SEX, PREGNANCY, AND A GREAT PAIR OF GENES: CRITICAL MEDIATORS
IN THE DEVELOPMENT AND PROGRESSION OF CNS AUTOIMMUNE INJURY

DISSERTATION

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By

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS thought to be due to an autoimmune response directed against myelin antigens. EAE is a commonly used animal model for MS and shares clinical, histopathologic and immunologic similarities with MS. Sex dimorphism has been well established in both MS and EAE. In MS, females are three times as likely as males to be affected; however male MS patients most commonly present with a more severe disease course with fewer effective treatments available. In EAE, certain mouse strains exhibit sex differences in response to myelin peptides. Sex-discrepant responses to disease have been attributed to sex and pregnancy hormones as well as genetics. Clinical trials are currently underway to investigate the role for sex hormones and multiple efforts are made to develop effective therapies to treat severe forms of chronic progressive MS. These studies have been aimed to investigate the impact of the pregnancy state on established EAE and to define the role of the PI3Kγ protein in immune-mediated protection from EAE disease.

Profound suppression of the MS and EAE relapse rate is observed during late pregnancy and is followed by a marked increase in disease severity postpartum. Earlier reports from our lab had demonstrated the importance of the immune microenvironment at the time of immunization. We found that mice immunized during late pregnancy had a decreased clinical severity, while mice immunized during the post partum period showed a rapidly severe clinical course. We have shown these findings to hold true across two different strains of mice. In SJL mice, we observed that histopathologic progression of EAE is diminished during late pregnancy and is exacerbated post partum. During EAE and late pregnancy, mice were found to have less mononuclear CNS infiltration, demyelination and axon severing than virgin controls. To explore
the hypothesis that decreased autoimmune disease during pregnancy is due to immunosuppressive serum factors, serum exosomes were isolated from late pregnant mice, imaged by electron microscopy and used in \textit{in vitro} settings. T cell proliferation assays were carried out using naïve or EAE immunized mouse spleen cells stimulated with anti-CD3 in the presence of 3% late pregnancy serum, exosome free serum or purified exosomes. Serum from age and sex matched virgin mice served as serum controls. While pregnancy serum demonstrated a greater suppression of T cell proliferation, pregnancy exosomes alone caused a substantial suppression of T cell proliferation as compared to virgin exosomes. These results demonstrate the broad suppressive potential of late pregnancy exosomes and may be responsible for amelioration of clinical signs of EAE during late pregnancy. These studies utilized relapsing-remitting (SJL/J mice) EAE model to help determine to role for pregnancy specific factors in immunosuppression during established EAE.

To investigate factors related to disease progression, we used C57Bl/6 mice immunized for EAE using myelin oligodendrocyte glycoprotein (MOG) 35-55. These mice demonstrate a clinical EAE course that is reminiscent of primary progressive MS. We hypothesized that disease progression in this model is mediated by factors that influence immune cell trafficking and inflammatory cytokine production. We investigated the role for phosphoinositide 3-kinases (PI3K) in inflammatory signaling pathways during EAE using the C57Bl/6 mouse strain. PI3Ks are intracellular signaling proteins involved in cellular responses such as chemotaxis, proliferation, and apoptosis. These kinases have been studied in the past for their roles in cancer and diabetes. However, recent studies investigating the immune specific functions of PI3K-$\gamma$ have shown this isoform to be a critical mediator in limiting autoimmune disease severity. Based on these studies and the restricted expression of the $\gamma$-isoform to hematopoietic cells, we hypothesized that targeting this isoform would show relevance for the PI3K$\gamma$ pathway in EAE disease development. We injected C57Bl/6 \textit{wt} or PI3K$\gamma^{-/-}$ \textit{ko} mice with MOG 35-55 to induce EAE and observed the clinical outcomes for up to 61 days. PI3K$\gamma^{-/-}$ mice exhibited a delayed onset and less severe disease course, which was characterized by decreased T cell activation and inflammatory cytokines relative to \textit{wt} controls. Interestingly, male \textit{ko} mice were significantly more protected than female \textit{ko} mice. We utilized adoptive transfer techniques to determine the
role for PI3Kγ in priming and effector functions in recipients during EAE. Our studies found that PI3Kγ−/− males mice are resistant to passive disease induction with significantly fewer CNS-infiltrating cells relative to wt controls. To ensure that the observed protection was specific to PI3Kγ protein function, we used a PI3Kγ selective inhibitor, AS-605240, which resulted in less severe disease when administered prophylactically, and a dramatic recovery when treated therapeutically. These findings suggest that depletion of PI3Kγ results in a down-regulation of the CNS inflammatory response and a suppression of development of encephalitogenic T cells.

Understanding factors that influence sex differences in autoimmunity are important for designing therapies in immune-mediated disorders. In MS, sex influences disease susceptibility as well as clinical outcomes. The studies outlined in this document are directed to: 1) Uncover pregnancy specific changes in a murine model reminiscent of the most common form of MS, relapsing-remitting (RRMS), that largely affects females, and 2) Define the role for PI3Kγ in sex-discrepant susceptibility to EAE disease using a murine model reminiscent of the most severe form of MS, primary progressive (PPMS), primarily seen in males.
DEDICATION

“Dedicated to those for whom the path to success was not clear,
Yet they arrived and unselfishly,
They left a trail.”

--- Na Tosha N Gatson, PhD (2007)

On this journey, I’ve learned that a PhD is earned when one has found comfort and clarity of mind while seated on the edge of insanity, toes dangling in the torrents of humility.

--- Na Tosha N Gatson, PhD (2007)
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My entire family has earned the degree and left their footprints on the trail.
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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract ................................................................. ii</td>
</tr>
<tr>
<td>Dedication ................................................................. v</td>
</tr>
<tr>
<td>Acknowledgments ........................................................ vi</td>
</tr>
<tr>
<td>Vita ................................................................. vii</td>
</tr>
<tr>
<td>List of Tables ........................................................ xi</td>
</tr>
<tr>
<td>List of Figures ........................................................ xii</td>
</tr>
<tr>
<td>List of Abbreviations ................................................. xiv</td>
</tr>
</tbody>
</table>

### Chapters:

1. Introduction ................................................................. 1
   1.1 Multiple Sclerosis (MS) ........................................... 1
   1.2 Experimental Autoimmune Encephalomyelitis (EAE) ........ 3
   1.3 Sex Differences and Clinical Forms of MS ................. 7
   1.4 Studies in CNS Autoimmune Injury and Pregnancy ......... 9
   1.5 Antigen Presenting Cells of Encephalomyelitis .......... 15
   1.6 Phosphoinositide-3-Kinases .................................... 17
   1.7 PI3Kγ and Autoimmune Disease ............................... 19
   1.8 Therapies in MS .................................................... 21
   1.9 Objective ......................................................... 22
2. Materials and Methods

2.1 Animals

2.2 Antigens

2.3 Induction and assessment of EAE

2.4 Pregnancy induction

2.5 Histopathology & Immunohistochemistry (IHC)

2.6 Cell proliferation analysis

2.7 Analysis of secreted cytokines by CBA, ELISA or ELISPOT

2.8 Exosome isolation, quantification and imaging

2.9 Reverse transcription PCR

2.10 Prophylactic and therapeutic treatment using AS-605240

2.11 Hormone pellet implantation

2.12 Statistical analysis

3. Pregnancy Serum Factors Prevent CNS Injury during Ongoing Experimental Autoimmune Encephalomyelitis (EAE)

3.1 Results

3.1.1 Pregnancy suppresses disease activity during ongoing EAE

3.1.2 Pregnancy reduces CNS demyelination during ongoing EAE

3.1.3 Pregnancy affects spleen size and histology during ongoing EAE
3.1.4 Late Pregnancy serum factors are responsible for suppression of EAE........49

3.2 Discussion........................................................................................................58

4. Sex Specific Role for PI3Kγ in Mediating the Development and Progression of CNS Autoimmune Injury ............................................................63

4.1 Results........................................................................................................63

4.1.1 PI3Kγ deficient mice are protected from EAE.................................63

4.1.2 PI3Kγ deficient males have reduced CNS pathology during EAE........67

4.1.3 PI3Kγ deficient males are resistant to passive transfer of EAE...........70

4.1.4 Impaired mononuclear cell infiltration into the CNS of PI3Kγ deficient males.................................................................71

4.1.5 Sex differences in PI3Kγ deficient mice in response to EAE..............73

4.1.6 Blockade of PI3Kγ is protective in EAE..............................................76

4.2 Discussion.....................................................................................................82

5. Summary........................................................................................................86

Bibliography.....................................................................................................112
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 1.</strong> Approved therapies for the treatment of MS</td>
<td>22</td>
</tr>
<tr>
<td><strong>Table 2.</strong> Primers used for rt-PCR</td>
<td>34</td>
</tr>
<tr>
<td><strong>Table 3.</strong> Clinical Averages – Corresponding table for Figure 8</td>
<td>45</td>
</tr>
<tr>
<td><strong>Table 4.</strong> Semi-quantitative scoring – Corresponding table for Figure 9</td>
<td>46</td>
</tr>
<tr>
<td><strong>Table 5.</strong> PI3Kγ deficiency delays onset and decreases progression of EAE</td>
<td>65</td>
</tr>
<tr>
<td><strong>Table 6.</strong> Testosterone is protective in PI3Kγ deficient females during EAE</td>
<td>73</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1. Animals induced for pregnancy during ongoing EAE show full recovery from disease without relapses until after delivery.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 2. Mice with ongoing EAE induced for pregnancy exhibit decreased TNF-α and IL-17 production</td>
<td>39</td>
</tr>
<tr>
<td>Figure 3. Mice with ongoing EAE induced for pregnancy have no differences in IL-2 and IFN-γ production</td>
<td>39</td>
</tr>
<tr>
<td>Figure 4. Late pregnancy during ongoing EAE reduces mRNA levels of inflammatory cytokines <em>in vivo</em></td>
<td>40</td>
</tr>
<tr>
<td>Figure 5. Th2 cytokines IL-4 and IL-5 not increased in mice induced for pregnancy during ongoing EAE</td>
<td>41</td>
</tr>
<tr>
<td>Figure 6. Proliferation is not decreased in mice induced for pregnancy during ongoing EAE</td>
<td>42</td>
</tr>
<tr>
<td>Figure 7. No difference in IL10 production (ELISPOT, ELISA and CBA)</td>
<td>42</td>
</tr>
<tr>
<td>Figure 8. Animals induced for pregnancy during ongoing EAE show decreased CNS demyelination and worsening post partum</td>
<td>45</td>
</tr>
<tr>
<td>Figure 9. Animals induced for pregnancy during ongoing EAE show decreased CNS Infiltration</td>
<td>46</td>
</tr>
<tr>
<td>Figure 10. Pregnancy during ongoing EAE results in increased splenic germinal center formation and fewer PALS</td>
<td>48</td>
</tr>
<tr>
<td>Figure 11. Pregnancy serum suppresses T cell proliferation to PLP 139-151</td>
<td>50</td>
</tr>
<tr>
<td>Figure 12. Pregnancy serum suppresses T cell proliferation to anti-CD3</td>
<td>51</td>
</tr>
<tr>
<td>Figure 13. Exosomes from pregnant mice are larger as compared to virgin controls</td>
<td>53</td>
</tr>
<tr>
<td>Figure 14. Late pregnancy exosomes suppress nonspecific T cell proliferation</td>
<td>54</td>
</tr>
</tbody>
</table>
Figure 15. Pregnancy suppressive phenomenon is not strain specific..........................57
Figure 16. PI3Kγ ko mice are protected from EAE.........................................................64
Figure 17. PI3Kγ ko LNC can be induced for TH17 differentiation ..............................66
Figure 18. PI3Kγ ko males produce less IL-2, MCP-1 and IL-6 relative to wt controls.......66
Figure 19. Reduced CNS pathology in PI3Kγ ko mice..................................................67
Figure 20. PI3Kγ +/- males are mildly susceptible to EAE induction............................69
Figure 21. Absence of PI3Kγ in donors or recipients protects mice in two different adoptive transfer systems.................................................................71
Figure 22. Activated wt lymphocytes fail to accumulate in ko CNS..............................72
Figure 23. Testosterone reduces disease in ko females...............................................74
Figure 24. Estrogen does not increase male ko susceptibility. Castration exacerbates disease. 75
Figure 25 Female ko donors transfer disease to wt and ko males...............................76
Figure 26 Blockade of PI3Kγ in wt males reduces CNS injury during EAE.................78
Figure 27 Blockade of PI3Kγ in wt males reduces TNFα and IFNγ production in EAE....80
Figure 28 Blockade of PI3Kγ in wt males reduces IL-6 but not MCP-1 production in EAE..81
Figure 29 WT males treated with AS-605240 do not exhibit reduced T cell proliferation........81
Figure 30 Class I PI3K signaling cascade.....................................................................107
Figure 31 Aged PI3Kγ null males exhibit profound end organ damage.....................111
ABBREVIATIONS

5α-DHT  5 alpha dihydrotestosterone
Ab    Antibody
ANOVA  Analysis of Variance
APC   Antigen Presenting Cell
APC   Allophycocyanin
AS-605240  Specific PI3Kγ inhibitor (Merck proprietary)
BBB   Blood brain barrier
CBA   Cytometric bead array
CCR2  CC chemokine receptor 2
CDI   Cumulative Disease Index
CFA   Complete Freund’s adjuvant
CNS   Central nervous system
Cop1  Copolymer-1
CORT  Corticosterone
CPM   Counts per minute
CSF   Cerebrospinal fluid
CXC   CXC chemokine receptor
DEX   Dexamethasone
DMSO  Dimethylsulfoxide
E2    17-beta estradiol
E3    Estriol
EAE   Experimental autoimmune encephalomyelitis
ELISA Enzyme linked immunosorbent assay
ELISPOT Enzyme linked immunosorbent spot assay
ESR   Estrogen receptor
FBS   Fetal Bovine serum
FITC  Fluorescein isothiocyanate
FOX3  Forkhead box P3
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GP3   Green fluorescent protein
GPCR  G-protein coupled receptor
H&E   Hematoxylin and eosin
HLA   Human Leukocyte Antigen
IDDM  Insulin dependent diabetes mellitus
IDO   Indoleamine 2,3 dioxygenase
IFN   Interferon
IL    Interleukin
LFB   Luxol fast blue
LN    Lymph Node
MBP   Myelin basic protein
MOG   Myelin oligodendrocyte glycoprotein
mRNA  Messenger ribonucleic acid
MS    Multiple sclerosis
NF-κB Nuclear factor kappa B
PALS  Periarteriolar Lymphoid Sheath
PBMC  Peripheral blood mononuclear cell
PBS   Phosphate buffered saline
PDL-1 Programmed cell death 1 ligand
Definitions

Exosome: Small (~40-100 nm) vesicles secreted into serum by mammalian cells usually as a result of activation. These vesicles are released from cells by microvesicular shedding and most often contain Hsc 70 and other components of the parent cell of origin. Exosomes have recently been studied for their potential biological functions outside of removal of membrane proteins such as tumor immunotherapy, induction of immune tolerance and immune invasion.


Encephalomyelitis: is a general term for inflammation of the brain and spinal cord and can be used to describe a number of disorders. Encephalomyelitis can be caused post infectious or virally induced (acute disseminated) or immune mediated (autoimmune). Animal models, EAE, are induced in rodent strains to mimic the CNS demyelinating and inflammatory effects of MS.
CHAPTER 1

INTRODUCTION

The reasonable man adapts himself to the world; the unreasonable one persists in trying to adapt the world to himself. Therefore, all progress depends on the unreasonable man.

---George Bernard Shaw (1856-1950)

1.1 Multiple Sclerosis

MS is the most common demyelinating disease of the central nervous system (CNS) and affects more than 2.5 million individuals worldwide. Although the etiology of MS has not been clearly defined, it is thought to be autoimmune in nature based on the presence of T cells directed against antigens in the myelin sheath (Noseworthy 2000; Wingerchuk 2001). Also, the presence of autoantibodies to myelin antigens in the CSF of suspected MS patients is suggestive of an autoimmune response (Olek 1999).

Patients usually present with a myriad of symptoms which makes MS difficult to diagnose. Clinicians are largely limited to patient history of symptoms and MRI imaging. Clinical presentation of MS ranges from mild to severe (paralysis), and no two patients can be said to demonstrate the same clinical course. Frequently, the initial complaint is optic neuritis
(unilateral painful vision loss). Other common symptoms include muscle weakness and spasticity, incontinence, loss of coordination and difficult speech. It has been reported that an initial presentation of optic neuritis, for example, suggests a better overall clinical outcome (Olek 1999).

MS is characterized by demyelinating white matter lesions commonly found in the optic nerve, cerebrum, cerebellum, brain stem and the spinal cord (Lassmann 2007, Olek 1999). Periods of inflammation and edema are often superimposed on neurodegenerative processes (Lassmann 2007, Bjartmar 2003). Neurodegeneration, the loss of nerve fibers, is the irreversible consequence of ongoing and severe MS, and is responsible for increasing clinical disability in chronic phases of disease (Bjartmar 2003, Olek 1999).

A genetic predisposition to MS has been proposed since susceptibility to disease often increases in families with other autoimmune diseases, like rheumatoid arthritis (RA) and type I diabetes (formerly known as insulin dependent diabetes mellitus, IDDM) (Marrie 2004). While, the chance of developing multiple sclerosis is less than one-tenth of 1 percent, in families where one family member has multiple sclerosis, that person's first-degree relatives have a 1-3 percent risk of getting multiple sclerosis. No specific gene has been identified for MS; however, researchers have found that people with MS share regions on individual chromosomes more often than people without multiple sclerosis. For example, recent studies are aimed at investigating the human leukocyte antigen (HLA) class II region, specifically the allele HLA-DRB1*15 on chromosome six, in MS susceptibility. Other alleles have been studied for prognostic value in predicting MS severity (Zivadinov 2007). Furthermore, the concordance among monozygotic twins is approximately 33%, similar to RA and IDDM. Also, it has been
found that there is a significantly lower risk among African (black) and Native American populations. Of major interest to MS researchers is the fact that an environmental factor based on a geographical observation demonstrates an increased risk of developing MS in populations farther from the equator. Other studies reference exposure to occupational or environmental toxins as increasing MS susceptibility (Marrie 2004). These findings in MS demonstrate a complex and not well understood etiology with clear environmental and genetic factors for disease susceptibility.

