PREGNANCY AND THE POST-PARTUM PERIOD REGULATE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS THROUGH IMMUNOREGULATORY CYTOKINE PRODUCTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Women with multiple sclerosis (MS) experience a decrease in relapse rate over the course of pregnancy, with the sharpest decline occurring during the third trimester. Abruptly following parturition, however, disease activity flares before returning to its baseline level three to six months later. As a result of these dramatic changes in disease, pregnancy and the post-partum period offer a unique opportunity to study both disease amelioration and disease exacerbation. We examined the effect of pregnancy on experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Our investigations focused on the effect of the different gestational stages on both the induction of disease as well as progression of pre-existing disease. We found that when EAE was induced in pregnant animals the clinical signs of disease were prevented while immunization with neuroantigen during the post-partum period led to increased relapse severity. These effects were not associated with alterations in lymphocyte activation or with changes in Th2 cytokine (IL-4 and IL-5) production. Instead, we observed a decrease in TNF-α and an increase in the immunoregulatory cytokine, IL-10, when animals were immunized during pregnancy, while a decrease in IL-10 occurred when the mice were immunized post-partum.
Similar effects were observed when pregnancy was induced during pre-existing EAE. When pregnancy was induced prior to the onset of EAE clinical signs, disease was delayed until after parturition. When pregnancy was induced after the onset of clinical signs, the severity of disease was decreased. Thus, regardless of when pregnancy was induced, suppression of pre-existing EAE was observed. Similarly, this protection from disease was associated with an increase in IL-10.

We extended these studies into a transgenic mouse model in order to determine the effect of the different gestational stages on autoreactive lymphocytes in an adjuvant free system. Each gestational stage was characterized by its own unique immunological environment, including a Th2 bias during mid pregnancy and lymphocyte hyperactivation in the post-partum period. The number of autoreactive cells present in the lymphoid organs, however, did not change. Together these investigations reveal that each gestational stage has its own distinctive effect on autoimmunity and demyelinating disease.
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropin hormone</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>BCL-2</td>
<td>B cell leukemia 2</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
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<tr>
<td>CCR5</td>
<td>CC chemokine receptor 5</td>
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<tr>
<td>CD</td>
<td>Cluster Designation</td>
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<tr>
<td>CDI</td>
<td>Cumulative Disease Index</td>
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<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CPM</td>
<td>Counts per minute</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
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<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
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<tr>
<td>E1</td>
<td>Estrone</td>
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<tr>
<td>E2</td>
<td>17-beta estradiol</td>
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<td>E3</td>
<td>Estriol</td>
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<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>ELISPOT</td>
<td>Enzyme linked immunosorbent spot assay</td>
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<td>ESR</td>
<td>Estrogen receptor</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine serum</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FOXP3</td>
<td>Forkhead box P3</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>ICOS-L</td>
<td>Inducible T-cell co-stimulator ligand</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2,3 dioxygenase</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>iκB</td>
<td>Inhibitor of nuclear factor κB</td>
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<td>IL</td>
<td>Interleukin</td>
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LN      Lymph Node
MBP     Myelin basic protein
MHC     Major Histocompatibility complex
MOG     Myelin oligodendrocyte glycoprotein
MRI     Magnetic resonance imaging
mRNA    Messenger ribonucleic acid
MS      Multiple sclerosis
NF-κB   Nuclear factor kappa B
p       Peptide
PBMC    Peripheral blood mononuclear cell
PBS     Phosphate buffered saline
pc      Post-conception
PD1     Programmed cell death 1
PDL-1   Programmed cell death 1 ligand
PDL-2   Programmed cell death 2 ligand
PE      Phycoerythrin
PLP     Proteolipid protein
PPD     Purified protein derivative
SEM     Standard error of the mean
SI      Stimulation index
SPL     Spleen
TCR     T cell receptor
Tg      Transgenic
TGF-β   Transforming growth factor beta
Th      T helper cell
TNF-α   Tumor necrosis factor alpha
VCAM    Vascular cell adhesion molecule
VLA-4   Very late antigen-4
1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that affects over 300,000 people in the United States. The disease is predominantly characterized by white matter lesions, or areas of demyelination, in the optic nerve, brain stem, cerebellum and spinal cord. An inflammatory response seemingly potentiates MS as cellular infiltrates and edema are commonly observed in the CNS of MS patients. In the later stages, however, the loss of axons around the initial lesion site contributes to the disease progression [1].

Several lines of evidence suggest that MS is an autoimmune disease. MS often occurs in families that have an increased prevalence of other autoimmune diseases, like rheumatoid arthritis and type I diabetes. It occurs more frequently in individuals who express particular MHC alleles and immunity related genes, and it exhibits great similarities with the induced autoimmune disease, experimental autoimmune
encephalomyelitis (EAE). The presence of autoantibodies to myelin antigens in the serum and CSF of MS patients is also suggestive of an autoimmune response. Although most people demonstrate autoreactive T cells in their immune repertoire, the activation of these cells is significantly increased in MS patients[2]. Thus, the inflammation observed in MS lesions is likely propagated by self-antigen.

The inflammation and subsequent axon loss associated with MS lead to impaired nerve conduction, resulting in a myriad of symptoms. Clinical presentations of the disease can range from mild to severe (paralysis) and exhibit great variability. Frequently, individuals first develop optic neuritis (or blurred and/or double vision). Other common symptoms include muscle weakness and spasticity, vertigo, loss of coordination and in the later stages, paralysis [3]. Most patients, however, report extreme fatigue, pain and uncomfortable sensory sensations as the most bothersome clinical presentations.

Since patients often present with variable symptoms, it is difficult to diagnosis MS. Medical examination and neurological history are used to identify the disease although MRI remains the most useful diagnostic tool. MS lesions are visible on MRI as a result of the local edema surrounding them. They are visualized as oval plaques appearing as areas of increased signal intensity on T2-weighted images. Such MRI findings have been found in 70-95% of patients with clinically definite MS and in approximately 50% of patients with an initial presentation of optic neuritis [3]. Despite success with MRI, there is no MS specific test, and other conditions like myelopathy and disseminated encephalomyelitis can present with similar findings.
Interestingly, the type of symptoms first experienced by a patient can be predictive of their prognosis. An initial presentation of optic neuritis, for example, suggests a better clinical outcome and is associated with the relapsing/remitting form of disease. Four forms of MS have been described. Relapsing remitting MS is the only type in which patients show little disease progression. This form is characterized by clearly defined relapses (or bouts of symptoms) followed by full or partial recovery and a period of no disease worsening. In contrast, patients with the progressive relapsing type of MS experience a continual worsening of disease during the periods between relapses. Primary progressive MS, the third form of disease, occurs when the disease progresses from the onset with few or no remittances. The fourth form of MS, termed secondary progressive, develops when a relapsing/remitting course converts to a progressive course [3].

MS is a complex disease, with both environmental and genetic factors appearing to play a role in its development. Genetics are implicated since monozygotic twins show a higher concordance (30%) of disease than dizygotic twins (5%). Individuals that have relatives with MS have a greater risk of developing disease than the general population, while half-siblings of an affected person have roughly half the risk of full siblings in acquiring the disease[4]. Certain ethnic groups tend to be at low risk (African blacks and native Americans), and an association between the HLA-DR2 MHC haplotype and MS has also been observed. Genetics, however, can not completely explain MS susceptibility [3]. In fact, there have been epidemics as well as clusters of disease development. For instance, Kurtze and colleagues described an apparent outbreak of MS in the Faroe Islands after British occupation in the 1940’s. An environmental factor also
seemingly explains the decreased risk of developing MS one acquires as one migrates closer to the equator. In fact, MS is rarely found in tropical or subtropical locations. Other evidence for environmental contributors includes associations between MS development and particular infections as well as exposure to specific occupational toxins[4]. Together, these findings suggest both genetic and environmental factors contribute to the development of MS.

Despite its heterogeneity and complex pathogenesis, several treatment options have been utilized in patients with MS. Most pharmacological agents have an immune modifying or suppressing effect and are largely non-specific in nature. The interferon-β related treatments, including IFN-β1a, IFN-β1b and Rebif, with their anti-inflammatory, anti-viral, and immunomodulatory activity, are often used in relapsing/remitting MS. It decreases T cell activation and limits migration to the CNS. Glatiramer acetate, or Copaxone, consists of a random polymer of four amino acids designed to mimic myelin basic protein and is thought to activate regulatory T cells. Mixoxantranec is another drug used to treat MS and suppresses a variety of immune cells through its cytolytic effects. All of these pharmacological agents, however, exhibit many side effects and have only partial efficacy in MS patients as a group[5]. Controversy exists as to whether any of these particular agents are beneficial in chronic progressive disease. For women with MS, the greatest modulation in disease activity has not been associated with a pharmacological treatment but rather with the physiological states of pregnancy and the post-partum period.
1.2 Multiple Sclerosis and Pregnancy

Historically, physicians advised their patients with MS to avoid pregnancy as it was thought to worsen the disease. Most doctors had based their belief on anecdotes from patients describing how their symptoms exacerbated abruptly after delivery. When investigators examined pregnancy and the post-partum period separately, changes in disease activity between the different stages were observed. In 1950, Tillman and colleagues reported for the first time that MS disease activity seemed to decline over pregnancy and increase post-partum [6]. Later studies, which differentiated pregnancy into trimesters, noted differences between the distinct stages. In Israel, one study following 66 women through 199 pregnancies noted that the relapse rate per woman in a non-pregnancy year was much higher than the rate recorded during the 3rd trimester of pregnancy. Several other investigations noted a sharp increase in relapse rate during the first few months of the post-partum period.[7-10] The first large, multinational study to examine MS disease activity during the different stages of pregnancy and to use stringent MS diagnostic criteria was conducted by Confavreux et al. in 12 European countries. This investigation studied 254 women over 269 pregnancies. The women were followed throughout the course of pregnancy and for 12 months after delivery in order to determine the rate of relapse per trimester. The rate of relapse recorded for each pregnancy stage was then compared to the rate of relapse for the year prior to pregnancy. This investigation found that 0.7 relapses occurred per woman per year during the year prior to pregnancy. A rate of 0.5 relapses was recorded for the 1st trimester of pregnancy, 0.6 for the 2nd trimester; and a rate of only 0.2 relapses was noted for the 3rd trimester. A
rate higher than baseline (1.2 relapses/year) was observed during the first three months post-partum. These studies reveal that MS disease activity is greatly influenced by the different gestational stages.

The cause of these changes in disease activity during pregnancy remains unknown. The observed alterations in relapse frequency, however, do correlate with changes in MRI. An investigation in the Netherlands reported on two patients that became pregnant while participating in a protocol involving serial MRI. Throughout pregnancy, both women experienced a reduction in the number of active lesions, with complete resolution occurring in the 3rd trimester. Shortly after delivery, however, lesion activity spiked for a few weeks before returning to baseline. Despite the low number of individuals in this study, the data supports the clinical observations noted during pregnancy and the post-partum period.

Although MS activity does worsen briefly following parturition, the relapse rate returns to baseline within a few months. A few investigations have even found pregnancy to be protective with regard to long-term prognosis. For instance, the risk of onset of MS is greatly reduced during pregnancy. Moreover, a decreased risk of MS in parous women compared to nulliparous women has been reported. Further studies have found a decreased risk of the relapsing/remitting form of disease converting to progressive MS in women who become pregnant after disease onset[11]. Most studies, therefore, suggest that pregnancy is protective with regard to the onset and progression of MS. In fact, the improvement in disease activity noted with pregnancy is greater than any therapeutic benefit observed with currently accepted treatments[12].
1.3 Experimental autoimmune encephalomyelitis (EAE)

Experimental autoimmune encephalomyelitis (EAE) is a CD4 T cell-mediated autoimmune disease studied as an animal model of MS. It is induced in laboratory animals by provoking an immune response to a neuroantigen or a myelin component. The disease exhibits many similarities to MS, including CNS perivascular inflammatory infiltrates, demyelination and axon loss. EAE presents with ascending paralysis, starting with a limp tail, leading to ataxia and partial hind limb weakness, before culminating in complete hind limb paralysis. The disease was first induced in mice in 1949 and has been extensively employed as an MS model in which to examine the basic pathogenesis of autoimmune disease as well as to investigate the efficacy of various treatments[13].

