicles released from microglia stimulate synaptic activity via enhanced sphingolipid metabolism. *EMBO J* 2012;31:1231–1240.
39. Dawson G, Qin J. Gileya (FTY720) inhibits acid sphingomyelinase by a mechanism similar to tricyclic antidepressants. *Biochem Biophys Res Commun* 2011;404:321–323.
40. Cohen JA, Chun J. Mechanisms of fingolimod's efficacy and adverse effects in multiple sclerosis. *Ann Neurol* 2011;69:759–777.
41. Ransohoff RM, Cardona AE. The myeloid cells of the central nervous system parenchyma. *Nature* 2010;468:253–262.
42. MacKenzie A, Wilson HL, Kiss-Toth E, et al. Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* 2001;15:825–835.
43. Pizzirani C, Ferrari D, Chiozzi P, et al. Stimulation of P2 receptors causes release of IL-1beta-loaded microvesicles from human dendritic cells. *Blood* 2007;109:3856–3864.
44. Centonze D, Muzio L, Rossi S, et al. Inflammation triggers synaptic alteration and degeneration in experimental autoimmune encephalomyelitis. *J Neurosci* 2009;29:3442–3452.
45. Faure J, Lachenal G, Court M, et al. Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 2006;31:642–648.
46. Taylor AR, Robinson MB, Gifondorwa DJ, et al. Regulation of heat shock protein 70 release in astrocytes: role of signaling kinases. *Dev Neurobiol* 2007;67:1815–1829.
47. Ostrowski M, Carmo NB, Krumeich S, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* 2010;12:19–30; sup pp 11–13.

48. Schwartz M, Shechter R. Systemic inflammatory cells fight off neurodegenerative disease. *Nature reviews. Neurology* 2010;6:405–410.
49. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* 2010;11:889–896.
50. Centonze D, Muzio L, Rossi S, et al. The link between inflammation, synaptic transmission and neurodegeneration in multiple sclerosis. *Cell Death Differ* 2009;17:1083–1091.
51. Thomas LM, Salter RD. Activation of macrophages by P2X7-induced microvesicles from myeloid cells is mediated by phospholipids and is partially dependent on TLR4. *J Immunol* 2010;185:3740–3749.
52. Matute C, Torre I, Perez-Cerda F, et al. P2X(7) receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. *J Neurosci* 2007;27:9525–9533.
53. Sharp AJ, Polak PE, Simonini V, et al. P2x7 deficiency suppresses development of experimental autoimmune encephalomyelitis. *J Neuroinflammation* 2008;5:33.
54. Kappos L, Antel J, Comi G, et al. Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* 2006;355:1124–1140.
55. Fujino M, Funeshima N, Kitazawa Y, et al. Amelioration of experimental autoimmune encephalomyelitis in Lewis rats by FTY720 treatment. *J Pharmacol Exp Ther* 2003;305:70–77.