

# **Exosomes: Mediators of Pregnancy-associated Immune Modulation and Neuroprotection in a Model of Multiple Sclerosis**

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## **Introduction**

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterized by inflammation, primarily mediated by CD4<sup>+</sup> T cells, leading to damage and eventual destruction of the cells that are critical to normal sensory and motor function (1). Currently, MS is one of the most common chronic, disabling neurologic disorders and is the primary cause of non-traumatic disability in young adults (2, 3). MS disease course is often heterogeneous among patients; nearly two thirds suffer from relapsing-remitting MS in which patients experience intermittent periods of disease exacerbation, often over a period of hours to days, followed by remission. During an MS relapse, inflammatory lesions form within the CNS and consist of several types of immune cells, including T cells (4). MS disease relapses are markedly reduced during pregnancy, with the greatest suppression in disease activity observed during the third trimester. This period of pregnancy-associated disease suppression is followed by an abrupt return of relapses post partum (5). This suggests that disease suppression is mediated by a factor specific to the gestation period. Exosomes are small vesicles secreted by cells that function as facilitators of cell-to-cell communication and, are more abundant in the serum during pregnancy (6). Further, exosomes from the serum of pregnant mice have been shown to inhibit inflammatory processes of T cells (7). Exosomes are well poised to orchestrate MS recovery during pregnancy, as they are able to modulate both inflammatory immune cells, as well as targeted cells in the CNS (8). Therefore, the goal of this study is to elucidate the role of serum exosomes in pregnancy-associated suppression of MS.

## Materials and Methods

*Mice.* Age-matched male and female C57Bl/6 mice were purchased from The Jackson Laboratory. Myelin oligodendrocyte glycoprotein peptide (MOG<sub>35-55</sub>) T cell receptor transgenic mice on a C57Bl/6 background (2D2) were a kind gift from Dr. Vijay Kuchroo (Harvard Institute of Medicine, Boston, MA). Male mice were introduced to females for 3 days for the induction of pregnancy. Animals used in experiments were 6-10 weeks of age. Mice were maintained on a 12-hour light/dark cycle and given food and water *ad libitum*. All mice were housed in specific pathogen-free conditions in individually ventilated cages and cared for according to The Ohio State University IACUC approved protocols.

*Experimental autoimmune encephalomyelitis (EAE) immunization.* C57Bl/6 mice were immunized subcutaneously with 200 µg of MOG<sub>35-55</sub> emulsified in adjuvant containing 200 µg heat-killed *Mycobacterium tuberculosis*, Jamaica strain. Pertussis toxin (List Biological Laboratories) was injected on the day of immunization and 2 days post immunization (dpi). Mice were monitored daily for clinical signs of disease and were scored as follows: 0, no observable signs; 1, limp tail; 2, limp tail and ataxia; 3, paralysis of one hind limb; 4, complete hind limb paralysis; and 5, death.

*Exosome isolation and quantification.* Exosomes were isolated from the serum of virgin (control) and pregnant female mice using differential centrifugation as previously described (9). To remove excess serum proteins, the exosomes were washed for 1 hour. A bicinchoninic acid assay (Thermo Scientific) was used for exosomal protein quantification.

*Differential (2D) gel electrophoresis (DIGE) and mass spectrometry.* Serum exosomes were isolated from control and late pregnant mice and exosomal protein was extracted. Protein samples were labeled with cy2 (internal standard), cy3 and cy5 (GE Healthcare). Proteins were

then separated based on isoelectric point and molecular weight. Gels for DIGE analysis were rinsed and scanned in a Typhoon 9400 scanner (GE Healthcare). Analysis of the gel images was performed using the Decyder image analysis software (GE Healthcare). After differing spots were identified between control and pregnant samples, the average ratio of spot volumes between groups were compared. Only spots that were significantly different ( $p < 0.01$ ) were subject to in-gel robot digestion. The Ettan Spot Handling Workstation was used to core protein spots of interest. Gels were digested and peptides were extracted. Capillary-liquid chromatography-nanospray tandem mass spectrometry was performed using a mass spectrometer.

*Proliferation analysis.* Spleen cells from female 2D2 mice were harvested and cultured in the presence of MOG<sub>35-55</sub> alone or in combination with control- or pregnant-derived serum exosomes for 48 hours, including an 18-hour pulse with [<sup>3</sup>H] thymidine. Cultures were harvested and analyzed by liquid scintillation using TopCount NXT (Perkin Elmer).

