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Induction of pregnancy during established EAE halts progression of CNS autoimmune injury via pregnancy-specific serum factors

NaTosha N Gatson, MD PhD[*], **Jessica L Williams**[*], **Nicole D Powell, PhD**[*], **Melanie A McClain, PhD**[*], **Teresa R Hennon, MD**[†], **Paul D Robbins, MD**[†], and **Caroline C Whitacre, PhD**[*]

[*]Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, 760 Biomedical Research Tower, 460 W. 12th Avenue, Columbus, Ohio 43210-1239

[†]Department of Microbiology and Molecular Genetics, University of Pittsburgh, 450 Technology Drive, Pittsburgh, Pennsylvania 15219

Abstract

Multiple sclerosis (MS) is a demyelinating disease of the CNS involving T cell targeting of myelin antigens. During pregnancy, women with MS experience decreased relapses followed by a postpartum disease flare. Using murine experimental autoimmune encephalomyelitis, we recapitulate pregnancy findings in both relapsing and progressive models. Pregnant mice produced less TNF-α, IL-17 and exhibited reduced CNS pathology relative to nonpregnant controls. Microparticles, called exosomes, shed into the blood during pregnancy were isolated and found to significantly suppress T cell activation relative to those from nonpregnant controls. These results demonstrate the immunosuppressive potential of pregnancy and serum-derived pregnancy exosomes.

Keywords

Multiple Sclerosis; Experimental Autoimmune Encephalomyelitis; Pregnancy; Exosomes; Autoimmunity

1. Introduction

Multiple sclerosis (MS)[3] is a chronic demyelinating disease of the CNS thought to involve an autoimmune response directed against myelin antigens (Wingerchuk et al., 2001). MS typically presents during the reproductive years and shows a female preponderance (3:1). Females typically demonstrate a relapsing-remitting (RRMS) clinical course (Whitacre et

[2]Address correspondence and reprint requests to Dr. Caroline C. Whitacre, Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, 208 Bricker Hall, 190 North Oval Mall, Columbus, OH 43210. whitacre.3@osu.edu.

[3]Abbreviations used in this paper: MS, multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; MOG, myelin oligodendrocyte glycoprotein; LNC, lymph node cell; CBA, cytometric bead array.

al., 1999), while males tend to have a more rapidly progressive form. Importantly, one of the most profound clinically suppressed periods in MS is observed during late pregnancy. This phenomenon has been observed in other immune-mediated disease states such as rheumatoid arthritis, and is thought to represent immune changes occurring to maintain the fetal allograft (Chaouat, 1987). Multiple studies have reported a general reduction in MS clinical disease activity during pregnancy (Birk et al., 1990,Bernardi et al., 1991,Davis and Maslow, 1992,Runmarker and Andersen, 1995,Damek and Shuster, 1997). Confavreux et *al.* (1998) conducted a series of large-scale human studies in women with RRMS who subsequently became pregnant. Results showed a pronounced decrease in the rate of relapse during late pregnancy followed by a postpartum flare in disease. Recently, animal models have been used to identify specific pregnancy factors (Langer-Gould et al., 2002,McClain et al., 2007).

Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model for MS, sharing clinical, histopathologic and immunologic similarities with the human disease (Paterson, 1998). EAE is inducible in susceptible rodent strains by immunization with myelin proteins or peptides such as myelin basic protein, proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). Susceptibility to disease induced by the various peptides is strain dependent and, as with human autoimmune disease, there is a demonstrable sex dimorphism noted within mouse stains (Encinas et al., 1996, Papenfuss et al., 2004). For example, SJL female, but not male mice immunized with the immunodominant epitope of PLP show a relapsing-remitting course of EAE that is similar to the clinical course observed in patients with RRMS. Conversely, C57Bl/6 males and females immunized with MOG peptides both exhibit a similar clinical course reminiscent of primary progressive MS (Papenfuss et al., 2004). Targeting the variability within these strains is useful to answer questions related to sex differences and disease progression in autoimmunity.

Several studies have examined the role of pregnancy factors such as hormones in mediating the immunosuppressive effects during EAE. Langer-Gould et *al.* (2002) reported a reduced incidence of EAE in SJL mice immunized for EAE during pregnancy and also demonstrated a decrease in clinical signs during pregnancy in SJL mice with pre-existing EAE raising the question of an early pregnancy factor contributing to the observed suppression. Offner et *al.* (2004) reported decreases in inflammatory infiltrates in the spinal cord in C57Bl/6 mice with EAE treated with estrogen derivatives. We recently reported the effects of pregnancy on the development and progression of EAE when induced during late pregnancy or the post-partum period (McClain et al., 2007). We observed a decreased incidence and severity of EAE in SJL mice that were in the mid or late stages of pregnancy at the time of immunization, and increased clinical severity when immunization was performed post partum.

This study focuses on the clinical, immunologic, and histopathologic changes that take place during late pregnancy in mice with established EAE and offers new insight into the role for serum based factors in treating established EAE. Understanding the suppressive potential of the pregnancy state could improve therapeutic modalities for managing MS and other immune-mediated diseases.

2. Materials and Methods

Mice

Age-matched female SJL/J and C57Bl/6 mice were purchased from The Jackson Laboratory. Mice were immunized for EAE at 6–10 wk of age. Mice were housed in the Ohio State University Laboratory Animal Facilities (ULAR) in accordance with approved

ULAR regulations, maintained on a 12-h light/dark cycle and given food and water *ad libitum*. Male SJL/J and C57Bl/6 mice, age 8–12 wk, were used for mating.

Antigens

The following peptide: PLP 139–151 (HCLGKWLGHPDKF) was purchased from Sigma-Genosys, and MOG 35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was purchased from Princeton Biomolecules. Peptides were purified by HPLC, purity >90%.