1.2 Experimental Autoimmune Encephalomyelitis (EAE)

EAE is a CD4+ T cell mediated CNS demyelinating autoimmune disease commonly used as an animal model for MS. EAE is induced in susceptible rodent strains (including guinea pigs, rabbits, rats and certain strains of mice) and in macaques, rhesus monkeys and marmosets by immunization with myelin proteins or peptides such as myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). Different species are used for purposes related to life span, reproductive and gestational periods and for their specific clinical response to EAE immunization. Mice are rats, however, are by far the most commonly used animal models for EAE. Since early 1920’s, studies that now are recognized as the origins for modern EAE models have been exploited to better understand the human disease MS. In 1925, Koritschoner and Schweinburg induced inflammatory process in rabbits by injecting homogenized human spinal cord in these animals (referenced in Gold 2006). The addition of mineral oil-based adjuvants by Dr. Jules Freund in combination with brain extracts allowed for a
more rapid induction of clinical signs after a single injection (Kabat et al. 1951 referenced in Gold 2006). EAE has proven to be a great tool for understanding CNS injurious processes in MS based on similar immunologic and histological findings.

Today, EAE is usually induced by active immunization with the whole or portions of myelin antigens in combination with an adjuvant (i.e. complete Freund’s adjuvant, pertussis toxin) or by adoptive transfer of activated cells, donor cells are commonly from an actively immunized mouse. Researchers employ different immunization techniques based on the experimental question. Active immunization is used to evaluate both priming and effector phases in the immunized host. Clinical disease can be seen as early as 9-10 days post immunization (dpi). However, it is sometime difficult to interpret the significance of the latter phase in the host if the priming phase was compromised. To answer questions about the effector response in the host, researchers employ the adoptive transfer model of disease. In adoptive transfer, the priming phase has taken place in the donor, and the transferred cells are entering the effector phase at the time of transfer into recipients. Recipients typically show signs of disease within 6-10 days post transfer (Zamvil 1990, McRae 1992).

Majority of EAE studies have been performed in susceptible mouse strains such as C57BL/6, B10.PL and SJL). Different strains of mice exhibit variations in clinical course in response to myelin peptide/protein immunization and show different patterns of sex dimorphism (Encinas 1996, Papenfuss 2004). Differences in EAE susceptibility to specific myelin components depends on antigen processing and presentation by particular MHC histocompatibility gene products and the frequency of the autoreactive T cells present in the
different strains. For example, relapsing remitting EAE (R-EAE) can be induced in female SJL/J mice immunized with PLP peptide 139-151 (Papenfuss 2004). SJL mice have a higher circulating repertoire of PLP responsive T cells and while they are the only strain sensitive to PLP, they exhibit low levels of disease in response to MOG peptide immunization (Zamvil 1990). A chronic progressive EAE (P-EAE) can be induced in C57Bl/6 mice immunized with MOG_{35-55} peptide and is used to model human PPMS. There are no reported differences in cumulative disease between sexes in this strain, however. B10.Pl mice, on the other hand, develop EAE with MBP immunization.

Investigation of specific cytokines that mediate EAE disease continues to offer new insights into mechanisms controlling disease susceptibility. EAE induction results in an autoimmune proinflammatory Th1 and Th17 response associated with cellular production of cytokines such as IFN-γ, IL-2, TNF-α and IL-17 (Gocke 2007). Changes in predominating cytokines in the host microenvironment can be used to regulate EAE responses. For example, biasing toward Th2 using IL-4 or IL-10 at the time of immunization prevents the induction of EAE (Shaw 1997, Bettelli 1998). These cytokine changes also mediate histopathologic outcomes of EAE that are similar to findings in MS include CNS perivascular inflammatory infiltrates, demyelination and axon severing (Zamvil 1990, Bjartmar 2003).

There are a number of benefits and caveats to using the EAE model to study MS. One benefit is the obvious moral issue with respect to investigations that withhold treatment or allow prolonged disability which would not be an acceptable standard for human research subjects.
There are a great number of animal rights organizations and nationally and institution based ethics committees that ensure ethical treatment of these experimental animals, however. Other advantages is that large numbers of animals can be bred specifically for research purposes, helping to achieve large populations for testing experimental treatments and alternative EAE induction techniques.

Another popular murine model for MS is the Theiler’s murine encephalomyelitis virus (TMEV), first presented in a classic study by Theiler, M. in 1937 (J. Exp. Med. 65:705–719, cited in Clatch et al. 1986). TMEV is a single-stranded RNA picornavirus that produces a chronic persistent central nervous system infection characterized by inflammatory demyelinating lesions of the spinal cord. After intracerebral inoculation of Theiler's murine encephalomyelitis virus (TMEV), certain mouse strains develop a persistent central nervous system (CNS) infection and inflammatory demyelinating lesions containing infiltrates of mononuclear cells and macrophages. These lesions are similar to those of demyelinating plaques seen in MS. It is important to note that in TMEV, CNS demyelination is mediated by the immune system and is not directly in response to viral infection (Clatch et al. 1986).

Disadvantages to using EAE include the increased risk for assumptions that specific findings in EAE are well correlated to what is seen by the clinician or experienced by the MS patient. A good researcher never over-interprets the data as being directly translational to the human situation. Ergo, it is important to understand the limitations of the animal model used. A less ethical and more scientific caveat to using the EAE model is that it requires large doses of potent immune adjuvants that have obvious impacts on the serologic findings in the disease. In
MS, it is unlikely for the patient to have sustained a similar immune insult at the beginning stages of disease. Finally, EAE is largely studied in inbred models, which negates the well established genetic heterogeneity that is characteristic of MS populations.

1.3 Sex Differences and Clinical Forms of MS

Female predominance in autoimmune disease states has been extensively studied; however, the immune or gene-based mechanisms that guide this difference remain unclear. Female to male ratios in rheumatic illnesses range from 10:1, as seen in Lupus to 3:1 in diseases like MS and rheumatoid arthritis (RA). While there are autoimmune diseases that predominantly affect males (i.e. ankylosing spondylitis), females are far more likely to suffer from autoreactive disease states. Sex chromosome effects such as bar-body or imprinting on X chromosomes or Y chromosome (SRY region) as well as hormone specific protective factors have been investigated as potential mediators in sex-discrepant responses to autoimmune disease (Lockshin 2006).

Sex dimorphism in MS is well established (Whitacre 1999; Voskhul 2002; Pelfrey 2002), since women are 3 times more likely to be affected than men and generally present with a relapsing remitting multiple sclerosis (RRMS) course. Men more often present with primary progressive multiple sclerosis (PPMS) characterized by rapid progression of clinical symptoms and pathology (Ebers 2004). Individuals with PPMS experience the most severe disease course; yet, fewer effective treatment options are available to these patients. RRMS is characterized by periods of disease exacerbation followed by defined periods of remission. This form of MS is commonly develops into a secondary progressive form. RRMS patients make up about 85% of
the MS population. PPMS patients are mostly males and make up about 10% of the MS patient population (Ebers 2004, Olek 1999).

There has been great emphasis placed on the potential role for sex hormones in therapeutic approaches for MS. Female hormones such as estrogen and estrogen derivatives have long since been reported to influence immune responses. Special attention has been paid to understanding the neuroprotective role for estrogens in CNS injury in multiple rodent strains (Offner 2004, Bebo 2001, Trooster 1993). Several studies have shown that supplementation with 17-β estradiol and estriol prior to EAE immunization reduces clinical onset and severity of disease (Bebo 2001). It is still unclear, however, whether estrogen effects on EAE are mediated through a direct or indirect mechanism. The male hormone testosterone has recently been investigated for potential neuroprotective effects in MS. The fact that males are less susceptible to disease lends to the possibility that obvious sex related factors such as testosterone and/or sex chromosomes influence protection from disease. Testosterone treatment has been reported to reduce clinical disease in experimental allergic encephalomyelitis in male rats (Macció 2005); however no definitive human studies had been conducted. A recent study done by Sicotte et al. (2007) investigated neuroprotective qualities of phasic exogenous testosterone supplementation in men with RRMS. This study found that atrophic changes in CNS common during MS were slowed during therapeutic periods and discontinuation of testosterone therapy resulted in return of cognitive deficits and increased brain atrophy (Sicotte 2007). Understanding how sex hormones and genetic differences between the sexes regulate immune responses may have implications in multiple sex-discrepant autoimmune and inflammatory diseases.
1.4 Studies in CNS Autoimmune Injury and Pregnancy

The first human studies on the suppressive effects of pregnancy on MS were done in the mid 1900’s. Damek et al. (1997) discussed some of the initial findings that demonstrate a pregnancy suppressive effect on MS activity. Since that time, multiple reports have confirmed the observation of reduced clinical signs in MS patients during the course of the pregnancy and increased clinical disease at some point after the postpartum period. Many studies focused specially on the gestational stage at lowest clinical disease (Korn-Lubetzki 1984, Birk 1990, Bernardi 1991).

A sentinel study often referenced by obstetricians and MS researchers and clinicians was conducted by Confavreux et al. (1998). This study remains the largest pregnancy study completed in the field of MS as it spanned 12 European countries and followed 254 women over 269 pregnancies. Clinical charts were reviewed to obtain a mean baseline for the four trimesters prior to pregnancy, and were followed for 9 months of pregnancy and up to 12 months after delivery. Confavreux (1998) and colleagues reported a baseline rate of relapse of 0.7 per woman per year which dropped to 0.2 relapses per woman per year in the third trimester of pregnancy. Postpartum flare in disease activity was noted in this study, finding an increase of relapses to 1.2 per year; however this number returned to the lower baseline level within the first three months post-partum (Confavreux 1998). Runmarker et al. (1995) reported the long-term protective potential of pregnancy in patients with established MS. These studies found an overall decreased risk of onset of MS in parous women as compared to nulliparous women. Furthermore, women who became pregnant after disease onset demonstrated a reduced risk of progression of MS
symptoms over time. A smaller study looking at changes in MRI in women MS patients who became pregnant, found a decreased number of active gadolinium enhancing lesions in the CNS during late pregnancy (Runmarker 1995). However, the study reports that while there were fewer new lesions, the existing brain lesions did not resolve. MRI scans in these women showed increased active lesions post partum (Runmarker 1995). These investigations provide evidence of a late-stage pregnancy suppressive effect followed by a post partum flare, and supports studies investigating operative immunosuppressive mechanisms during pregnancy.

The immunomodulatory effects of pregnancy on many autoimmune disease states remains puzzling topic for clinicians and researchers alike. Researchers have offered several explanations as to why pregnancy suppresses autoimmune Th1 type diseases; however, there is yet controversy as to how pregnancy impacts Th2 type diseases such as systemic lupus erythematosus (SLE). A study published in the New England Journal of Medicine done by Branch and colleagues (1985) found links between early pregnancy elevations in the antiphospholipid antibodies and increased abortions in women with established SLE. Another study done in 1992 by Ginsberg et al. observed 42 women with SLE that had histories of multiple unsuccessful pregnancies. In this study, these women were followed for 1 or more new pregnancies where serum levels of antiphospholipid and lupus anticoagulant were obtained at several intervals during early pregnancy. Results of this study showed a positive correlation between increased levels of these important coagulation factors and failed pregnancy rates (Ginsberg 1992).

Prior to the above findings being widely reported, physicians and researchers had already begun treating pregnant patients with SLE with increased doses of corticosteroids and aspirin to
reduce suspected risk of pregnancy loss (Lubbe 1983; Parke 1986). A study done by Johns et al. (1998) found that while the number of women that experienced flares in disease during pregnancy were not always matched for elevations in lupus anticoagulant and antiphospholipid antibodies; a significant number of the women experienced unfavorable pregnancy outcomes. Negative outcomes found in several similar studies included abortions, low birth weights, increased perinatal mortality and lower mean gestational ages at delivery (Tincani 1991; Johns 1998; Clowse 2005). It has been well established that women with SLE are in a high risk pregnancy category. Today, the debate remains as to whether there is a statistically relevant associated flare in clinical SLE disease activity during pregnancy or if the disease course remains unchanged. There are, however, no reports of SLE symptoms resolving during any of the pregnancy trimesters as reported in MS.

Another autoimmune based disease that has been observed worsen during pregnancy is Guillain-Barre syndrome (GBS). GBS is a rare neurological disorder associated with infectious processes resulting in antiganglioside antibody production and clinical symptoms of weakness, parasthesias and possible ventilatory collapse (Hurley 1991). As of 2006, slightly more than 30 cases of GBS during pregnancy had been reported in the obstetric literature (Hurley 1991; Vijayaraghavan 2006). Interestingly, these studies reported that GBS disease activity progressively worsened over the gestational period and thereby complicated pregnancy in these women (Hurley 1991; Vijayaraghavan 2006). These studies were not aimed at determining serologic markers increased during pregnancy, but focused on treatment of symptoms that were not detrimental to the pregnancy itself. Other autoimmune diseases, such as psoriasis and rheumatoid arthritis, however, have also been reported to resolve during mid-late pregnancy and
demonstrate a postpartum flare in disease activity similar to MS (Hench 1983; Weatherhead 2007).

Many have postulated that sex hormones play a major role in pregnancy immunomodulation of autoimmune disease states. Others attribute changes in disease activity to cytokine shifts or to the induction of tolerance. Estrogens and estrogen derivatives are increased in cyclic fashion in non-pregnant post pubertal women until the time of menopause; however, during pregnancy, serum levels of estrogen are increased for the duration of the pregnancy. Estrogens are known to influence immune responses and have been investigated for their suppressive effects on EAE during pregnancy (Offner 2004).

There are three active forms of estrogen which increase over the course of pregnancy: estrone, 17-β estradiol and estriol. In early pregnancy, these hormones are produced by the ovaries. This job is taken over by the placenta in late pregnancy and produced in even larger amounts with estriol being the predominant hormone produced almost exclusively by the placenta. Most of these hormone levels decrease dramatically postpartum. Prolactin levels, however, progressively increases over the pregnancy trimesters and remains elevated postpartum for the duration of the lactation period (Jenkins 1978, Gregg 2007). Prolactin has also been reported to be involved in neuroprotection during CNS inflammatory diseases (Gregg 2007).

Bebo and colleagues (2001) investigated the effectiveness of 17-β estradiol and estriol treatment prior to EAE immunization and found a significant delay in onset and suppression of disease activity. Estriol is the focus of a new RRMS clinical trial that is currently recruiting new patients (personal communication, Rhonda Voskuhl, PhD 2007). A pilot trial published in the *Annals of Neurology* included ten RRMS patients and had shown that patients treated with oral
estriol [8mg daily] had increased anti-inflammatory immune shifts, a nearly 80% reduction in MRI enhancing lesions and a reported improvement in cognition in patients (Sicotte 2002). A larger trial is needed to obtain significance. Similarly, estriol studies in EAE have shown that treatment with near pregnancy levels of estriol result in significant reduction of clinical signs (Kim 1999; Liu 2003).

Interestingly, estriol has been shown to regulate the expression of NF-κB and pregnancy has been found to cause a profound and sustained down-regulation of this transcription factor throughout the pregnancy period (Keith 1978). Human pregnancy serum studies have shown a nonspecific suppressive potential on T cell proliferation, inflammatory cytokine production and regulatory effects on transcription factor nuclear factor κ B (NF-κB). NF-κB is responsible for the upregulation of many inflammatory markers such as IL-12, TNF-α, IFN-γ and IL-2, as well as specific chemokines and chemokine receptors (Nicholas 1984; Nicholas 1986; Wolf-Levin 1996). Other studies have focused on the production Th2 cytokines and T regulatory cell products during pregnancy such as TGF-β and IL-10. During pregnancy, tissues at the maternal-fetal interface during the first trimester has been found to express high levels of IL-10 but not IFN-γ, IL-2, TNF-α or IL-1 (Wegmann 1993, Bennett 1999). However, there are many pregnancy associated factors found to be increased that also have immunomodulatory effects. These include proteins such as alpha-fetoprotein, early pregnancy factor, pregnancy-specific glycoproteins, and the enzyme, indoleamine 2,3 dioxygenase (IDO). Of these factors, IDO has been the most well studied and has been shown to impact maternal immunity through its role in
tryptophan catabolism. Tryptophan has been found to be critical to T cell activation and cell survival, thus the interest in pregnancy immunosuppression (Mellor 2007, Munn 1999).

This suppressive hormone response was shown to be pervasive across strain and species in studies done using several mouse strains (SJL/J, B10.PL and C57Bl/6J) as well as in the Lewis rat model (Trooster 1993; Bebo 2001). The SJL mouse strain most closely mimicked the RRMS disease course, and is therefore commonly used in pregnancy studies. However, investigations in Lewis rats, which demonstrate a monophasic disease response, have been useful in establishing the late pregnancy immunosuppressive potential (Harness 2001, Langer-Gould 2002). Langer-Gould et al. (2002) found that SJL females immunized for EAE during late pregnancy showed a nearly 50% reduction in disease incidence as compared to nonpregnant immunized mice. This study evaluated the proliferative response by lymphocytes stimulated in the presence of pregnancy sera, and found a decreased proliferation and the production of IL-2. Another study using Lewis rats immunized for EAE investigated mRNA expression of Th2 and Th1 cytokines in the spinal cords of animals immunized during pregnancy (Harness 2001). To date, limited evidence for the presence of an immunosuppressive serum factor in pregnant animals has been reported. Most importantly, there are few reports that deal with the effect of pregnancy induced during established EAE. This would more closely model the clinical findings reported by Confavreux et al. (1998).

With regard to reduction in overall clinical MS disease activity and systemic immune responses, pregnancy remains the best known therapy (Confavreux 1998). The problem, however, is that the pregnancy state is sex specific! Identification of the immunosuppressive factors in pregnancy may offer new nonsex-discrepant treatment modalities.
1.5 Antigen Presenting Cells in Encephalomyelitis

Proposed mechanisms for CNS injury in MS, based on studies done in the EAE model, are largely T cell centric. The leading hypothesis identifies CD4\(^+\) autoreactive T cell targeting of myelin antigens as the primary culprit. Alternatively, the role for CD8\(^+\) myelin-specific T cells in the pathogenesis of EAE has been described as having pathologic and clinical features of MS not reported in CD4\(^+\) models (Ji et al., 2007; Huseby et al., 2001). Effector T cell based mechanisms focus on the T cell as one of the initiating events driving the inflammatory autoimmune response. This research, however, places emphasis on the role of macrophages in the progression and maintenance of autoimmune CNS injury.

Macrophages are antigen presenting cells with important roles in innate and adaptive immunity. Adaptive immune responses involve activation of macrophages and natural killer cells in order to target intracellular pathogens and to produce cytokines that influence the function of other cells in both the adaptive and innate immune responses. Macrophages are a part of system that includes circulating monocytes, their bone marrow precursors and multiple tissue specific cells that are involved in host defense. Tissue-fixed macrophages are identified by their organ specificity. Lung macrophages are exposed to a larger variety of antigens than CNS macrophages. For this reason, the final phenotype and ultimately the function of macrophages are highly dependent upon the tissue microenvironment. For example, macrophages specific to the liver are called Kupffer cells, Langerhans cells in the skin, osteoclasts in bone, alveolar macrophages in the lung, dendritic or antigen-presenting cells in lymphoid tissues, and microglia in the CNS. In either case, all macrophages retain the ability to
phagocytize foreign particles, secrete inflammatory and anti-inflammatory cytokines and process and present antigen.

