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Pregnancy Suppresses Experimental Autoimmune Encephalomyelitis through Immunoregulatory Cytokine Production¹

Melanie A. McClain, NaTosha N. Gatson, Nicole D. Powell, Tracey L. Papenfuss, Ingrid E. Gienapp, Fei Song, Todd M. Shawler, Aaron Kithcart, and Caroline C. Whitacre²

Women with multiple sclerosis (MS) often experience a decrease in relapse rate during pregnancy, most notably during the third trimester, with a flare of disease activity 3–6 mo postpartum. Studies in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, have shown that pregnancy delays the onset and decreases the incidence of disease. We investigated the effect of pregnancy and the postpartum period in a remitting-relapsing model of murine EAE. When immunization occurs during pregnancy, mice show a reduction in the incidence of EAE as well as a decrease in clinical severity, while mice immunized during the postpartum period exhibit more severe disease. No differences in lymphocyte proliferation or expression of activation markers were noted when immunization occurred during pregnancy as compared with the nonpregnant controls. Mice immunized during pregnancy produced less TNF- α and IL-17, and showed an increased number of IL-10-secreting cells within the CD11b⁺, CD11c⁺, CD19⁺, and CD4⁺/CD25⁺ populations. No differences were noted in the production of IFN- γ , IL-2, IL-4, and IL-5. These results suggest that when an Ag is introduced during pregnancy, an immunoregulatory rather than an immunosuppressive or Th2 environment predominates. *The Journal of Immunology*, 2007, 179: 8146–8152.

Multiple sclerosis (MS)³ is a chronic demyelinating disease of the CNS thought to involve an autoimmune response directed against myelin Ags (1). MS typically presents during the reproductive years and shows a female preponderance (3:1). The disease course has been observed to differ between men and women, with males typically showing a primary progressive course and females displaying a relapsing/remitting form. These sex differences have been attributed to many factors, the most prominent of which are sex hormones (2).

During pregnancy, women usually experience a decrease in MS clinical disease activity (3–7). Confavreux et al. (8) observed that the rate of MS relapse declines over the course of pregnancy, with the fewest number of relapses occurring during the third trimester. Following parturition, however, the rate of relapse increases sharply before returning to prepregnancy levels 3–6 mo postpartum. Because both disease amelioration and exacerbation occur within a relatively short time frame, investigators have been particularly interested in the physiological and immunological changes that occur between late pregnancy and the postpartum period.

Experimental autoimmune encephalomyelitis (EAE), an animal model of MS, is induced by neuroantigen injection and is mediated by CD4⁺ proinflammatory cells. In acute models of EAE using rats, rabbits, and guinea pigs, pregnancy reduces the incidence of disease and delays the day of onset (9–11). Pregnancy was observed to reduce the incidence of relapsing/remitting EAE in the SJL mouse (12). This protection was not associated with a decrease in CNS histopathological changes nor any change in cytokine secretion relative to nonpregnant EAE controls, but rather the presence of an immunosuppressive serum factor (12). Little is known about the precise mechanism(s) by which pregnancy (and its associated factors) suppress clinical disease. A particularly intriguing question which has not been explored is the cause for the increase in disease activity during the postpartum period in EAE.

The purpose of this study was to examine the effect of different gestational stages on EAE and to explore the immunological mechanisms operative at each stage. Our investigations focused on pregnancy (late stage) as well as the postpartum period, to determine why disease activity decreases most dramatically during the third trimester and increases abruptly following parturition. We also examined a number of immunological parameters to ascertain whether disease suppression during pregnancy is due to generalized immune suppression, a Th2 bias, or the production of immunoregulatory cytokines. We found that each gestational stage results in a unique immunomodulatory environment that has specific influences on EAE.

Materials and Methods

Mice

Age-matched timed pregnant and non-pregnant female SJL/J and C57BL/6 mice were purchased from The Jackson Laboratory. The mid-pregnancy gestational stage mice were obtained on days 9–11 post conception (pc), allowed to acclimate for 2 days, and then immunized on days 11–13 pc. Late-pregnancy mice were obtained on days 13–15 pc, allowed to acclimate for 2–3 days, and then immunized on days 15–18 pc. Postpartum mice were obtained before parturition on days 13–15 pc, allowed to acclimate and give birth followed by immunization on 22–25 days pc. Mice

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³ Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; PLP, proteolipid protein; pc, post conception; MOG, myelin oligodendrocyte glycoprotein.

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were used at 6–10 wk of age. Mice were maintained on a 12-h light/dark cycle and given food and water ad libitum.