EAE immunization and scoring

SJL mice were immunized subcutaneously over four sites on the flank with 0.2 ml of an emulsion containing 150 μg of PLP 139–151 in PBS and an equal volume of CFA (containing 200 μg of heat-killed *Mycobacterium tuberculosis*, Jamaica strain). C57Bl/6 mice immunized with MOG peptide 35–55 also received Pertussis toxin (200 ng) (List Biological Laboratories) injected i.p. at the time of immunization and 48 h later. Mice were monitored daily for clinical signs of disease and were scored as follows: 0, no signs; 1, limp tail or mild ataxia; 2, complete ataxia; 3, paralysis of one hindlimb; 4, complete hindlimb paralysis, 5, moribund or death. Mice showing intermediate signs of disease were scored at increments of 0.5 between 0 – 4.

Pregnancy induction

Age-matched female SJL/J and C57Bl/6 mice were housed separately from strain matched males until mating. On d 8–10 post-EAE immunization (3–5 d prior to mating), ~ 1 tablespoon (3 g) soiled bedding from male cages was mixed into the female cages to familiarize the females with the male pheromones, increase receptivity and stimulate estrous (protocol adapted from Halem et *al.*, 2001). Male bedding was added twice weekly until late pregnancy (d 16–18). Virgin control females were likewise exposed to male bedding over the course of the study period but were never caged with males or mated for pregnancy. Female bedding was mixed into the male cage 2–3 h prior to introducing them into the female cages for mating (protocol adapted from Kelliher et al., 1999). Males were housed with females in a ratio of 1:3 for mating and were left paired in cages for 72 h. Pregnancy was confirmed by presence of a vaginal plug and serial weights (increase ≥25% over baseline) to ensure pregnancy maintenance.

Histopathology

Spinal cords were removed from SJL/J mice at varying times after pregnancy induction during ongoing EAE. Tissues were fixed in 10% phosphate buffered formalin and then dissected and embedded in paraffin. Three 2 mm sections were taken from the lumbar, thoracic and cervical portions of the cord and cut at 4–10 microns depending on staining technique used. The brain was sectioned at the frontal cortex, thalamus, and pons and stained similarly for comparison. Sections were then processed for H and E and luxol fast blue staining. For all H and E stained sections the degree of cellular infiltration was quantified by assigning scores based on the number of perivascular cuffs observed in the section as well as the number of cell layers (thickness of cells) surrounding each cuff. Sections were scored as follows: 0, absence of infiltrates; +, small, rare perivascular lesions; ++, small, numerous perivascular lesions; +++, numerous perivascular lesions and parenchymal infiltration; and ++++, severe, confluent lesions. Areas of pathology were scored in each of the 9 sections of the spinal cord and 6 sections of brain per mouse per group.

Reverse transcription PCR

Lymph node cells (LNC) were removed from EAE immunized SJL/J mice during late pregnancy, and from virgin and naïve pregnant only controls. Single cell suspensions were prepared and suspended in 4 mL of Trizol Reagent (Invitrogen) for 15 min at room temperature. RNA was isolated using RNeasy Kit (Qiagen) and reverse transcription was completed using SuperScript III reverse transcription reagents (Invitrogen). Primers were developed in our lab for short-range PCR experiments (Table I): 95°C − 60 s; and 30 cycles of 95°C − 30 s, 56°C −30 s, 72°C −30 s; final extension of 72°C − 4 min before cooling to 4°C. Negative agarose gel images were semi-quantitatively evaluated using NIH Scion imager scanning software.

ELISPOT analysis for cytokine-producing cells

Frequencies of cytokine-secreting cells were determined for IL-10 and IFN-γ (R&D Systems). Briefly, microtiter plates with nitrocellulose bottoms (Millipore) were coated overnight at 4°C with capture antibody. After washing, plates were blocked with 1% BSA (Sigma) for 2 h at room temperature. Splenocytes or LNCs were resuspended in HL-1 medium and then cultured in triplicate with medium alone or with the following stimulants: PLP 139–151 (30 μg/ml) or anti-CD3 (2 μg/ml). Cells were cultured according to assay protocol, plates were washed and cytokine-specific biotinylated antibodies were added. After overnight incubation, Streptavidin-AP was added to the plates for 2 h. After a final wash, plates were developed with BCIP/NBT chromogen. Image analysis of ELISPOT plates was performed using the KS ELISPOT system (Zeiss). Data are expressed as the mean number of cytokine-producing cells per million +/− SEM for all animals in a group.

Cytometric bead array (CBA)

IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were measured using the mouse Th1/Th2 and IFN-γ, TNF-α, IL-10, MCP-1 and IL-6 were assayed using the mouse inflammation cytokine CBA detection system (BD Biosciences) according to manufacturer's instructions. Standard curves were generated for each cytokine and the concentration of cytokine in the cell supernatant was determined by interpolation from the appropriate standard curve. All samples were analyzed by flow cytometry (FACS Calibur, BD Biosciences).

ELISA

OPT-EIA Sandwich ELISA kits were used to determine the levels of IL-12p40 (Pharmingen) and IL-17 (R&D Systems) in culture supernates as described above. The optical density was determined using the SpectraMax Plus^{384} high throughput microplate spectrophotometer and analyzed using SoftMax Pro software (Molecular Devices).

Exosome isolation, quantification, and Western blot

Blood was collected via retro-orbital eye bleed from late pregnant (16–18 d post conception) and virgin mice without EAE and allowed to clot at room temperature for 30 min. Exosomes were collected by differential centrifugation at 4°C: 4,000 × *g* for 5 min and 15,000 × *g* for 10 min to remove pelleted cells; then 14,000 × *g* for 20 min reserving the supernate. The serum supernatant was diluted 2× in PBS and ultracentrifugation was completed using a Beckman ultracentrifuge with a swinging bucket rotor (SW55 Ti) at 116,000 × *g* for 1.5 h at 4°C to pellet the exosomes. The supernatant was saved for an exosome-free sample and the pellet was washed with sterile PBS and resuspended in 100–200 ml PBS. A small aliquot was used for protein quantification using the Bradford assay (Biorad). Samples (10 μg) were separated on 5–20% gradient SDS–PAGE, transferred onto nitrocellulose, and mouse anti-heat shock congnate-70 (Abcam) was used for detection by Western blot using an enhanced

chemiluminescence detection kit (Amersham) as described (Kim et al., 2006). Aliquots were stored at −20°C avoiding multiple freeze-thaw cycles.