In encephalomyelitis, macrophages function in disease processes through presentation of the myelin antigen to autoreactive T cells, elaboration of inflammatory cytokines and damage causing proteases, and cleaning up debris or engulfing foreign particles. Localization is one of the most important steps in macrophage function and antigen presentation is another. Peripheral macrophages migrate to a site of inflammation plays a large role in removing a foreign invader, in the case of infection, or promoting disease, in the case of autoimmunity. Non-resident macrophages are called to the site of inflammation. Their entry into the CNS can be accomplished by several means including areas of decrease BBB integrity and upregulation of adhesion molecules (Karpus 1995).

Events downstream of macrophage activation are propagated by effector cells that secrete IFNγ, which bind IFNγ-receptors on macrophages. Macrophages then produce increased amounts of inflammatory cytokines such as TNFa, IL-1, IL-12 and chemokines such as CCR2 and MCP-1. Elaboration of these factors promote the differentiation of naive T cells to a Th1 phenotype, stimulates T cell proliferation and recruits cells to accumulate at the site serving as an inflammatory positive feedback loop. In the case of autoimmune encephalomyelitis, these processes lead to damage of CNS tissues directly or through the production of matrix metalloproteases (MMPs). MMPs decrease the integrity of the blood brain barrier resulting increased local inflammatory responses. Adhesion molecules and selective cell entry may be altered under these conditions until the integrity of the BBB is restored. When B cells are
present in the cascade, they produce myelin antibodies that directly attack the neuron coatings (Karpus 1995, Tompkins 2002).

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 characterized by activated T cells and macrophages migrating into the CNS and causing tissue damage. The effector phase outcomes are responsible for the overt clinical symptoms seen in MS and EAE patients. (Bjartmar 2003, Tompkins 2002). There is a large body of evidence supporting the necessity for macrophage accumulation in the CNS for the chronic progression of encephalomyelitis (Bennett 2007, Tompkins 2002). Studies using another murine model for MS, Theiler’s murine encephalomyelitis virus–induced demyelinating disease (TMEV-IDD), demonstrate a reduction in disease severity and progression of disease in CCR2-/- mice. Here, there was diminished macrophage accumulation in the CNS due to decrease chemottractant response (Bennett 2007). Macrophages play a detrimental role in autoimmune encephalomyelitis both directly and indirectly, and while they play a clear role in disease induction, they are critical for encephalitogenic disease progression.

1.6 Phosphoinositide-3-Kinases (PI3K)

In mammals, there have been eight identified PI3K catalytic isoforms, divided into three different classes based on their substrate/lipid selectivity and regulation: class IA and IB, class II
and class III. However, class I enzymes are the most well studied for their role in mammalian immune responses based on their ability to generate phosphotidyl-inositides such as the critical second messenger PIP3 [PtdIns(3,4,5)P3] (Fruman 2004). Class I PI3Ks are involved in intracellular signaling pathways which promote cell survival, proliferation, differentiation and chemotaxis (Cantley 1997; Vanhaesebroeck 2005). These enzymes have been studied extensively in cancer research due to their role in cell survival and proliferation. Neoplastic cells have enhanced PI3K signaling which has been attributed to increased expression of oncogenes that activate PI3K pathways (Vivanco 2002). Researchers have also been able to attribute PI3K gene amplification to mutations or deletions in class IA regulatory subunits. These tumor cells have decreased levels of signals which antagonize PI3K pathways such as phosphatase and tensin homologue deleted on chromosome-10 (PTEN). Of the Class I subunits, class IA has been the most well studied in these conditions (Vivanco 2002).

Class IA PI3K isoforms (α, β and δ) have been studied for their roles in cancer and diabetes as these isoforms regulate inflammatory processes (Mehrian-Shai 2007; Taniguchi 2007; Ueki 2002). The p85α regulatory subunit and the p110δ catalytic subunit of class IA PI3K are essential in the development and activation of murine B cells and mice deficient in these subunits mount ineffective antibody responses. It is important to note that mice deficient for the α, β catalytic subunits are early embryonic lethal and therefore no PI3K α or PI3Kβ -/- mice are available for research (Fruman 2004).

Class IB PI3K isoform (γ, PI3Kγ) is only expressed on hematopoietic cells and function mainly downstream of GPCRs (G-protein-coupled receptors) (Vanhaesebroeck 2001; Bi 2002, Fruman 2004). PI3Kγ and δ -/- mice are viable and the PI3Kγ -/- mice show no overt adverse
phenotype (Hirsch 2000). The p110γ, the GPCR-activated isoform has been reported to be essential for optimal T cell proliferation, however, not all PI3Kγ −/− strains demonstrate this finding. (Li 2000, Hirsch 2000).

1.7 PI3Kγ and Autoimmune Disease

Recent studies report critical roles for PI3Kγ in generation of the immune response, specifically in the context of autoimmunity (Hirsch 2000; Camps 2005; Rommel 2007). Restricted expression and limited mechanism for activation of the γ-isoform makes PI3Kγ a potential therapeutic target for immune mediated diseases. PI3Kγ deficient mice exhibit defects in migration of antigen presenting cells (APC) with subsequent impaired T cell activation (Sasaki 2000; Hirsch 2000; Del Prete 2004). In addition, there is a clear dependence on intracellular PI3Kγ for chemoattractant-mediated signal transduction and APC cell motility (Li 2000; Del Prete 2004). Both antigen presentation by APC and T cell recognition of antigen are key in initiating an antigen specific immune response. Furthermore, antigen representation and APC inflammatory cytokine elaboration in the CNS are necessary for progressive phases of disease. These processes have been targeted in the development of therapies for inflammatory and autoimmune diseases (Hemmer 2006; Sweet 2007; Pomel 2006). The recent development of a potent selective PI3Kγ-isoform inhibitor (AS-605240) allows for specific clinical investigation of PI3Kγ in inflammatory autoimmune diseases such as RA and MS (Camps 2005).

Theories regarding the pathogenesis of RA and MS have focused largely on T cells emphasizing the role of autoreactive T cells in the initiation and perpetuation of chronic
inflammation. Camps et al. (2005) investigated the role for PI3Kγ inhibitors, which reduce infiltration of neutrophils and limit disease in animal models for RA. RA and MS share similarities in several features of the disease process, including sex-discrepancy, prominent Th1 inflammatory cytokine profiles, and the involvement of reactive T cells targeted against self-antigens (Lockshin 2006). There are, however, notable pathogenetic and therapeutic differences between RA and MS. In MS, neutrophils are not indicated as primary culprits for the observed CNS injury. This is also not the mechanism of damage targeted in commonly used animal models for MS (Holmoy 2007). Additionally, RA is often associated with elevated levels of cytokines produced by macrophages such as IL-1β and TNFα (Hosaka 2005). The predominant disease mediating cytokines in MS are of mixed leukocyte origin such as IFNγ, TNFα, IL-23 and IL-17 (Gocke 2007). Furthermore, recent studies have shown that anti-TNFα therapies (adalimumab, infliximab and etanercept) are beneficial in treating patients with RA (Perper 2006). Conversely, treatment with anti-TNFα worsens disease in animal models for MS (Kollias 2002) and is contraindicated for patients with pre-existing MS due to increased risk for adverse neurologic events (Mohan 2001). TNFα is a critical player in CNS demyelinating disease processes having dual opposing functions. It is both necessary for the process of remyelination at low levels and causes increased differentiation of CD4+ T cells toward a Th1 phenotype at higher doses (Arnett 2001). PI3Kγ inhibitors mitigate various features of CNS injury that indicate pathogenic mechanisms of MS that are not well understood, underscoring the importance of investigating this potential therapy in both disease processes. Understanding the protective mechanisms in this model could help improve therapeutic modalities for managing chronic progressive forms of MS.
1.8 Therapies in MS

Of the six pharmacological therapies available to MS patients, the most widely used, beta-interferon, has a greater efficacy in treatment of RRMS (Dudesek 2006). Few of the therapies offer significant relief in PPMS patients. Most of the treatment options can be characterized as being immune modifying or immunosuppressive and are largely non-specific in nature. The interferon-β related treatments, including IFN-β1a and IFN-β1b have anti-inflammatory and immunomodulatory activity and work by decreasing T cell activation. Glatiramer acetate (Copaxone), is a random polymer of four amino acids and is thought to mimic myelin basic protein and thereby activate a regulatory T cells population subverting an autoimmune Th1 response. Another therapy, Mitoxantrone, is used to treat MS and broadly suppresses immune cells through cytolysis. The recent return to the market of Natalizumab (Tysabri) after a temporary withdrawal due to three reported cases of progressive multifocal leukoencephalopathy offers another alternative treatment for RRMS patients (Khalili 2007; Jeffery 2006). Tysabri, a humanized monoclonal antibody to alpha-4 integrin subunits, has been shown in clinical trials to delay the progression and frequency of clinical exacerbations in RRMS (Sweet 2007). All of these pharmacological agents, however, exhibit many side effects and have only partial efficacy in MS patients as a group. There remains a demonstrable need for therapeutic advancement in PPMS.

Ultimately, when treating patients with MS, it is important to communicate the long-term goal to the patient, as well as have a full understanding of the limits of each treatment. A table (Table 1) listed below as adapted from Noseworthy et al. (2000) is provided with information
about dosing schedules and routes of administration. This information is important to the patient and the physician when determining the best therapy to fit lifestyle and increase compliance.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Active Ingredient</th>
<th>Course</th>
<th>Schedule</th>
<th>Dose</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVONEX®</td>
<td>IFNβ-1a</td>
<td>RRMS</td>
<td>Once a week</td>
<td>30 mcg</td>
<td>IM</td>
</tr>
<tr>
<td>Betaseron®</td>
<td>IFNβ-1b</td>
<td>RRMS</td>
<td>Every other day</td>
<td>250 mcg</td>
<td>SC</td>
</tr>
<tr>
<td>Copaxone®</td>
<td>GA</td>
<td>RRMS</td>
<td>Daily</td>
<td>20 mg</td>
<td>SC</td>
</tr>
<tr>
<td>Novantrone®</td>
<td>Mitoxantrone HCl</td>
<td>Acute Disease</td>
<td>Once in 3 months</td>
<td>12 mg/m²</td>
<td>IV</td>
</tr>
<tr>
<td>Rebif®</td>
<td>IFNβ-1a</td>
<td>RRMS</td>
<td>3x per week</td>
<td>132 mcg</td>
<td>SC</td>
</tr>
<tr>
<td>Tysabri®</td>
<td>Natalizumab</td>
<td>RRMS</td>
<td>Monthly</td>
<td>300 mg</td>
<td>IV</td>
</tr>
</tbody>
</table>

Table 1. Approved therapies for the treatment of MS. Intramuscular (IM); subcutaneous (SC); glatiramer acetate (GA); hydrochloride (HCl); Intravenous (IV).

1.9 Objective

Multiple Sclerosis is the most common demyelinating disease of the CNS world-wide, and is thought to be autoimmune in nature. However, there are two striking clinical issues that need further attention with respect to immunomodulation by both researchers and clinicians alike. One of those issues is based on the repeated observation that pregnancy affords a level of protection from disease that is far greater than any therapeutic modality offered today. The
second issue deals with the fact that of the few pharmacologic therapies available to MS patients, none have been deemed effective in offering relief to PPMS patients.

The time between mid-pregnancy and parturition provides a window of opportunity to investigate changing circulating factors that might be responsible for immunosuppressive outcomes. Previous studies done in our lab and by others have only addressed disease induction during or after pregnancy time points and they have specifically looked at hormone and cytokine changes during these times. To address the former issue, we used immunized SJL mice for EAE (PLP 139-151) *prior* to pregnancy induction to evaluate clinical, histopathologic and cytokine changes that more closely mimic the human clinical picture. Our studies demonstrate a murine model of MS that has disease responses to pregnancy induced in established EAE that is reminiscent of the human disease during pregnancy. Through our investigations in collaboration with Dr. Paul Robbins at the University of Pittsburgh, we have also been able to report the identification of cellular and serum based immunosuppressive pregnancy derived factors.

While PPMS makes up only 10% of the world’s population of MS patients, it is the most rapidly progressive and clinically severe form of multiple sclerosis. Treatments available to MS patients are largely directed to decrease the frequency of relapses and increase the duration of remission periods, and are therefore primarily effective in RRMS patients. C57Bl/6 mice immunized for EAE using MOG 35-55 peptide exhibit clinical disease that is reminiscent of PPMS. We hypothesized that chronic progressive disease is mediated by continuous antigen representation and lymphocyte recruitment. We aimed our studies in this case to address factors that would influence progressive or chronic phases of disease. PI3Ks are useful in cell survival, proliferation and cellular motility. Impairment of this signaling cascade leads to apoptosis,
decrease cellular differentiation and reduced chemotactic responses to inflammatory signals. C57Bl/6 PI3Kγ -/- males are 83% protected from disease induction by active and passive immunization. PI3Kγ ko males have no CNS pathology and produce very little Th1 and Th17 cytokines in response to EAE immunization as compared to wt C57Bl/6 controls. Female ko mice, while exhibiting reduced clinical signs, were not as protected as male ko mice. Interestingly, female ko mice supplemented with testosterone showed a reduced clinical course in response to EAE as compared to placebo treated ko controls. Through a collaboration with Merck Serono Pharmaceutical, prophylactic use of a specific PI3Kγ inhibitor, AS-605240, affords a reduced induction and less severe non-progressive disease course, while therapeutic use of AS-605240 after acute EAE results in dramatic recovery from disease. This study offers new and hopeful prospects for treatment of chronic progressive forms of MS and an interesting combination therapy using PI3Kγ blockade plus testosterone supplementation may prove even more effective. Further understanding of sex differences in autoimmunity might also be gained from the findings of these studies. Treatment of MS in all its forms and across the sexes has been the driving force behind these studies.
CHAPTER 2
MATERIALS AND METHODS

2.1 Animals

Age-matched female SJL/J and C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and mated with strain matched males for pregnancy experiments. C57Bl/6 PI3Kγ−/− mice were obtained from Dr. Bao Lu at Harvard University, developed as described (Barbier 2001), and housed at The Ohio State University. All mice used for experiments were between 6-10 weeks of age. Mice were maintained on a 12-hour light/dark cycle and given food and water ad libitum.

2.2 Antigens

Peptides used in this study were: PLP 139-151 (HCLGKWLGHPDKF) purchased from Sigma-Genosys (The Woodlands, Texas), and MOG 35-55 (MEVGWYRSPFSRVVHLRNGK) purchased from Princeton Biomolecules (Langhorne, Pennsylvania), and were purified by HPLC, purity >90%.
2.3 Induction and assessment of EAE

SJL

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 (containing 200μg of heat-killed Mycobacterium tuberculosis, Jamaica strain).

C57Bl/6 and PI3Kγ-/-

Mice were immunized subcutaneously in the four flanks with 100 μl of emulsified antigen made up of 200 μg MOG 35-55 combined with complete Freund’s adjuvant (containing 200 μg heat-killed Mycobacterium tuberculosis Jamaica strain). 200 ng of pertussis toxin (List Biological Laboratories, California USA) was injected intraperitoneally in 0.2 ml PBS on the day of immunization and 48 hours post immunization.

For adoptive transfer of disease, wt C56Bl/6 or C56Bl/6-GFP+ (Jackson Laboratories) mice were immunized as described above with MOG 35-55 and spleen cells were harvested 10 days post immunization and cultured in T-75 tissue culture flasks (6 X 10^6 cells/ml) with either MOG peptide 35-55 (20ug/ml) in complete RPMI 1640 media containing 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5 x 10^-5 M 2-ME . After 72 hours in culture, 6 X 10^6 spleen cells suspended in 200ul 1X PBS were injected i.v. into the tail vein of sex and age matched naïve recipient mice.

For adoptive transfer of MOG Vα3.2 and Vβ11 TCR Tg T cells, spleens were harvested from naïve MOG Tg mice obtained from Dr. Vijay Kuchroo at Harvard University. Spleen cells
were pooled from two Tg mice (\(V\alpha 3.2: 33.3+/37.8+\) and \(V\beta 11 33.0+/40.3+\)) and suspended in cold MACs buffer containing PBS, 0.5% BSA and 2mM EDTA and stained with FITC conjugated anti-CD4 mAb (BD Pharmingen, San Diego, CA.). 10 X 10^6 cells were washed twice to remove any unbound mAb by adding 1-2mL MACs buffer and centrifuged at 300Xg for 10 minutes. Supernates were discarded and cellular pellet was resuspended in 90\(\mu\)l MACs buffer per 10 X 10^6 total cells. 10\(\mu\)l anti-FITC MicroBeads (Miltenyi Biotec, Auburn, CA) were added per 10 X 10^6 total cells, vortexed and incubated for 15 minutes at 4°C and washed as described above. 100 X 10^6 cells were suspended in 500\(\mu\)l of MACs buffer. The CD4- cells were then negatively selected for by magnetic separation using large LS MACs columns and a MACs separator (Miltenyi Biotec). Briefly, LS columns were placed on the magnetic field of a MACs separator. Columns were rinsed twice with 3mls MACs buffer, and labeled cells were added to the separation column. The negative fraction containing the CD4- cells was collected in a collection tube. The separation column was removed from the MACs separator and the positive fraction was collected in a separate collection tube. The purity of the negative and positive fraction was assessed by flow cytometry analysis for CD4+/ \(V\alpha 3.2+/V\beta 11+\) cells. 1 X 10^6 T cells suspended in 200ul 1X PBS were injected i.v. into the tail vein of age matched naïve male recipients. Mice were actively immunized for EAE 24hrs post transfer of Tg cells using MOG 35-55 as described above.

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. Wilcoxon Rank-Sum test was used to determine significant differences in clinical disease scores, p<0.05.
2.4 Pregnancy Induction

Established EAE prior to pregnancy: Mice were induced for pregnancy after acute disease (approximately 15-19 days post immunization). Late pregnancy at the time of immunization for EAE: Mice were induced for pregnancy and EAE is induced at late pregnancy (approximately 16-18 days post conception). EAE immunization during post partum period: Mice were induced for pregnancy and allowed to go full term to delivery. Mice were immunized for EAE approximately 3-5 days post parturition. Virgin age/sex matched mice, exposed to male bedding, were immunized for EAE to serve as disease only controls for all groups.