Ags

Peptides were purchased from Sigma-Genosys and Princeton Biomolecules and were purified by HPLC. Peptides used in this study were: PLP 139–151 (HCLGKWLGHDPKF), PLP 178–191 (NTWTTCQSIAPFSK), PLP 258–273 (IAATVNFAVLKMGGR), MOG 35–55 (MEVGWYRSPFSRV-VHLYRNGK), MBP 87–99 (VHFFKNIVTPRTP), and MBP 84–104 (VHFFKNIVTPRTPPSQKGGR). All peptides had a purity >90%.

EAE immunization

SJL mice were immunized subcutaneously over four sites on the flank with 0.2 ml of an emulsion containing 150 μ g of PLP 139–151 in PBS mixed with an equal volume of CFA containing 200 μ g of heat-killed *Mycobacterium tuberculosis*, Jamaica strain. C57BL/6 mice received 200 μ g of MOG 35–55 in PBS together with CFA, given in two separate immunizations at the base of the tail, 7 days apart. No pertussis toxin was used in either immunization scheme because of the risk of spontaneous abortion. 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.

Histopathology

Spinal cords and brains were removed from mice in all groups (control, pregnant and postpartum) at varying times after immunization, including day 15 for all groups, day 25 for control and pregnant mice, and day 35 for control and postpartum mice. Tissues were fixed in 10% phosphate-buffered formalin and then dissected and embedded in paraffin. Sections were then processed for H&E staining. Sections were scored as follows: 0, absence of infiltrates; 1, small rare perivascular lesions; 2, small numerous perivascular lesions; 3, numerous perivascular lesions and parenchymal infiltration; and 4, severe confluent lesions.

Proliferation analysis

Peripheral lymph nodes (inguinal, axillary, brachial, cervical, popliteal, and periaortic) and spleens were removed from mice on days 15, 25, and 35 post immunization. Single cell suspensions were prepared and suspended in RPMI 1640 containing 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 5×10^{-5} M 2-ME in round-bottom 96-well plates (4×10^5 cells/well). Cells were cultured with medium alone or with PLP 139–151 (30 μ g/ml), PLP 178–191 (15 μ g/ml), PLP 258–273 (15 μ g/ml), MBP 87–99 (15 μ g/ml), MBP 84–104 (15 μ g/ml), or anti-CD3 (2 μ g/ml). Cultures were incubated for 72 h at 37°C and 7% CO₂, including an 18 h pulse with [³H]thymidine (1 μ Ci per well). Cultures were harvested onto glass-fiber filter mats using a Skatron harvester and were counted by liquid scintillation on a Wallac betaplate (LKB, Wallac). Results are expressed as cpm \pm SEM.

ELISPOT analysis for cytokine-producing cells

Frequencies of cytokine-secreting cells were determined for IL-10 (R&D Systems). In brief, microtiter plates with nitrocellulose bottoms (Millipore Corporation) were coated overnight at 4°C with capture Ab. After washing, plates were blocked with 1% BSA (Sigma-Aldrich) for 2 h at room temperature. Spleen cells were resuspended in HL-1 medium and then cultured in triplicate with medium alone or with the following stimulants (15–30 μ g/ml): PLP 139–151, PLP 178–191, PLP 258–273, MBP 87–99, MBP 84–104, or anti-CD3 (2 μ g/ml). Cultures were maintained at 37°C for 72 h. Plates were washed and cytokine-specific biotinylated Abs were added. After overnight incubation, streptavidin-alkaline phosphatase 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 \pm SEM for all animals in a group.

Cytometric bead array (CBA)

IFN- γ TNF- α , IL-2, IL-4, and IL-5 were detected using the mouse Th1/Th2 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 (FACSCalibur; BD Biosciences).

IL-17 ELISA

SJL/J mice were immunized for EAE during late pregnancy and observed for 27 days post immunization. Age matched nonpregnant SJL/J mice were included as EAE immunized controls. Splenocytes were cultured (4×10^5 cells/well) with PLP (30 μ g/ml), anti-CD3 (2 μ g/ml) or medium alone and supernatants were collected after 72 h for ELISA. DuoSet ELISA kits (R&D Systems) were used to determine the levels of IL-17 cytokine production. The OD was determined using SpectraMax Plus384 high throughput microplate spectrophotometer and analyzed using SoftMax Pro software (Molecular Devices).

Intracellular cytokine staining

Spleens were removed from pregnant and nonpregnant control mice on day 15 post immunization. Single cell suspensions were prepared and suspended in complete RPMI 1640 medium containing 10% FBS in round-bottom 6-well plates (20×10^6 cells/well). Cells were cultured with PLP139–151 (30 μ g/ml) for 48 h, including exposure to the Golgi inhibitor, monensin, for the last five hours. Intracellular cytokine staining for IL-10 was performed following manufacturer's instructions (EBioscience) and analyzed using flow cytometry.