Exosome electron microscopy

After exosomes were purified by differential centrifugation, they were loaded on a Formvar/carbon-coated grid, stained with neutral 1% aqueous phosphotungstic acid, and viewed using a JEOL-1210 computer-controlled high-contrast 120-kV transmission electron microscope as described previously (Kim et al., 2006).

Charcoal inactivation of serum

Dextran coated charcoal was prepared using 3.3 g activated charcoal (protocol adapted from Welhua et al., 2001). Charcoal was washed in deionized water a total of three times with final resuspension in PBS. Dextran T70 (0.33 g) was dissolved in PBS, mixed with charcoal, and the mixture was centrifuged for 20 min at 750 × *g*. The supernatant was removed and 5 ml of serum was added and incubated at 37° C for 1 h. After centrifugation for 10 min at 750 × *g*, the serum supernatant was filtered prior to use in T cell proliferation assays.

Proliferation analysis

Peripheral lymph nodes (inguinal, axillary, brachial, cervical, popliteal and periaortic) and spleens were removed from mice 10 d post immunization. Single cell suspensions were prepared and suspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol in round-bottom 96-well plates (4×10^5 cells/well). Cells were cultured in medium alone or with PLP 139–151 (30 μg/ml), MOG 35–55 (10 μg/ml) or anti-CD3 (2 μg/ml). Cultures were incubated for 72 h at 37°C and 7% CO_2, including an 18 h pulse with [^{3}H] thymidine (1 μCi per well). For serum suppression assays, 3% pregnant or virgin serum was added for the duration of the culture period. Cultures were harvested onto glass-fiber filter mats using a Skatron harvester (Skatron) and were counted by liquid scintillation on a Wallac betaplate (LKB, Wallac, MD). Results are expressed as CPM ± SEM.

Statistical analysis

A two-tailed Student's *t* test was used to determine statistical differences when comparing two groups with parametric data as in the ELISA, ELISPOT and proliferation assays. χ^2 analysis was used for determining differences in disease incidence. Analyses of continuous data such as clinical scores were determined using nonparametric Wilcoxon Rank-Sum test with a $p < 0.05$ significance level. Sample size calculations were carried out using nQuery Advisor v. 3.0. Simulations using sample size estimates were performed using SAS v8.2.

3. Results

Pregnancy halts the progression of clinical disease in mice with established EAE

To determine the effects of pregnancy on established EAE, we immunized SJL mice with the immunodominant epitope of PLP (p139–151), and induced pregnancy after the acute phase of EAE. We reduced the stress of mating by familiarizing the females with male pheromones and introduced male bedding into the female cages prior to mating. This practice resulted in an 80–90% pregnancy induction rate. We observed suppression of disease throughout the later stages of pregnancy as compared to virgin controls. The third trimester of pregnancy, however, afforded a near full recovery from disease, and the entire pregnancy period was free from relapses in >95% of the pregnant females (Fig. 1). To evaluate the utility of exposing the females to male bedding prior to mating, we compared

EAE clinical signs with and without presensitization. We observed a more rapid remission, reduced clinical score, and lower rate of relapse in presensitized females as measured by lower cumulative disease index and mean peak score in this group (Table II). Spontaneous abortion events in mice previously confirmed as pregnant led to early relapse and these females were subsequently excluded from the study. As shown in Figure 2, disease activity in mice that carried the pregnancy to term increased dramatically within 3 d of parturition, resulting in profound morbidity and increased mortality. Disease was observed to return to the baseline pre-pregnancy score within 5–7 d post partum (Fig. 2).

Pregnancy reduces CNS demyelination and limits cell infiltration when induced during established EAE

To determine the effect of pregnancy on CNS pathology, we evaluated inflammatory cell infiltration and demyelination of the spinal cord during EAE at three time points: acute disease, late pregnancy, and the early post partum period. The clinical course which corresponds to the point of sacrifice for tissue harvesting is depicted in figure 2 (arrows). Mice were sacrificed 15–18 d post immunization (acute), 35–40 d post immunization (late pregnancy), and 48–51 d post immunization (post partum). Mean clinical score at the time of sacrifice was as follows: acute (3.4), late pregnancy (0.4), and post partum (2.7).

To evaluate mononuclear cell infiltration and perivascular cuffing in acute, late pregnant and post partum immunized mice, the dorsal and ventral funiculi of the spinal cord were analyzed by H and E. Fewer infiltrating cells and areas of perivascular cuffing were observed in late pregnancy as compared to acute and post partum samples (Fig. 2A–C). To evaluate demyelination, the lower cervical, mid-thoracic and lumbar spinal cord sections were stained by luxol fast blue. Here we observed significant demyelination in the dorsal (position sense) and ventral (voluntary motor movements) areas of the spinal cord during the acute phase of disease in virgin mice (Fig. 2D) followed by a reduction in demyelination in these areas during late pregnancy (Fig. 2E). There was an increase in demyelination ~10 d post partum in mice that previously exhibited fewer clinical signs of disease during late pregnancy (Fig. 2F).

Reduced secretion of IL-17 and TNF-α but not IFN-γ during late pregnancy

To determine the role of specific inflammatory mediators such as IL-17, TNF-α, IFN-γ and IL-2 in the initiation and progression of EAE during the pregnancy period, changes in the levels of these cytokines were measured. Lymph node cells were isolated from both virgin and late pregnant (gestation day 16–19) EAE-immunized mice, then restimulated with the immunizing peptide PLP 139–151 *in vitro*. Cells from pregnant mice produced significantly less IL-17 and TNF-α relative to virgin controls (Fig. 3). MCP-1 and IL-6 also were found to be decreased in late pregnancy (data not shown). IFN-γ (Fig. 3) and IL-2 (data not shown) production was not significantly different between groups. However, at the RNA level, expression of IL-2, IFN-γ, TNF-α and the macrophage inflammatory marker IL-12p40 were all down-regulated in late pregnancy relative to virgin controls (Fig. 4).