*Intracellular cytokine staining.* 2D2 spleen cells were collected and suspended in the presence of MOG<sub>35-55</sub> alone or in combination with control- or pregnant-derived serum exosomes for 48 hours. Cell surfaces were stained with anti-CD4 antibodies. After cells were permeabilized and fixed (BD Biosciences), intracellular interferon-gamma (IFN- $\gamma$ ) was stained using anti-IFN- $\gamma$  antibodies (BD Biosciences). All samples were analyzed by flow cytometry.

*Primary oligodendrocyte precursor cell (OPC) cultures.* Mouse pups were sacrificed on postnatal day 2 by rapid decapitation and relevant portions of the CNS were removed and the tissue was dissociated. Cells were cultured in poly-L-lysine-coated T-75 flasks and incubated for 8-10 days or until cells were confluent. Irrelevant cells were reduced in cultures by placing flasks on an orbital shaker for 2 hours and discarding the supernatant. Cultures were then allowed to equilibrate for 2 hours. After equilibration, flasks were placed on an orbital shaker at

250 rpm for 15 hours to detach OPCs. OPCs were plated onto poly-L-lysine-coated glass coverslips. To maintain OPCs, medium was changed to neurobasal (Invitrogen) media. After 48 hours, neurobasal media alone or media with control- or pregnant-derived exosomes was added for 72 hours. Cells were then fixed with 4% paraformaldehyde and stained appropriately.

*Bromodeoxyuridine (BrdU) administration.* BrdU (Roche Diagnostics) was dissolved in warm, sterile saline solution. Mice with EAE were injected daily from 15-21 dpi.

*Immunohistochemistry.* Following treatment with exosomes, OPC coverslips were double-labeled with anti-myelin basic protein (Aves Labs) and -A2B5 (Millipore) antibodies for 2 hours. Coverslips were then incubated with Alexa Fluor 488- and Alexa Fluor 546-conjugated antibodies (Molecular Probes) for 30 minutes, washed, and incubated with DAPI for 20 minutes. Coverslips were mounted onto slides using Immu-mount (Thermo Scientific). Cells of the oligodendrocyte lineage (A2B5/MBP<sup>+</sup>) were confirmed and quantified. The relative number of cells in each treatment (DAPI<sup>+</sup> nuclei) were examined using MCID image analysis software (Interfocus Imaging). The MCID software controlled the motorized X-Y stage of an Axioplan 2 imaging microscope (Zeiss) and 3% of each coverslip was counted.

Mice were perfused with 4% paraformaldehyde, and spinal cords were removed. Sequential longitudinal sections were cut and slide mounted. For BrdU staining, sections were rinsed and incubated in 2N HCL at 37°C for 30 minutes. Anti-NG2 (USBiological) and anti-BrdU (AbD Serotec) antibodies were applied for 20 hours. Sections were then incubated with Alexa Fluor 488- and Alexa Fluor 546-conjugated antibodies (Molecular Probes) for 1 hour and coverslips were applied using Immu-mount. Images were acquired using the Olympus FluoView™ FV1000 Spectral confocal microscope. A minimum of 5 lesions per animal were analyzed, lesion data for each animal were averaged, and analysis was performed on treatment means.

*Statistical analysis.* A Mann-Whitney *U* test was used to determine significant differences in mean clinical disease score. The spot volume ratio between samples following DIGE was subject to independent student's *t* tests with false discovery rate correction applied. A two-tailed student's *t* test was used to determine differences in flow cytometric and immunohistochemistry data. A paired *t* test for repeated measures was used to analyze proliferation data. All data are graphed as mean  $\pm$  SEM. GraphPad Prism software (GraphPad Software) was used to analyze data.

## **Results and Discussion**

Since pregnancy is able to suppress MS disease activity (5), we examined the effects of pregnancy in a murine model of MS, experimental autoimmune encephalomyelitis (EAE), which shares many histological and clinical characteristics with MS. To recapitulate the dynamic observed between MS and pregnancy, we induced EAE in mice and after the onset of disease, mated a portion of the females with males for pregnancy induction. Disease severity was significantly reduced during pregnancy compared to unmated female controls, with a post partum flare in disease course consistent with the observation in humans (Figure 1).