EAE is induced in one of two ways: 1) by active immunization or 2) by adoptive transfer. Active immunization requires the injection of a neuroantigen combined with adjuvant (i.e. complete Freund’s adjuvant, Pertussis toxin) while adoptive transfer involves restimulating and transferring spleen cells from an actively immunized animal into a naïve recipient animal. Depending on the experimental question, each induction method can be used to focus on a particular phase of EAE. The establishment of EAE consists of two phases, the induction phase and the effector phase. During the induction phase, initial antigen processing and presentation occurs, followed by the primary activation of lymphocytes. The effector phase is when the activated cells (i.e. T cells, macrophages, etc.) mediate their functional responses and migrate to the CNS where tissue injury is evident. Active immunization allows both phases to occur in the same animal, while the adoptive transfer method of EAE induction permits only the effector phase to take place in the recipient.
Myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and myelin basic protein (MBP) are antigens commonly used to induce EAE. The disease can be induced in rats, guinea pigs, monkeys, rabbits and mice, but only in genetically susceptible strains. Frequently employed murine strains include SJL/J, B10.PL, PL/J and C57BL/6 mice. Successful EAE induction in each of the strains depends upon the nature of the immunogen used. C57BL/6 mice develop EAE when immunized with a peptide of MOG (35-55) while the SJL strain responds poorly to this antigen. Instead, SJL mice show a high incidence of EAE when immunized with a peptide component of PLP (amino acids 139-151). B10.Pl mice, on the other hand, develop EAE with MBP immunization [13, 14]. These disparities in EAE susceptibility to different myelin components relate to antigen processing and presentation by particular MHC histocompatibility gene products as well as the frequency of the autoreactive cells present in the different strains.

Regardless of the antigen or peptide used to induce EAE, an autoimmune Th1 response mediates EAE. Th1 immunity, associated with cell mediated responses, leads to production of cytokines such as IFN-γ, IL-2 and TNF-α while Th2 immunity is linked with the production of IL-4, IL-5 and IL-10 [15]. EAE is regarded as a Th1 disease since large amounts of IFN-γ, IL-2 and TNF-α are produced by lymphocytes in immunized animals. In fact, neutralizing antibodies directed against TNF-α can protect against both active EAE and the adoptive transfer of disease[16]. Immune deviation therapies that promote a shift in immune responses away from Th1 and toward Th2 responses often suppress EAE. For instance, the intraperitoneal injection of MBP combined with incomplete Freud’s adjuvant (a known Th2 biasing agent) prevents EAE induction.
Exogenous administration of soluble IL-4 as well as the delivery of IL-4 or IL-10 by retrovirus-transduced T cells can prevent or treat the disease [17]. IL-10 transgenic mice are completely resistant to the development of EAE, while IL-10 knockout mice exhibit more severe EAE and increased proinflammatory cytokine production [18]. Overall, these studies illustrate that Th1 producing cells are required to initiate disease, while Th2 cytokines play an immunomodulatory role in EAE.

Although EAE is readily inducible in genetically susceptible strains, particular research questions have required the development of T cell receptor transgenic mice for the study of particular aspects of EAE. One frequently used transgenic mouse strain overexpresses the Vα4/Vβ8.2 T cell receptor (TCR), which is specific for the immunodominant N-terminal 1-11 amino acids of MBP. With 95% of the CD4+ T cells expressing the autoreactive TCR in this strain, this transgenic animal permits investigation of the immunobiology of autoimmune disease in an in vivo setting. Moreover, it serves as a useful strain in which to explore the limits of therapeutic modalities [19].

1.4 EAE and Pregnancy

Most studies in EAE have examined the effect of pregnancy on acute or monophasic forms of disease. In these models, animals exhibit a limited bout of clinical signs followed by a complete resolution of disease. The earliest study with regard to EAE and pregnancy examined the effect of pregnancy on monophasic disease in guinea pigs and rats. In this investigation, EAE developed approximately 11 days after immunization in the non-pregnant guinea pigs, while the onset of disease was delayed until 25-29 days for the animals immunized during pregnancy. A similar effect was
observed in rats with EAE, as the onset of disease was greatly delayed in those immunized during pregnancy. Interestingly, CNS histopathological infiltrates were observed in all immunized animals regardless of pregnancy state[20]. Other studies in acute models have reported that the onset of EAE is often delayed in pregnant animals until after parturition. Even then, however, the incidence of animals developing disease is markedly reduced and the severity of clinical score dramatically decreased [21, 22]. Investigations in Lewis rats have revealed that the greatest protection from disease occurs when animals are immunized during late pregnancy rather than in the early stages [23, 24]. SJL mice, which develop chronic relapsing disease show similar results. The incidence of disease in this strain is reduced from 71% in control non-pregnant mice to 32% in mice immunized during late pregnancy. The latter half of pregnancy also has a greater suppressive effect on pre-existing EAE than the earlier stages [25].

Little is known about the mechanism by which pregnancy offers protection from EAE. An investigation with Lewis rats found increased mRNA expression for both Th2 (IL-4, IL-10) and Th1 cytokines in the spinal cords of animals immunized during pregnancy. Limited evidence also exists for the presence of an immunosuppressive serum factor in pregnant animals [24, 25]. For instance, Langer-Gould et al found no differences in the ability of lymphocytes from pregnant animals to produce Th1 and Th2 cytokines or proliferate in response to antigen. However, when lymphocytes were stimulated in the presence of pregnancy sera, proliferation and the production of IL-2 were decreased [25]. Most investigation in this area has explored the effect of pregnancy hormones on EAE.
1.5 Hormones and EAE

Pregnancy is a dynamic and complex physiological state that results in alterations to numerous hormones. The changes in autoimmune disease that occur over pregnancy and the post-partum period are accompanied by marked changes in hormone levels. With regard to MS and EAE, investigations have primarily focused on the major reproductive hormones. Of particular interest are those known to undergo abrupt changes between the 3rd trimester and the post-partum period, including the estrogens, progesterone, ACTH, cortisol and prolactin.

The estrogens exist in three active forms: 1) estrone, 2) 17-β estradiol and 3) estriol. All three increase over the course of gestation. They are predominantly produced by the maternal ovaries until 5-8 weeks of gestation when their production switches to the fetal-placental unit. Both estrone and 17-β estradiol increase to supra-physiological concentrations (from < 1ng/ml to over 10 ng/ml) during gestation. Although estriol is produced in low concentrations in non-pregnant women, it is viewed as primarily a hormone of the placenta, and it increases over 1000-fold in pregnant women relative to levels found in the menstruating woman. Serum levels of progesterone also rise over pregnancy, exhibiting a 6-8 fold increase in comparison to non-pregnant levels. Like the estrogens, progesterone production also comes from the fetal-placental unit starting around 7 weeks of gestation. Other important hormones known to increase over pregnancy include ACTH and cortisol. Whether their biological activity also increases during gestation remains unknown. Abruptly following parturition, the levels of most of these hormones
decrease dramatically. Prolactin, however, increases over the course of pregnancy and remains elevated post-partum. In fact, in lactating females, its levels can be increased for several months [26].

Because the estrogens are known to influence immune responses and they undergo such significant changes in concentration during pregnancy, their effects on EAE have been a predominant focus [27]. These studies have largely found the estrogens to offer protection from disease [28-30] Bebo et al found that the implantation of 17-β estradiol and estriol pellets lowers the incidence of disease when given prior to EAE immunization. Of the estrogen treated mice that did develop EAE, the onset of disease was delayed and the peak score and cumulative disease indices were reduced. These effects were stronger in high dose estrogen treated mice, but protection from disease could still be demonstrated with diestrus concentrations of the hormones. The two hormones, 17-β estradiol and estriol, were found to be equally effective in decreasing the incidence of disease and lowering the severity of EAE [28]. These protective effects have been observed in SJL, B10.PL and C57BL/6 mouse strains as well as in the Lewis rat, suggesting that estrogen’s ameliorating properties are not strain or species specific [28, 29, 31].

There is controversy as to whether estrogen effects on EAE are mediated through a direct or indirect mechanism. Steroid hormones, as lipid soluble substances, produce their effects through the binding of a nuclear receptor. 17-β estradiol has two known receptors: 1) estrogen receptor alpha and 2) estrogen receptor beta [32, 33]. Knockout
mice have been used to study the effect of the different receptors on EAE. These investigations have found that the ESR1 or estrogen receptor alpha is necessary for estrogen to suppress EAE while the absence of ESR2 has no effect on disease [34-36].

The mechanism by which the estrogens alter disease through ESR1 is unknown. Alterations in cytokine production have been reported following treatment with estrogen. For instance, spleen cells from estrogen treated mice produce less TNF-α and IFN-γ when stimulated ex vivo. Decreases in these cytokines have also been noted in the CNS of immunized mice receiving estrogen [31, 36-39]. The levels of the Th2 cytokines, IL-4 and IL-5 remain low and do not seem to increase with estrogen treatment [36]. In fact, the ameliorating effects of estrogen have been observed even in the absence of IL-4 and IL-10. [31] These investigations suggest that estrogen may lower Th1 cytokines, but have minimal impact on the Th2 cytokines. Moreover, animals receiving estrogen treatment show a reduction in the expression of chemokines and their receptors, and in some cases have smaller CNS infiltrates.[38, 40] Thus, estrogen seems to promote a reduction in the inflammatory environment.

1.6 Hormones and MS

As a result of estrogen’s ameliorating effects on EAE, some studies have examined the effect of pregnancy-associated hormones on MS. Correale and Gilmore have explored the effect of several hormones in vitro on the cytokine secretion of T cell clones from MS patients and normal control subjects. They have reported that the three estrogens (E1, E2 and E3) affect cytokine production differently than either progesterone or dexamethasone. All three major forms of the estrogens were observed to increase
levels of IL-10 and IFN-γ production in a dose-dependent manner. TNF-α modulation, however, occurs in a biphasic fashion, with low concentrations of the estrogens enhancing production and high concentrations inhibiting secretion. The estrogens were found to have no effect on the production of IL-4 or TGF-β. Progesterone, on the other hand, was not found to influence IFN-γ or TNF-α, but does potently upregulate IL-4 production. Dexamethasone, like the estrogens, lowered the levels of the Th1 cytokines IFN-γ and TNF-α, but unlike the other hormones, increased TGF-β. Overall, these effects were observed in all the T cell clones regardless of whether the cell line was classified as Th1 or Th2. Thus, each of the pregnancy associated hormones seems to have its own unique effects on cytokine secretion [41, 42].

A small clinical trial was recently conducted to determine whether estriol had an ameliorating effect on MS similar to the effects noted during pregnancy. This investigation found that the number and volume of gadolinium enhancing lesions significantly decreased in estriol treated patients compared to their 6-month pretreatment baseline. This ameliorating effect disappeared when hormone therapy was stopped, but quickly returned when estriol treatment resumed. To address whether cell-mediated responses were altered during the estriol treatment, DTH responses to tetanus toxoid were measured throughout the study and PMBCs were collected for analysis of proliferation and cytokine production. DTH responses decreased during estriol treatment when compared to the pretreatment baseline. No differences in proliferation were noted in the PBMCs, but estriol treatment lead to increased production of IL-5 and decreased
Although little is about the mechanism of estriol action in vivo, this investigation illustrates that harnessing the pregnancy environment could offer effective MS treatments.

1.7 Mechanisms by which pregnancy may modulate immunity

1.71 Immune suppression

The evolution of viviparity has long puzzled immunologists. The fetus in this situation is an allograft (and non-self) and avoids being targeted by the maternal immune system. One hypothesis that has been proposed to explain this paradox is that the maternal immune system simply becomes anergic during pregnancy. Indeed, several investigations have found evidence for pregnancy associated immune suppression. Early studies noted that mixed lymphocyte reactions (MLR) are inhibited during pregnancy [45]. In particular, pregnancy sera has been reported to decrease proliferative responses in autologous MLR and to suppress CD3 zeta chain expression in T cells [46, 47]. Other investigations have revealed that the cytolytic activity of T lymphocytes in response to Epstein-Barr virus is also inhibited during all three trimesters of pregnancy, but not during the post-partum period [48]. Even decidual cells exert immunosuppressive activity as they have been found to suppress cytotoxic T cell responses in both primary and secondary CTL assays as well as the proliferation of T cell lines [49].