2.5 Histopathology & Immunohistochemistry (IHC)

Histopathology

SJL

Spinal cords, brains and spleens were removed from SJL/J mice EAE immunized pregnant and virgin controls at varying times after pregnancy induction during ongoing EAE (prior to disease induction, during acute disease, during late pregnancy, and post partum). Tissues were fixed in 10% phosphate buffered formalin and then dissected and embedded in paraffin. CNS sections were then processed for hematoxylin and eosin staining (H&E), luxol fast blue (LFB), and Bielschowsky silver stain. Spleen tissues were only processed for H&E
staining. Scoring of stained sections were as follows: Infiltrating cells “Infiltrate” 0=None to minimal; + = mild; ++ = moderate; +++ = severe. Perivascular cuffing layers “Cell layers” 0=None; + = single layer; ++ = two layers.

C57Bl/6 and PI3Kγ-/-

Spinal cords and brains were removed from ko and wt mice at various times during disease to assess tissues: macrophage infiltration (19 days post immunization [dpi]); demyelination and mononuclear cell infiltration (10-49dpi), and axon severing (49dpi). For AS-605240 (PI3Kγ-specific inhibitor) versus saline treated wt mice, tissues were collected after 30 days of treatment to assess CNS histology changes. Tissues were fixed in 10% phosphate buffered formalin and then dissected and embedded in paraffin. Sections were then processed for hematoxylin and eosin staining (H&E), luxol fast blue (LFB), and Bielschowsky silver stain. Kidney and Liver tissues were processed as above. Liver and kidney sections were taken from naive 8 month old wt and ko males and stained using either H&E (liver) or Congo Red (kidney).

For all H&E stains of brain and spinal cord, we quantified the degree of cellular infiltration by assigning scores based on the number of perivascular cuffs seen in the section as well as the number of cell layers (thickness of cells) surrounding each cuff. Sections were scored as follows: 0, absence of infiltrates; +, small, rare perivascular lesions; ++, small, numerous perivascular lesions; +++, numerous perivascular lesions and parenchymal infiltration; and ++++, severe, confluent lesions. Due to the ascending pathologic nature of EAE, spinal cord tissues were evaluated as follows: Mice were selected during defined clinical periods, scored independently and sacrificed for tissue harvesting. Three 2mm sections were taken from the
lumbar, thoracic and cervical portions of the cord and cut at 4-10 microns depending on staining technique used. Each section was studied under light microscopy using 4x – 100x objectives. Areas of pathology were graded in each of the 9 sections per mouse per group and one representative section was selected for publication in this document.

**Immunohistochemistry**

For immunohistochemistry, C57Bl/6 mice were perfused with PBS and 4% paraformaldehyde prior to removal of tissues. Tissues were fixed for 2hrs in 4% paraformaldehyde, placed in 15% sucrose overnight and transferred to 30% sucrose prior to freezing in OCT blocks for sectioning. Infiltration of inflammatory macrophages from the periphery was assessed using rat anti-mouse CD11b antibodies (MCA74G, Serotec) at 10 days post immunization. GFP trafficking studies were performed using rat anti-mouse GFP antibody (gift from Dr. Andrew J. Fischer, Ohio State University) at 10 days post transfer of cells.

**2.6 Cell Proliferation Analysis**

Peripheral lymph nodes (inguinal, axillary, brachial, cervical, popliteal and periaortic) and spleens were removed from mice during late pregnancy in SJL mice and 10 days post immunization for C57Bl/6 studies. 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 (SJL, 30ug/ml), MOG 35-55 (C57Bl/6, 10ug/ml) or anti-CD3 (2ug/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). Results are expressed as CPM ± SEM.

**Th17 Differentiation**

Peripheral lymph nodes were harvested as described above from naive and 10 days post immunization of C57Bl/6 males. 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 X10⁵ cells/well). Cells were cultured with medium alone or with 2μg/ml anti-CD3, 10 ng/ml IL-23, 100ng/ml IL-6 and 5 ng/ml TGF-β. 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 as described above.

### 2.7 Analysis of secreted cytokines by CBA, ELISA or ELISPOT

**CBA.** IFN-γ, TNF-α, IL-2, IL-4, and IL-5 were detected using the mouse Th1/Th2 Kit (SJL studies) or IFN-γ, TNF-α, IL-12p70, IL-6, MCP-1, and IL-2 Inflammation Kit (C57Bl/6 studies) CBA detection systems (BD Biosciences, San Jose, CA) according to manufacturer’s instructions. Standard curves were generated for each cytokine and the concentration of cytokine
in the cell supernatant was determined by interpolation from the appropriate standard curve. All samples were analyzed by flow cytometry (FACS Calibur, Becton-Dickinson, San Jose, CA).

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

**ELISPOT.** Frequencies of cytokine secreting cells were determined for IL-10 (R & D Systems, Minneapolis, MN). Briefly, microtiter plates with nitrocellulose bottoms (Millipore, Bedford, MA) were coated overnight at 4°C with capture antibody. After washing, plates were blocked with 1% BSA (Sigma, St. Louis, MO) for two hours at room temperature. LNC were resuspended in HL-1 medium and then cultured in triplicate with medium alone or with the following stimulants (30ug/ml): PLP 139-151 or anti-CD3 (2ug/ml). Cultures were maintained at 37°C for 72 hours. Plates were washed and cytokine specific biotinylated antibodies were added. After overnight incubation, Streptavidin- AP was added to the plates for two hours. After a final wash, plates were developed with BCIP/NBT chromogen. Image analysis of ELISPOT plates was performed using the KS ELISPOT system (Zeiss, Oberkochen, Germany). Data are expressed as the mean number of cytokine-producing cells per million +/- SEM for all animals in a group.
2.8 Exosome isolation, quantification and imaging

Whole blood was collected via terminal retro-orbital eye bleed from naïve late pregnant (16-18 days post conception) and virgin mice and allowed to coagulate at RT for 30min. Serum was collected by differential centrifugation carried out at at 4°C for several cycles as specified: 4,000g for 5 minutes; 15,000g for 10 minutes; and 14,000g for 20 minutes. Serum was diluted 2:1 in PBS and ultracentrifugation of the serum was completed using a Beckman ultracentrifuge with swinging bucket rotors (SW55(Ti) rotor [5 mL bucket]) spin at 116,000g for 1.5 hours at -20°C. A small aliquot was used for exosome quantification and electron microscopy. The supernatant was saved for exosome-free studies and pellet was washed with sterile PBS and resuspended in 100-200μL PBS and stored at 4°C. Protein concentration was determined by microtiter-plate Bradford assay (Biorad) and remainder was stored at -20°C avoiding repeated freeze-thaw cycles. Imaging was done by electron microscopy.

2.9 Reverse transcription PCR

Lymph nodes were removed from EAE immunized SJL/J mice during late pregnancy, and virgin and naïve pregnant only controls. C57Bl/6 wt and ko mouse LNC were harvested at day 19 post immunization. Single cell suspensions were prepared and suspended in 4mL of Trizol Reagent (Invitrogen) for 15min at RT. RNA was isolated using RNeasy Kit (Qiagen) and
reverse transcription was completed using SuperScript III RT reagents (Invitrogen). Primers were developed in our lab for short range polymerase chain reaction (PCR) experiments using Primer 3® primer design software (See Table 2): 95°C – 60s; and 30 cycles of 95°C – 30s, 56°C –30s, 72°C –30s; final extension of 72°C – 4min before cooling to 4°C.

<table>
<thead>
<tr>
<th>TARGET</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>AAC TTT GGC ATT GTG GAA GG</td>
<td>ACA CAT TGG GGG TAG GAA CA</td>
</tr>
<tr>
<td>IFNγ</td>
<td>ACT GGC AAA AGG ATG GTG AC</td>
<td>TGA GCT CAT TGA ATG CTT GG</td>
</tr>
<tr>
<td>IL12p40</td>
<td>AGG TGC GTT CCT CGT AGA GA</td>
<td>AAA GCC ACC AAG CAG AAG A</td>
</tr>
<tr>
<td>TNFα</td>
<td>CGT CAG CCG ATT TGC TAT CT</td>
<td>CGG ACT CCG CAA AGT CTA AG</td>
</tr>
<tr>
<td>IL-2</td>
<td>TTTGGAGGAAAAAGTGGGAAGA</td>
<td>AACATTCATACATCCT-GGC</td>
</tr>
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Table 2. List of primers. Primers were developed in our lab and used for short range rt-PCR. Cycling conditions were as specified in the text.

2.10 Prophylactic and Therapeutic Treatment using AS-605240

PI3Kγ inhibitor, AS-605240, was synthesized as previously described (Camps 2005), reconstituted in sterile saline, and treatments were administered as described (Barber 2005). WT mice were injected i.p. with 0.1 mL of either AS-605240 10mg/kg and 30mg/kg doses or vehicle (saline) twice daily at 10hr intervals or dexamethasone (Calbiochem, San Diego, CA) 1 mg/kg once daily as a positive control. For prophylactic studies, mice were treated with the drugs one day prior to immunization for EAE. For therapeutic treatment studies, drug treatment was initiated after the acute phase (15-20 days post immunization) during ongoing disease. Clinical
disease was assessed for 30 days after initiation of treatment. Treatment was then discontinued and mice were observed for clinical signs for 15-20 days thereafter. The recently synthesized AS-605240 has been characterized in great detail to include X-ray crystallographic structure, key pharmacokinetic features for potency and selectivity of the \( \gamma \)-isoform (Class IB), oral and injection bioavailability and \textit{in vivo} toxicity profiles as described by Pomel \textit{et al.} (2006). We are currently investigating CNS permeability of this agent, as this is the premier study involving the use of AS-605240 CNS injurious diseases.

\textbf{2.11 Hormone pellet implantation}

Hormone pellet implantation was performed using 12.5mg/pellet 5 alpha dihydrotestosterone (5\( \alpha \)-DHT) (Innovative Research of America, Sarasota, FL) placed s.c. between the scapulae of \textit{wt} and PI3K\( \gamma \) \textit{ko} females 7 days prior to EAE immunization. Male \textit{wt} and \textit{ko} mice were castrated and supplemented with 0.72mg/pellet of 17\( \beta \)-estradiol prior to EAE induction. Noncastrated or sham surgerized and placebo pellet implanted controls were used. All animals were observed daily for clinical signs and scored as described above.

\textbf{2.12 Statistical Analysis}

A two-tailed Student’s \( t \) test was used to determine statistical differences when comparing two groups with parametric data as in the Elisa, Elispot and proliferation assays. A one-way ANOVA was used for the percent expression assays. \( \chi^2 \) analysis was used for
determining differences in disease incidence. Analyses of continuous data such as clinical scores will be determined by using nonparametric Wilcoxon Rank-Sum test with a \( p < 0.05 \) significance level. Sample size calculations were carried out using nQuery Advisor v. 3.0 (Sangus, MA), an industry accepted software used to determine sample size. Simulations using sample size estimates were performed using SAS v8.2 (Cary, NC).
CHAPTER 3

PREGNANCY SERUM FACTORS PREVENT CNS INJURY DURING ONGOING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

3.1 RESULTS

3.1.1 Pregnancy suppresses disease activity during ongoing EAE.

Confavreux et al. (1998) reported that women with established MS who subsequently became pregnant experienced a reduced frequency and severity of relapses during late pregnancy followed by a post partum flare in MS activity. To determine the effect of pregnancy during established EAE, we immunized SJL mice with the immunodominant epitope of PLP (p139-151) prior to pregnancy. After the acute phase of disease, we mated females with age and strain matched males (~21 dpi). We observed a suppression of disease during pregnancy, which was most profound during mid-late pregnancy, followed by a post partum flare in disease activity (Fig 1). Third trimester pregnancy afforded a full recovery from disease. Disease activity
increased dramatically within 2 days of parturition, resulting in profound disability in the mothers and a subsequent inability to nurse the pups. As shown in figure 1, disease progression in previously protected mice was observed to stabilize after peaking around day 7 post partum. To determine if the observed clinical suppression during pregnancy exhibited a corollary change in inflammatory cytokine production, we measured the inflammatory cytokines IL-17 and TNFα, IL-2 and IFNγ production by lymph node cells from EAE immunized mice virgin and late pregnant mice at the late pregnancy time point. Of the inflammatory cytokines tested, IL-17 and TNFα were found to be significantly decreased during late pregnancy in mice with EAE (Fig. 2). IL-2 and IFNγ production were not significantly different between groups (Fig. 3).

Figure 1. Animals induced for pregnancy during ongoing EAE show full recovery from disease without relapses until after delivery. SJL mice were immunized with PLP 139-151 and CFA then induced for pregnancy after the acute phase of disease during the first remission (~20-23 days post immunization, dpi), with non-pregnant EAE immunized mice serving as controls. Animals were monitored for clinical signs for up to 51 dpi, including late pregnancy and post partum period. Data is representative of three experiments. (Virgin n=23; Pregnant n=17)
Figure 2. Mice with ongoing EAE induced for pregnancy exhibit decreased TNF-α and IL-17 production. Mice were immunized with PLP 139-151 and CFA, induced for pregnancy after acute disease and sacrificed during late pregnancy (16-18 days post conception). Lymph node cells were cultured with PLP 139-151 for 72 hours. Cytokines were measured: a) TNF-α (CBA) and b) IL-17 (ELISA). Data are representative of three experiments. * p<0.05 compared to non-pregnant EAE disease only controls. (n=3 per group)

Figure 3. Inflammatory cytokines IL-2 and IFNγ are not decreased during pregnancy in established EAE. Mice were immunized with PLP 139-151 and CFA, induced for pregnancy after acute disease and sacrificed during late pregnancy (16-18 days post conception). Lymph node cells were cultured with PLP 139-151 for 72 hours. Cytokines were measured: a) IFNγ and b) IL-2 (CBA). Data are representative of three experiments (n=3-5 per group per experiment).
To determine if there was an observable difference in the production of inflammatory markers (IL-2, IL-12p40, IFNγ and TNFα) at the RNA expression level during late pregnancy, we isolated total RNA from lymph node cells of late pregnant (16-18 days post conception) and virgin control mice. Virgin mice immunized for EAE showed higher levels of all inflammatory markers as compared to mice that became pregnant (Fig 4). Pregnancy thus appears to downregulate the expression of these inflammatory mediators. Contrasting protein and RNA findings in these cytokines suggest post transcriptional processing or breakdown events.

**Figure 4. Pregnancy during ongoing EAE reduces RNA levels of inflammatory cytokines in vivo.**
Mice were immunized with PLP 139-151 and CFA, induced for pregnancy after acute disease and sacrificed during late pregnancy. LNC were placed in 4mL of Trizol reagent at room temperature for 5 minutes. RNA was isolated using RNeasy Kit (Invitrogen, Carlsbad, California) and reverse transcription was completed using Superscript III RT (Invitrogen). PCR was completed using IL-2, IL12p40, IFNγ, TNF-α, and GAPDH primers designed in our laboratory. Images are displayed as agarose gel image (top) and in numeric value graphed as an area under the curve using NIH Scion Gel Imager Software for quantitation. One representative experiment of three experiments.
We considered the possibility that anti-inflammatory Th2 cytokine production contributed to clinical suppression during pregnancy. Using CBA assays, we evaluated the production of IL-4 and IL-5 under the same conditions as listed in the above CBA studies. IL-4 and IL-5 were found to be produced in negligible amounts in both pregnant and control groups (Fig 5). These data demonstrate that a Th2 cytokine shift is not likely to be the mechanism of protection.

**Figure 5.** Th2 anti-inflammatory cytokines IL-4 and IL-5 are not responsible for observed protection during pregnancy in established EAE. Mice were immunized with PLP 139-151 and CFA, induced for pregnancy after acute disease and sacrificed during late pregnancy (16-18 days post conception). Lymph node cells were cultured with PLP 139-151 for 72 hours. Cytokines were measured using CBA analysis. Data are representative of three experiments (n=3-5 per group per experiment).

To evaluate the affect of pregnancy on lymphocyte proliferation in response to the immunizing antigen (PLP), we sacrificed mice during late pregnancy or at a comparable time in control mice and cultured whole spleen cells with PLP 139-151 for 72hrs. We found that lymphocytes from late pregnant mice proliferated significantly more than cells from the virgin mice in response to PLP peptide (Fig 6). This finding was likely due to increased growth factors present normally to aid in the maintenance and development of the fetus during the pregnancy period.
Figure 6. Proliferation is increased in mice induced for pregnancy during ongoing EAE. SJL mice were immunized with PLP 139-151 and CFA and pregnancy was induced (16-18 days post immunization), with non-pregnant diseased mice serving as controls. Lymph node cells were harvested during late pregnancy and cultured with PLP 139-151 for 72 hours, including an 18 hour pulse with [3H] thymidine. Data is shown from one representative experiment of four. (n=6-10 per group)

Figure 7. No difference in IL-10 production (ELISPOT, ELISA and CBA). SJL mice were immunized with PLP 139-151 and CFA and pregnancy was induced (15-18 days post immunization), with non-pregnant diseased mice serving as controls. Spleen cells were harvested during late pregnancy and cultured with PLP 139-151 for 72 hours. IL-10 was measured by ELISA (a) ELISPOT (b) and CBA (c). Representative of 2 experiments (n=3-4 per group).
IL-10 is a regulatory T cell product and has been shown to be a powerful mediator of inflammatory disease suppression. Our lab, as well as others, has reported a significant up regulation of IL-10 during pregnancy. Past studies have shown that up regulation of IL-10 during pregnancy reduces EAE disease susceptibility in the late and post partum periods. Those studies evaluated affect of increased levels of IL-10, in the context of pregnancy, prior to or at the time of immunization for EAE. In contrast, this study focused on cytologic changes during ongoing EAE in mice that were later induced for pregnancy in order to determine the affects of pregnancy on established EAE. We measured changes in IL-10 in mice immunized for EAE and subsequently induced for pregnancy. To determine if modulation of IL-10 was involved in the observed clinical suppression during pregnancy and EAE, we employed several assays: ELISA, CBA and ELISPOT. The ELISA and CBA techniques measure the net amount of secreted protein remaining in the supernatants at the time of assay and therefore can not provide information about per cell cytokine production or cellular reuptake and protein degradation which occurs over the assay period. ELISPOT analysis provides information about per cell production of the cytokines and binds the cytokine to a substrate immediately after it is secreted by the cells. Overall, production of IL-10 during late pregnancy (16-18 days post conception) was not found to be significantly different between groups when measured by either levels in supernatants from cells cultured with PLP peptide for 72hrs (ELISA, Fig. 7a; CBA, 7c) or by determining the frequency of cells that secrete IL-10 (ELISPOT, Fig. 7b). IL-10 does not appear to be a primary mediator of disease suppression in this model.
3.1.2  *Pregnancy reduces CNS demyelination and infiltration during ongoing EAE.*

We evaluated the histopathologic progression of EAE during pregnancy. The Luxol Fast Blue staining technique is used to evaluate areas of demyelination as observed as unstained tissues that are expected to have an abundance of myelinated fibers. We used Luxol Fast Blue to stain lower cervical and upper thoracic spinal cord sections, and observed dramatic demyelination in the dorsal and ventral areas of the spinal cord during the acute phase (15-17dpi) of disease (Fig 8a) followed by a reduction in demyelination in the same areas during late pregnancy (16-18 dp conception) in established EAE (Fig 8b). Figure 8c demonstrates a post partum increased in demyelination just 10-13 days post partum. These mice were immunized for EAE, induced for pregnancy and followed through the post partum period. Increased demyelination was found, in these mice, to correspond with the increased clinical picture. Although, it is understood that identifiable changes in immunological and histological lesions do not necessarily predict clinical signs during EAE. The histology images used in these studies are not intended to be used as a graded measure of clinical function or disability. Dorsal spinal cord structures, such as the fasciculus gracilis and fasciculus cuneatus contain fibers involved in proprioception or “position sense,” that is, being aware of where the limbs are in space at a given time. Ventral structures of the spinal cord, such as the lateral and anterior corticospinal systems, contain nerve tracts that are important in basic voluntary motor movements. As the corticospinal motor and somatosensory fibers exit the brain and tract through the pons (i.e., corticopontine fibers) headed for the spinal cord. This area also contains postural tracts that are essentially necessary for maintenance of posture against gravity – requiring many small muscle adjustments during motion. All of these
pathways are important in ambulation and balance and if affected might cause some of the disability of gait that we observed in clinically diseased mice. The corresponding table for figure 6 shows mean clinical scores and dates post immunization (dpi) and conception [dpc] at the time of sacrifice (Table 3).