Flow cytometric analysis

Single cell suspensions derived from lymph nodes and spleens were stained for CD4, CD8, CD11b and CD11c using FITC-conjugated mAb and CD25, CD28, CD44, CD62L, CD69, CD80, CD86, PD-1, PD-L1, PD-L2, ICOS, ICOS-L with PE-conjugated mAb. Isotype control mAbs (Pharmingen) were matched for fluorochrome and used for cursor placement. Lymphocytes were gated based on forward vs side scatter and a total of 10,000 events were analyzed by three color flow cytometry (FACSCalibur).

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. A one-way ANOVA was used for the percent expression assays. χ^2 analysis was used for determining differences in disease incidence.

Results

Pregnancy prevents the development of EAE while disease induction during the postpartum period increases relapse severity

To determine the effect of pregnancy on the induction of EAE, we immunized SJL mice with PLP peptide during late pregnancy (15–18 days pc), with nonpregnant female mice serving as controls. When EAE was induced during pregnancy, few mice developed clinical signs of EAE (Table I). Specifically, 89% of the

Table I. Pregnancy suppresses EAE and prevents disease relapse

	Incidence	Day of Onset \pm SEM	CDI ^a \pm SEM	Max Score ^b \pm SEM	Relapse ^c
Control	89%(63/71)	16.1 \pm 1.8	22.0 \pm 5.2	2.5 \pm 0.2	38%
Late pregnancy	13%(3/24)*	28.5 \pm 0.5*	6.0 \pm 1.0	2.0 \pm 0.0	0%

^a Cumulative Disease Index (mean sum of clinical scores over the entire observation period).

^b Mean maximum score obtained for the group over the entire observation period.

^c The percentage of mice exhibiting a relapse (≥ 1 increase in clinical score for ≥ 2 days) after the initial bout of disease.

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

immunized nonpregnant mice showed clinical signs of disease, while only 13% of the mice immunized during late pregnancy developed EAE. Of the few mice that did develop EAE, the day of disease onset was markedly delayed (Fig. 1). Interestingly, no relapses were observed when mice were immunized during late pregnancy (Table I and Fig. 1). We also examined the effect of EAE induced during mid-pregnancy (11–13 days pc) and found that there was some reduction in disease incidence and severity (data not shown). However, EAE induced during mid-pregnancy resulted in a large number of resorptions or aborted pregnancies. To explore the effect of EAE induction during the postpartum period, we immunized mice 2–3 days following parturition. Both postpartum and control groups showed comparable clinical disease during the acute phase of EAE, with no difference observed in the day of disease onset (Table II and Fig. 2). Mice immunized during the postpartum period, however, showed increased relapse severity (Fig. 2). These results indicate that EAE induction during late pregnancy results in protection from disease, while EAE induction during the postpartum period causes exacerbation of clinical signs.

Pregnancy does not offer protection from EAE through generalized immune suppression or by interfering with lymphocyte trafficking

Delayed type hypersensitivity (DTH) responses, mixed lymphocyte reactions and the expression of immune system related transcription factors are all suppressed during pregnancy (13–15). We therefore sought to determine whether immunization during pregnancy suppressed EAE as a result of a global decrease in lymphocyte activation. We first examined the levels of activation and costimulatory marker expression on the surface of T lymphocytes and APCs because these molecules have been shown to be required for T cell activation. No differences between the pregnant and nonpregnant groups were noted in the expression of CD28, CTLA-4, ICOS or PD-1 on the surface of CD4⁺ T cells, or in the expression of CD80, CD86, ICOS-L, PDL-1 or PDL-2 on the surface of macrophages or dendritic cells (data not shown). These results indicate that leukocytes from mice immunized during pregnancy express cell surface markers necessary for T cell activation. We next examined the total number of cells present in the lymphoid organs during pregnancy and ascertained whether these cells could proliferate in response to the immunizing Ag. The total number of cells present in the peripheral lymph nodes was similar between groups. Mice immunized during pregnancy, however, exhibited nearly a 2-fold increase in the number of splenocytes relative to control animals (Fig. 3). Interestingly, lymphocytes from mice immunized during late pregnancy showed no reduction in