One means by which pregnancy may promote an immunosuppressive environment is through the downregulation of specific transcription factors. Peripheral blood mononuclear cells from pregnant women show decreased activity of nuclear factor κB (NF-κB). This molecule is a rapid response transcription factor that is expressed in a variety of cells and is involved in the generation of multiple gene products. Many of the
proteins modulated by this factor are associated with inflammation and autoimmunity, including several cytokines, chemokines, adhesion molecules and immunoreceptors. NF-κB serves an important role in the signaling cascade of TNF-α and is also involved in the induction of the pro-inflammatory cytokines IL-2, IL-6 and IL-12. The p65/p50 components of NFκB, in particular, have been found to be decreased in T lymphocytes from pregnant women. Higher levels of IκB (an inhibitor of NF-κB) have been noted during pregnancy. This decrease in NFκB activity remains throughout pregnancy and cannot be overcome even upon stimulation with a strong mitogen, such as PMA [20]. Interestingly, stimulation of MBP specific T cells with estriol has also shown reduced NF-κB activity. This effect can be reversed through the addition of the estrogen receptor antagonist, tamoxifen, which leads to the degradation of IκB. Thus, pregnancy may modulate immunity by altering the biological activity of an important inflammatory transcription factor.

Pregnancy may also suppress immunity through the production of TGF-β. This cytokine has highly pleiotropic properties and its three isoforms (TGF-β 1,2,3) are instrumental in regulating immunity. TGF-β 1 knockout mice, for example, exhibit severe multi-organ inflammation and autoimmunity. Although it can affect almost all immune cells, TGF-β largely influences T cells. It inhibits T cell proliferation as well as Th cell differentiation, preventing both Th1 and Th2 development [50]. Cells in murine allogeneic pregnancy have been found to secrete a novel immunosuppressive factor similar to TGF-β2. This molecule is released by murine decidua into supernatant cultures in a biologically active form but its molecular weight is distinct from TGF-β [51]
Interestingly, this factor can be produced in Scid mice, indicating that T and B cells are not necessary for its production [52]. Regardless of its source, TGF-β effectively suppresses primary and secondary CTL responses during pregnancy [53]. In addition to this novel form of TGF-β, both fetus and mother make nascent TGF-β [54]. In fact, all three TGF-β isoforms are made by decidual and villus tissue [55]. The inhibition of PBMC proliferation associated with amniotic fluid has been correlated with increased production of both TGF-β1 and TGF-β2 [56].

TGF-β, however, is just one of many factors upregulated during pregnancy that have immunosuppressive properties. Other pregnancy associated products with immune altering effects include alpha fetoprotein, early pregnancy factor, pregnancy-specific glycoproteins, and the enzyme, indoleamine 2,3 dioxygenase or IDO. In particular, IDO has been shown to have potent effects on maternal immunity. This enzyme serves as the first and rate-limiting factor in the oxidative degradation of tryptophan. Since activated T cells require unrestricted access to tryptophan during the first G1 phase following activation, IDO can limit T cell survival. As a result, IDO can prevent in vitro T cell proliferation and lead to local microenvironments which are suppressive for T cells [57-58]. IDO seemingly plays a large role in pregnancy, as mice exposed to IDO inhibitors often reject allogeneic fetuses [59]. Incidentally, the concentration of tryptophan decreases over pregnancy [60]. These results suggest that IDO may be an essential regulator of maternal immunity during pregnancy.

1.72 Th2 Bias

Although evidence exists for pregnancy associated immune suppression, Thomas Wegmann postulated that during gestation there is simply a shift in the type of immune
response generated. Specifically, he proposed that during pregnancy, immunity is directed away from cell-mediated (Th1) responses and toward antibody driven (Th2) responses. He theorized that cytokines like IL-12, IFN-\(\gamma\), TNF-\(\alpha\), and IL-2 triggered fetal absorption, while the Th2 cytokines IL-4, IL-5, IL-10 and IL-13 promoted a successful pregnancy [61]. Wegmann based this Th2 hypothesis largely on his observations that IL-4, IL-5 and IL-10 are spontaneously secreted by placental and decidual cell cultures during all three trimesters of pregnancy, while IFN-\(\gamma\) is only secreted for a few days during early pregnancy[62]. The idea that a Th2 bias occurs during pregnancy has been supported by similar investigations examining other maternal/fetal tissues. For instance, first trimester human chorionic villi have been found to express high levels of IL-6 and IL-10 but not IFN-\(\gamma\), IL-2, TNF-\(\alpha\) or IL-1[63]. Additionally, other studies have noted the spontaneous secretion of IL-4 and IL-10 in decidual cells as well as localization of IL-10 to the maternal fetal interface[64-67].

The functional relevance of these cytokines has been explored in both successful and unsuccessful pregnancies. When injected into pregnant mice, Th1 cytokines TNF-\(\alpha\) and IFN-\(\gamma\) have been found to cause abortions, while Th2 cytokines have no untoward effects [68]. In human investigations, Picinni et al found that decidual T cells from women with unexplained recurrent abortions produce less IL-4 and IL-10 when compared to T cells from women with successful pregnancies [69]. PBMCs from women with a history of normal pregnancies have also been noted to produce more Th2 cytokines than cells from women with recurrent spontaneous abortion[68]. The effects of Th1 and Th2 cytokines have been studied extensively in the abortion prone CBA X DBA/2 mating combination. Chaouat et al showed that placentas from this mating
produce less IL-4 and IL-10 than other mating combinations. In fact, injection of recombinant IL-10 into pregnant females of this mating combination reduced the resorption rate, while injections of anti-IFN-γ only partially restored pregnancy success[70]. These results suggest that Th1 cytokines are detrimental for pregnancy while Th2 cytokines may be necessary for a successful environment.

When a pregnant animal must mount a systemic Th1 immune response, the risk of resorption greatly increases. Krishnan et al have demonstrated that when C57BL/6 mice are infected with *Leishmania major* (which provokes Th1 immunity), they exhibit an increased number of implantation failures as well as an increase in resorptions. These unsuccessful pregnancies are associated with increased production of IFN-γ and TNF-α and decreased production of IL-4 and IL-10 when compared to non-infected pregnant controls. Interestingly, compromised pregnancies have not been observed in BALB/c mice, which typically produce Th2 cytokines upon infection with *L major* [71].

Using the same C57BL/6 *L major* infection model, investigators noted that although the infected pregnant mice did produce more Th1 cytokines than their non-infected pregnant counterparts, it was not enough to clear the bacteria. Pregnant mice developed larger cutaneous lesions that showed no signs of resolution for over 2 months, while non-pregnant infected mice began to show reductions in lesion size within 30 days. This inability to clear the infection was associated with increased levels of IL-4, IL-5 and IL-10 in the pregnant mice[72]. These findings are corroborated with studies using *Neospora caninum*, an intracellular parasite. Mice infected with this organism produce more Th2 cytokines than their infected non-pregnant counterparts[73]. Thus, pregnancy seems to be associated with global Th2 cytokine production.
Several hormones that are elevated during pregnancy can elicit Th2 type responses. Although stimulatory at low doses, increased levels of estrogen have been reported to decrease Th1 or cell mediated responses, including DTH reactions to oxazolone, keyhole limpet hemocyanin, collagen and purified protein derivative (PPD). Estrogens can also reduce the number of leukocytes present in the draining lymph nodes and spleen 24 hours after DTH induction. Moreover, the leukocytes present proliferate less to antigen and produce increased levels of IL-4 and IL-10. Interestingly, estrogens have also been shown to augment antibody responses to several autoantigens as well as increase the number of plasma cells in the spleen [74]. Th2 responses may also be promoted by the estrogens through an increased expression of the survival factor BCL-2 in Th2 T cell clones [75].

In addition to estrogen, progesterone also has been shown to enhance Th2 responses. IL-4, for instance, has been shown to increase during the luteal phase of the menstrual cycle, correlating to increases in progesterone [76]. Studies investigating the differentiation of T helper cells have revealed that progesterone treatment inhibits IFN-γ production in a dose dependent manner and increases intracellular IL-10 expression [77]. Thus, several hormones that increase over pregnancy have Th2 promoting properties.

Although many different lines of evidence suggest that Th2 immunity is more prevalent during pregnancy, there are a few studies that indicate that the modulation is more complex. For instance, IL-4 and IL-10 are not necessary for the successful completion of murine allogeneic pregnancy [78, 79]. In addition, cytokines like IFN-γ are observed in healthy pregnancies [80]. The cytokines IL-12 and IL-18 appear in
maternal-fetal tissues at precise locations and times, suggesting that they too play a role in successful pregnancy [81]. Although a Th2 bias may occur during pregnancy, this shift may not be completely exclusive.

1.73 Regulatory cells

Another mechanism by which pregnancy has been shown to alter immune responses is through the upregulation of regulatory T cells. There are many types of these cells, but they all share the ability to limit immune responses and prevent inflammation and/or autoimmune mediated pathology. Such cells have been classified into five major groups: natural CD4+/CD25+ T cells, T regs or Th3 cells, anergic T cells, T suppressor cells and double negative (CD4-/CD8-/CD3+) cells. There is substantial overlap in characteristics as well as function of these cell types, making specific designations difficult. Despite difficulties in categorizing regulatory T cells, most of them inhibit the proliferation and activation of other T lymphocytes. They frequently produce the anti-inflammatory cytokine IL-10 and to a lesser degree TGF-beta. Cell-to-cell contact has been noted as a possible mechanism by which these cells suppress activation. Anergic T cells seem to be antigen-specific, while the majority of regulatory T cells are not specific in nature and seem to mediate their effects through bystander suppression. The most well-characterized of the regulatory T lymphocytes are the naturally occurring CD4+/CD25+ cells that typically comprise 5-7% of all leukocytes. In addition to their expression of CD4 and CD25, these cells abundantly express the transcription factor FoxP3 and are easily isolated from naïve animals [82, 83].
Chaouat and colleagues were the first to report an association between pregnancy and the presence of regulatory T cells. In particular, their studies noted that splenocytes from pregnant animals suppressed mixed lymphocyte reactions [84]. These cells were later identified as CD4-/CD8-/CD3+ cells that produced suppressive soluble factors [85]. More recently, increases in the naturally occurring CD4/CD25 T regulatory cells have been noted during pregnancy. A progressive increase in the number of CD4+/CD25+ cells occurs in the blood of pregnant women, with cell numbers peaking during the 2nd trimester, plateauing through the 3rd trimester and then declining following delivery. These cells express high levels of FoxP3 and can suppress CD3 induced proliferation [86]. Increases in the number of CD4+/CD25+ cells have also been noted along the maternal-fetal interface as well as in umbilical cord blood of pregnant women [87-89]. Interestingly, this increase in CD4+/CD25+ cells also has been noted in the blood of pregnant women with MS [90].

Aluvihare and colleagues reported that the levels of CD4+/CD25+ cells also increase in the spleen and lymph nodes of pregnant animals and that these cells are essential for successful pregnancy. Specifically, they showed that transferring splenocytes from pregnancy mice into pregnant nude BalB/c mice (which lack T cells) does not affect the outcome of pregnancy. Transferring CD25 depleted splenocytes into the pregnant nude mice, however, results in complete pregnancy failure. These results suggest that CD4+/CD25+ cells increase during pregnancy to prevent fetal allograft rejection [91]. The hormones associated with pregnancy, may be responsible for these
increases in regulatory cells, as studies treating immune cells with estrogen have found increases in the expression of FoxP3 expressing cells as well as other novel regulatory cells [92, 93].