To determine whether serum exosomes alone, outside of the pregnancy environment, are able to suppress disease, we administered serum exosomes to mice with established EAE. Importantly, with a single treatment of exosomes, we observed reduced clinical severity of EAE compared to mice that were treated with a vehicle control (Figure 2).

Since MS has immunological and neurological components, we sought to determine how exosomes function in both of these facets. We focused first on the effects of exosomes on immune cells that are thought to have a critical role in disease, T helper type-1 cells (Th1). Th1 cells are inflammatory immune cells that are positive for the molecule CD4 and are known to

mediate MS and EAE progression through the release of soluble mediators like IFN- $\gamma$ . Using T cells that recognize a portion of the myelin sheath, myelin oligodendrocyte glycoprotein (MOG<sub>35-55</sub>), we observed a dose-dependent decrease in proliferation of Th1 cells following incubation with pregnancy-derived exosomes (Figure 3A), indicating these exosomes are able to suppress the activation of T cells. Using flow cytometry, a technique that allows the analysis of individual cells, we also demonstrated that pregnancy exosomes are able to suppress the inflammatory potential of Th1 cells. This was indicated by reduced expression of the Th1-specific inflammatory mediator, IFN- $\gamma$ , specifically in disease perpetuating CD4<sup>+</sup> T cells (Figure 3B). This observation was confirmed over several experiments after analyzing the compiled suppression of IFN- $\gamma$  in T cells following treatment with pregnancy-derived serum exosomes (Figure 3C). Taken together, these data suggest that serum exosomes specific to pregnancy are able to inhibit Th1-mediated inflammatory processes that contribute to MS and EAE pathogenesis.

Next, we explored the effects of pregnancy-associated exosomes on the targeted cell type in MS and EAE, oligodendrocytes. Oligodendrocytes are derived from oligodendrocyte precursor cells (OPCs) and function within the CNS to produce the protective myelin sheath that insulates nerves for efficient nerve conduction. The production of IFN- $\gamma$  has been shown to directly destroy oligodendrocytes (10). The loss of this cell type results in motor and sensory function deficits commonly seen in MS patients. Additionally, the adult CNS is unable to initiate repair without the presence of OPCs (11). Importantly, when OPCs were incubated with pregnancy-derived serum exosomes, we observed a profound increase in maturation (Figure 4I-L) compared to when OPCs were vehicle-treated (Figure 4A-D). This is evidenced by the up-regulation of MBP, a marker of OPC maturity (Figure 4C, G, K). The change in cell distribution, from

spacious to clustered, with exosome treatment (Figure 4A, E, I) led us to determine if exosomes were increasing the proliferation of OPCs. We observed an increase in cell number, quantified by DAPI staining, when OPCs were treated with exosomes (Figure 4M). These data suggest that serum exosomes enhance the maturation and proliferation of OPCs *in vitro*, poising them for reparative processes.

To examine the effects of exosomes on proliferation of OPCs *in vivo*, we immunized mice for EAE and treated them with serum exosomes from control and pregnant mice 15 dpi. Following the administration of exosomes, BrdU was injected daily for 6 days to label actively dividing cells. The spinal cords of vehicle- and exosome-treated mice were then removed and processed for immunohistochemistry to label proliferating (BrdU<sup>+</sup>) OPCs (NG2<sup>+</sup>) (Figure 5A-C).

Interestingly, mice with EAE that were treated with pregnancy-derived serum exosomes had an increase in the number of proliferating OPCs within CNS lesions compared to control-treated mice (Figure 5D). These data indicate that pregnancy exosomes promote reparative processes within the CNS in areas of damage.

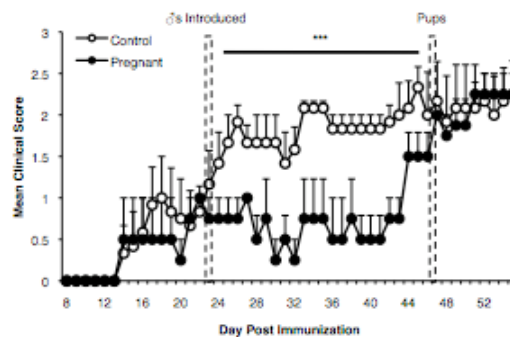
Exosomes can contain a variety of proteins depending on the cell from which they were derived. To identify proteins that may be responsible for mediating the effects of serum-derived pregnancy exosomes as compared to control exosomes, we performed differential gel electrophoresis followed by mass spectrometry, techniques used to identify specific proteins that are differentially expressed between treatments. Proteins enriched in pregnancy exosomes include corticosteroid binding globulin, which facilitates the local action of anti-inflammatory molecules; ceruloplasmin, a scavenger of tissue damaging oxygen-derived free radicals; alpha-2 macroglobulin, an inhibitor of enzymes required for entry of immune cells into the CNS; and

leukemia inhibitory factor receptor, which provides survival and growth signals to oligodendrocytes (Table I).