### Table 3. Corresponding clinical scores and pregnancy status during periods of increased cellular infiltration and demyelination (Table for Fig 8.)

<table>
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<th>ACUTE</th>
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<tr>
<td>DPC</td>
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Figure 8 (a-c). Animals induced for pregnancy during ongoing EAE show decreased CNS pathology and worsening post partum. SJL mice were immunized with PLP 139-151 and CFA and induced for pregnancy ~18dpi, with virgin EAE immunized mice serving as controls. At ~40 days post immunization, brain and spinal cords were removed, photographed, and fixed in 10% formalin for 5 days before paraffin embedding. Luxol Fast Blue staining was completed and cervical-lumbar sections [Virgin n=14, Pregnant n= 5, Postpartum n=11].
Figure 9 (a-c) Animals induced for pregnancy during ongoing EAE show decreased CNS infiltration. Animals were immunized as stated (Fig 8) above and H&E staining was completed in similar sections with a high powerd view of ventral spinal cord structures. Infiltration was quantified using a scoring system as detailed in the methods Histopathology. Data is representative of two experiments. (Virgin n=3; Virgin EAE  n=7, Pregnant n= 7).

<table>
<thead>
<tr>
<th>Infiltrate</th>
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<tr>
<td>Naive</td>
<td>+</td>
</tr>
<tr>
<td>EAE Virgin</td>
<td>+++</td>
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<tr>
<td>EAE Pregnancy</td>
<td>++</td>
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Table 4. Decreased inflammatory cells present in the CNS of EAE immunized pregnant mice relative to virgin controls (Table for Figure 9). SJL mice were immunized with PLP 139-151 and CFA, induced for pregnancy and sacrificed at late pregnancy (15-18 days post conception), virgin mice served as controls. Tissues were processed for hematoxylin and eosin staining and scored for inflammatory cell infiltration and perivascular cuffing “layers”. (Experimental n=7, control n=3).

We also assessed mononuclear cell infiltration using H&E staining in the ventral funiculi of the spinal cord and found that pregnancy during ongoing EAE (Fig 9b) reduces cell infiltrates relative to EAE immunized virgin controls (Fig 9c) as compared to naive mice (Fig 9a). Table 4 provides a semi-quantitative analysis of Figure 9.
3.1.3 Pregnancy affects spleen morphology and histology during ongoing EAE.

Multiple reports have shown increases in regulatory cell numbers from lymphoid organs during pregnancy as an essential aspect of pregnancy maintenance. Our lab has recently reported increased levels of CD4+/CD25+ cells in the spleens of mid-late pregnant mice. In addition, those animals had a two fold increase in overall splenocyte number (personal communication, McClain et al. 2007 J Immunol in press). This is likely due to increased hematopoesis which takes place with volume expansion during pregnancy. Extramedullary hematopoesis is known to take place during pregnancy in an effort to accomadate the newly expanded hemodynamic demands on the cardiovascular structures as the fetus develops. The spleen and liver have been noted to be dramatically increased in size specifically during the third trimester of pregnancy (Boldorini et al., 2006). Excessive expansion of the splenic blood compartments are pathogenetic and has been reported to lead to fatal splenic rupture in the 3rd trimester of human pregnancy in mothers with blood dyscrasias (Boldorini et al., 2006). Upon sacrificing the pregnant and virgin mice from the above experiment, we observed that the pregnant mice had markedly larger spleens than their virgin counterparts (Fig. 10 top). These physiologic changes are likely signaled in response to the changing hormone environment, with the primary hormone producer being the placenta.
SJL spleens were removed and photographed 18 days post conception. Pregnant mouse spleen (left) and virgin mouse spleen (right). (a-c) SJL mice were immunized with PLP 139-151 and CFA and induced for pregnancy at 16 days post immunization. Virgin EAE immunized mice served as controls. At ~40 days post immunization/18 days post conception, spleens were removed and processed for H&E staining. Naïve virgin (a), EAE pregnant (b), and EAE virgin (c) spleens are depicted. EAE pregnant spleens (b) show increased germinal center formation and reduced PALS relative to (c) EAE virgin controls.

Figure 10. Pregnancy during ongoing EAE results in increased germinal center formation and fewer PALS. Pregnancy results in increased spleen size (photo top). SJL spleens were removed and photographed 18 days post conception. Pregnant mouse spleen (left) and virgin mouse spleen (right). (a-c) SJL mice were immunized with PLP 139-151 and CFA and induced for pregnancy at 16 days post immunization. Virgin EAE immunized mice served as controls. At ~40 days post immunization/18 days post conception, spleens were removed and processed for H&E staining. Naïve virgin (a), EAE pregnant (b), and EAE virgin (c) spleens are depicted. EAE pregnant spleens (b) show increased germinal center formation and reduced PALS relative to (c) EAE virgin controls.

In this study, we compared actual size of spleens in virgin and pregnant mice with EAE and looked at the histologic architecture of the spleens during acute disease and late pregnancy using a standard H&E staining technique. Hematoxylin is a basic stain that stains basic cellular components such as chromatin and ribosomes a deep blue color providing a picture of the cell nuclei. Eosin stains acidic structures of the cell a red color, and provides a picture of the cellular membrane. H&E was used in this study to provide information about cell clustering patterns on low magnification and can be used to distinguish cell types (i.e. neutrophil, monocyte, lymphocyte) on higher magnification based on chromatin structures. We observed that pregnant
mice had overall larger spleens on gross imaging. However, in the mice impregnated during ongoing EAE, there was a decrease in the size of the T cell compartments (periarteriolar lymphocyte areas and white pulp) and increased B cell compartments (germinal centers) on H&E staining relative to EAE virgin controls (Fig. 10 bottom). While the aims of our pregnancy studies were not centered on the role for B cells; it was important to recognize other likely contributing cell types and lymphoid organs in the pregnancy suppression of disease. The production of immunosuppressive Ig’s during pregnancy by newly expanded B cells is likely the result the body’s attempt to neutralize destructive factors targeted against the growing fetal allograft. Ongoing studies are being conducted in the lab to investigate the role for B cells during pregnancy suppression of EAE.

3.1.4 Late Pregnancy serum factors are responsible for suppression of EAE

Langer-Gould et al. (2002) first reported that relapsing-remitting EAE in the SJL mouse was maximally suppressed during late pregnancy and proposed the existence of a protective serum factor during pregnancy, which was not identified. Past studies that have investigated the protective serum factors have used two experimental designs that evaluate hormones and cytokines present in the serum during: 1) ongoing pregnancy in the absence of disease, or 2) disease induced at the time of pregnancy. Those studies answer questions related the protective properties of pregnancy that prevent disease induction. In this study, we evaluate mice with established EAE and measure clinical and cytologic affects of pregnancy induction during
ongoing disease. These studies are therefore intended to answer questions related to protective properties of pregnancy that suppresses ongoing disease.

To determine the relative suppressive potential of late pregnancy serum alone, we collected lymph node cells from virgin naïve and virgin EAE immunized mice and plated them as effector cells for a T cell proliferation assay. Cells were cultured with PLP 139-151 for 72 hours in the presence of 3% virgin or late pregnancy serum. Cells supplemented with pregnant mouse serum were more suppressed than virgin serum treated cells (Fig. 11). Naïve cells were more readily suppressed by pregnancy serum (53% suppression as compared to medium alone). Virgin serum only mildly suppressed in this case. In mice that had been previously immunized with PLP, suppression was 34% as compared to medium alone (no serum), and virgin mouse serum suppressed about 22%. Pregnancy serum was also observed to suppress lymphocyte proliferation to anti-CD3 with similar significant differences (Fig. 12).

Figure 11. Pregnancy serum suppresses T cell proliferation. LNC from virgin naïve and virgin 10dpi EAE immunized mice were collected and plated as effector cells for a T cell proliferation assay. Cells were cultured with PLP 139-151 for 72 hours in the presence of 3% virgin or late pregnant serum. Cells supplemented with pregnant mouse serum were more suppressed than virgin serum treated cells. * p<0.05 compared to virgin serum. † p < 0.05 as compared to media treatment only (n=3 per group).
The prevailing view is that suppression of immune reactivity during pregnancy somehow involves hormones such as estrogen, which is neuroprotective. Many studies have shown that estrogen reduces MS and EAE severity when given exogenously. Presently, there are no treatments available or completed hormone supplementation trials that can be observed to provide levels of suppression equal to that of the pregnancy state. For this reason, we suspected that there non-hormone serum factors are also present and aid in the suppression of disease seen in our previous results (Fig. 1). In collaboration with Dr. Paul Robbins, University of Pittsburgh, serum exosomes were separated from whole serum using serial ultracentrifugation, quantified by Bradford protein assay (Biorad, Hercules, CA), and imaged using electron microscopy. Exosomes have recently been studied for their role in adjuvant therapies.... We observed phenotypic differences between exosomes derived from pregnant mice as compared to virgin controls. Pregnant exosomes were larger and more numerous than those from virgin mice (Fig 13). Exosomes were positive for Hsc 70 and FasL as determined by Western blot. However, continued studies are needed to determine the cellular source of these exosomes as well as other molecules contained within or expressed by these vesicles. To test the hypothesis that non-hormone factors of pregnancy play a role in the observed suppression of ongoing EAE, we
resuspended purified exosomes derived from virgin and late pregnant mice in PBS and used them in T cell proliferation assays. In these studies, we compared T cell proliferative responses when cultured with whole serum, purified exosome isolates and serum only (exosomes removed). In all comparisons, we used media as a negative control for normalization of the experimental groups (virgin or late pregnant serum products). Whole serum from late pregnant mice suppressed anti-CD3 stimulated T cell proliferation by 35% as compared to a 7% suppression by virgin serum (Fig. 14a). All components of serum from late pregnant mice were present in the assay and provide a baseline of suppression including soluble and non-soluble factors. To determine if the exosomes alone were capable of suppressing T cell proliferation, we added equivalent volumes of purified exosomes resuspended in sterile PBS. Purified exosomes from late pregnant mice cultured with T cells stimulated with anti-CD3 results in a 26% suppression, whereas virgin serum derived exosomes afford only a 10% suppression in proliferation (Fig. 14b). These findings suggest that pregnancy derived exosomes are powerful at suppressing T cell proliferation and likely package immunosuppressive factors (PDL-1, TGFβ, estrogens). This does not rule out the possibility that these packaged factors could be leaked into the serum in whole serum preparations. Alternatively, factors present in the serum could be similar to the exosomal packaged factors, however, present in varying amounts or having variable half-lives or stability in serum. Finally, serum from pregnant mice that was cleared of exosomes resulted in a 25% suppression of T cell proliferation, while virgin serum without exosomes only caused an 8% suppression (Fig. 14c). These results could be interpreted to support the idea that soluble (hormones, cytokines) factors present in the serum are powerful at suppressing T cell proliferative responses independently of the exosome. However, the
possibility that removal of exosomes from the serum resulted in damage to exosomal membranes and released their contents into the serum could not be ruled out. Taken together, these findings support the hypothesis that pregnancy serum is more potent at suppressing T cell activation than virgin serum. These studies also demonstrate the capacity for identifiable nonsoluble component of serum, such as the exosome, to suppress inflammatory T cell responses. Studies to further characterize the exosomes are ongoing.

**Figure 13.** Exosomes from late pregnant mice are larger as compared to virgin control exosomes. Virgin and late pregnant mice were sacrificed 16 days post conception and serum was collected via retro-orbital eyepipette. Exosomes were harvested via ultracentrifugation, resuspended in PBS and imaged using electron microscopy at University of Pittsburgh.
Figure 14. Late pregnancy serum factors suppress T cell proliferation. Virgin and late pregnant mice were sacrificed 16 days post conception and serum was collected via retro-orbital eye bleed. Whole serum (V/P serum, A), exosomes (V/Pexo, B) and exosome free serum (V/Pexfree, C) were harvested from serum and stored at 4°C. 5×10⁵ spleen cells were cultured with anti-CD3 and serum components for 72 hrs and T cell proliferation was measured as compared to cells alone. Percent suppression is shown in box to the right of each graph. * p<0.05 compared to non-pregnant controls. (n=3 mice x 9 wells per mouse per group).
To this point, we had shown that pregnancy is suppressive in SJL mice with relapsing EAE when induced during ongoing disease. We hypothesized that the pregnancy suppressive phenomenon would be pervasiveness across strain and disease type. To test this hypothesis, we chose to study another well established mouse strain for EAE, the C57Bl/6 mouse strain, which exhibits a primary progressive form of disease. C57Bl/6 mice, however, are susceptible to a different myelin antigen, MOG 35-55, and have been historically known to require pertussis toxin as an additional adjuvant for significant disease induction. In contrast with SJL mice, the C57Bl/6 mice do not demonstrate a relapsing-remitting disease course. These mice exhibit a disease course that is reminiscent of primary progressive MS disease.

To examine the impact of pregnancy in the C57Bl/6 strain, we used three experimental designs: 1) Pregnancy induced during established – after the acute phase of disease, 2) Immunization for EAE during the post partum phase, and 3) Immunization for EAE during late pregnancy. The first method addresses the impact of pregnancy during clinical disease. The second method is useful to determine outcomes on disease course when EAE is initiated during an inflammatory period. Finally, the third method evaluates clinical outcomes when disease is initiated during anti-inflammatory microenvironments. First, we repeated the original model tested above in the SJL mice, where mice with established EAE were later induced for pregnancy. Similarly, we found that pregnancy reduced disease most profoundly during the 3rd trimester and reduced the overall severity of disease post parturition (Fig. 15a). Second, we tested whether signs of EAE would be increased during the post partum period in the C57Bl/6 mice. Immunizing for EAE during the post partum stage was not found to be protective (Fig.
Attempts to induce disease during late pregnancy in C57Bl/6 mice using MOG 35-55 + CFA + Pertussis toxin (PT) on days 0 and 2 post immunization resulted in abortion of pregnancy and a more severe disease course (Fig. 15c).

Pertussis toxin is an exotoxin produced by the bacterium *Bordetella pertussis* and released in an inactive form. Once bound to cell membrane surfaces, PT becomes activated. PT then catalyzes the ADP-ribosylation of G-protein inhibitory subunits and interrupts intracellular signaling pathways. In EAE, it is useful as an adjuvant to promote the development of tissue specific autoimmune disease by increasing Th1 cell mediated responses and enhancing vascular permeability. Using the PT as an adjuvant in pregnant mice revealed possible abortifacient properties of the toxin. To test this idea and rule out the possibility of other factors related to EAE immunization could be causing abortions, we immunized mice with CFA and MOG only during late pregnancy period and observed no preterm delivery and an overall decreased susceptibility and severity of disease in C57Bl/6 mice (Fig. 15d).
Figure 15. Pregnancy suppression of disease is not strain specific. C57Bl/6 mice were immunized with MOG 35-55 and CFA + Pertussis toxin (PT) and observed for signs of disease. Pregnancy induced during ongoing EAE results in decreased severity of EAE (a). Disease activity is shown to increase during the post partum period (b). Immunizing mice during late pregnancy using PT resulted in abortion of pregnancy and no observed suppression of disease (c). Revision of the immunization protocol to exclude the PT injection prevented preterm delivery of pups and resulted in less severe disease during pregnancy as compared to virgin controls (d). [EP= EAE Pregnant; EO= EAE virgin; EPpp= EAE post partum; EOpp= EAE virgin post partum controls]. Representative of two experiments; n=5-7 per group.
3.2 DISCUSSION

Pregnancy profoundly impacts MS and EAE disease activity, with the third trimester being the most suppressive period. The post partum period represents an inflammatory phase during disease, which is observed to increase in severity. The time between late pregnancy suppression and the post partum flare provides a window of opportunity to investigate the mechanisms that influence pregnancy suppressive effects. Previous studies on pregnancy and EAE were not aimed at addressing the mechanisms that guide the human MS clinical scenario, but rather were focused on determining the impact of pregnancy on immunization as opposed to the impact of pregnancy on disease. By immunizing mice during various gestational periods, researchers have addressed the question of the effect of the microenvironment at the time of disease induction. In the case of humans with MS, this would address what happens to the disease course of MS if disease is initiated during pregnancy. More than likely, this is not the clinical case. Typically, MS is established prior to pregnancy. Since this is the more common clinical scenario, we aimed our studies to address the outcome on the MS disease course if one becomes pregnant. Other studies have looked at the pregnancy effect in acute disease models, such as the Lewis rat. This too is a rare clinical presentation. To date, there are few reports about the factors of pregnancy that suppress MS or EAE.