**Objectives**

Pregnancy and the post-partum period dramatically alter the clinical course of MS, providing a unique opportunity to study both disease amelioration and exacerbation. The improvement in disease activity that occurs during pregnancy is more significant than any known pharmacological treatment to date, deeming it essential that we explore how and why these clinical alterations occur. We chose to conduct our examinations in the animal model EAE. Our goal was to examine the effect of the different gestational stages on the clinical course of EAE as well as to explore the immunological mechanisms by which their effects are mediated. Specific objectives for our investigations were: 1) to determine the effect of pregnancy and the post-partum period on the induction of EAE; 2) to determine the effect of pregnancy and the post-partum period on pre-existing EAE; and 3) to determine the effect of the different gestational stages on the encephalitogenic capacity of naïve autoreactive T cells. In exploring these three fundamental areas of investigations, we have examined several hypotheses with regard to how pregnancy and the post-partum period mediate their effects. We have addressed whether pregnancy results in immune suppression, a Th2 bias or an immunoregulatory environment. Our studies have revealed that each gestational stage has its own unique influences on EAE.
CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

SJL/J

Age-matched timed pregnant and non pregnant female SJL/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 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 days, and then immunized on days 15-18 pc. Post-partum mice were obtained on days 13-15 pc, allowed to acclimate and undergo parturition (days 20-21) and then the mice were immunized 22-25 days p.c.

Vα4/Vβ8.2 MBP TCR Tg

Vα4/Vβ8.2 MBP TCR transgenic mice were obtained from Dr. Charles Janeway and then maintained in our breeding colony at the Ohio State University animal facility [19]. Progeny were screened by flow cytometry for expression of the Vβ8.2 transgene on
CD4+ peripheral blood lymphocytes. Transgene positive animals were used at 6-10 weeks of age. All mice were maintained on a 12-hour light/dark cycle and given food and water *ad libitum*.

### 2.2 Pregnancy Induction

For the studies regarding the effect of pregnancy on EAE induction, timed pregnant mice were ordered from the Jackson Laboratories. For all other investigations, pregnancy was induced at the Ohio State University animal facilities. Pregnancy was induced by housing a single male mouse with 3-4 female mice for 3 days. Confirmation of pregnancy was performed by dissection in mice sacrificed prior to parturition or by daily inspection for pups in non-terminal investigations.

### 2.3 Antigens

Peptides were purchased from Sigma-Genosys (The Woodlands, Texas) and were purified by HPLC, including PLP 139-151 (HCLGKWLGHPDKF), PLP 178-191 (NTWTTCQSIAFPSK), PLP 258-273 (IAATVNFAVLKMGGR), MBP 87-99 (VHFFKNIVTPRP), MBP 84-104 (VHFFKNIVTPRTPPSQGKGR) and MBP NAc 1-11 (ASQKRPSQRHG). All peptides had a purity >90% by HPLC.

### 2.4 EAE Immunization

Mice were immunized subcutaneously over four sites on the flank with 0.2ml of an emulsion containing 150 µg of PLP 139-151 in PBS and an equal volume of complete Freund’s adjuvant (CFA) containing 200 µg heat-killed *Mycobacterium tuberculosis*,
Jamaica strain. 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.

2.5 Histopathology

Intact spinal cords and brains were removed from mice in all groups (control, pregnant and post-partum) at varying times after immunization, including day 15 for all groups, day 25 for control and pregnant mice, and day 35 for control and post-partum mice. Tissues were fixed in 10% phosphate buffered formalin and then dissected and embedded in paraffin. Sections were then processed for hematoxylin and eosin staining. Scores were assigned based on the number of cell layers of infiltration.

2.6 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% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 5 x 10^{-5} M 2-ME in round-bottom 96-well plates (4 X10^5 cells/well). Cells were cultured with medium alone or with PLP 139-151 (30µg/ml), PLP 178-191 (15µg/ml), PLP 253-271 (15µg/ml), MBP 87-99 (15µg/ml), MBP 84-104 (15µg/ml), or anti-CD3 (2µg/ml). Cultures were incubated for 72 hours at 37°C and 7%CO₂, including an 18 hour pulse with [³H] thymidine (1uCi per well). Cultures were harvested onto glass-fiber filter mats using a Skatron harvester (Skatron, Sterling, VA) and were counted
by liquid scintillation on a Wallac betaplate (LKB, Wallac, MD). Stimulation indices were calculated by dividing the CPM from peptide-stimulated cultures by the CPM from unstimulated cultures.

2.7 Supernatant Collection for Cytokine Determination

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% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 ug/ml streptomycin and 5 x 10^{-5} M 2-ME in round-bottom 96-well plates (4 X10^5 cells/well). Cells were cultured with medium alone or with PLP 139-151 (30ug/ml), PLP 178-191 (15ug/ml), PLP 253-271 (15ug/ml), MBP 87-99 (15ug/ml), MBP 84-104 (15ug/ml), or anti-CD3 (2ug/ml). Supernantants were collected after 24, 48 or 72 hours of culture at 37°C with 7% CO₂.

2.8 ELISPOT analysis for cytokine-producing cells

Frequencies of cytokine secreting cells were determined for IFN-γ, IL-2, IL-4, IL-5, IL-10 and TNF-α. Microtiter plates with nitrocellulose bottoms (Millipore, Bedford, MA) were coated overnight at 4°C with anti-IFN-γ (R46A2), anti-IL-2 (JES6-1A12), anti-IL-4 (11B11) or anti-IL-5 capture antibody (at 2-5ug/ml). After washing, plates were blocked with 1% BSA (Sigma, St. Louis, MO) for two hours at room temperature. Spleen and lymph node cells were resuspended in HL-1 medium and then cultured in triplicate at 4 x 10^5 cells per well with medium alone or with the following peptides (15-
30ug/ml): PLP 139-151, PLP 178-191, PLP 253-271, MBP 87-99, MBP 84-104, or anti-CD3 (2ug/ml). Cultures were maintained at 37°C for 24 hours (IFN-γ, IL-2 and TNF-α), 48 hours (IL-4 and IL-5) or 72 hours (IL-10). Plates were washed and cytokine specific biotinylated antibodies were added. After overnight incubation, an alkaline phosphatase-conjugated goat anti-biotin IgG ((Vector, Burlingame, CA) was added to plates for two hours. After a final wash, plates were developed with 5-bromo-4-chloro-3-indolyolphosphatase/nitro blue tetrazolium phosphatase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Frequencies of TNF-α and IL-10 secreting cells were determined using cytokine specific kits from R and D systems. Image analysis of ELISPOT plates was performed using the KS ELISPOT system (Zeiss, Oberkochen, Germany). Data are expressed as the number of cytokine-producing cells per million +/-SEM for all animals in a group.

2.9 Cytometric Bead Array (CBA)

IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were detected using the mouse Th1/Th2 cytokine CBA kit from BD Biosciences (San Jose, CA). Fifty µl of culture supernatant was mixed with 50 µl of the mixed capture beads and 50 µl of the mouse phycoerythrin detection reagent. Samples were incubated at room temperature for 2 hours in the dark, washed, and then resuspended in 400 µl of wash buffer before flow cytometric analysis (FACSCalibur, Becton-Dickinson, San Jose, CA). Data was analyzed using CBA.
software. Standard curves were generated for each cytokine using the mixed bead standard provided in the kit, and the concentration of cytokine in the cell supernatant was determined by interpolation from the appropriate standard curve.

2.10 Flow Cytometric Analysis

Single cell suspensions of lymphoid cells derived from lymph nodes and spleens were stained for CD4, CD8, CD11b and CD11c with FITC-conjugated mAb and CD25, CD28, CD44, CD62L, CD69, CD80, CD86, PD-1, PD-L1, PD-L2, ICOS, ICOS-L with PE-conjugated mAb using two or three color flow analysis. Suspensions of $10^6$ cells were incubated with labeled antibodies diluted in PBS plus azide with 2% mouse serum. After 30 minutes, cells were washed and fixed with 1% paraformaldehyde. Isotype control mAbs (Pharmingen) were matched for fluorochrome and used for cursor placement. Lymphocytes were gated based on forward versus side scatter and a total of 10,000 events were analyzed by flow cytometry (FACS Calibur, Becton-Dickinson, San Jose, CA).

2.11 Intracellular Cytokine Measurement

Spleens were removed from mice on day 15 post immunization. Single cell suspensions were prepared and suspended in RPMI 1640 containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 ug/ml streptomycin and 5 x
$10^{-5}$ M 2-ME in round-bottom 6-well plates (20 X$10^6$ cells/well). Cells were cultured with PLP p139-151 (30ug/ml) for 48 hours, including a pulse with the Golgi inhibitor, monensin, for the last five hours. Intracellular cytokine staining was performed following manufacturer’s instructions (IL-10 intracellular cytokine staining protocol Ebioscience). Briefly, cells were fixed with fixation buffer (ebioscience) and then permeabilized. Cells were then stained with anti-IL-10 mAb. Samples were analyzed using flow cytometry.

### 2.12 Statistical Analysis

A two-tailed Student’s t test was used to determine statistical differences when comparisons were made between two groups with parametric data. A one-way ANOVA was used to determine statistical significance when more than two groups were compared. $\chi^2$ analysis was used to determine statistical significance when comparing the incidence of disease between groups.
CHAPTER 3

THE EFFECT OF DIFFERENT GESTATIONAL STAGES ON EAE INDUCTION

3.1 Results

3.11 Pregnancy

_Pregnancy reduces the incidence and severity of EAE._

In order to determine the effect of different pregnancy stages on the induction of EAE, we immunized SJL mice with the immunodominant epitope of PLP (p139-151) during mid pregnancy (8-11 days post conception) and late pregnancy (days 15-18 post conception), with non-pregnant female mice serving as controls. We found that when EAE was induced during pregnancy, few mice developed clinical signs of disease (Table 3.1). Most of the non-pregnant mice showed clinical signs of EAE, while only 33% of the mice immunized during the mid stage of pregnancy developed signs of disease. When immunization occurred during the late stage of pregnancy, even fewer mice (13%) exhibited disease. Of the few mice that did develop EAE in the late pregnancy group, the day of disease onset was delayed approximately 12 days. The disease severity, as
<table>
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<th></th>
<th>Incidence</th>
<th>Day of Onset ±SEM</th>
<th>CDI±SEM</th>
<th>Max Score±SEM</th>
<th>Relapse</th>
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<tr>
<td>Control</td>
<td>89% (63/71)</td>
<td>16.1 +/- 1.8</td>
<td>22.0 +/- 5.2</td>
<td>2.5 +/- 0.2</td>
<td>38%</td>
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<tr>
<td>Mid pregnancy d</td>
<td>33% (2/6)</td>
<td>18.5 +/- 0.5</td>
<td>8.0 +/- 3.0</td>
<td>2.0 +/- 0.0</td>
<td>0%</td>
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<tr>
<td>Late pregnancy</td>
<td>13% (3/24)*</td>
<td>28.5 +/-0.5*</td>
<td>6.0 +/- 1.0</td>
<td>2.0 +/- 0.0</td>
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Table 3.1 Summary of clinical disease characteristics for pregnancy and EAE induction

a. Mean Cumulative Disease Index (the sum of clinical scores for the entire observation period)
b. Mean Maximum clinical score recorded throughout observation period
c. Percentage of animals that exhibited relapses (an increase in clinical score greater than or equal to 1 for more than one day after the initial bout of disease)
d. Data reported for animals with a confirmed pregnancy; four animals resorbed/aborted (100% disease induction)

* p<0.05 when compared to non-pregnant controls
Figure 3.1 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 mid (8-11 days post conception) and late pregnancy (15-18 days post conception), with non-pregnant mice serving as controls. Animals were monitored for 50 days for clinical signs of disease. Control N=24; Mid pregnancy N=6; Late pregnancy N=11; Graph is representative of three experiments.
measured by cumulative disease index, was significantly reduced when EAE induction occurred during the course of pregnancy (Table 3.1). Interestingly, none of the animals immunized during pregnancy displayed relapses (Figure 3.1). Thus, pregnancy (particularly the late stage) offers protection from EAE.

Pregnancy does not suppress EAE through general immune suppression or by interfering with lymphocyte trafficking.

DTH responses, mixed lymphocyte reactions and the expression of immunity related transcription factors are all suppressed during pregnancy[94, 95]. We therefore sought to determine whether the suppression of EAE occurred because of decreased lymphocyte activation. Our investigations focused on day 15 and day 25 post immunization, as these time points immediately precede the onset of disease (Figure 3.1). We first examined the levels of activation and costimulatory marker expression on the surface of T lymphocytes and antigen presenting cells since these molecules have been shown to be necessary for T cell activation to occur. No differences between the pregnant and non-pregnant 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. These results indicate that leukocytes from mice immunized during pregnancy exhibit adequate expression of important cell surface markers necessary for T cell activation.