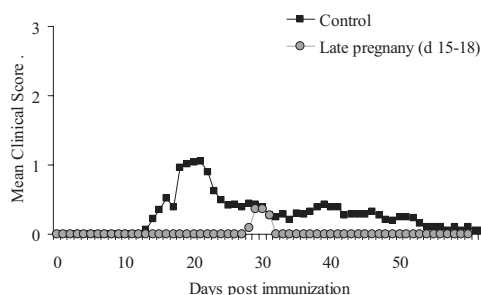


FIGURE 1. Animals immunized for EAE during pregnancy show a delayed onset of disease and no relapses. SJL mice were immunized with PLP 139–151 and CFA during late pregnancy (15–18 days post conception), with nonpregnant mice serving as controls. Animals were monitored for 60 days for clinical signs of disease. Data is representative of three experiments (control $n = 24$; late pregnancy $n = 11$).

proliferative response to PLP 139–151 relative to controls (Fig. 4). There was also no significant difference in the proliferative response to the next epitope in the inflammatory spreading cascade, PLP 178–191. Taken together, this data shows that lymphocyte activation does occur in mice immunized for EAE during pregnancy. Although leukocytes from animals immunized during pregnancy clearly recognized and responded to the immunizing Ag, the possibility existed that the inflammatory cells were not reaching their target organ. All CD4⁺ T cells examined, however, expressed CD11a (LFA-1) and most expressed CD44, CCR5, CD49d (VLA4) and CD54 (ICAM) on their surface in both the nonpregnancy and the late pregnancy groups (data not shown). Moreover, inflammatory infiltrates were present in the CNS of both control mice and those immunized during pregnancy. Both control and pregnancy-immunized groups of mice showed similar levels of infiltration with a mean inflammatory score of 2 (small, numerous perivascular lesions), in agreement with the previous report by Langer-Gould et al. (12). Thus, lymphocytes from animals immunized during pregnancy show evidence of activation and can traffic to the CNS.

Pregnancy reduces TNF- α and IL-17 production and increases the frequency of IL-10-secreting cells

Even though lymphocytes from mice immunized during pregnancy become activated and traffic to the CNS, clinical signs of EAE are rarely observed in these animals. Several lines of evidence suggest that Th1 cytokine production is reduced during pregnancy while Th2 cytokine secretion is increased (15). We therefore examined the ability of lymphocytes from control and pregnant mice to produce Th1, Th2 and Th17 cytokines in response to the immunizing Ag. We found that the nonpregnant mice and those immunized during pregnancy produced a similar level of IFN- γ and IL-2 in response to the immunizing Ag (Fig. 5, A and C). A similar but minimal production of the Th2 cytokines, IL-4 and IL-5, were also observed in both groups (data not shown). Lymphocytes from mice immunized for EAE during late pregnancy, however, produced significantly less TNF- α and IL-17 relative to control mice in response to PLP 139–151 (Fig. 5, B and D). We also determined the frequency of lymphocytes secreting IL-10 and found that mice immunized during pregnancy had a three-fold increase in these cells (Fig. 6).

We also examined the activation state and cytokine production of lymphocytes from mice immunized during the postpartum period. No differences were noted in the ability of these cells to proliferate in response to the immunizing Ag or any other epitopes in the inflammatory spreading cascade. Lymphocytes from mice immunized during the postpartum period also produced similar levels of the Th1 cytokines IFN- γ and IL-2 as well as the Th2 cytokines, IL-4 and IL-5 (data not shown). Mice immunized during the postpartum period, however, produced less IL-10 (Fig. 7). Thus, increased IL-10 production was observed in the animals immunized during pregnancy and decreased IL-10 production was observed in the animals immunized postpartum.

Multiple cell types up-regulate production of IL-10 in the mice immunized during pregnancy

IL-10 is considered a Th2 cytokine as well as an immunoregulatory cytokine, and can be produced by a number of cell types, including Th2 T cells, T regulatory cells, CD4⁺/CD25⁺ cells, B cells, macrophages and dendritic cells (16). To further delineate the type of environment induced when immunization occurs during pregnancy, we used intracellular cytokine staining to determine

Table II. Postpartum induction of EAE increases disease severity and the number of relapses

	Incidence	Day of Onset	CDI ^a	Relapse ^b	Multiple Relapses ^c
Control	89%(63/71)	16.1 ± 1.8	22.0 ± 5.2	38%	25%
Postpartum	48%(11/23)	18.5 ± 1.8	36.4 ± 3.0	44%	100%*

^a Cumulative Disease Index (mean sum of clinical scores over the entire observation period).
^b The percentage of mice exhibiting a relapse (≥1 increase in clinical score for ≥2 days) after the initial bout of disease.
^c The percentage of mice exhibiting more than one relapse (≥1 increase in clinical score for ≥2 days) after the initial bout of disease.
*, *p* < 0.05 when compared with controls; Student's *t* test.

what cell type produced IL-10 in mice immunized during pregnancy. As shown in Fig. 8, several cell types significantly up-regulate IL-10 production during pregnancy, including CD4⁺/CD25⁺, CD11c⁺, CD11b⁺ and CD19⁺ cells. Interestingly, no differences were observed in the ability of total CD4⁺ cells to produce IL-10.