## Conclusions and Implications

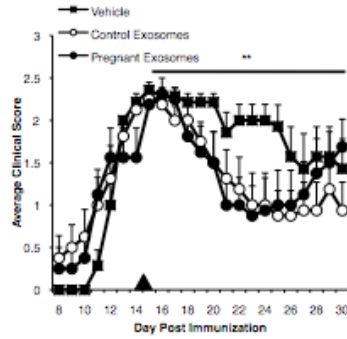
Taken together, these data suggest that pregnancy-derived serum exosomes mediate pregnancy-associated suppression of EAE and MS via inhibition of inflammatory processes and neuroprotection. Harnessing the mechanism by which pregnancy exosomes suppress immunity, enhance the function of OPCs, and consequently suppress clinical EAE, can have extraordinary implications for therapy development in MS.

## Figures

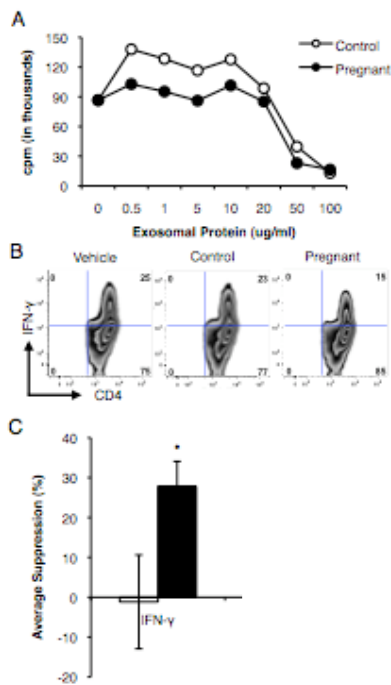


**Figure 1:** Pregnancy suppresses the severity of established EAE. Mice were immunized for EAE and female mice in the pregnant group were mated for 3 d, 22-27 dpi, with immunized, strain-matched virgin female mice serving as controls. The gestation period is represented as the time between the hatched bars, when males were introduced until pups were delivered. Clinical signs of EAE were monitored ( $*** p < 0.0001$ ).