We next examined the total number of cells present in the lymphoid organs and ascertained whether these cells could proliferate in response to the immunizing antigen. If insufficient activation of leukocytes were occurring in the animals immunized for EAE
during pregnancy, their lymphoid organs would have fewer cells and show a decreased ability to proliferate. The total number of cells present in the peripheral lymph nodes was similar between groups (Figure 3.2). Mice immunized during pregnancy, however, showed nearly twice as many splenocytes relative to control animals at day 15 post immunization. This difference was no longer apparent by day 25 post immunization as similar cell numbers were observed in both organs at that time. We next analyzed the ability of T lymphocytes from both groups to recognize the immunizing antigen. As shown in Figure 3.3, lymphocytes from mice immunized during pregnancy show no reduction in their proliferative response to PLP 139-151 relative to controls. There was also no significant difference in the response to the next epitope in the inflammatory cascade, PLP 178-191. Together this data shows that lymphocyte activation does occur in mice immunized for EAE during pregnancy.

Although leukocytes from animals immunized during pregnancy clearly recognize and respond to the immunizing antigen, the possibility exists that inflammatory cells may not reach the target organ. We measured the level of adhesion molecule expression on the surface of CD4+ T cells in both groups. All CD4+ T cells expressed CD11a (LFA-1) and most expressed CD44, CCR5, CD49d (VLA4) and CD54 (ICAM) on their surface (Table 3.2). Thus, CD4+ T cells from both groups showed high levels of adhesion molecule expression. We then examined the brains and spinal cords of immunized animals from both groups for evidence of inflammatory cell infiltration into the CNS. As shown in Table 3.3, inflammatory cells were noted in the CNS of non-pregnant control
Figure 3.2 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 non-pregnant mice serving as controls. At day 15 post immunization, the spleen and peripheral lymph nodes were removed and cells counted. (*) p<0.05 compared to non-pregnant controls N=3 per group; representative of 5 experiments

Figure 3.3 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 non-pregnant mice serving as controls. At day 15 post immunization, lymph node cells were cultured with PLP 139-151 or PLP 178-191 for 72 hours, including an 18 hour pulse with [³H] thymidine (1uCi per well). N=3; representative of 5 experiments
Table 3.2 Mice immunized for EAE during pregnancy show similar levels of adhesion molecule expression as controls. SJL mice were immunized with PLP 139-151 and CFA during late pregnancy (15-18 days post conception), with non-pregnant mice serving as controls. At day 15 post immunization, lymph node cells were stained with directly conjugated antibodies specific for cell surface adhesion molecules. Samples were then analyzed by flow cytometry. N=3; representative of 2 experiments

<table>
<thead>
<tr>
<th>Adhesion Molecule Expression</th>
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<tr>
<td></td>
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<tr>
<td>CD11a+</td>
</tr>
<tr>
<td>CD44</td>
</tr>
<tr>
<td>CCR5</td>
</tr>
<tr>
<td>CD49d</td>
</tr>
<tr>
<td>CD54</td>
</tr>
</tbody>
</table>

Table 3.3 Inflammatory cells enter the CNS of 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 non-pregnant mice serving as controls. On day 15 and day 25 post immunization, brain and spinal cords were removed. Tissues were processed for hematoxylin and eosin staining and scored for inflammatory cell infiltration. N=6, representative of 2 experiments

<table>
<thead>
<tr>
<th></th>
<th>Day 15</th>
<th>Day 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Late Pregnancy</td>
<td>0</td>
<td>++</td>
</tr>
</tbody>
</table>
Mice at day 15 post immunization, while infiltrates were not observed in the pregnant group until day 25 post immunization. While lymphocyte trafficking was delayed in the pregnant animals, inflammatory cells did ultimately reach the target organ. 

*Pregnancy reduces TNF-a production and increases the frequency of IL-10 secreting cells.*

Lymphocytes from mice immunized during pregnancy become activated and traffic to the CNS, yet 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 [96]. Since EAE is predominantly a Th1 driven disease, a shift to Th2 cytokine production would likely suppress EAE development. We therefore examined the type of cytokines produced in the control and pregnant groups in response to the immunizing antigen. We examined cytokine production in the two groups at days 15 and 25 post immunization. No differences were noted in the levels of the Th1 cytokines, IFN-γ and IL-2 (Figure 3.4 A and B). Lymphocytes from mice immunized for EAE during pregnancy, however, produced significantly less TNF-α relative to control mice in response to PLP 139-151. We also measured the levels of the Th2 cytokines IL-4 and IL-5. Minimal but similar levels of these cytokines were observed (Table 3.4). We also determined the frequency of lymphocytes secreting IL-10 and we found that mice immunized during pregnancy had a three-fold higher frequency of these cells (Figure 3.5). Since IL-10 can be considered a Th2 cytokine as well as an immunoregulatory cytokine, we next examined which cells were producing IL-10 through intracellular cytokine staining. We found that multiple cell types, including
Figure 3.4 Pregnancy decreases TNF-α production.
Non-pregnant and late stage pregnant mice were immunized with PLP 139-151 and CFA. At day 15 (A and B) and day 25 (C) post immunization, mice were sacrificed and lymph node cells harvested. Cells were cultured with PLP 139-151 and supernatants collected after 24 hours. Cytokines were measured by ELISA. * p<0.05 when compared to non-pregnant controls; N=3/group; representative of four experiments.
Table 3.4 Minimal levels of Th2 cytokines are produced when immunization occurs in pregnant or nulliparous mice. Non-pregnant and late stage pregnant mice were immunized with PLP 139-151 and CFA. At day 15 post immunization, mice were sacrificed and lymph node cells harvested. Cells were cultured with PLP 139-151 and supernatants collected after 48 hours. Cytokines were measured by CBA. N=3/group; representative of four experiments.

<table>
<thead>
<tr>
<th></th>
<th>IL-4</th>
<th>IL-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5 +/- 3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Late Pregnancy</td>
<td>2.8 +/- 2.9</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 3.4 Minimal levels of Th2 cytokines are produced when immunization occurs in pregnant or nulliparous mice. Non-pregnant and late stage pregnant mice were immunized with PLP 139-151 and CFA. At day 15 post immunization, mice were sacrificed and lymph node cells harvested. Cells were cultured with PLP 139-151 and supernatants collected after 48 hours. Cytokines were measured by CBA. N=3/group; representative of four experiments.
Figure 3.5. EAE induction during pregnancy results in an increased frequency of IL-10 secreting cells. Non-pregnant 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 hours. The frequency of IL-10 secreting cells were measured via ELISPOT. * p<0.05 compared to non-pregnant controls. N=3/group; representative of 3 experiments.
Figure 3.6 Multiple cell types increase their production of IL-10 when immunization occurs during pregnancy. Non-pregnant 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 hours, in the presence of momensin for the last 4 hours. Percent of cells expressing IL-10 was measured by intracellular cytokine staining. * p<0.05 compared to non-pregnant controls. N=3/group
macrophages, dendritic cells, B cells and CD4/CD25 T cells all upregulate their secretion of IL-10. No differences, however, were noted in the ability of CD4+ cells to produce IL-10 between the pregnant and control groups (Figure 3.6).

3.12 Post-partum

*EAE induction during the post-partum period leads to increased relapse severity.*

To determine the effect of the post-partum period on the induction of EAE, we immunized mice 22-25 days after conception. Since mice have a 21 day gestation cycle, PLP peptide immunization occurred approximately 1-3 days after delivery. As shown in Figure 3.7, mice immunized post-partum show a similar acute phase of disease as compared to nulliparous controls. During the relapse phase of EAE, however, the post-partum group exhibits increased severity of clinical signs. All mice immunized during the post-partum period show multiple relapses as compared to only 25% of control mice (Table 3.5).

*Mice immunized during the post-partum period exhibit decreased immune regulation rather than increased immune reactivity.*

Prolactin, a hormone produced in response to lactation, has potent immunostimulatory properties [97]. Since mice immunized during the post-partum period show increased relapse severity, we hypothesized that their lymphocytes would show enhanced activation and could potentially 1) exhibit enhanced epitope spreading or 2) show increased levels of immunostimulatory cytokine production in response to the immunizing antigen. We first examined the proliferative response of the post-partum group and controls in response to PLP139-151 (the immunizing antigen) at day 15 post
Figure 3.7 Immunization for EAE during the post-partum period results in increased relapse severity. Non-pregnant and post-partum (days 22-25 post conception) SJL mice were immunized with PLP 139-151 and CFA. Animals were then monitored for 50 days for clinical signs of disease. Control N=24, Post-partum N=11; representative of two experiments.

<table>
<thead>
<tr>
<th>Days post immunization</th>
<th>Mean Clinical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3.5 Summary of disease characteristics for post-partum EAE induction

<table>
<thead>
<tr>
<th></th>
<th>Day of Onset</th>
<th>CDIa</th>
<th>Relapseb</th>
<th>Multiple Relapses^{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.1 +/- 1.8</td>
<td>22.0 +/- 5.2</td>
<td>38%</td>
<td>25%</td>
</tr>
<tr>
<td>Post-partum</td>
<td>18.5 +/- 1.8</td>
<td>36.4 +/- 3.0</td>
<td>44%</td>
<td>100%*</td>
</tr>
</tbody>
</table>

a. Cumulative Disease Index (sum of the clinical scores for the observation period).

b. Percent of animals exhibiting at least one relapse

c. Percent of animals exhibiting more than one relapse.

* p<0.05 compared to controls; Fisher Exact Probability Test
immunization. No differences were noted in the proliferative response to the initiating peptide (Figure 3.8A). On day 35 post immunization, we assessed their ability to respond to other PLP and MBP epitopes. Relapses are thought to occur, in part, as the result of new responses initiated to other antigens or epitopes. It is thought that inflammation from the primary response causes tissue damage, releasing the other antigens. The epitope spreading cascade has been established in the SJL mouse strain, and we tested the whether lymphocytes from the post-partum group exhibited increased proliferation to the secondary epitopes as compared to controls. As shown in Figure 3.8B, both groups show minimal proliferation in response to the secondary epitopes. This suggests that at day 35, epitope spreading has not yet occurred and therefore, does not contribute to the increased relapse severity noted in the mice immunized during the post-partum period.

Since responses to other epitopes were not noted day 35, we next determined whether lymphocytes from mice induced for EAE during the post-partum period were producing increased levels of immunostimulatory cytokines. Since prolactin upregulates both Th1 and Th2 cytokine production, we examined levels of both types. Both groups exhibited a similar frequency of cells producing IFN-γ, IL-2 and IL-4 (Figure 3.9). We found, however, that lymphocytes from mice immunized during the post-partum period produce a decreased level of the immunoregulatory cytokine IL-10 (Figure 3.10).
Figure 3.8 Increased relapse severity is not caused by enhanced epitope spreading. Non-pregnant and post-partum (days 22-25 post conception) SJL mice were immunized with PLP and CFA. Animals were sacrificed and on day 15 (A) and day 35 (B), lymph node cells were harvested and placed into culture with PLP 139-151 or subsequent epitopes in the inflammatory cascade for 72 hours, including an 18 hour pulse with $[^3]$H thymidine. CPM’s were counted by liquid scintillation. N=3/group; representative of two experiments.
Figure 3.9 Post-partum induction does not result in increased Th1 cytokine production. Non-pregnant and post-partum (days 22-25 post conception) SJL mice were immunized with PLP and CFA. Animals were sacrificed day 15 and spleen cells were harvested and placed into culture with PLP 139-151 for 24 hours (IFN-γ and IL-2) or 48 hours (IL-4). The frequency of cells secreting a given cytokine were determined by ELISPOT. N=3/group; representative of two experiments.
Figure 3.10 Decreased IL-10 production when EAE is induced post-partum. Non-pregnant and post-partum (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 hours then supernatants were collected. IL-10 was measured by ELISA. * p<0.05 when compared to the nulliparous controls. N=3/group; representative of two experiments.
3.2 Summary

These investigations indicate that EAE induction during pregnancy results in protection from disease while EAE induction during the post-partum period causes exacerbation of clinical signs. When EAE was induced in either stage of pregnancy (mid or late) a reduction in the incidence of disease was observed. The late stage of pregnancy, however, offered the greatest protection from EAE development. This protective effect was not associated with any decreases in lymphocyte activation or trafficking. Mice immunized during pregnancy did not exhibit a decrease in production of Th1 cytokines IFN-γ and IL-2, nor did they show an upregulation of the Th2 cytokines IL-4 and IL-5. Instead, lymphocytes from mice immunized during pregnancy produced lower levels of the pro-inflammatory cytokine TNF-α while exhibiting an increased frequency of cells secreting the immunoregulatory cytokine, IL-10.