Pregnancy Suppresses EAE during Pregnancy in the C57BL/6 Strain

To determine whether suppression of EAE during pregnancy is a generalized phenomenon, we conducted similar experiments in the C57BL/6 mouse strain. Female C57BL/6 mice were immunized for EAE using MOG 35–55 during late pregnancy (Fig. 9). Our results show that C57BL/6 mice treated in this manner showed suppression of disease activity when immunized during late pregnancy relative to virgin controls. Thus, both SJL and C57BL/6 mice, which differ in sex dimorphism effects on EAE, both exhibit suppression of EAE clinical signs when immunized during late pregnancy.

Discussion

MS disease activity undergoes dramatic changes over the course of pregnancy and the postpartum period. During pregnancy, the rate of relapse decreases, with the sharpest decline occurring during the third trimester. Abruptly following parturition, disease activity flares before returning to prepregnancy levels three to six months later (8). Within this relatively short window of time, one can observe opposing effects on disease. As a result, pregnancy and the postpartum period offer a unique opportunity to study both disease improvement and disease exacerbation.

Most experimental investigations addressing the effect of pregnancy on demyelinating disease have focused on EAE. These studies have found that pregnancy either prevents the development of disease, delays disease onset, or reduces clinical severity (10–11). The majority of work in this area, however, has been conducted in acute models of EAE in guinea pigs, rats or rabbits. To date, only

one report has examined the effect of pregnancy on relapsing/remitting EAE, and no studies have addressed the effect of the postpartum period on disease development. Consequently, little is known about how these hormonally diverse gestational stages affect chronic relapsing/remitting EAE in a comparative fashion.

To develop a thorough understanding of the effects of pregnancy and the postpartum period on EAE, our investigations included multiple gestational stages. We studied two timepoints during pregnancy (mid and late) as well as one from the postpartum period so that we could evaluate the degree of protection offered throughout pregnancy. Nonoverlapping, discrete 3 day ranges were chosen (e.g., mid pregnancy = days 11–13 post conception and late pregnancy = days 15–18 post conception), allowing a distinct characterization of each stage.

Our results showed that each gestational stage has its own unique effect on disease. When mice were immunized during pregnancy, the incidence of EAE was reduced (Table I). This effect was most pronounced when EAE induction occurred during the late stage of pregnancy. These results agree with the findings of Langer-Gould et al. (12) who reported that immunization during the latter half of pregnancy is more protective than earlier in gestation. Interestingly, of the few mice that did develop EAE, none exhibited relapses (Fig. 1). When mice were immunized during the postpartum period, however, the incidence of EAE remained similar to nulliparous mice, but the animals exhibited increased relapse severity (Fig. 2). These results suggest that both the incidence of disease, as well as the clinical course of EAE can be dramatically affected, depending upon the gestational stage in which immunization occurs.

To delineate the mechanism(s) by which the different gestational stages mediated the observed effects on EAE, we explored three alternatives. We first examined whether immunization during the late stage of pregnancy resulted in leukocyte activation. Some evidence has shown that certain aspects of immunity are suppressed

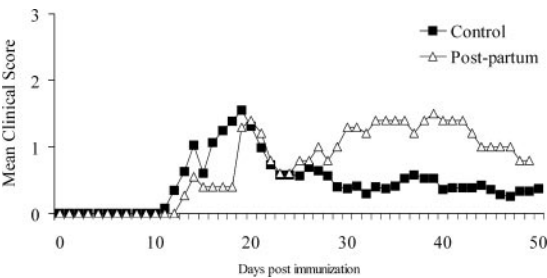


FIGURE 2. Immunization for EAE during the postpartum period results in increased relapse severity. Nonpregnant and postpartum (days 22–25 post conception) SJL mice were immunized with PLP 139–151 and CFA. Animals were monitored for 50 days for clinical signs of disease. Data is representative of two experiments (control *n* = 24, postpartum *n* = 11).

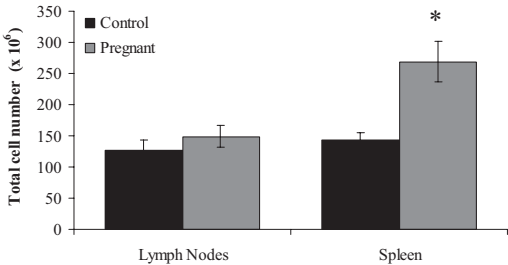


FIGURE 3. Total splenocyte number increases in mice immunized for EAE during pregnancy. SJL mice were immunized with PLP 139–151 and CFA during late pregnancy (15–18 days post conception), with nonpregnant mice serving as controls. At day 15 post immunization, the spleen and peripheral lymph nodes were removed and cells counted. Data is shown from one representative experiment of five (*n* = 3 per group).