Th2 cytokine shifts and increased secretion of IL-10 are not primary mediators of disease suppression during pregnancy in established EAE

To determine if anti-inflammatory Th2 cytokines contributed to suppression of clinical disease observed during pregnancy, lymph node and splenocyte production of IL-4 and IL-5 was examined. Both IL-4 and IL-5 were found to be produced in negligible amounts in both pregnant and control groups (data not shown). There also was no difference in proliferation following antigen stimulation of T cells from late pregnant versus virgin mice (data not shown). IL-10 has been shown to be a powerful mediator of inflammatory disease suppression, specifically in mice immunized for EAE during late pregnancy (McClain et al.,

2007). Analysis of the levels of IL-10 by ELISA, ELISPOT and CBA showed that there was not a significant difference in IL-10 production between the pregnant and virgin mice when pregnancy is induced after EAE is established (Fig 5).

Pregnancy suppression of disease is pervasive across mouse strains

C57Bl/6 mice usually exhibit a progressive disease course without remissions, which is in contrast to the relapsing remitting course in the SJL mouse. To evaluate this strain's response to pregnancy, C57Bl/6 mice were immunized with MOG 35–55/CFA/pertussis toxin to elicit EAE. Females were mated after the initial acute phase of disease. Mice that subsequently became pregnant exhibited a profound remission of clinical disease, and a gradual return of clinical signs during the postpartum period (Fig. 6). In a parallel experiment, C57Bl/6 mice were immunized during the postpartum period (6 d after parturition) and a slightly more severe cumulative disease course and increased mortality were observed compared to virgin immunized mice (data not shown). These findings are consistent with pregnancy suppression of disease observed in SJL/J mice.

Late pregnancy serum suppresses T cell activation *in vitro*

SJL/J mice have an increased frequency of T cells specific for PLP 139–151, such that a proliferative response to this antigen can be detected in naïve T cells. To determine the relative suppressive potential of late pregnancy serum, we cultured LNC from virgin naïve and virgin EAE immunized SJL/J mice with PLP peptide plus 3% late pregnancy serum or virgin serum and measured T cell proliferation. Antigen stimulated naïve cells cultured with late pregnancy serum proliferated significantly less than those cultured with virgin serum (data not shown). To more closely recapitulate the *in vivo* EAE pregnancy experiments, LNCs from EAE-immunized SJL mice were harvested 10 d after immunization and cultured with PLP or anti-CD3. Cells exposed to pregnancy serum were more suppressed than virgin serum treated cells (Fig. 7). Spleen cells were cultured under similar conditions and demonstrated similar findings (data not shown). A similar trend toward pregnancy serum suppression of T cell responses was observed in the C57BL/6 strain using splenocytes harvested from MOG TCR transgenic mice cultured with MOG35–55 and 3% pregnancy versus virgin serum (Fig. 7). Importantly, treatment of pregnancy and virgin serum with dextran coated charcoal no longer offered a significant suppression of T cell proliferation in response to anti-CD3 stimulation (data not shown).

Isolated pregnancy exosomes are larger and more numerous relative to virgin mouse serum exosomes

Exosomes are circulating small particles formed from late endosomal or cytosolic budding that usually carry proteins from the parent cell. We reasoned that serum exosomes could possibly be a serum factor responsible for pregnancy-induced suppression of immune responses. Serum was separated from whole blood collected at the time of late pregnancy or from age-matched SJL female virgins. After isolation, exosomes were visualized using electron microscopy. While there was variation in exosomal size in virgin mouse serum, late pregnancy serum-derived exosomes were consistently more numerous and larger in size as compared to virgin serum exosomes (Fig. 8). Both virgin and pregnancy exosomes were positive for Hsc 70, a marker of exosomes, as determined by Western blot (data not shown).

Isolated pregnancy exosomes suppress T cell activation *in vitro*

To determine if pregnancy-derived serum was able to suppress T cell proliferation, we cultured splenocytes stimulated with anti-CD3 in the presence of whole serum. A serum dose-titration was performed and 3% serum was found to be optimal for proliferation analysis (data not shown). Figure 9A represents control cultures with unfractionated serum

from which the exosomes were purified. Whole serum from late pregnant mice (Pserum) suppressed T cell proliferation by 35% as compared to 7% suppression by virgin serum (Vserum) (Fig. 9A). To determine if purified exosomes were capable of suppressing T cell proliferation in the absence of serum, we cultured splenocytes from SJL/J mice stimulated with anti-CD3 and added 10 μg of exosomes purified from either virgin or pregnancy serum. We found that purified pregnancy exosomes were significantly more suppressive as compared to virgin exosomes or cells cultured without any exosomes. Pregnancy purified exosomes (Pexo) suppressed proliferation by 26%, whereas virgin exosome (Vexo) cultures only led to 10% suppression (Fig. 9B). We also compared suppression by exosome-depleted serum and found that pregnancy serum devoid of exosomes resulted in significant suppression of T cell proliferation as compared to virgin serum, indicating that exosomes may not be the only source of suppression (Fig. 9C). Exosomes represent a non-soluble fraction of serum and provide the opportunity to gain insight into a novel set of factors responsible for immune modulation during pregnancy.

4. Discussion

Pregnancy has been shown to have suppressive effects on several autoimmune diseases including MS and rheumatoid arthritis. A large prospective study reported by Confavreux *et al.* (1998) recorded clinical improvements particularly during the third trimester of pregnancy in women with relapsing MS. Relapse rate increased following parturition but eventually returned to the pre-pregnancy level months after delivery. Worse clinical outcomes in nulliparous women with MS as compared to parous or multiparous women have been reported (Kaplan, 2005). Our study focused on recapitulating the human clinical scenario in the EAE mouse model in order to investigate specific mechanisms of pregnancy suppression.