Immunization during the post-partum period resulted in a clinical disease course that differed from controls only during the relapse phase of EAE. This increased relapse severity was not because of enhanced epitope spreading or elevated production of immunostimulatory cytokines in response to the immunizing antigen. A decrease in IL-10 production, however, was noted in the mice immunized for EAE during the post-partum period. Overall, these results suggest that IL-10 plays a pivotal role when EAE induction occurs during different gestational stages.
CHAPTER 4

THE EFFECT OF PREGNANCY AND THE POST-PARTUM PERIOD
ON PRE-EXISTING EAE

4.1 Results

4.11 Pregnancy induction soon after EAE immunization but prior to the onset of clinical disease.

Pregnancy suppresses EAE when induced prior to the onset of disease.

The majority of human studies have focused on the effect of pregnancy and the post-partum period on the rate of disease relapse in women who have already been diagnosed with MS. Thus, these studies have predominantly emphasized the effect of pregnancy and the post-partum period on pre-existing disease. As the most clinically relevant situation, we explored the effect of pregnancy on the effector phase of disease. As shown in Figure 4.1, pregnancy was induced 5-8 days after EAE immunization. This window of time was chosen in order to determine if pregnancy induction early in the course of disease could prevent the development of clinical signs of EAE. We found that when pregnancy was initiated prior to the onset of disease, the appearance of clinical
Figure 4.1 Pregnancy induced soon after EAE immunization reduces clinical severity during the acute phase. Female mice were immunized with PLP 139-151 and CFA. Pregnancy was induced between 5-8 days post immunization, with nulliparous mice serving as controls. Animals were monitored for 50 days for clinical signs of disease. N=13 controls, N=6 pregnant
<table>
<thead>
<tr>
<th></th>
<th>Incidence&lt;sub&gt;a&lt;/sub&gt; (≤d29)</th>
<th>Incidence&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Day of Onset&lt;sub&gt;±SEM&lt;/sub&gt; (d 0-29)</th>
<th>CDI &lt;sub&gt;±SEM&lt;/sub&gt; (d 0-29)</th>
<th>CDI &lt;sub&gt;±SEM&lt;/sub&gt; (d 29-50)</th>
<th>CDI &lt;sub&gt;±SEM&lt;/sub&gt; (d 0-50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>77% (10/13)</td>
<td>77% (10/13)</td>
<td>13.5 +/- 0.7</td>
<td>22.9 +/- 3.1</td>
<td>16.5 +/- 5.6</td>
<td>41.3 +/- 7.3</td>
</tr>
<tr>
<td><strong>Pregnancy</strong></td>
<td>33% (2/6)</td>
<td>66% (4/6)</td>
<td>23.5 +/- 2.8*</td>
<td>4.5 +/- 2.1*</td>
<td>19.5 +/- 6.8</td>
<td>23.4 +/- 7.4</td>
</tr>
</tbody>
</table>

Table 4.1 Summary of disease characteristics for pre-existing EAE when pregnancy is induced prior to disease onset.

a. Percentage of animals developing EAE before day 29 post immunization
b. Percentage of animals developing EAE over the entire observation period of 50 days
* p<0.05 when compared to nulliparous controls
signs was delayed. The nulliparous control mice exhibited a mean day of onset of 13.5 days post immunization while the pregnant mice showed a mean day of onset of 23.5 days (Table 4.1). While 77% of the nulliparous control mice developed EAE before day 29, only 33% of the pregnant mice developed EAE during gestation (i.e. before day 29). The cumulative disease index (CDI) was significantly lower during pregnancy relative to nulliparous controls. The overall CDI was lower in the mice that had been pregnant, but the protective effect of pregnancy was temporary. The CDI for days 29-50 (when the mice were not pregnant) was similar between the groups, and the incidence of disease for the entire observation period did not differ greatly between the parous and nulliparous mice (Table 4.1).

4.12 Pregnancy induction after disease onset.

*Pregnancy reduces the severity of relapses when induced after the onset of clinical disease.*

In order to determine the effect of pregnancy on the relapsing phase of disease, we induced pregnancy 25-27 days post immunization. Unlike our previous studies, this time frame was chosen so that pregnancy would occur immediately after the acute phase of disease but before the animal experienced relapses (see Figure 4.2). Pregnant mice exhibited a reduction in disease severity, but this effect was most pronounced during the latter half or late stage of pregnancy (Figure 4.2). As a result, the pregnant mice exhibited a lower CDI over the gestational period (d24-46) than nulliparous mice in the
Figure 4.2 Pregnancy reduces relapse severity of pre-existing EAE when induced after the acute phase of disease. Female mice were immunized with PLP 139-151 and CFA. Pregnancy was induced 25-27 days post immunization, with nulliparous mice serving as controls. Animals were monitored for 50 days for clinical signs of disease. N=13 controls, N=7 pregnant; representative of three experiments.
<table>
<thead>
<tr>
<th></th>
<th>Incidence</th>
<th>Day of Onset ± SEM</th>
<th>CDI ± SEM (d 6-24)</th>
<th>CDI ± SEM (d 24-46)</th>
<th>CDI ± SEM (d 0-50)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>77% (10/13)</td>
<td>13.5 +/- 0.7</td>
<td>22.9 +/- 3.1</td>
<td>19.9 +/- 4.2</td>
<td>41.3 +/- 7.3</td>
</tr>
<tr>
<td><strong>Pregnancy</strong></td>
<td>71% (5/7)</td>
<td>14.2 +/- 0.4</td>
<td>22.2 +/- 6.0</td>
<td>9.2 +/- 3.2*</td>
<td>31.2 +/- 8.4</td>
</tr>
</tbody>
</table>

Table 4.2 Summary of disease characteristics for pre-existing EAE when pregnancy is induced after the acute phase of disease.

* p<0.05 when compared to nulliparous control
same stage of disease (9.2 compared to 19.9 for the pregnant and nulliparous mice respectively; Table 4.2). The suppressive effect of pregnancy was again temporary as the mean clinical score for the pregnant group increased after delivery (days 46-50 on Figure 4.2).

*Pregnancy suppresses EAE through the production of an immunoregulatory cytokine rather than immune suppression or a Th2 bias.*

Pregnancy was observed to suppress pre-existing EAE regardless of when the gestational period occurred (before or after disease onset). Since we were predominantly interested in the clinically relevant situation, we focused our immunological investigations on the model in which pregnancy was induced after the onset of disease. In this situation, the greatest suppression of EAE occurred during the latter half of pregnancy, so day 40 post immunization was chosen for further exploration (see Figure 4.2). We first determined whether lymphocytes from immunized mice were still responding to the immunizing antigen at this time, and whether lymphocytes from pregnant mice showed any evidence of decreased activation. As shown in Figure 4.3, lymphocytes from both groups proliferated in response to the immunizing antigen (PLP 139-151), but not respond to the next epitope in the cascade (PLP 178-191). No differences were noted in the proliferative abilities between control and pregnant mice, suggesting that pregnancy did not result in a generalized immune suppression. To further delineate whether pregnancy resulted in decreased activation, we also examined the level of costimulatory molecule expression on the surface of macrophages. Both control and pregnant mice, however, expressed similar levels of CD80 and CD86 (Table 4.3).
Figure 4.3 Lymphocytes from pregnant mice proliferate to the immunizing antigen. Female mice were immunized with PLP 139-151 and CFA. Pregnancy was induced between 25-27 days post immunization, with nulliparous mice serving as controls. On day 40 post immunization, lymph node cells were harvested and cultured for 72 hours in the presence of PLP 139-151, including an 18 hour pulse with $[^3]$H thymidine (1uCi per well). N=3/group; representative of two experiments.
<table>
<thead>
<tr>
<th></th>
<th>CD11b/ CD80+</th>
<th>CD11b/ CD86+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45.5 +/- 1.6</td>
<td>70.5 +/- 5.1</td>
</tr>
<tr>
<td>Pregnant</td>
<td>46.7 +/- 0.6</td>
<td>69.1 +/- 1.4</td>
</tr>
</tbody>
</table>

Table 4.3 Costimulatory molecule expression on splenic macrophages from control and pregnant mice. N=3/group

Figure 4.4 Percent of CD4+ T cells expressing TIM-3. Female SJL mice were immunized with PLP 139-151 and CFA. Pregnancy was induced 25-27 days after immunization and at day 40, mice were sacrificed and lymph nodes were removed. Cells were stained with CD4-Fitc and TIM-3 – PE. Graph above is gated on CD4+ cells. N=3/group; representative of two experiments
We next determined whether EAE was reduced in the pregnant animals as a result of a Th2 shift. Frequencies of lymphocytes secreting the Th1 cytokines IFN-γ and IL-2 as well as Th2 cytokines IL-4 and IL-5 were determined. Minimal (<0.001% of cells) but similar frequencies of lymphocytes were found to secrete these cytokines between the nulliparous control and pregnant mice. Furthermore, CD4+ cells from pregnant and non-pregnant mice were observed to express similar levels of the Th1 inhibitory molecule TIM-3 (Figure 4.4).

Although no differences were observed in the number of cells secreting the conventional Th1 and Th2 cytokines between the two groups, pregnant mice showed an increased frequency of IL-10 secreting cells (Figure 4.5). This difference, however, was only noted in lymphocytes not stimulated with PLP ex vivo.
Figure 4.5 Lymphocytes from pregnant mice have an increased frequency of IL-10 secreting cells. Female mice were immunized with PLP 139-151 and CFA. Pregnancy was induced 25-27 days post immunization, with nulliparous mice serving as controls. On day 40 post immunization, lymph node cells were harvested and cultured for 72 hours. The frequency of IL-10 secreting cells was measured by ELISPOT. P<0.05 when compared to controls. N=3/group; representative of two experiments
4.2 Summary

The data presented in this chapter shows that pregnancy suppresses pre-existing EAE. When pregnancy was induced prior to the onset of clinical disease, the appearance of clinical signs of EAE was delayed, with few mice developing disease until after parturition. This protection only lasted for the duration of pregnancy, as the incidence and severity of disease were similar between the groups after the completion of the gestational period. When pregnancy was induced after the onset of clinical disease, the relapse phase of EAE was suppressed. This reduction in disease severity, however, did not occur until the late stage of pregnancy. Regardless of when pregnancy was induced (i.e. before or after disease onset), its protective effects were only temporary.

Our immunological investigations focused on the effect of pregnancy on the relapse phase of disease. We chose a time point that corresponded to the late stage of pregnancy and tested whether lymphocytes from the pregnant mice exhibited signs of immune suppression. Neither reductions in proliferation nor costimulatory molecule expression were noted in the lymphocytes from pregnant animals. Although we expected to see an increase in the frequency of cells secreting Th2 cytokines in the pregnant mice, both groups exhibited minimal but similar levels of these cytokines. The pregnant mice, however, did show an increase in the frequency of IL-10 secreting cells upon removal from the in vivo environment. These results suggest that pregnancy reduced the severity of pre-existing EAE through the production of the immunoregulatory cytokine IL-10.
CHAPTER 5

THE EFFECT OF PREGNANCY AND THE POST-PARTUM PERIOD ON THE ENCEPHALITOGENIC CAPACITY OF AUTOREACTIVE LYMPHOCYTES

5.1 Results

Immunological adjuvants are required for the induction of EAE, as part of the active immunization regimen as well as part of the donor immunization scheme in an adoptive transfer. The microbial components of adjuvants are necessary because naïve antigen-specific T cell populations are present in low frequencies and responses are insufficiently robust for detection. No studies to date have investigated the effect of pregnancy on lymphocyte activation without the use of adjuvants. Our studies have employed a MBP TCR transgenic mouse in which 95% of the CD4+ T cells recognize and respond to the immunodominant encephalitogenic epitope of MBP. With such a large and homogenous population of autoreactive cells, a considerable antigen-specific response can be generated even in a naïve setting. Using this unique mouse, we have examined how the encephalitogenic capacity of naïve lymphocytes changes over the
course of pregnancy and the post-partum period. Specifically, we induced pregnancy in
the TCR Tg mouse and during mid pregnancy (8-11 days post conception), late
pregnancy (15-18 days post conception) and the post-partum period (1-2 days after
delivery), we harvested lymphocytes for ex vivo analysis.