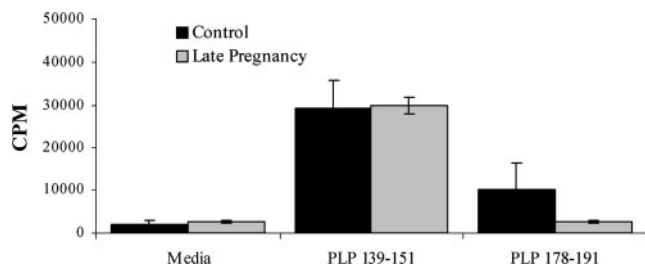


FIGURE 4. Proliferation is not decreased in mice immunized for EAE during pregnancy. SJL mice were immunized with PLP 139–151 and CFA during late pregnancy (15–18 days post conception), with nonpregnant mice serving as controls. At day 15 post immunization, lymph node cells were cultured with PLP 139–151 or PLP 178–191 for 72 h, including an 18 h pulse with [3 H]thymidine. Data is representative of five experiments ($n = 3$ per group).

during pregnancy. If insufficient activation of leukocytes were occurring in mice immunized for EAE during pregnancy, we predicted that their lymphoid organs would have fewer cells and show a decreased ability to proliferate. The total number of cells present in the lymphoid organs was not reduced in the mice immunized during pregnancy. In fact, the pregnancy group actually showed an elevated number of splenocytes, likely due to increased hematopoiesis during pregnancy (Fig. 3). Leukocytes from animals immunized during pregnancy showed similar levels of activation marker and costimulatory molecule expression compared with the immunized nulliparous controls, and cells from both groups proliferated similarly in response to the immunizing Ag (Fig. 4). $CD4^+$ T cells from the pregnancy group expressed similar levels of adhesion molecules as the controls and were able to infiltrate the

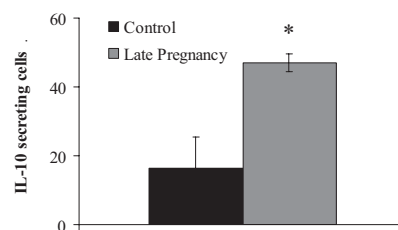


FIGURE 6. EAE induction during pregnancy results in an increased frequency of IL-10-secreting cells. Nonpregnant and late-stage pregnant mice were immunized with PLP 139–151 and CFA. On day 15 post immunization, mice were sacrificed and spleens were harvested. Splenocytes were cultured with PLP 139–151 for 72 h. The frequency of IL-10-secreting cells were measured via ELISPOT. *, $p < 0.05$ compared with nonpregnant controls. Graph is representative of three experiments. ($n = 3$ per group)

CNS. Thus, leukocyte activation in response to neuroantigen immunization clearly does occur when EAE is induced during pregnancy.

We also explored the alternative that when EAE induction occurs during pregnancy, there is a shift in cytokine production away from proinflammatory Th1 cytokines and toward Th2 cytokines. Increased levels of Th2 cytokines have been found at the maternal/fetal interface and pregnant animals have a decreased ability to clear intracellular infections that require a Th1 response (1–3). Because EAE is predominantly a Th1 driven disease and Th2 type responses are associated with protection, a shift in cytokine production toward Th2 could prevent mice from displaying clinical signs. To determine whether a Th2 shift occurred, we compared the production of proinflammatory cytokines (IFN- γ , IL-2, TNF- α and IL-17) and Th2 cytokines (IL-4 and IL-5) between nonpregnant immunized mice and those immunized during pregnancy. The two groups exhibited similar levels of IFN- γ and IL-2 and both displayed minimal secretion of IL-4 and IL-5. Thus, no differences were observed in the typical Th1 and Th2 cytokine profiles. Lymphocytes from mice immunized during pregnancy, however, produced significantly less TNF- α and IL-17, while displaying an increased frequency of IL-10-secreting cells when compared with control mice. TNF- α is frequently characterized as a Th1 cytokine (17), while IL-17 is considered to be the product of a newly described lineage of $CD4^+$ effector cells. Therefore, an increase in IL-10 with concurrent decreases in TNF- α and IL-17 could potentially be classified as a Th2 shift in the mice immunized during pregnancy. Yet, IL-10 also has roles independent of the Th cell developmental pathway. IL-10 inhibits the synthesis of IFN- γ and suppresses the maturation and activation of APCs. Although Th2