Pregnancy maintenance involves sustained immunosuppression and increased immune tolerance throughout the pregnancy period (Chaouat, 1987). Regulation of this process has been the focus of clinical investigations aimed at reducing spontaneous abortions and modulating autoimmune disease (Sabapatha et al., 2006). We previously demonstrated the importance of the host microenvironment at the time of disease induction when pregnancy was initiated prior to EAE. Clinical signs of EAE were suppressed when neuroantigen immunization occurred during late pregnancy, while increased clinical disease was seen with immunization during the post partum period (McClain et al., 2007). Because MS is often preexisting at the time of pregnancy, we focused this study on pregnancy effects on established EAE.

Langer-Gould *et al*. (2002) reported a decrease in clinical signs during pregnancy in SJL mice with pre-existing EAE. However, they also reported mid-pregnancy relapses of disease in mice and no decreases in inflammatory cell infiltration in the brain relative to virgin EAE controls. Our studies reported here show a >95% relapse-free pregnancy period with complete remission of disease in >90% of pregnant mice. We also note decreased demyelination, cellular infiltration and perivascular cuffing in the spinal cord during late pregnancy in mice with preexisting EAE. We attribute the differences in our study findings to the pregnancy induction technique, methodologic differences in EAE induction, and the focus on spinal cord pathology. Familiarizing females with male pheromones prior to mating increased their receptivity to males and likely stimulated estrous. Continuous addition of soiled male bedding likely helped to stimulate hormone secretion and maintain pregnancy, consequently limiting relapses and resulting in decreased CNS pathology during pregnancy. Trafficking of inflammatory cells into the CNS is a highly programmed event in EAE. Activated cells move to the meninges and adjacent white matter in an identifiable pattern along fiber tracts established during CNS development. The meninges and spinocerebellar

systems are the early targets of inflammatory cells in murine EAE, followed by white matter infiltration including distal axon microglial activation and ultimately a more widespread influx of cells into the CNS via vascular beds (Brown and Sawchenko, 2007). The spinal cord and brain parenchyma are infiltrated differentially based on their composition of white matter and gray matter. The spinal cord has peripheral white matter and central gray and the reverse is true for the brain. Importantly, we propose that the obvious early clinical deficits measured when scoring EAE are related to declining proprioceptive (position sense), sensorimotor and motor reflexive responses and less so to injuries to higher cortical brain structures. Offner et *al.* (2004) reported decreases in inflammatory infiltrates of the spinal cord in mice treated with estrogen derivatives. The pregnancy hormone, estrogen, has been extensively studied for its neuroprotective properties involving stimulating nerve growth factors, promotion of synaptogenesis and support of neurite arborization (McEwen, 2001). Taken together, using the spinal cord in our studies offered a more relevant picture of timed CNS injurious events. Injury to axons leads to the irreversible neurologic deficits observed in MS and EAE. Understanding the chronology of target tissue damage is important to prevent these irreversible changes.

A number of mechanisms have been proposed to explain how pregnancy influences clinical outcomes in autoimmune disease. The more prominently articulated notions are related to a pregnancy-specific Th2 cytokine shift, pregnancy-induced T cell anergy, or pregnancy hormone-mediated suppression of inflammation. We observed that there were no significant changes in Th2 cytokines between pregnant and virgin mice with preexisting EAE. The regulatory T cell product, IL-10, has been shown to provide a powerful suppressive influence in mice immunized for EAE during pregnancy or in response to estrogen in mice with EAE (Gilmore et al., 1997, Bebo et al., 2001). We found that IL-10 did not play a significant role in suppression of established EAE in pregnancy. Clinical trials are currently underway to investigate the role of sex hormones that predominate during pregnancy, such as estrogen and estrogen derivatives based on positive results using estriol in EAE and MS pilot trials (Soldan et al., 2003). While estrogens have been found to be neuroprotective, there is concern for increased risk of cardiovascular and breast cancer in patients treated with unopposed estrogen (Li et al., 2008, Newton et al., 2008). Furthermore, there are clear undesirable side effects such as decreased libido and gynecomastia related to elevated estrogen levels in male patients (Abaci and Buyukgebiz, 2007). However, the current clinical trials use a particular estrogen that is believed to have a reduced side-effect profile, and the results of these trials would be highly beneficial to therapeutic advancement in MS.

Given that none of the proposed mechanisms can adequately explain the profound suppression of MS/EAE by pregnancy, we sought to identify and characterize a nonsoluble serum factor(s) that had been proposed previously. The immunomodulatory properties of human pregnancy serum have been demonstrated by adding small aliquots to mixed leukocyte cultures (Kaskura, 1971). More recently, Langer-Gould et *al.* (2002) reported that addition of 3% pregnancy serum to cultures of T lymphocytes suppressed proliferation. Here, we have identified nonsoluble circulating exosomes in late pregnancy serum as one of the immunosuppressive factors. Exosomes are generated by inward or reverse budding of late endosome vesicles, resulting in particles that contain cytosol and expose the extracellular domains of certain membrane-bound proteins. Exosomes are not to be confused with apoptosomes or microvesicles shed from the cell plasma membrane. While multiple cell types generate exosomes, including lymphocytes, dendritic cells and epithelial cells, suppression of autoimmune disease by pregnancy is most likely mediated by placental cell-derived exosomes similar to those reported by Sabapatha et *al.* (2006). In our study, exosomes isolated from the serum of mice during late pregnancy were found to be more numerous than those isolated from virgin mice. Furthermore, in T cell cultures, unfractionated pregnancy serum, purified pregnancy exosomes, and exosome-free

pregnancy serum were found to suppress T cell activation. This suggests that both soluble serum components, like hormones and pregnancy-specific proteins, as well as nonsoluble serum factors, such as exosomes, are responsible for T cell suppression during EAE and pregnancy. We have conducted a preliminary proteomic analysis of exosome components and found differences between pregnancy and control exosomes with regard to specific proteins. Further analysis of exosomal components may reveal the mechanism by which exosomes mediate pregnancy-associated disease suppression. In conclusion, our findings advance the understanding of pregnancy in MS through demonstration of the markedly suppressive influence of pregnancy on established EAE. A profound suppression of both clinical and histopathologic indices of disease was observed. We also identified the circulating serum exosome as one possible mediator of disease suppression during late pregnancy. Harnessing the suppressive potential of the pregnancy state could help improve therapeutic modalities for managing MS and other immune-mediated disease.