5.11 Pregnancy

_Pregnancy does not suppress activation of MBP TCR Tg lymphocytes._

We first determined whether lymphocytes from two different gestational stages of
pregnancy (mid and late) showed any indication of immune suppression when compared
to non-pregnant controls. The total number of Tg (CD4+/VB8.2+) cells present in the
lymph nodes of mice during pregnancy was determined. Approximately 40-45% of the
lymphocytes from non-pregnant, mid pregnant and late pregnant mice expressed CD4
and VB8.2 (Figure 5.1 and Table 5.1). These results show that pregnancy does not result
in a decrease in the number of autoreactive T cells. We also measured the expression of
the Vβ8.2 molecule on the surface of CD4+ T cells during each gestational stage. As
reported for this mouse strain, approximately 95% of the CD4+ cells expressed the Vβ8.2
T cell receptor chain regardless of pregnancy stage.

We next examined whether the transgenic cells differed in their ability to
undergo activation. The expression of the activation and costimulatory molecules CD28,
CD62L and CD69 on the surface of CD4+ T cells was analyzed between the gestational
stages. No differences were observed in expression levels of these markers. We next
explored whether cells from the different stages of pregnancy could recognize and
proliferate in response to the autoantigen. As shown in Figure 5.2, lymphocytes from
Figure 5.1 Similar percentage of lymphocytes express both CD4 and Vβ8.2. Naïve MBP TCR transgenic mice were sacrificed and peripheral lymph nodes were harvested. The percentage of lymphocytes expressing both CD4-FITC and Vβ8.2-PE were analyzed using flow cytometry. Representative mice are displayed from A. non-pregnant control, B. mid pregnancy, C. late pregnancy.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mid Pregnancy</th>
<th>Late Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+/Vβ8.2+</td>
<td>40.8 +/- 1.6</td>
<td>44.3 +/- 2.8</td>
<td>40.0 +/- 2.1</td>
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Table 5.1 Percentage of CD4+Vβ8.2+ lymphocytes during pregnancy
Figure 5.2 Proliferation is not suppressed during pregnancy. Pregnancy was induced in naïve MBP TCR Tg mice. At the mid stage of pregnancy (8-11 days post conception) and the late stage of pregnancy (15-18 days post conception), mice were sacrificed and lymph nodes were harvested. Cells were cultured with 10ug/ml Nac 1-11 for 72 hours, including an 18 hour pulse with $^3$H thymidine. CPMs were measured via liquid scintillation. N=3/group; representative of three experiments.
both the mid and the late stages of pregnancy proliferated robustly in response to the NaC 1-11 peptide, showing no reduction in ability to recognize antigen when compared to the non-pregnant control mice. Overall, these results show no evidence for an immunosuppressive effect of pregnancy.

_A Th2 bias in cytokine production occurs in the mid stage but not during the late stage of pregnancy._

Since we observed no decrease in activation of lymphocytes from pregnant mice, we next determined whether pregnancy in the absence of adjuvant exposure resulted in a shift in cytokine production. It has been proposed for many years that Th1 immunity (IFN-γ and IL-2) decreases during pregnancy, while Th2 type responses (IL-4 and IL-5) are amplified. In order to determine the type of cytokine environment present during pregnancy, we harvested lymphocytes at each gestational stage and stimulated the cells in culture with the NaC 1-11 peptide. We then measured the frequency of cells secreting a given cytokine via ELISPOT. Although we expected lymphocytes from the pregnant mice to show a decreased frequency of Th1 secreting cells, no differences were observed between the gestational stages and nulliparous mice (Figure 5.3). Lymphocytes from both the mid and late stages of pregnancy exhibited a similar frequency of IFN-γ and IL-2 producing cells as the non-pregnant control mice and neither group produced TNF-α. These results suggest that Th1 cytokine production is not altered during the different gestational stages of pregnancy. We predicted that we would observe an elevated frequency of Th2 secreting cells throughout pregnancy. Interestingly, we observed an
Figure 5.3 Transgenic lymphocytes from the mid stage of pregnancy exhibit increased Th2 cytokine production. Pregnancy was induced in MBP TCR transgenic mice. At different gestational stages (mid or late pregnancy) mice were sacrificed and lymph nodes were harvested. Cells were stimulated in culture with NaC 1-11 for 24 hours (IFN-γ and IL-2) or 48 hours (IL-4 and IL-5). Cytokine secretion was measured by ELISPOT. * p<0.05 ANOVA N=3/group; representative of two experiments.
increased frequency of both IL-4 and IL-5 secreting cells only in the mid stage of pregnancy. Lymphocytes from the late stage of pregnancy exhibited a similar frequency as the nulliparous controls. This data suggests that a bias towards Th2 cytokine production occurs only during the mid stage of pregnancy and only in the absence of adjuvant stimulation.

5.12 Post-partum

*Increased overall immunoreactivity occurs during the post-partum period*

Since the relapse rate in women with MS increases sharply following delivery, we next examined the effect of the post-partum period on the activation status of our MBP TCR transgenic lymphocytes. To generate post-partum lymphocytes, we induced pregnancy in the MBP TCR Tg mouse and harvested peripheral lymph nodes approximately 1-3 days after delivery. Similar to our investigations of lymphocytes during pregnancy, we also determined whether alterations occurred in the overall levels of autoreactive T cells present during the post-partum period. As shown in Figure 5.4 and Table 5.3, lymphocytes obtained during the post-partum period exhibited a similar percentage of cells expressing both CD4 and Vβ8.2 as the nulliparous controls. 95% of the CD4+ cells in both groups expressed the transgenic TCR (Vβ8.2). Thus, no upregulation of the autoreactive TCR was observed in the post-partum lymphocytes.

We next determined whether lymphocytes from the post-partum period displayed increased activation. We first analyzed the expression of the cell surface molecules CD28, CD62L and CD69. We predicted that if the post-partum lymphocytes had an increased activation potential, they would show elevated levels of CD69 while displaying decreased levels of CD28 and CD62L. No differences, however, were noted between
Figure 5.4 Similar percentage of lymphocytes express both CD4 and Vβ8.2. Non-pregnant or post-partum MBP TCR transgenic mice were sacrificed and the percentage of lymphocytes expressing both CD4-FITC and Vβ8.2-PE were analyzed using flow cytometry. Representative mice are displayed from A. non-pregnant controls, B. post-partum.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Post-partum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+/Vβ8.2+</td>
<td>40.8 +/- 1.6</td>
<td>44.9 +/- 2.1</td>
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Table 5.3 Percentage of CD4+/Vβ8.2+ lymphocytes in mice during the post-partum period.
Figure 5.5 Lymphocytes from the post-partum period exhibit increased proliferation to MBP NaC 1-11. Pregnancy was induced in naïve MBP TCR Tg mice. During the post-partum period (1-2 days after delivery), mice were sacrificed and lymph nodes were harvested. Cells were cultured with 10ug/ml Nac 1-11 for 72 hours, including an 18 hour pulse with ³H thymidine. CPMs were measured via liquid scintillation. Nulliparous MBP TCR Tg lymphocytes served as controls. *p<0.05 N=3; representative of three experiments.
lymphocytes of the post-partum period and nulliparous controls. Despite the similar expression of cell surface markers, post-partum lymphocytes were found to proliferate more in response to stimulation with NaC 1-11 than lymphocytes from the nulliparous controls (Figure 5.5). This increased proliferative response was observed at three different concentrations of peptide.

We next analyzed the type of cytokines produced by the post-partum lymphocytes. Since these cells displayed an increased ability to proliferate to MBP Nac 1-11, we predicted that they would display an increased frequency of Th1 secreting cells in response to peptide. We found that lymphocytes from the post-partum mice had an increased frequency of both Th1 and Th2 cytokine secreting cells. An increase was observed in IFN-γ and IL-2 secreting cells as well as in IL-5 producing cells when compared to lymphocytes from nulliparous control mice. These results show that post-partum lymphocytes exhibit an overall more vigorous response to peptide stimulation through the TCR than lymphocytes from nulliparous mice.
Figure 5.6 Transgenic lymphocytes from post-partum exhibit increased overall immunoreactivity. Pregnancy was induced in MBP TCR transgenic mouse. During the post-partum period, mice were sacrificed and lymph nodes were harvested. Cells were stimulated in culture with NaC 1-11 for 24 hours (IFN-g and IL-2) or 48 hours (IL-4 and IL-5). Cytokine secretion was measured by ELISPOT. *P<0.05
5.2 Summary

The purpose of the studies in this chapter was to determine how the encephalogenic capacity of autoreactive T cells changes over the course of pregnancy and the post-partum period. We observed dynamic and distinct immunological alterations for each gestational stage. No changes, however, occurred in the percentage of autoreactive transgenic T cells present nor in the expression of the TCR Vβ8.2 chain on the lymphocytes. Differences between the groups were observed in cellular activation and the cytokine environment produced. Cells from neither pregnancy stage (mid or late) showed any evidence of immune suppression. Both groups expressed similar levels of costimulatory and activation molecules as controls. Moreover, the lymphocytes from pregnant mice proliferated robustly in response to specific antigen. Although we predicted that a Th2 bias would be observed throughout pregnancy, only lymphocytes from the mid stage of gestation showed an increased frequency of IL-4 and IL-5 secreting cells. Interestingly, lymphocytes from the post-partum period did not show differences in their expression of activation markers, but they did show an overall increase in immunoreactivity. These cells showed increased proliferation and elevated Th1 and Th2 cytokine production. Thus, each gestational stage was characterized by its own unique immunological environment.
MS disease activity undergoes dynamic changes over the course of pregnancy and the post-partum period. Throughout gestation the rate of relapse decreases, with the sharpest decline occurring during the third trimester. Abruptly following parturition, however, disease activity flares before returning to its pre-pregnancy level three to six months later. Within this relatively short window of time, one can observe opposing effects on disease. As a result, pregnancy and the post-partum 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 prevent the development of disease, delays its onset, or reduces clinical severity. The majority of research in this area, however, has been conducted in acute models of disease. To date, only one report has examined the effect of pregnancy on
relapsing/remitting EAE, and no studies have addressed the effect of the post-partum period. Consequently, little is known about how these gestational stages affect chronic relapsing/remitting EAE.

In order to develop a thorough understanding regarding the effects of pregnancy and the post-partum period on EAE, our investigations included multiple gestational stages. We included two timepoints from pregnancy (mid and late) as well as one from the post-partum period so that we could evaluate the degree of protection offered throughout gestation. Non-overlapping, discrete 3 day ranges were chosen within each stage (e.g. mid pregnancy= days 8-11 post conception), allowing us to characterize each distinctly and separately. As the literature regarding MS and pregnancy has shown, each gestational stage has its own unique but transient effect on clinical outcome.

Over the course of our studies employing the multiple gestational stages, we addressed three fundamental areas of investigation. First, we examined the effect of pregnancy and the post-partum period on the induction of clinical EAE. These experiments explored the effect of the different gestational stages on the initial antigen presentation and T cell priming events associated with disease onset. Second, we addressed the effect of pregnancy on pre-existing disease, in order to determine the effect of the different gestational stages on the later effector phase of EAE. Lastly, we investigated the effect of pregnancy and the post-partum period on the encephalitogenic capacity of naïve autoreactive cells. This model permitted us to examine the effect of pregnancy on inflammatory responses in the absence of adjuvants, which strongly bias
the immune response. Together, these studies have allowed us to develop a broad and integrative understanding of how pregnancy and the post-partum period alter demyelinating disease.