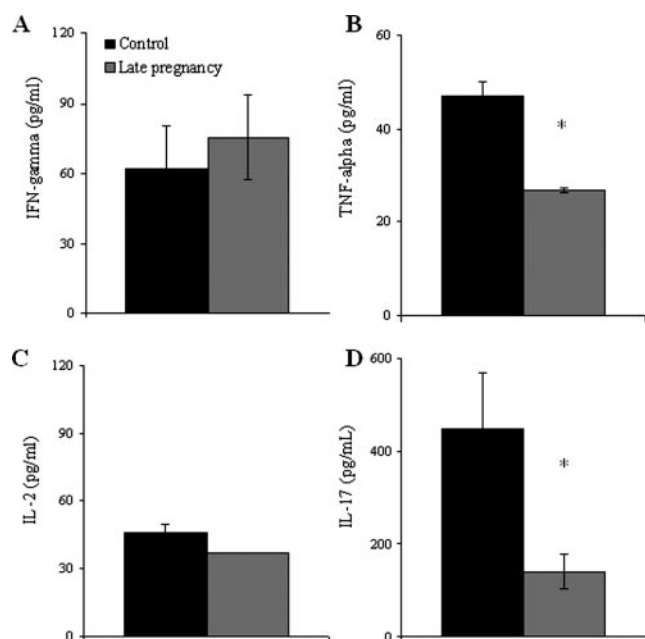


FIGURE 5. Mice immunized during pregnancy exhibit decreased TNF- α and IL-17 production. Nonpregnant and late-stage pregnant mice were immunized with PLP 139–151 and CFA. Mice were sacrificed and lymph node cells were harvested for culture with PLP 139–151. A, IFN- γ production day 15 post immunization; B, TNF- α production day 25 postimmunization; C, IL-2 production day 15 post immunization; D, IL-17 production day 15 post immunization as measured from spleen cells after 72 h culture with PLP 139–151. Cytokines were measured by CBA for IFN- γ , TNF- α and IL-2. IL-17 was measured via ELISA. *, $p < 0.05$ compared with nonpregnant controls ($n = 3$ per group).

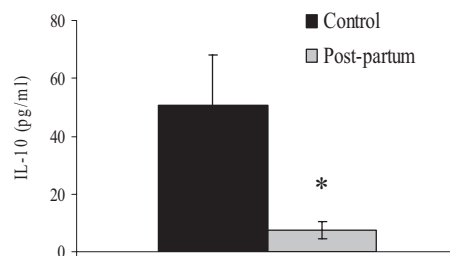


FIGURE 7. Decreased IL-10 production when EAE is induced postpartum. Nonpregnant and postpartum (days 22–25 post conception) SJL mice were immunized with PLP and CFA. Animals were sacrificed on day 35 and lymph nodes were harvested. Cells were placed into culture for 72 h then supernatants were collected. IL-10 was measured by ELISA. *, $p < 0.05$ when compared with the nulliparous controls ($n = 3$ per group).