Using SJL/J mice with R-EAE, we were able to recapitulate the human MS pregnancy scenario where the patient has established disease and subsequent pregnancy suppresses disease activity. In this study, we also provided information about the pervasive effects of pregnancy in that the suppressive phenomenon is not strain specific. Here we offer insight into two separate
questions. First, pregnancy suppressive effects are not MHC or strain specific. This lends to the potential broad application of our findings to human disease states, as humans are highly variable in HLA (MHC) specificity. Second, is pregnancy can afford profound suppression in both relapsing-EAE and progressive-EAE. R-EAE in the SJL mouse is studied as a model that mimics RRMS while P-EAE is the C57Bl/6 mouse model for PPMS, with the former affecting more women and the latter affecting more men.

After developing a model that shows a similar effect across strains, we studied different aspects of the pregnancy state when induced during ongoing EAE. We observed a similar clinical picture in pregnancy during R-EAE to that of pregnancy during RRMS as reported by Confavreux et al. (1998). We evaluated cytokine changes at the level of mRNA, protein, and serum in *in vitro* and *in vivo* settings. We also observed an overall increase in cells in the spleen in late pregnant mice relative to virgin controls. Changes in the gross size as well as the histology of the spleen during pregnancy were seen. It will be important to investigate localization of specific spleen cells in both B cell and T cell compartments within the spleen. It is possible that pregnancy affects multiple cell types responsible for the overall inflammatory cascade seen in EAE and MS.

We found an overall decrease in TNFα and IL-17, inflammatory cytokines. However, there was no significant difference in IFNγ, IL-10, IL-2 and T cell proliferation between the groups. Furthermore, IL-5 and IL-4 were not found to be significantly increased during pregnancy as compared to virgin controls. This information is important because it helps us to understand if a Th2 bias is protective, or if other mechanisms are operative. For example, IL-10 is classified as an immunoregulatory cytokine that is produced by a number of cell types,
including Th2 T cells, T regulatory cells, CD4+/CD25+ cells, and many APCs (Mocellin 2003). Increased levels of IL-10 have also been reported in studies examining protective effects of pregnancy hormones, such as estrogen, during EAE (Offner 2004). While some studies cite a cytokine shift during pregnancy based on an increase in levels of Th2 cytokines found at the maternal-fetal interface (Chaouat 1999), our findings do not match this and cannot be directly compared due to the ongoing EAE at the time of pregnancy induction. Since EAE is predominantly a Th1 driven disease and pregnancy is a Th2 biased state, the observation of cytokine shifts may be complicated by masking or inhibition of one type by the other. There may be higher levels of Th2 cytokines produced during pregnancy and ongoing EAE, but, the pre-existing Th1 cytokines accompanying acute disease may limit the detection of the protective cytokines. Regardless of their levels, Th1 vs Th2, there appears to be other factors at play that can be identified as having a role in suppression of disease during the pregnancy state induced after EAE onset.

Interestingly, we found a non-soluble serum derived factor that is capable of suppressing T cell proliferation in response to the immunizing antigen, PLP, and a nonspecific T cell stimulant, anti-CD3. One of the non-soluble factors of late pregnancy, we suspect is the serum derived exosome. Exosomes have been thought of as byproducts or waste post cell death. However, these are also microvesicles shed from activated cell types. These cell types include tumor cells, fetal cells and cells of the immune system (Taylor 2006). It is thought that fetal cells establish a state of microchimerism in the mother during pregnancy (Whitacre 1999) and possibly suppress reactive T cells in an effort to maintain a successful pregnancy. Women in the late stages of pregnancy produce nearly twice as many exosomes and increased expression of Fas
ligand than women who undergo early delivery or abortion (Taylor 2006). The placenta is thought to be a source of these circulating exosomes. The placenta is known to produce immunoregulatory hormones such as estrogen and progesterone. It has been proposed that the role of the placental derived exosome is to modulate the activity of effector T cells by activating apoptotic cascades and downregulating CD3 zeta chain expression. Furthermore, the presence of the inhibitory costimulatory molecule PDL-1 on placental derived exosomes was shown (Sabapatha 2006). It is our goal in these exosome studies to identify the contents of the pregnancy exosome, the mechanism by which exosomes delivery its contents to other cells and to determine whether the exosome is truly responsible for suppression of immune responses. These findings could lead to development of better treatment modalities that are not pregnancy or hormone specific, yet are efficacious in suppressing inflammatory aspects of disease.

There are few reports in the literature addressing the specific questions we address here, with respect to disease and pregnancy effects. One such report by Langer-Gould et al. (2002) showed that immunization for EAE during the latter half of pregnancy is more protective than during early pregnancy. We showed that this effect can be seen in C57Bl/6 mice and our lab previously demonstrated this finding in SJL R-EAE as well. Accompanying this is a decrease in inflammatory Th17 cytokines that have major implications for suppression of disease induction and progression.

In human reproductive immunology, the focus has been on fetal or placental derived factors that play a role in pregnancy maintenance by suppressing the natural immune response to reject the growing fetal allograft (Taylor 2006, Sabapatha 2006). Exosome studies have been directed to determine the specific cell type from which these vesicles are released after cell
activation. Many have looked at exosomes derived from dendritic cells, focusing on the ability of the exosomes to suppress in an antigen specific manner (Kim 2006). While it has been proposed that most the exosomes of pregnancy are derived from the placenta – it is likely that exosomes are produced from splenocytes as it increases in size in response to pregnancy hormones. It is also likely that hepatocytes shed exosomes into the serum in response to physiologic changes during pregnancy, as detailed by Linzer and et al. (1999). In future studies, it will be important to determine the cell types from which these exosomes are derived as well as the surface markers expressed and the contents of the vesicles.

In agreement with previous studies, we found that late pregnancy is the most protective in EAE, while the post-partum period was disease enhancing or afforded no protection. We also found that pregnancy induced during ongoing EAE resulted in a decreased encephalitogenic capacity of CNS infiltrating cells characterized by decreased production of Th1 and Th17 cytokines as well as reduced T cell proliferation. This break in inflammatory responses in the target organ resulted in less demyelination at the dorsal and ventral funiculi. We have shown a clear down regulation of key inflammatory cytokines such as TNFα and IL-17 in response to pregnancy induced during ongoing EAE. Finally, we have demonstrated the ability to separate out exosomes from pregnancy serum that exhibit a suppressive capacity in response to PLP or anti-CD3 T cell stimulation. These results are useful in directing future studies in our lab and others to further characterize this pregnancy specific molecule that can be found in abundance in the serum during late stage pregnancy. The ability to use a molecule that is not sex-discrepant, such as estrogens, or a therapy that has less potential for adverse side effects, as seen in dexamethasone based therapies, will help to advance new therapies in autoimmune disease.
CHAPTER 4

PI3Kγ IS A CRITICAL MEDIATOR IN THE DEVELOPMENT AND PROGRESSION OF CNS AUTOIMMUNE INJURY

4.1 RESULTS

4.1.1 PI3Kγ deficient mice are protected from EAE.

To determine if PI3Kγ plays a role in immune-mediated demyelinating disease, EAE was induced in C57Bl/6-PI3Kγ deficient (ko) mice. We observed a clear delay in onset and decreased severity of disease as compared to wt mice (Fig. 16a, Table 5) with disease being more profoundly suppressed in male ko than female ko littermates.

We assessed T cell activation properties such as proliferation, Th1 and Th17 inflammatory cytokine production in response to the immunizing antigen (MOG35-55). KO mice demonstrate a marked reduction in T cell proliferation and production of the Th1 inflammatory cytokines IFNγ, TNFα, and IL-12p40 (Fig. 16b, 16d-f). IL-17 production 10 days post EAE immunization was significantly reduced in ko males; however, there was no significant difference observed between ko and wt females in production of IL-17 (Fig. 16c).
Figure 16. PI3Kγ ko mice are protected from EAE. (a) EAE was induced in C57Bl/6 (wt) males and females and PI3Kγ −/− (ko) males and females by immunization with MOG35-55 in complete Freund’s adjuvant (CFA). EAE course shown as mean clinical score (n=6). (b) T cell proliferation (lymph node (LN) cells harvested 10 days post immunization (dpi) and stimulated with 10μg MOG35-55 for panels b-f). (c) IL-17 production (ELISA) (d) IFNγ production (CBA) (e) TNFα production (CBA), and (f) IL-12p40 production (ELISA) (g) IL-10 production (CBA).
Table 5. PI3Kγ deficiency delays onset and decreases progression of EAE.

WT and ko males and females were immunized for EAE and observed for 50 days (see clinical graph Fig 1a). This data is representative of 4 experiments.

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<tr>
<td>C57Bl/6 male</td>
<td>5/5 (100%)</td>
<td>10.8 (+ 0.33)</td>
<td>3.2 (+ 0.12)</td>
<td>94.6 (+ 7.60)</td>
<td>3.00 (+ 0.45)</td>
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<td>C57Bl/6 female</td>
<td>4/4 (100%)</td>
<td>11.8 (+ 0.88)</td>
<td>3.9 (+ 0.20)*</td>
<td>72.8 (+ 19.70)</td>
<td>3.25 (+ 0.25)</td>
</tr>
<tr>
<td>PI3Kγ/- female</td>
<td>6/6 (100%)</td>
<td>22.8 (+ 1.53)</td>
<td>1.9 (+ 0.37)*</td>
<td>22.1 (+ 5.77)‡</td>
<td>2.00 (+ 0.26) *‡</td>
</tr>
<tr>
<td>PI3Kγ/- male</td>
<td>1/6 (17%)* ‡</td>
<td>26*‡†</td>
<td>0.2 (+ 0.17)*‡</td>
<td>0.50 (+ 0.50)* ‡</td>
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* p< 0.05 compared to wildtype male controls  
‡ p< 0.05 compared to wildtype female controls  
† p< 0.05 PI3Kγ -/- males compared to PI3Kγ -/- females

However, we found that both naïve wt and ko mice lymph node cells can be induced in vitro to produce IL-17 to the same levels in response to anti-CD3 (Fig. 17), which demonstrates an intact capacity to produce Th17 inflammatory cytokines equal to that of wt males. Th17 differentiation protocol is described in methods section as per personal communication with Dr. Daniel Cua.

IL-10 production did not appear to be involved in the observed protection in ko mice (Fig. 16g). Significant decreases were also noted in the production of IL-2, IL-6 and the chemokine MCP-1 in ko vs wt males (Fig. 18), suggesting that reduced proinflammatory cytokine production in PI3Kγ/- mice protects from EAE disease. IL-6 and MCP-1 specifically are important due to their role in maintaining the integrity of the blood brain barrier (Stamatovic 2005; Paul 2003).
Figure 17. Naive PI3Kγ ko and wt cells can be induced to undergo Th17 differentiation equally in response to anti-CD3. LNC were harvested from wt and ko mice for T cell proliferation assay as described above. Cells were cultured under special Th17 driving conditions (as detailed in methods section) for 72 hrs before counting cells. Representative of 2 experiments (n= 6-7).

Figure 18. PI3Kγ ko mice produced less IL-2 (a), IL-6 (b) and MCP-1 (c) after immunization for EAE. EAE was induced in C57Bl/6 (wt) males and females and PI3Kγ -/- (ko) males and females as described in figure 16 above. Cytokines were measured using an inflammatory CBA Kit. Representative of three independent experiments. N= 5-7 per group.
### 4.1.2 PI3Kγ deficient males have reduced CNS pathology during EAE.

*WT* and *ko* males were immunized for EAE and sacrificed 19 days post immunization to assess inflammatory cell infiltration into CNS and demyelination. Spinal cords were processed and evaluated as specified in the methods section of this document. We found fewer areas of focal demyelination in cross sections of the lumbar spinal cord in *ko* males (Fig. 19a,b).

**Figure 19.** Reduced CNS pathology in PI3Kγ *ko* mice. EAE was induced in *wt* and *ko* males, brains and spinal cords were harvested 19 days post immunization (dpi) and stained: (a) [Luxol Fast Blue] Increased demyelination in *wt* ventral lumbar spinal cord as compared to (b) *ko* spinal cord, (c) [H&E] Increased perivascular cuffing in vascular areas of *wt* mesencephalon as compared to (d) *ko* mesencephalon, (e) [CD11b] Increased macrophage infiltration in *wt* ventral lumbar spinal cord as compared to (f) *ko* spinal cord, and (g) Increased axon severing in *wt* pons as compared to (h) *ko* pons at 49 dpi. Low power images were rotated similar orientation with high power views.

PI3Kγ deficient males had decreased perivascular cuffing observed in sections of the mesencephalon as compared to *wt* males (Fig. 19c,d). Moreover, *ko* males demonstrated a marked reduction in macrophage infiltration into the ventral spinal cord when compared to *wt* males (Fig. 19e,f).
The Bielschowsky silver stain is commonly used to stain nervous tissue, especially in diagnosing neurodegenerative diseases. We utilized the Bielschowsky stain due to availability and trained staff at the histology core, significant number of references detailing the utility of the stain in neurodegeneration, and cost effectiveness (100+ slides made). It stains axons, neurofibrillary tangles and senile plaques a dark black/brown color and background will be yellow/brown. Amyloid beta-protein as well as the SMI-32 (neurofilament epitope) immunohistochemical stains were recognized and considered as two other sensitive stains for axon pathology; however, were not used in these experiments.

We selected the pons for imaging axons using Bielschowsky silver stain. The pons is a structure of the hindbrain which also includes the cerebellum and medulla. Usually, the pons and medulla are included with portions of the midbrain and referred to as the brainstem. The brainstem is responsible for basic vital autonomic functions such as blood pressure, heart beat and breathing. The pons is positioned superior to the medulla and is a direct rostral extention of the spinal cord – and is functionally similar to the spinal cord. Four pairs of cranial nerves originate from the pons (CN V-VIII) which influence functions such as eye movement and balance, both seen to be impacted in patients with MS. The pons is situated ventral to the cerebellum contains multiple neurons serving as a relay station between the cerebrum and the cerebellum. The cerebellum receives somatosensory, motor and balance input for various portions of the brain, spinal cord and vestibular organs. It is the job of the cerebellum to coordinate this input to maintain posture and balance during skeletal muscle movement among other functions. In this study, we evaluated the integrity of axons coursing through the pons for three reasons: 1) Function – these are axons that track from the spinal cord rostrally and between
the cerebrum to the cerebellum, 2) Location – the pons can be easily located and is not highly variable between subjects (as compared to spinal cord levels, cervical, thoracic, lumbar and sacral), and finally, 3) Organization – the architecture of the axons coursing through this area are variable in path and depth; therefore providing multiple planes for staining and comparing sections. Fewer areas of severed axons in the pons of ko mice were observed later in disease (49dpi) as compared to wt males (Fig. 19g,h). These findings correlate with the observed protection from EAE in PI3Kγ deficient mice. PI3Kγ heterozygous mice show low level disease (limp tail or ataxia) without progression as compared to PI3Kγ +/- having typical disease and PI3Kγ -/- protected littermates (Fig. 20). This finding demonstrates the relative suppressive potential of the PI3Kγ gene.

**Figure 20. PI3Kγ +/- heterozygotes are partially protected from EAE.** C57Bl6/male wt (+/+), and ko (-/-) and heterozygous (+/-) mice were immunized as described and monitored for clinical signs. Heterozygotes were found to be more susceptible to disease than complete ko mice, but exhibited significantly less severe disease relative to homozygous wt controls. [n = 4 – 5 per group]
Based on the finding that ko males are protected from disease induced by active immunization (see Figure 1a), we wanted to assess the effector phase response in these mice to better understand the level of protection. We employed the use of adoptive transfer of in vivo MOG activated lymphoid cells into naïve wt and ko recipients in order to bypass peripheral activation of T cells. Transfer of activated ko cells into either wt or ko mice did not lead to clinical disease (Fig. 21a). The most intriguing of the comparisons in figure 21a is the transfer of activated WT cells into KO mice. This demonstrates a level of protection within the CNS. In this transfer model, cell activation and initial antigen presentation were completed in the WT host which ensures normal activation. This is confirmed by the transfer of WT cells into WT recipients that become sick. Circulating activated T cells did not cause disease in the KO recipients, however. Peripheral autoreactive T cells are not sufficient to cause EAE in ko males.

Transfer of naïve MOG Tg T cells into wt and ko mice 24hr prior to active immunization of recipients with MOG 35-55 resulted in disease only in wt mice. KO recipients receiving either Tg cells or vehicle prior to active immunization were not susceptible to disease (Fig. 21b). These findings point to a possible defect at both the priming and effector phases of disease in ko mice. In this transfer method, mice are preloaded with 1 million T cells that already have the receptor for the immunizing antigen, MOG, prior to MOG immunization. It is expected that all mice should show clinical signs of disease. KO mice were still not susceptible to EAE induction, pointing to a defect in cell-to-cell communication and/or decreased access into the CNS.
4.1.4 Impaired mononuclear cell infiltration into the CNS of PI3Kγ deficient males.

To determine the fate of peripherally transferred activated T cells in ko recipients, we transferred MOG activated GFP⁺-C57Bl/6 wt T cells into wt and ko recipients. Ten days post transfer, we harvested spinal cords and the lung, as a peripheral organ, and completed immunohistochemical staining of the tissues using anti-GFP Ab. GFP⁺ cells were able to traffic to and penetrate into the parenchyma of lung (Fig. 22a) of both wt and ko mice equally. In the CNS, we could identify focal areas of GFP⁺ cells in the ventral motor portion of the spinal cord (Fig 22b) of wt
mice, however, these GFP⁺ cells were virtually absent in the CNS of ko mice (Fig 22b). Limited infiltration of cells into the CNS of PI3Kγ deficient mice also confers protection from EAE.