For our investigations exploring the effect of pregnancy and the post-partum period on the induction of EAE, we induced disease in animals of different gestational stages. These studies revealed that each gestational stage has its own unique effect on disease. For instance, when we immunized mice during pregnancy, the percentage of mice developing EAE was reduced (Table 3.1). 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. who reported that immunization during the latter half of pregnancy is more protective than earlier in gestation [98]. Interestingly, of the few mice that develop EAE, none exhibit relapses (Figure 3.1). When we immunized mice post-partum, however, the percentage of mice developing disease remained similar to nulliparous mice, but the animals exhibit increased relapse severity (Figure 3.6). This data suggests that not only is the incidence of disease affected when immunization occurs during different gestational stages, but the clinical course is also substantially altered.

In order to delineate the mechanism(s) by which these different gestational stages mediated their effects on EAE, we explored three predominant hypotheses. We first examined whether immunization during the late stage of pregnancy resulted in leukocyte activation. Evidence in the reproductive immunology literature has shown that at least some aspects of immunity are suppressed during pregnancy. We therefore, explored whether pregnancy was preventing EAE by inhibiting lymphocyte mobilization. When we evaluated the activation status of lymphocytes from mice immunized during
pregnancy, we found no evidence for inhibition. 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 exhibited an elevated number of splenocytes. Leukocytes from animals immunized during pregnancy showed similar levels of activation marker and costimulatory molecule expression compared to the immunized nulliparous controls, and they proliferated strongly in response to the immunizing antigen (Figure 3.3). CD4+ T cells from the pregnancy group expressed similar levels of adhesion molecules and clearly infiltrated the CNS (Table 3.2 and 3.3). Thus, leukocyte activation in response to neuroantigen immunization clearly does occur when EAE is induced during pregnancy.

We next hypothesized that when EAE induction occurs during pregnancy, there is a shift in cytokine production away from the pro-inflammatory Th1 cytokines and towards the Th2 cytokines. Increased levels of Th2 cytokines have been found at the maternal/fetal interface and pregnant animals have difficulty clearing intracellular infections that require a Th1 response [99-101]. Since EAE is predominantly a Th1 driven disease and Th2 type responses are associated with protection, a shift in cytokine production could prevent mice from displaying clinical signs. To determine if a Th2 shift occurred, we compared the frequency of cells secreting Th1 cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4 and IL-5) between non-pregnant immunized mice and those immunized during pregnancy. The two groups exhibited similar frequencies of Th1 secreting cells and both displayed a minimal frequency of Th2 producing cells. 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 displayed an increased frequency of IL-10 secreting cells when compared to control
mice. TNF-α is frequently characterized as a Th1 cytokine while IL-10 can be considered a Th2 cytokines. An increase in IL-10 and a decrease in TNF-α could potentially be classified as a Th2 shift in the mice immunized during pregnancy. Yet, TNF-α and IL-10 also have roles independent of the Th cell development pathway. TNF-α is the predominant cytokine produced in response to gram negative bacterial infections and plays an important role in innate immunity. This cytokine upregulates the expression of adhesion molecules and activates neutrophils and macrophages, serving as a pro-inflammatory mediator[102]. IL-10, on the other hand, is considered an immunoregulatory cytokine. It inhibits the synthesis of IFN-γ and suppresses the maturation and activation of antigen presenting cells. Although Th2 cells secrete IL-10, other cell types including macrophages, dendritic cells and B cells can produce IL-10 as well[103]. As a result of these pleiotropic effects, TNF-α and IL-10 are not only characterized as Th1 or Th2 cytokines. Some investigators regard the two cytokines as central mediators of innate immunity and thus, define TNF-α as pro-inflammatory and IL-10 as immunoregulatory.

Regardless of their designations, 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 increase their IL-10 production and decrease their TNF-α secretion when cultured in the presence of high doses of estrogen[104]. Similar results have been observed in PBMCs from MS patients receiving oral estriol [105]. Reductions in TNF-α production in the CNS as well as upon ex vivo stimulation with antigen have
reported with estrogen pre-treatment of EAE [106, 107] Pregnancy and its associated hormone environment, therefore, seem to result in consistent alterations in the cytokine milieu.

In order to explore whether these changes in cytokine production represented an immunoregulatory or a Th2 type environment, we utilized intracellular cytokine staining to determine which cells were producing the IL-10. We hypothesized that if the elevated IL-10 was the result of a Th2 type of 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 upregulated their production of IL-10 in mice immunized for EAE during pregnancy. Unexpectedly, similar numbers of CD4+ cells produced IL-10 between control mice and those induced 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.

Since immunization during pregnancy resulted in an immunoregulatory environment that offered protection from EAE, we next determined whether pregnancy was capable of altering pre-existing EAE. We first induced pregnancy between 5-7 days after immunization for EAE, and then monitored the animals for clinical signs of disease over the course of gestation and the post-partum period. We found that pregnancy reduced the incidence of disease when pregnancy was induced prior to the onset of clinical signs (Table 4.1). This effect, however, was only temporary as the suppression of EAE was observed only during pregnancy. These results indicate that pregnancy is also an effective modulator of the effector phase of EAE. To determine the extent of
protection mediated through pregnancy, we also induced pregnancy in after the acute
phase of disease. We predicted that the later in disease that pregnancy was induced, the
less effect it would have on clinical outcome. Once again, we found that pregnancy
suppressed EAE. This effect, however, did not occur until the last week of pregnancy.
Our results are similar to the effects observed in pregnant MS patients, where the last
trimester of pregnancy offers the greatest protection from disease. Therefore, regardless
of when pregnancy is induced (prior or after the onset of clinical signs), it suppresses
EAE. This protective effect, however, does not persist as it disappears shortly after
parturition.

When we immunized mice during pregnancy, an immunoregulatory environment
developed and EAE was either prevented or greatly reduced. In our studies investigating
the effect of pregnancy on pre-existing EAE, we expected our results to differ. Afterall,
when pregnancy is induced in mice with pre-existing EAE, the animals complete the
entire pregnancy course during the progression of disease. In our previously described
induction experiments, EAE was initiated during pregnancy, but the animal underwent
parturition a few days later. Thus, in those studies the animals are no longer pregnant
when disease begins to present clinically. We predicted that the mice with pre-existing
EAE would produce Th2 cytokines to the immunizing antigen during pregnancy.
Surprisingly, minimal cytokine production (Th1 or Th2) was observed in either the
nulliparous or the pregnant mice. The pregnant mice, however, did show an increase in
the frequency of cells producing IL-10. This data suggests that IL-10 also plays an
important role during the relapsing phase of EAE.
In both active disease models, an increase in IL-10 was associated with pregnancy. Interestingly, changes in this cytokine were associated with ameliorating effects in both the induction and the relapsing phases of EAE. This finding agrees with many investigations regarding IL-10 and EAE. For example, the incidence of EAE is increased in IL-10 knockout mice and is greater than the incidence observed in IL-4 knockout mice[108]. IL-10 transgenic mice, on the other hand, are relatively resistant to EAE[109]. In addition to these effects on the induction of EAE, IL-10 has also been found to be a significant modulator of the relapse phase of EAE. Increases in IL-10 correlate with recovery from EAE while decreases in IL-10 precede relapses[110, 111]. Indeed, IL-10 has been shown to be an important immunoregulatory cytokine in EAE.

Using an active disease model, we found that pregnancy modulates EAE through the production of an immunoregulatory cytokine in both the induction and effector phases of disease. We next explored the effect of pregnancy on the immune response in an adjuvant free system. In actively induced EAE, whether addressing the effect of pregnancy on the induction of disease or its effect on the clinical course of pre-existing EAE, we employed the use of the immune biasing agent, CFA. Adjuvants are used to boost or enhance the inflammatory response to antigen and are necessary for the establishment of active EAE. Such agents alter the immunological environment such that disease will develop, but adjuvants also modulate the immune environment associated with pregnancy. In order to develop a better understanding of how immune responses change over the course of pregnancy and the post-partum period, we utilized an MBP TCR Tg mouse. Since this mouse has an increased number of autoreactive cells, a robust response to antigen can be observed without the use of immune biasing adjuvants. With
this mouse we addressed how the encephalitogenic potential of autoreactive cells changes over the course of pregnancy and the post-partum period. We examined the ability of the lymphocytes to proliferate and produce cytokines in response to antigen at different time points through gestation. Interestingly, neither the percentage of autoreactive T cells nor their expression of the TCR changed during the different gestational stages. Yet, lymphocytes from pregnancy and the post-partum exhibited functional differences. During mid pregnancy an increase in the frequency of Th2 (IL-4 and IL-5) secreting cells was observed while no differences were observed in the frequency of Th1 (IFN-γ and IL-2) producing cells. During late pregnancy, however, no differences were observed in the ability of the lymphocytes to proliferate or to produce Th1 or Th2 cytokines. During the post-partum period, lymphocyte hyper reactivity was observed. These results suggest that each gestational stage has its own unique immunological environment.

Interestingly, a Th2 bias was observed during mid-pregnancy in the transgenic mouse. Yet, no Th2 bias was noted in studying the effect of pregnancy on active disease. These differing results are most likely to result of the adjuvant. CFA, composed of mineral oil and heat-inactivated mycobacterium, is a potent stimulator of pro-inflammatory responses. It is known to trigger toll-like receptors and upregulate TNF-α [112]. Thus, pregnancy may not be able to promote Th2 responses when a Th1 response is already established. Instead, the pregnancy environment results in regulatory type responses. The immunity generated during pregnancy may differ depending on whether the response is an established or naïve reaction.

The diversity observed throughout pregnancy and the post-partum period in the way lymphocytes respond to antigen most likely reflects the complex physiological
environment present during each gestational stage. The hormones associated with pregnancy are known to have immune altering effects. Since each gestational stage is distinctly characterized by different concentrations of hormones, the complex interplay between hormones could result in different inflammatory responses. For instance, the differences between mid and late pregnancy with regard to Th2 cytokine production might be the result of varying estrogen and progesterone levels. Estrogen has been reported to have a biphasic effect on immune responses. At low concentrations, estrogen is immune stimulatory while at high doses, it is either inhibitory or Th2 promoting [113]. Progesterone, when administered alone, promotes Th2 responses[114]. When the two interact, however, unique effects may develop. Bansil and colleagues reported that differences in MS disease activity as measured by MRI were found to differ between the follicular and luteal phases of the menstrual cycle. In particular, these alterations in disease correlated with the estrogen to progesterone ratio present during the menstrual cycle rather than to the direct concentration of each hormone alone. A high estrogen to progesterone ratio corresponded to decreased disease while a low estrogen to progesterone ratio was linked to more active disease [115]. Hormone concentration during the post-partum period may also play a role in the increased immune reactivity observed then. Prolactin, a hormone increased in response to lactation, is immunostimulatory[116, 117]. Consequently, prolactin may contribute to the IL-10 reduction observed in the mice immunized during the post-partum period. As a result of these synergistic and divergent effects, the complex hormone environments present at each gestational stage modulate disease in their own distinctive manner.
In our studies, we have found EAE to be a good model for studying the effects of pregnancy and the post-partum period on autoimmune demyelinating disease. We found that pregnancy suppressed both the induction and effector phases of EAE. Similar clinical findings have been noted with regard to the effect of pregnancy on EAE in this strain. Like the other report, we found little evidence for a Th2 shift. Rather, pregnancy seems to result in an immunoregulatory environment, with increased production of IL-10. The other investigation in this area attributed the protective effect of pregnancy to an unidentified serum factor. Since this report did not examine cytokine production in splenocytes of immunized animals, it is possible that their immunosuppressive serum factor was also IL-10. Indeed, an increase in IL-10 has been found in most studies examining pregnancy and its hormones.

Like the studies regarding pregnancy and MS, we found the later stage of pregnancy to be the most protective, while the post-partum period was disease enhancing. We also found that different gestational stages affected the encephalitogenic capacity of naïve cells in a distinct manner, with unique types of immune responses noted during each stage. Our results demonstrate that pregnancy and the post-partum period are not static physiological stages that cause uniform changes in disease, but rather, they are characterized by distinct, temporary alterations in disease. Harnessing the immune environment associated with the late stage of pregnancy would be of great therapeutic benefit for women with MS, as it represents an immune competent environment with unique regulatory properties.


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