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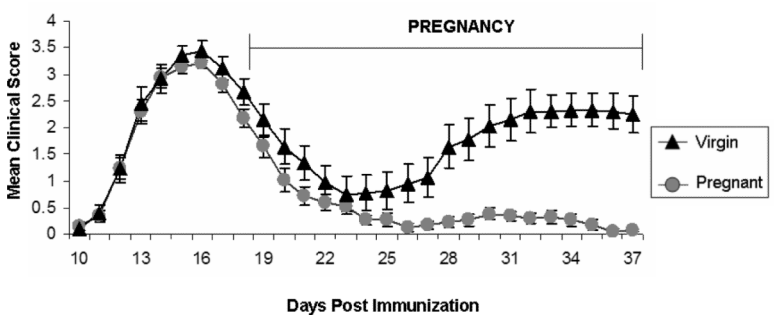

Figure 1. Mice with established EAE exhibit profoundly suppressed disease during late pregnancy

SJL mice were immunized with PLP 139–151 and CFA then induced for pregnancy after the acute phase of disease during the first remission (~18–22 d post immunization), with virgin EAE immunized mice serving as controls. All mice were monitored for clinical signs for 37 d post immunization. Data are representative of three independent experiments. (Virgin n = 18; Pregnant n = 35).

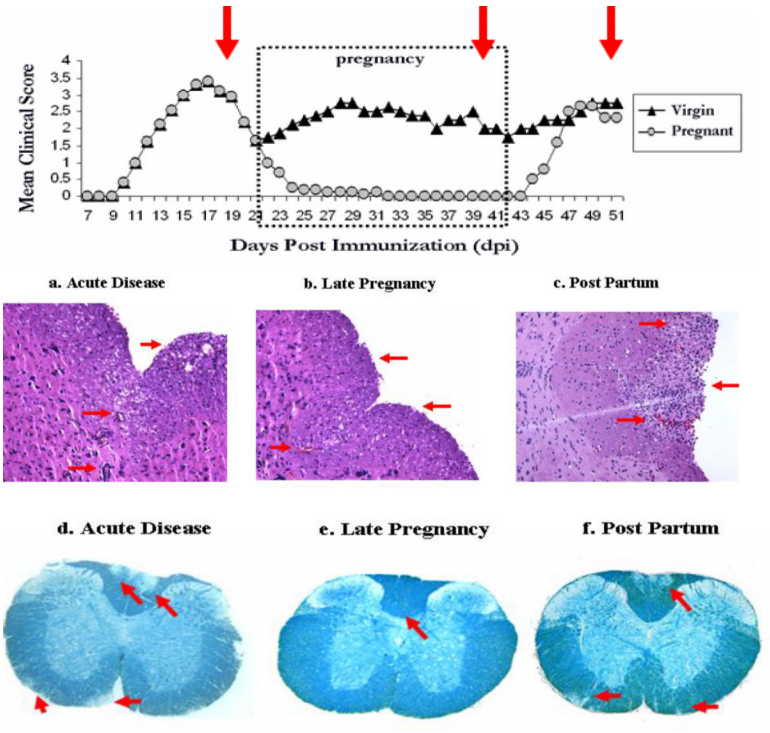

Figure 2. Animals with pre-existing EAE show decreased CNS infiltration, perivascular cuffing, and areas of demyelination during late pregnancy with worsening post partum

SJL mice were immunized with PLP 139–151 and mated for pregnancy and CNS tissue was harvested during acute disease, late pregnancy, and post partum period. H and E staining was completed at 18, 40, and 51 d post immunization and 20× light microscopic views of ventral lumbar spinal cord are shown. Infiltration was quantified as detailed in the histopathology methods (a–c). Luxol fast blue staining was completed at 18, 40, and 51 d post immunization. Spinal cords were removed and fixed in 10% formalin for 5 d before paraffin embedding. Light microscopic views (4×) of the cervical-lumbar sections are shown (d–f). See above clinical disease chart, red arrows demonstrate clinical score at the time of sacrifice. Data are representative of two independent experiments. (Virgin, $n = 7$; Pregnant, $n = 7$).

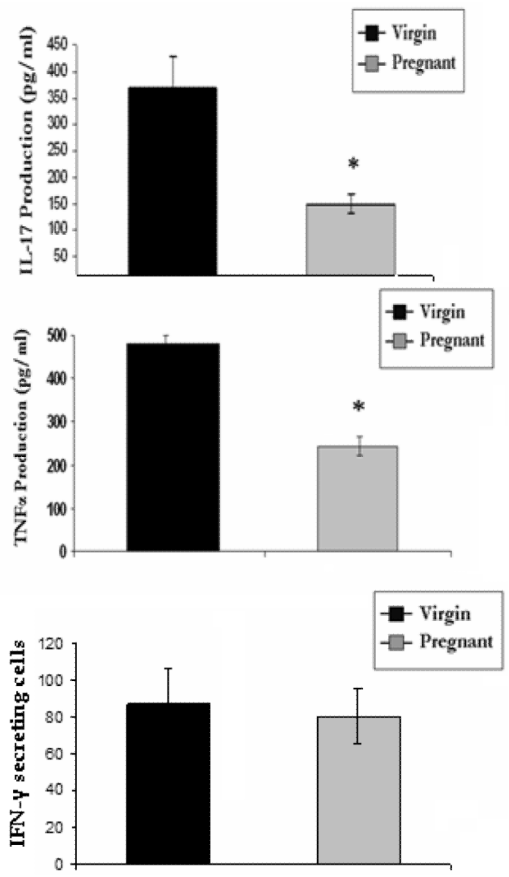

Figure 3. Mice with established EAE induced for pregnancy exhibit decreased IL-17 and TNF-α production

Mice were immunized with PLP 139–151 and CFA, induced for pregnancy after acute disease and sacrificed during late pregnancy. LNCs were cultured with PLP 139–151 for 72 h. Cytokines were measured: top) IL-17 (ELISA), middle) TNF-α (CBA), and bottom) IFN-γ (ELISPOT). *$p<0.05$ compared to virgin controls. (n = 3 mice per group).