**Figure 22.** Adoptive transfer of activated wt-GFP positive T cells. (a) No difference in infiltration of activated cells into peripheral organs (lung). (b) PI3Kγ ko male recipients have dramatically less T cell infiltration into CNS as compared to wt males at 10dpi.
Table 6. Testosterone is protective in PI3Kγ deficient females during EAE. PI3Kγ *ko* females supplemented with 7.5mg/pellet 60-day dose release 5α-DHT (testosterone) pellets 7 days prior to EAE have decreased severity and progression of disease as compared to placebo treated and untreated (sham) *ko* females and *wt* controls. Clinical scores were monitored for 30 days. (Standard deviations noted)

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<tr>
<td>C57Bl/6 Sham</td>
<td>3/3 (100%)</td>
<td>10</td>
<td>4 (± 0.87)</td>
<td>68.3 (± 21)</td>
<td>2.3 (± 0.58)</td>
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<td>C57Bl/6 Placebo</td>
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<td>65.9 (± 16)</td>
<td>2.5 (± 0.58)</td>
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<td>11.8 (± 1.6)</td>
<td>3.8 (± 0.29)</td>
<td>56.3 (± 8)</td>
<td>2.4 (± 0.55)</td>
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<tr>
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<td>2.8 (± 0.29)</td>
<td>37.7 (± 3)</td>
<td>1.7 (± 0.58)</td>
</tr>
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<td>1.8 (± 0.50)</td>
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<td>15.2 (± 1.8)</td>
<td>1.2 (± 0.65)*</td>
<td>7.4 (± 7)*</td>
<td>0.6 (± 0.55)*</td>
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Table 6. Testosterone is protective in PI3Kγ deficient females during EAE. PI3Kγ *ko* females supplemented with 7.5mg/pellet 60-day dose release 5α-DHT (testosterone) pellets 7 days prior to EAE have decreased severity and progression of disease as compared to placebo treated and untreated (sham) *ko* females and *wt* controls. Clinical scores were monitored for 30 days. (Standard deviations noted)

a. Percent of mice with clinical signs
b. First day of disease onset
c. Maximum Clinical Score averaged
d. Cumulative Disease Index; sum of clinical scores over observation period
e. Periods of Progressing Disease (increase in score ≥1.0 for 2+ days)
   (students t test was used to compare values b - e)
   * p< 0.05 compared to all control groups
   † p< 0.05 compared to wildtype controls

4.1.5 Sex differences in PI3Kγ deficient mice in response to EAE.

Female *ko* mice are not as protected as males (see Figure 1a) during EAE. However, female *ko* mice do exhibit a milder disease course than *wt* mice. Interestingly, treatment with testosterone (5α-dihydrotestosterone [5α-DHT]) significantly reduced clinical disease in PI3Kγ *ko* females relative to placebo treated *ko* females, control *wt* females or testosterone treated *wt* females (Table 6, Fig. 23a,b). This data was interesting due to its implication for the use of testosterone in combination with specific PI3Kγ inhibitors. Also, these data demonstrate the existence of hormone sensitive immune factors that are important during the priming phase of EAE. To determine is estrogen contributed to the exacerbated phenotype of the female, we
implanted castrated male mice with 17beta-estradiol pellets prior to immunization (see figure 24
design). Male ko mice were not made more susceptible to EAE with the addition of estrogen, however, gonadectomized males in both groups were significantly more susceptible to disease (Fig. 24 top-bottom). Males were castrated or sham surgerized 7 days prior to pellet implantation and immunized 7 days after this procedure to allow time to reduce surgery stress and distribution of the hormone. As reported in previous studies, wt males pretreated with low doses of estrogen were protected from EAE induction (Fig. top). Castration induced susceptibility also points to the protective role of testosterone in ko males.

a.  

![Figure 23(a-b). Female ko mice treated with testosterone have reduced susceptibility to EAE (b). Mice were implanted with 5α-DHT pellets 7 days prior to immunization for EAE using MOG (as described above). WT(a) females are not protected with testosterone treatment.](image)

Transfer of disease by female mice, though only transient in nature, was a novel and intriguing finding (Fig. 25). Use of the adoptive transfer technique has helped to divide the clinical picture into two separate phases of disease: priming and effector phases. This helped to develop directed questions regarding donor priming and recipient effector responses. In this study, when the priming phase took place in females wt or ko, disease was transferable to ko males although not sustainable (Fig 25a,b). However, if either the recipient or donor was
deficient for PI3Kγ (male or female) disease was not sustainable. This finding supports the idea that protection from CNS injury in ko mice takes place to some degree in both the periphery as well as in the CNS. The new data that male ko mice are susceptible to disease induction, albeit monophasic, is interesting and points to processes during the initiation phase of disease that is likely hormone dependent. Focused investigations of this finding will add significantly to the current understanding of sex-discrepant immune mediated disease research.

Figure 24. Castration of males wt or ko results in increased susceptibility to EAE and estrogen is protective in wt males. Castrated or non-castrated mice were implanted with 17β-estradiol (E2) pellets 7 days prior to immunization for EAE using MOG (as described above). KO males showed mild increase in susceptibility when castrated (as compared to ko non-castrated suppressed phenotype). Disease in wt males was profoundly suppressed when estrogen was implanted, with only mild disease in castrated estrogen pretreated males.
Figure 25. *WT* and *KO* female donors transfer disease to recipients, however PI3Kγ *ko* recipients do not exhibit disease progression. (a) Cells from *ko* females immunized for EAE were harvested 10 dpi and cultured with MOG Ag for 72hrs. Donor cells were transferred into *ko* and *wt* recipient males and females (6x10⁶). All recipients exhibited a less severe acute disease without progression. [n=3-5]. (b) Cells from *wt* females immunized for EAE were harvested 10 dpi and cultured with MOG Ag for 72hrs. Donor cells were transferred into *ko* and *wt* recipient males and females (6x10⁶). All *ko* recipients exhibited a less severe acute disease without progression while *wt* recipients exhibited a typical progressive disease course. [n=3-5].

4.1.6 Blockade of PI3Kγ is protective in EAE.

To evaluate the role of PI3Kγ in the progression of EAE, we used the recently developed small molecule PI3Kγ specific inhibitor AS-605240 in *wt* mice immunized for EAE. When AS-605240 therapy is initiated one day prior to immunization for EAE, mice demonstrate a less
severe acute phase of disease followed by a remission of signs without progression. Both 10mg/kg and 30mg/kg doses of AS-605240 showed similar levels of protection (Fig. 26a). When treatment was discontinued, AS-605240 treated mice exhibited disease exacerbation and progression while dexamethasone treated mice showed less progression. Dexamethasone therapy was used as a positive control and found to demonstrate a more profound suppression of disease when given prophylactically. After 30 days of treatment, we assessed histopathologic changes between treatment groups. Vehicle treated mice had increased mononuclear cell infiltration, perivascular cuffing and increased demyelination of the ventral white matter as compared to AS-605240 treated mice (Fig. 26b).

To assess the therapeutic potential of AS-605240 in treating ongoing EAE, we initiated treatment after acute disease (15-20 dpi). A 30mg/kg dose of AS-605240 inhibitor was sufficient to significantly reduce clinical signs from hind limb paresis to a mild ataxia or tail limpness (score 2.5 to 0.5). We observed an immediate drug effect with steady improvement of neurologic motor function in treated mice that was similar to the dexamethasone control treated mice. Vehicle treated mice developed a primary progressive EAE clinical disease course (Fig. 26c).
Figure 26. Blockade of PI3Kγ in wt males reduces CNS injury during EAE. Sections stained at conclusion of treatment. (a) Prophylactic treatment (n=10) with specific PI3Kγ-inhibitor, AS605240, prevents progression of EAE as compared to vehicle treated mice. (b) Prophylactic treatment CNS histopathology: top left [H&E] Increased mononuclear cell infiltration in spinal cord of vehicle treated mice as compared to bottom left AS605240 treated mice. top center [H&E] Increased perivascular cuffing in brain of vehicle treated mice as compared to bottom center AS605240 treated mice. top right [Luxol Fast Blue] Increased demyelination in spinal cord of vehicle treated mice as compared to bottom right AS605240 treated mice. (c) Therapeutic treatment (n=20) with AS605240 reduces clinical disease as effectively as dexamethasone with a profound suppression over that of vehicle treated mice. (d) Therapeutic treatment CNS histopathology: top left [H&E] Increased mononuclear cell infiltration in spinal cord of vehicle treated mice as compared to bottom left AS605240 treated mice. top center [H&E] Increased perivascular cuffing in brain of vehicle treated mice as compared to bottom center AS605240 treated mice. top right [Luxol Fast Blue] Increased demyelination in spinal cord of vehicle treated mice as compared to bottom right AS605240 treated mice. (e) Increased axon severing noted in the pons of vehicle treated mice as compared to right mice treated with AS605240 (severed axons noted at arrows).
We then compared the CNS histopathologic findings after 29 days of treatment with vehicle, PI3Kγ inhibitor or dexamethasone. We found a marked reduction in mononuclear cell infiltrates, perivascular cuffing and fewer focal areas of demyelination in AS-605240 treated mice as compared to vehicle treated controls (Fig. 26d). Silver stains of the pons after 29 days of treatment showed a significant increase in axon severing in vehicle treated mice as compared to AS-605240 and dexamethasone treated mice (Fig. 26c). Findings in the dexamethasone treated group were similar to those of 30mg/kg AS-605240 treated mice in all stains (data not shown). Discontinuation of treatment after 29 days resulted in slower progressive course in AS-605240 treated mice as compared to vehicle and dexamethasone treated groups (Fig. 26c).

To determine whether treated mouse cells retained their T cell activation capacity or were rendered inactive by the prolonged treatment, we designed several studies to measure T cell activation. On the last day of treatment (Vehicle, AS-605240, Dexamethasone), 3 representative mice from each group were sacrificed and spleens were harvested for analysis of inflammatory cytokine and chemokine production and for T cell proliferation analysis as described in the methods section. Cells from mice that had been treated with the AS-605240 PI3K-gamma specific inhibitor produced significantly less amounts of IFNγ (Fig. 27 top), TNFα (Fig. 27 bottom) as well as amounts of IL-6 (Fig. 28 top) relative to that of vehicle treated mice. In these comparisons, the dexamethasone treated group produced profoundly less of these cytokines as compared to the other treatment groups. Interestingly, the spleens in the vehicle and AS-605240 treated mice were normal size and appeared healthy prior to isolation of cells as compared to dexamethasone treated mice. The Dex treated mice had severely atrophied and contracted spleens with multiple grossly visible areas of infarction. This may have impacted
measured outcomes in these graphs. Alternatively, dexamethasone may have caused changes in
the cells capacity to respond to specific inflammatory stimuli related to cortisol regulation of
NFkB transcription.

In contrast, AS-605240 and dexamethasone treatment groups produced more of the
inflammatory chemokine MCP-1 as compared to the vehicle treated mice (Fig. 28 bottom).
PI3Kγ inhibitors are known to reduce chemokine receptor expression to reduce APC migration
to the site of inflammation. Inhibitor treatment likely caused a homeostatic upregulation of the
CCR2 receptor ligand MCP-1. There could be a similar response produced in the
dexamethasone treated group. There was not a significant difference between the level of T cell
proliferation in response to the immunizing antigen (MOG 35-55) between vehicle and AS-
605240 treatment groups (Fig. 29). However, dexamethasone treated mice had less T cell
proliferation in response to MOG for reasons described above.

Figure 27. Blockade of PI3Kγ reduces
TNFα and IFNγ production in vitro at 50dpi in response to MOG. EAE was induced in
C57Bl/6 (wt) males and treatment with either saline [vehicle], AS-605240 [inhibitor] or
dexamethasone [dex] was initiated after acute disease 19dpi. Treatment was continued until
day 50 post immunization and spleen cells were cultured with MOG immunizing antigen
for 72hrs. TNFα (top) and IFNγ (bottom)
were measured using CBA and determined to
be significantly reduced in inhibitor treated
cells relative to vehicle treated controls. * p =
< 0.5. (Cells were split into 6 wells per mouse
per group; n=5)
Figure 28. Blockade of PI3Kγ reduces IL-6 but increases MCP-1 production \textit{in vitro} at 50dpi in response to MOG. EAE was induced in C57Bl/6 (wt) males and treatment with either saline [vehicle], AS-605240 [inhibitor] or dexamethasone [dex] was initiated after acute disease 19dpi. Treatment was continued until day 50 post immunization and spleen cells were cultured with MOG immunizing antigen for 72hrs. IL-6 (top) was significantly reduced and MCP-1 (bottom) was increased in inhibitor treated mice relative to vehicle treated controls. * p = < 0.5. (Cells were split into 6 wells per mouse per group; n=5)

Figure 29. Blockade of PI3Kγ does not reduce T cell proliferative capacity at 50dpi in response to MOG immunizing antigen. T cell proliferation spleen cells harvested 50 days post immunization (dpi) and stimulated with 10μg MO\textsubscript{35,55}. Inhibitor treated mice T cells retained the capacity to proliferate in response to MOG immunizing antigen in the absence of the inhibitor. Dexamethasone treated mouse cells remained suppressed. (Cells were split into 6 wells per mouse per group; n=5)
4.2 Discussion

Class I PI3Ks are therapeutic targets for treatment of inflammatory and autoimmune disease processes (Wetzker 2004; Pomel 2006; Camps 2005). More importantly, isoforms expressed on hematopoetic cells, such as PI3Kγ, have been found to play specific roles in regulating immune responses (Katso, 2001). The C57Bl/6 mouse model of EAE is reminiscent of PPMS and has been useful to study the progressive phase of disease. During disease progression, demyelinating periods involve inflammatory and non-inflammatory neurodegeneration and subsequent accumulation of neurologic deficits (Bjartmar 2003; Lassmann 2007). C57Bl/6 mice deficient for the PI3Kγ gene demonstrated a marked protection from disease induction and progression, with a notable sex dimorphism during EAE, where males were more profoundly suppressed than were females. Sex differences in EAE have been well reported (Papenfuss 2004; Yu 2004). Papenfuss et al. (2004) reported no sex differences in response to EAE using C57Bl/6 mice. This study has shown a significant difference between male and female C57Bl/6 mice, with regard to Th1 cytokine production during the acute phase; however, without an overall cumulative difference in disease. C57Bl/6-PI3Kγ deficient males were protected from EAE induction, ~0.5 cumulative disease score, while ko females were more susceptible, ~22.0 cumulative disease score (Table 2). However, addition of the testosterone derivative, 5α-DHT, dramatically reduced the cumulative disease in females to ~7.0 (Table I). 5α-DHT did not protect wt females immunized for EAE, further emphasizing a role for the PI3Kγ gene in sex differences. Remarkably, the lack of sex dimorphism in C57Bl/6 mice
becomes obvious when PI3Kγ gene is deleted, therefore warranting further investigation of the role for PI3Kγ in autoimmune sex differences.

Development of the isoform-selective PI3Kγ inhibitor, AS-605240, has aided in understanding the role of chemokine-mediated intracellular signaling in chronic inflammatory diseases (Camps 2005). AS-605240 treatment mimicked the effect of PI3Kγ gene deficiency during EAE, inhibiting T cell activation and decreasing leukocyte migration into the CNS, thereby limiting induction and progression of disease. These findings suggest that inhibition of PI3Kγ impairs activation of auto-reactive T cells directed against the myelin sheath, thus blocking neurodegenerative processes during EAE. In this study, AS-605240 inhibitor was administered using two treatment protocols: prophylactic (treatment initiated one day prior to EAE induction) or therapeutic (treatment initiated during ongoing disease). In both models, AS-605240 treatment reduced disease severity and rate of progression. Interestingly, therapeutic treatment led to rapid remission of signs similar to treatment outcomes using dexamethasone, an effective broad immunosuppressive therapy.

Camps et al. (2005) reported that therapeutic treatment with AS-605240 led to reduced severity of disease in animal models for rheumatoid arthritis (RA). Theories regarding the pathogenesis of RA and MS have focused largely on T cells emphasizing the role of autoreactive T cells in the initiation and perpetuation of chronic inflammation. Camps et al. (2005) investigated the role for PI3Kγ inhibitors, which reduce infiltration of neutrophils and limit disease in animal models for RA. RA and MS share similarities in several features of the disease process, including sex-discrepancy, prominent Th1 inflammatory cytokine profiles, and the involvement of reactive T cells targeted against self-antigens (Lockshin 2006). There are,
however, notable pathogenetic and therapeutic differences between RA and MS. In MS, neutrophils are not indicated as primary culprits for the observed CNS injury. This is also not the mechanism of damage targeted in commonly used animal models for MS (Holmoy 2007). Additionally, RA is often associated with elevated levels of cytokines produced by macrophages such as IL-1β and TNFα (Hosaka 2005). The predominant disease mediating cytokines in MS are of mixed leukocyte origin such as IFNγ, TNFα, IL-23 and IL-17 (Gocke 2007). Furthermore, recent studies have shown that anti-TNFα therapies (adalimumab, infliximab and etanercept) are beneficial in treating patients with RA (Perper 2006). Conversely, treatment with anti-TNFα worsens disease in animal models for MS (Kollias 2002) and is contraindicated for patients with pre-existing MS due to increased risk for adverse neurologic events (Mohan 2001). TNFα is a critical player in CNS demyelinating disease processes having dual opposing functions. It is both necessary for the process of remyelination at low levels and causes increased differentiation of CD4+ T cells toward a Th1 phenotype at higher doses (Arnett 2001). PI3Kγ inhibitors mitigate various features of CNS injury that indicate pathogenic mechanisms of MS that are not well understood, underscoring the importance of investigating this potential therapy in both disease processes.

This study uses PI3Kγ gene knock out mice as well as the pharmacologic inhibitor to PI3Kγ, AS-605240, to demonstrate its critical role in both the initiation and progressive phases of disease by impairing T cell activation and APC migration. KO mice show a dramatic reduction in susceptibility to disease with decreased evidence for T cell activation as compared to wt mice. To bypass potential defects in peripheral T cell activation in ko mice, adoptive transfer of in vivo activated T cells into naïve ko mice was performed. KO recipients remained
resistant to disease indicating that the presence of activated autoreactive T cells alone was not sufficient to induce CNS injury. Trafficking studies in this transfer model reveal an inability of pathogenic cells to accumulate in the target organ. Without infiltration of peripheral APCs, antigen representation in the CNS was inhibited, thereby conferring protection. Blockade of PI3Kγ reduces the inflammatory phenotype of both T cells and APCs at the site of inflammation. Taken together, these findings underscore the critical role of infiltrating mononuclear cells in driving the chronic inflammatory processes in EAE.