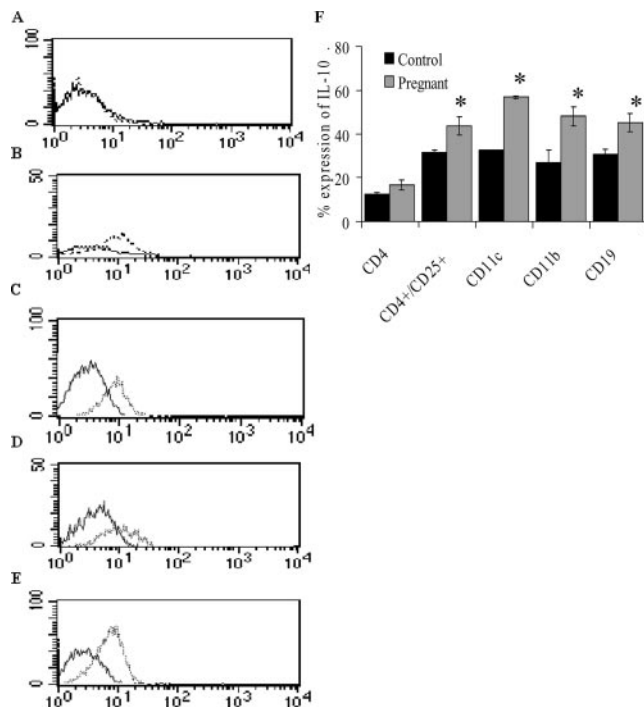


FIGURE 8. Multiple cell types increase production of IL-10 when immunization occurs during pregnancy. Nonpregnant and late-stage pregnant mice were immunized with PLP 139–151 and CFA. On day 15 post immunization, mice were sacrificed and spleens were harvested. Splenocytes were cultured with PLP 139–151 for 72 h, in the presence of monensin for the last 4 h. Percentage of cells expressing IL-10 was measured by intracellular cytokine staining. Histograms show percent expression of IL-10 on isotype control (solid line) and percent IL-10 expression on specific cell type (dashed line). A, CD4; B, CD4/CD25; C, CD11c; D, CD11b; E, CD19. The bar graph shows percentage of IL-10 expression on different cell types quantitatively (F). *, $p < 0.05$ compared with nonpregnant controls ($n = 3$ per group).

cells can secrete IL-10, other cell types including macrophages, dendritic cells and B cells can produce IL-10 as well (16). As a result of these pleiotropic effects, IL-10 is considered to have a role broader than just a Th1 or Th2 cytokine. Some investigators regard IL-10 as immunoregulatory.

Changes in TNF- α and IL-10 are frequently observed in MS and EAE during pregnancy or with estriol treatment. For instance, T cell clones isolated from MS patients and stimulated with PLP show increased IL-10 production and decreased TNF- α secretion when cultured in the presence of high dose estrogen (18). Similar results have been observed in PBMC from MS patients receiving oral estriol (19). Kim et al. (20) reported that implantation of estriol pellets in mice with EAE resulted in elevated levels of IL-10 derived from T cells. Moreover, a reduction in TNF- α production in the CNS as well as upon ex vivo stimulation with Ag has been observed with estrogen treatment in EAE (21, 22). Pregnancy and its associated hormone environment, therefore, seem to result in consistent alterations in the cytokine milieu.

To explore whether these changes in cytokine production represented an immunoregulatory or a Th2 type environment, we used intracellular cytokine staining to determine which cells were producing the IL-10. We reasoned that if the elevated IL-10 was the result of a Th2 response, we would observe an increase in this cytokine in the CD4⁺ cells of the mice immunized during pregnancy. We found that B cells, macrophages, dendritic cells and CD4⁺/CD25⁺ T cells all up-regulated their production of IL-10 in

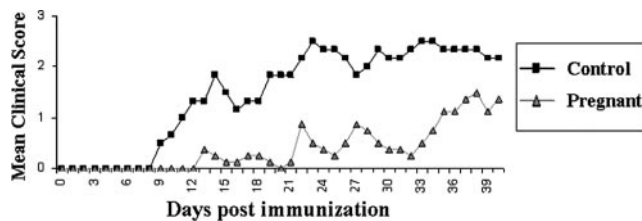


FIGURE 9. Late pregnancy suppresses EAE development in the C57BL/6 mouse. C57BL/6 mice were immunized with MOG 35–55 and CFA during late pregnancy (16–18 days pc), with nonpregnant mice serving as controls. Animals were monitored for clinical signs for a period of 40 days (control $n = 5$; pregnant $n = 7$).

mice immunized for EAE during pregnancy. Similar numbers of total CD4⁺ cells produced IL-10 between control mice and those immunized for EAE during pregnancy. These results, coupled with the lack of IL-4 and IL-5 production, suggest that when an animal is immunized during pregnancy, an immunoregulatory rather than a Th2 environment predominates.

Langer-Gould et al. (12) first reported that relapsing-remitting EAE in the SJL mouse was maximally suppressed during late pregnancy. We have confirmed these results and extended the observations into the postpartum period. Similar to the results of Langer-Gould (12), we found little evidence for a Th2 shift. Rather, pregnancy seems to result in an immunoregulatory environment, with increased production of IL-10. The prior study attributed the protective effect of pregnancy to an unidentified serum factor. We reasoned that the serum factor could indeed be IL-10 contained in the circulation. We measured the level of IL-10 in the serum of late pregnant mice, and found that it was below the limits of detection (unpublished observation). Thus, the protective factor identified by Langer-Gould is unlikely to be IL-10.

IL-10, however, has been found in many studies examining pregnancy and hormones, including investigations examining the protective effects of estrogen therapy on EAE (23). Similar to the findings in MS, we observed the later stage of pregnancy to be the most protective, while the postpartum period was disease enhancing. Interestingly, we observed a decrease in IL-17 when EAE is induced during pregnancy. Harnessing the immune environment associated with the late stage of pregnancy will likely be of great therapeutic benefit for women with MS, as it represents an immune competent environment with unique regulatory properties.

Disclosures

The authors have no financial conflict of interest.

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