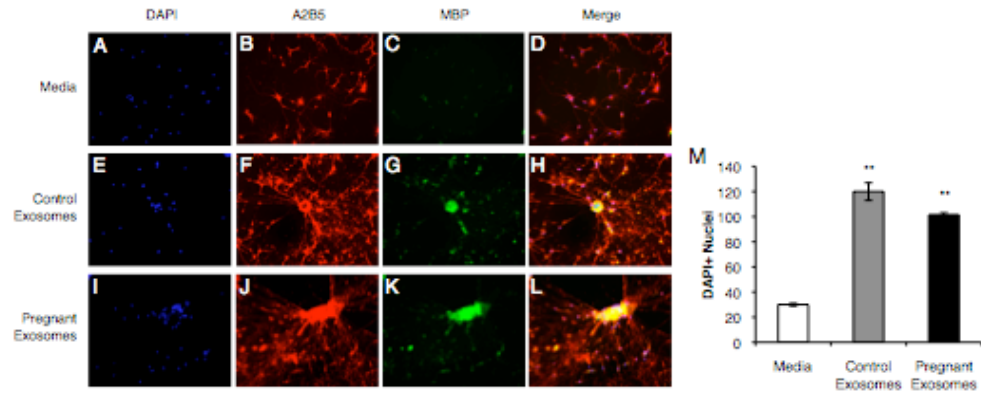




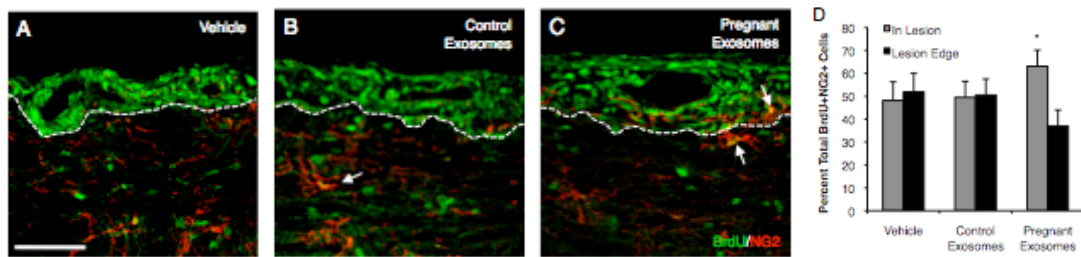
**Figure 2:** Exosome treatment is therapeutic in established EAE. Serum exosomes were isolated from control and late pregnant mice and either vehicle, control- or pregnant-derived exosomes were injected intravenously into female mice 15 dpi, indicated by the black arrow. Mice were monitored for signs of clinical EAE ( $p < 0.01$ , compared to vehicle-treated mice). Data are a combination 7-8 mice per treatment in 2 separate experiments.



**Figure 3:** Pregnancy-derived serum exosomes suppress T cell activation. MOG-specific spleen cells were cultured with MOG<sub>35-55</sub> in the presence of serum exosomes. (A) The proliferation of MOG-specific T cells was measured in the presence of increasing concentrations of exosomal protein. (B) Intracellular IFN- $\gamma$  expression in CD4<sup>+</sup> T cells was measured following culture with MOG<sub>35-55</sub> and vehicle or MOG<sub>35-55</sub> and exosomes. (C) Suppression of IFN- $\gamma$  in T cells by control (open bar) and pregnant (filled bar) exosomes was compiled and analyzed over 3 independent experiments ( $p < 0.05$ ).



**Figure 4:** Pregnancy exosomes augment the maturation and proliferation of OPCs. OPCs were cultured in (A-D) media alone or media supplemented with serum exosomes from (E-H) control or (I-L) pregnant mice and triple-labeled for DAPI (blue), myelin basic protein (MBP; green), and A2B5 (red) by immunofluorescence (A-L, 40x). (M) DAPI<sup>+</sup> nuclei were counted on 3% of each coverslip (86 random fields) (\*\*  $p < 0.01$ ).



**Figure 5:** The trafficking of proliferating OPCs into lesions is enhanced in mice with EAE treated with pregnancy-derived exosomes. Female mice were immunized for EAE and treated with either vehicle, control- or pregnancy-derived serum exosomes 15 dpi. Following treatment, mice were injected with BrdU daily for 6 days. Mice were then perfused and spinal cords were removed and processed for immunohistochemistry. Spinal cord sections from (A) vehicle-, (B) control exosome-, and (C) pregnancy exosome-treated mice were double-labeled for BrdU (green) and NG2 (red) by immunofluorescence, indicated by arrows (60x, scale bar, 50  $\mu$ m). (D) The proportion of total BrdU<sup>+</sup>NG2<sup>+</sup> cells was quantified within (above the hashed line) and

outside (below the hashed line) of lesions ( $p < 0.05$ ). Data are representative of 4 mice per treatment, 5 lesions per animal.

## Tables

**Table I:** Proteins are differentially expressed in control- versus pregnancy-derived exosomes.

Spot Number	Protein Name	Protein Accession (Mouse)	Fold Change (Pregnant/ Control)	<i>p</i> -value
595, 630, 634	Alpha-1B-Glycoprotein	A1BG	16.43	0.0002
765, 771, 811	Corticosteroid-binding Globulin	CBG	9.85	0.000005
330, 355, 369, 420	Ceruloplasmin	CERU	2.33	0.0001
1326	Serum Amyloid P-Component	SAMP	2.12	0.01
353, 362, 376, 429, 467	Leukemia Inhibitory Factor Receptor	LIFR	2.07	0.0006
615	Carboxypeptidase N Subunit 2	CPN2	-2.79	0.0009
1429, 1438, 1439, 1480	Apolipoprotein A-I	APOA1	-2.81	0.0006
327, 801, 821	Serine Protease Inhibitor A3K	SPA3K	-2.90	0.0001
181, 182, 321, 755, 764, 779	Murinoglobulin-1	MUG1	-3.03	0.0003
1478, 1480	Transthyretin	TTHY	-4.84	0.000001

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