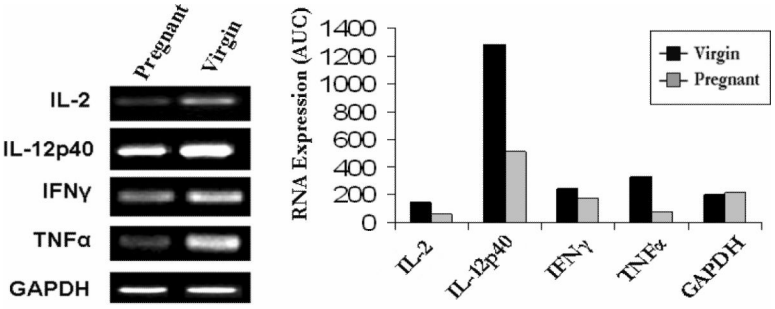

Figure 4. Pregnancy in established EAE reduces RNA levels of inflammatory cytokines *in vivo*

Mice were immunized with PLP 139–151, induced for pregnancy after acute disease and sacrificed during late pregnancy with disease time-matched virgin controls. RNA was isolated from LNCs and reverse transcription-PCR was completed using IL-2, IL12p40, IFN-γ, TNF-α, and GAPDH primers designed in our laboratory. Images are displayed as agarose gel image (left) and graphed as area under the curve using NIH Scion Gel Imager Software for quantification (right). (One representative band per group of $n = 3$ mice, completed in triplicate lanes).

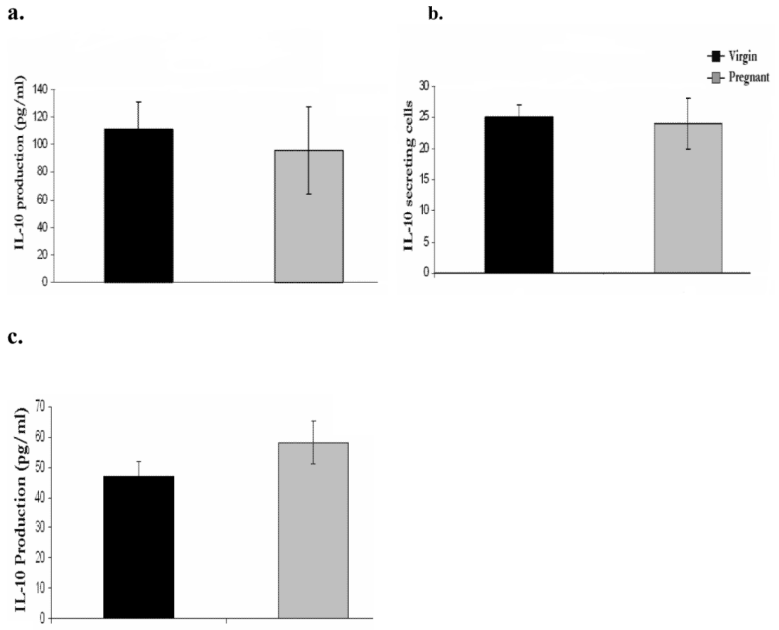

Figure 5. No difference in IL-10 production

SJL mice were immunized with PLP 139–151 and CFA and pregnancy was induced (15–18 days post immunization), with non-pregnant diseased mice serving as controls. Spleen cells were harvested during late pregnancy and cultured with PLP 139–151 for 72 h. IL-10 was measured by ELISA (a) ELISPOT (b) and CBA (c). Representative of 2 experiments (n = 3–4 per group).

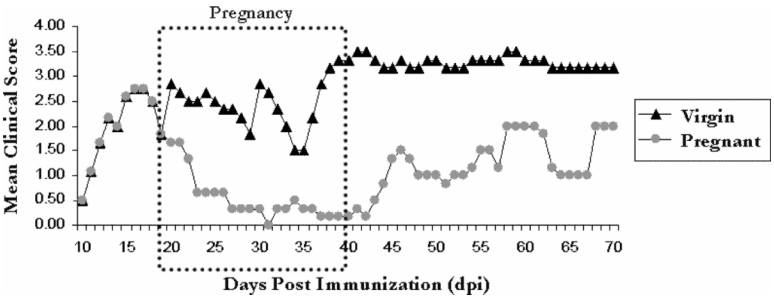

Figure 6. Pregnancy suppression of established disease is not mouse strain specific

C57Bl/6 mice were immunized with MOG 35–55 and induced for pregnancy at the first sign of disease remission. Mice that subsequently became pregnant demonstrated profound remission in disease and exhibited a decreased rate of disease progression in the post partum period. (Data are representative of one experiment, n = 9–10 per group).

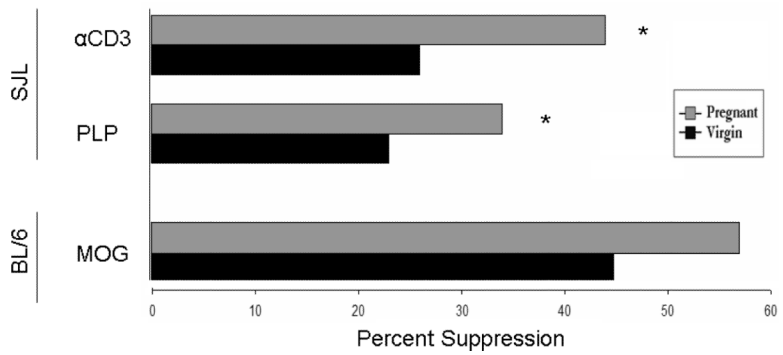

Figure 7. Pregnancy serum suppresses T cell proliferation

LNCs from SJL mice were collected 10 d post immunization and stimulated with PLP 139–151 or anti-CD3 for 72 h in the presence of 3% virgin or 3% late pregnant SJL mouse serum. T cell proliferation was significantly more suppressed by pregnancy serum as compared to virgin serum treated cells. *$p<0.05$ compared to virgin serum ($n = 3$ per group). A similar trend was seen using MOG TCR transgenic C57Bl/6 mice spleen cells cultured with MOG 35–55 in combination with 3% late pregnant or virgin C57BL/6 mouse serum. Data are representative of two combined experiments.

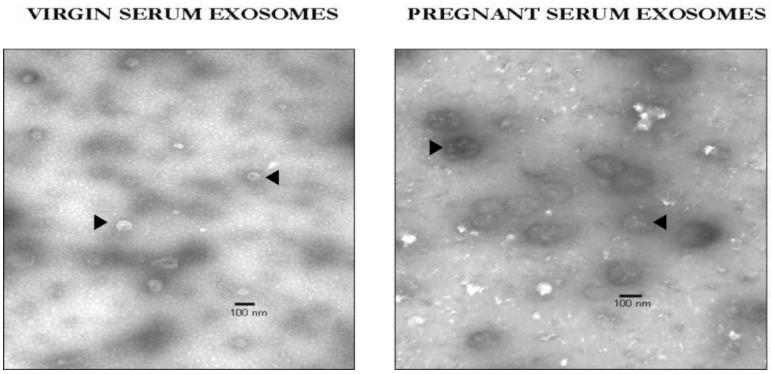

Figure 8. Exosomes from late pregnant mice are larger as compared to virgin control exosomes

Virgin and late pregnant mice were sacrificed 16–18 d post conception and serum was collected via retro-orbital eyebleed. Exosomes were harvested via ultracentrifugation, resuspended, and imaged using electron microscopy. Black arrows indicate representative exosomes from each image.

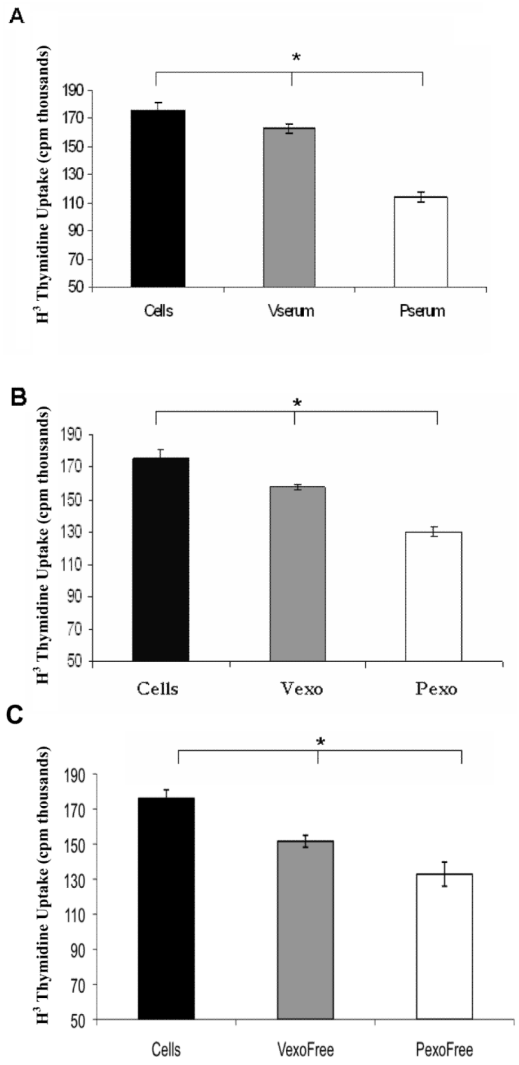

Figure 9. Late pregnancy serum factors suppress T cell proliferation

Virgin and late pregnant mice were sacrificed 16 d post conception and serum was collected via retro-orbital eyebleed. Whole serum (Vserum, Pserum, **A**) (3%), purified exosomes (Vexo; Pexo, **B**) (10 μg), and exosome-depleted serum (3%) (VexoFree, PexoFree, **C**) from virgin and pregnant mice were compared. Spleen cells (5×10^5/well) were cultured with anti-CD3 and serum components for 72 h and T cell proliferation was measured as compared to cells alone. *$p<0.05$ compared to non-pregnant controls. ($n = 3$ per group with 9 replicate determinations).

Table I

Primer sequences used for reverse transcription-PCR.

Target	Forward	Reverse
GAPDH	AAC TTT GGC ATT GTG GAA GG	ACA CAT TGG GGG TAG GAA CA
IFN-γ	ACT GGC AAA AGG ATG GTG AC	TGA GCT CAT TGA ATG CTT GG
IL12p40	AGG TGC GTT CCT CGT AGA GA	AAA GCC ACC AAG CAG AAG A
TNF-α	CGT CAG CCG ATT TGC TAT CT	CGG ACT CCG CAA AGT CTA AG
IL-2	TTTGGAGGAAAAGTGGAAGA	AACATTCCATACATCCT-GGC

Table II

Female mice pre-sensitized to breeders demonstrate a more profound remission.

	Pre-sensitized		Not Pre-sensitized	
	CDI[a] ± SEM	**MPS[b] ± SEM**	**CDI ± SEM**	**MPS ± SEM**
Pregnant	3.6 ± 1.0*	0.6 ± 0.2*	8.4 ± 2.8*	1.3 ± 0.3*
Virgin	25.8 ± 4.3	3.1 ± 0.4	21.1 ± 4.9	2.3 ± 0.4

[a] Cumulative disease index, the mean sum of disease scores during the gestation period.

[b] Mean peak score, the mean of the highest disease score during the gestation period.

* $p < 0.05$ when compared with virgin; student's *t* test.