Of the six common therapies available to patients with MS, many have unclear mechanisms of action, as with glatiamer acetate (copaxone) therapy, or come with many undesirable side-effects, as seen in interferon or steroid treatment. Treatment with the specific PI3Kγ inhibitor showed no signs of building tolerance to the drug and recipients did not show any overt adverse side-effects when studied in mouse models of MS. Prophylactic treatment of wt mice with AS-605240 results in reduced severity of clinical disease without further progression, and withdrawal of therapy restores disease progression. Therapeutic treatment with AS-605240 halts further progression of disease, and mice recover within 10 days of treatment. Withdrawal of therapy restores disease progression, in this case, albeit slower and less severe. KO mice produced less MCP-1, Th1 and Th17 cytokines and had dramatically reduced CNS pathology as compared to wt controls post immunization for EAE. In conclusion, we have shown that pharmacological inhibition of PI3Kγ can ameliorate ongoing EAE as well as impair the induction and progressive phases of disease, mimicking the PI3Kγ−/− mouse model. PI3Kγ therefore represents an ideal therapeutic target for mitigating inflammatory and neurodegenerative effects in MS.
CHAPTER 5

SEX, PREGNANCY AND GENETICS ARE CRITICAL MEDIATORS IN THE DEVELOPMENT AND PROGRESSION OF AUTOIMMUNE CNS INJURY

*It is incident to physicians, I am afraid, beyond all other men, to mistake subsequence for consequence.*  --Samuel Johnson, 1734

Sex, pregnancy and genetics are all critical mediators of autoimmune CNS injury. MS is a chronic disease of the CNS that involves both inflammatory demyelination and neuronal degeneration. MS is defined by a myriad of symptoms that are so different in onset and disease course, no two patients can be described as having the same clinical presentation. There are several variants of MS disease and clinicians have managed to group patients into descriptive classes based on the disease onset and cumulative course. The two most common presentations of MS are the relapsing remitting MS (RRMS) and the primary progressive MS (PPMS) forms. It is believed that sex hormones guide many clinical outcomes in MS. Clinical observation and
scientific evaluation of pregnancy suppression during MS and EAE drives these discussions. EAE has served as a suitable animal model for studying MS based on similarities in immunological and histological findings. Mouse models in EAE have also been shown to exhibit disease courses that are reminiscent of RRMS and PPMS with similar sex dimorphisms. The mechanisms that lead to the production of MS symptoms are yet unclear. With the knowledge that pregnancy provides the most profound suppression of disease, our lab has endeavored to investigate intracellular and extracellular processes thought to be influenced by pregnancy hormones. Development of future therapies in MS and other autoimmune disease states rely on continued research to offer new philosophies that guide clinical and scientific discussions. Below, I provide a cohesive summary of our findings in EAE as they relate to what is currently known about MS and the ongoing studies in the field.

**Diagnosing MS.** A clinician needs only a thorough medical history of the illness and confirmatory imaging in diagnosing MS. This includes the patient’s report of two episodes of neurological disturbances with physical signs indicating separate CNS lesions (e.g. limb numbness, visual changes, bowel or bladder incontinence, etc...). Magnetic resonance imaging (MRI) is useful to determine the presence of old lesions and with special contrast can determine if there are new or active inflammatory lesions. Evaluation of cerebrospinal fluid (CSF) and measuring evoked potentials (EP) have also been helpful in diagnosing MS. Techniques such as EP and CSF evaluation are not well developed or widely used in murine EAE studies. The use
Specialized MRI studies have been done to monitor inflammatory cell trafficking into the CNS during EAE. However, histopathological and clinical correlates have been only poorly linked to MRI changes in MS making it difficult to predict clinical outcomes of disease (Rausch 2003; Brochet 2006). MRI atlases of murine CNS structures, as developed by Duke University and the University of California Los Angeles, have helped in the interpretation of EAE lesions. Reports have shown increased clinical utility of MRI to predict tissue damage and clinical outcomes of EAE by monitoring the evolution of CNS lesions over time (Nessler 2007). Nessler and colleagues (2007) matched lesion cellularity, Ig deposition and myelin loss to hyporesonant areas on MRI. They reported that this type of lesion was shown to progress and cause more tissue damage and clinical disability relative to hypocellular hypointense type lesions that were observed to resolve on serial MRI (Nessler 2007). Difficulty in acquiring high-quality images and the cost of the technology and specialization of skill to interpret MRI scans has been limiting factors for many EAE laboratories. No MRI imaging, EP or CSF studies were completed in the studies discussed in this dissertation. However, plans to place a 7-tesla MRI scanner for research purposes in our research facility will be a useful tool for our future EAE studies.

The Kurtzke Expanded Disability Status Scale (EDSS) is used to quantify MS disability in eight functional systems on a scale from 1 to 9.5. These functional systems include: pyramidal, cerebellar, brainstem, sensory, visual, cerebral, bowel and bladder and other. The
Clinical features of MS. RRMS patients experience episodes of disease exacerbation followed by periods of remission. A clinical relapse is defined as a period of at least 24 hours with an increase in neurologic symptoms that is without fever or concomitant infection in a patient previously diagnosed with MS (Poser 1983). Within the RRMS group, some patients may experience more severe exacerbations and shorter remission phases, while other RRMS patients report fewer relapses and longer periods of remissions. There are also patients who fall somewhere between the two previously described clinical courses of RRMS. None the less, these RRMS patients slowly develop into a progressive disease type (Weinshenker 1989; Runmarker 1995). Therapies have been targeted to reduce the severity of relapses and prolong the remission period to improve the quality of life for these patients. Our studies have found that the greatest and most predictable suppression can be observed in R-EAE during late pregnancy,
which will be discussed in more detail later. Typically, R-EAE studies were carried out using SJL female mice immunized with PLP 139-151. Papenfuss et al. (2004) reported clinical variants and sex differences in mouse models of EAE. Those studies demonstrated the utility of the SJL female in mimicking RRMS as males showed little response to immunizing peptides and other strains such as the C56Bl/6 and B.10 mice exhibited a more progressive form of EAE.

PPMS patients describe a severe and sudden onset of symptoms. These patients have a steady and relatively rapid progression of disease with increasing neurologic deficits over time. There are no clear periods of remission in PPMS although some patients report slight decreases in severity or periods of no change in symptoms. Many of these findings can also be found in patients with secondary progressive MS (Thompson 1997; Confavreux 2000). Many PPMS patients have been found to be refractory to standard MS therapies. As such, there are no definitive treatments available for patients with PPMS (Thompson 1997). There remains a demonstrable need to develop effective therapies for PPMS patients. Our studies investigating the role of a specific intracellular signaling protein, PI3Kγ, offer some therapeutic insight into treatments for PPMS. C57Bl/6 mice exhibit a progressive EAE, in response to MOG 35-55 immunizing antigens and are suitable to study PPMS.

Papenfuss et al. (2004) reported no sex differences between C57Bl/6 males and females in response to MOG EAE immunization. Our studies report for the first time a difference in acute clinical and immunologic responses in these males as compared to females. Papenfuss and colleagues (2004) evaluated the long-term cumulative clinical differences between males and females. In contrast, our studies evaluated both clinical and immunologic parameters in early and late stages of disease. We report that C57Bl/6 females produce less inflammatory cytokines
acutely and have a slower clinical onset – but agree with Papenfuss (2004) that there is no cumulative difference in clinical outcomes. The information that females have a more insidious onset in P-EAE may be useful evaluating the initiation of therapies with respect to the sex of the patient. MS studies have reported that initiating therapies early in the disease results in delayed progression of disability. Our studies compared responses between C57Bl/6 males treated with either saline (vehicle) or PI3Kγ inhibitor treatment. However, it would also be beneficial to determine if there is a difference in histological and clinical response to PI3Kγ inhibitor therapy between C57Bl/6 males and females.

**Mechanisms of symptom production.** We utilized the EAE model in pregnancy to elucidate some of the mechanisms by which pregnancy reduces relapses. Pregnancy maintenance is the body’s continuous efforts to avoid rejecting the fetal allograft (Hunziker 1986). While this is more the case in outbred models, there are both fetal and non-fetal antigens related to pregnancy in inbred models. Nonfetal antigens include those of the male (stud) sperm and the newly formed maternal-fetal interface (placenta). Fetal antigens are constantly being presented to the host (mother) circulation throughout the pregnancy in a condition called microchimerism (Whitacre 1999).

(PRL) and the PRL family of hormones, including prolactin like hormones I and II (PL-I, PL-II), as they are released from the placenta to drive physiologic changes during pregnancy. This study argues that prolactin interacts with the placental derived proliferin-related protein (PRP) to induce this well orchestrated series of vascular and hemodynamic changes. PRP is an angiogenic hormone that has receptors in spongiotrophoblasts and diploid cells within the placenta. PRP as well as proliferin receptors are found in nonpregnant women; however, the increased levels of placentally produced hormones increase receptor binding in pregnant women as well as the increased availability of receptors in the newly formed placenta modulates the actions of PRP (Linzer and, 1999). This may help to explain how the placenta works to temporarily orchestrate certain physiologic changes during pregnancy. However, it is unclear why the fetal allograft is not rejected during successful pregnancies.

Several ideas have been put forth to why fetal antigens do not sensitize the maternal immune system. One such idea was that the maternal immune system is maximally suppressed during pregnancy. The fact that pregnant women can mount sufficient immune responses to infections and tissue grafts contradicts this hypothesis. Another idea is that the fetal tissues do not present foreign antigens due to the lack of major histocompatibility antigen expression on the syncytiotrophoblast and non-villous cytotrophoblast (components of the vascular interface). However, these antigens are present on the cells of the fetus and the stromal tissues of the placenta (Hunziker 1986; Chaouat 1987). Also, the MHC antigens are present on the previously described fetal red and white blood cells found in maternal circulation. This makes this explanation less plausible as well. Other researchers have proposed that local decidual barriers prevent immune recognition of the fetus by the mother or serve as an impermeable barrier to
competent maternal immune cells thereby protecting the fetus. While there is a functioning decidual immune barrier, it is often breached due to mild trauma. Furthermore, the existence of a protective barrier does not explain the reported cases of extrauterine/intraabdominal pregnancies that have gone to term without other complications (Chaouat 1987).

An interesting idea put forth by our studies is the shedding of microvesicles, exosomes, from the placental tissue with surface fetal antigens and possibly packaging immunosuppressive cytokines (TGFβ), hormones (estriol) or other unidentified factors. Exosomes shed into the serum may come into contact with competent maternal immune cells; however, in the absence of co-stimulation would lead to tolerance of the presented fetal antigens. Alternatively, fusion of the exosomes with the maternal immune cells and subsequent delivery of internal suppressive contents cause anergy in the T cells. These anergic T cells could be subsequently rendered nonencephalitogenic and lead to the observed suppression of clinical MS and EAE.

While we did not specifically test for placental vascular endothelial cell expression of PI3Ks, it is plausible that the vasoconstrictive events downstream of estrogen stimulation of Class IA PI3Ks provide some protection by securing the placental barrier. It could be argued that increased vasoconstrictive effects described above could be paralleled in the CNS vascular endothelial cells in response to increased estrogen induction of class IA PI3Ks – further reducing clinical signs of MS and EAE.

Studies using the PI3Kγ inhibitor and gene ko mice were focused to evaluate other critical factors in the development of clinical signs. The disease initiating steps of T cell
activation and antigen presentation within the CNS were targeted in these studies. Th1 and Th17 markers of T cell activation provide only a snapshot of what is happening at particular time points within the evolution of clinical signs. Histological evaluation of the specific motor and sensory portions of the CNS can sometimes serve as a correlate to immunological findings. The combination is often tied to measurable clinical signs in EAE. We have used these overlapping immune and histological findings to help target mechanisms of symptom development in EAE. We selected time points during average maximal disability or alternatively maximal suppression of disease to study immune responses and histopathology. Identification of targeted CNS structures during different phases of disease is critical for understanding clinical progression of EAE and MS.

A key study by Brown and Sawchenko (2007) mapped the neurodegenerative and underlying inflammatory events in various parts of the brain during progressive EAE. The study found that sensory pathways were targeted disproportionately to those of motor and cognitive tracts. Consistent with findings in early stages of MS, visual, auditory/vestibular, somatosensory and proprioceptive fibers were found to be targeted (Brown 2007). Inflammatory cell infiltration, microglial activation and neurodegeneration were found to be the predominating pathology in these mice (Brown 2007).

In our studies using both the progressive and relapsing models of EAE, we report dorsal column (sensory) structures were largely demyelinated with correlative increases in cellular infiltrates. We also evaluated neurodegeneration using a silver stain in C57Bl/6 mice, while Brown and Sawchenko (2007) used a fluoro-jade and SMI-32 immunohistochemical staining for this purpose. Their study did not evaluate demyelination, but rather microglial activation in
the sensory areas targeted in our research as well as in Brown et al. (2007) lead to deficits in position sense (proprioception), hearing and balance (vestibular) and visual tracts. It is clear why disturbances of the sensorium bring patients in to the clinical setting. In mice, it is arguable that these same sensory disturbances could manifest as imbalance and gait ataxia along the spectrum of scored signs in EAE.

The study done by Brown et al. (2007) was not an exhaustive study of the spinal cord, however, offered full detail of motor thalamic tracts in the brain. Our studies focused on neurodegenerative changes in the lower brainstem (pons) and the ventral and lateral structures of the spinal cord to assess motor functions and offer some correlative data for loss of function. As Brown et al. (2007) demonstrated, there are early inflammatory events that take place in menengial tracts and adjacent structures. We evaluated the pons at later time points for neurodegeneration and found significant axon severing at 60dpi. The work done by Brown et al. (2007) supports the idea that the pons is a suitable area to study for increased inflammation based on its proximity to early inflammatory white matter tracts. Timing and distribution of inflammatory events such as cellular infiltration and microglial activation as evaluated by Brown et al. (2007) were not evaluated for clinical correlates, as they did not report average scores along with relative immunohistochemical pathology. In both the PI3Kγ inhibitor and pregnancy studies, we link average clinical score with changes in demyelination, cellular infiltration and neurodegeneration. However, it is clear that many immunological changes take place in the absence of clinical signs in both MS and EAE. It was our goal to evaluate mice at time points that were matched to maximal suppression of disease activity, in late pregnancy, and maximal disability in both studies. The distribution of immunological change was more difficult to tie to
clinical signs in the relapsing EAE (SJL) model due to the cyclic nature of the disease course. In our PI3Kγ studies, we timed the use of the inhibitor to induce suppression and evaluate changes in inflammatory markers. We report a decrease in demyelinated areas during late pregnancy (SJL) and PI3Kγ null or PI3Kγ inhibitor treated (C57Bl/6) mice immunized for EAE matched with decreased average clinical score relative to controls.

It is important to note three differences between our studies and those done by Brown et al. (2007). The first was Brown evaluated primarily brain structures and we looked at brainstem and spinal cord structures. While both the brain and spinal cord consist of white matter and gray matter, in the spinal cord, the white matter is at the surface and the grey matter is inside – this pattern is reversed the brain. This would impact the timing and distribution of demyelinating and other inflammatory events. The functional differences between the two structures also have an effect on clinical presentations of disease. The spinal cord connects the peripheral nervous system to the brain and coordinates certain reflexes, while the brain is the primary center for processing these sensory inputs and coordinating motor outputs, among other major functions.

A second difference between the two studies was that Brown et al. (2007) used a two-step immunization that involved two separate injections of a lower dose CFA-MOG plus pertussis toxin over the period of 14 days. This is important to note as in their studies they did not see clinical signs until day 14 post initial immunization. Our lab has found that varying a single dose injection of CFA-MOG from 100 μg to 400 μg does not change the clinical severity of the EAE course very dramatically. However, it is likely that two separate low dose injections prolonged the inflammatory period leading to a more pronounced histological picture. In their studies, Brown and Sawchenko (2007) did not aim to make clinical corollaries, and therefore this
dual injection may have been a more suitable immunization technique to answer the questions they proposed. Our studies used a single higher dose of CFA-MOG and first clinical signs were observed around days 8-10.

The third difference was the use of female C57Bl/6 mice by Brown et al. (2007). Our studies have found that while there is no overall accumulative clinical difference in disease between male and female C57Bl/6 mice, females produced less inflammatory cytokines and have slightly more insidious disease onset as compared to males. Using females may have confounded attempts to make good clinical correlations with periods of increased inflammation and microglial activation. However, it has been reported that microglial activation is often distant to the inflammatory and neurodegenerative sites. Local axon damage is known to produce distant ipsilateral activation of microglial cells, thereby, initiating Wallerian degeneration. It would be interesting to determine if the same patterns of CNS histopathology can be observed between male and female mice using the same CNS mapping design.

**Treatment of relapse and progression of MS:** To investigate operative factors in disease progression, we focused our studies on regulation of inflammatory cell responses. While clinical relapses have been targeted in therapies for RRMS, it is yet unclear why majority of patients in this group are female. Alternatively, of the male MS population, most exhibit the progressive MS clinical variant. Interestingly, sex dimorphism has not been addressed with regard to the selection of treatment or to determine variable responsiveness between sexes. By-in-large, treatments for MS are directed to do one of three things: 1) reduce the severity of an ongoing flare-up to enhanced speed of recovery [corticosteroids]; 2) reduce the frequency of
relapses [beta-interferons & copaxone]; and 3) reduce development of new MRI lesions [beta-interferons] (Noseworthy 2000). Unconfirmed relief from disability progression has been listed as a possible therapeutic benefit of the beta-interferons; however, this has not been consistently reported. IVIg treatment and plasmapheresis is reserved for treating those patients that have been refractory to corticosteroids during an acute flare in disease activity. Overall, these therapies are most effective in the treatment of RRMS.

The SJL mouse strain exhibits relapsing EAE in response to PLP immunizing antigens. SJL mice demonstrate a sex dimorphism where males have been found to be less susceptible to disease and females show typical relapsing remitting course (Papenfuss 2004). Our studies describe a clinically relevant model of RRMS that demonstrates a pregnancy induced suppression of disease similar to that described in MS (Confavreux 1998). Previous studies in our lab demonstrate an increase in the production of IL-10 and a reduction in TNFα production when disease is initiated during late pregnancy (McClain 2007, J Immunol, in press). However, there were no reports that examined the pregnancy affect on established/ongoing R-EAE to serve as an animal equivalent to the findings published by Confavreux et al. (1998). In these studies, we aimed to harness the pregnancy potential to reduce relapses and uncovered both soluble non-soluble components of pregnancy serum that likely influence disease outcomes.

Understanding sex differences in the treatment of disease are very real challenges in academic and clinical medicine. Pregnancy is clearly a sex specific condition. Presently, pregnancy hormone trials predominate with respect to research efforts to mimic the suppressive potential of the pregnancy state. We submit that purification of small identifiable immunosuppressive factors from pregnancy serum, such as exosomes, may present a unique
opportunity to isolate sex-independent factors from a sex specific state. Ultimately, our goal is to determine if exosomes act independently of sex hormones and modulate immune responses using mechanisms present in both men and women such as induction of tolerance and immune cell anergy. This would reduce the reliance on presently proposed sex specific treatments that use pregnancy/sex hormones.

Exosomes have been studied in the past as nonspecific markers for ongoing or recent inflammation. It was thought that the presence of these microvesicles in the serum were evidence for cell death. However, the use of exosomes in therapies has increased the recognition of these particles as having a positive role in disease suppression (Sabapatha 2006). There are three major advantages to the exosome system of packaging proteins in reducing disease activity: 1) the bilipid layer coat around the contents of the exosome provide a temporary shelter from serum based degrading enzymes, 2) these microvesicles can move through the serum and communicate with multiple cell types – based on size and ease in crossing barriers, and 3) in the absence of costimulatory markers/receptors on these exosomes, contact with nearby inflammatory T cells would result in tolerance inductions. The ideas listed above will be useful to study the dynamics of pregnancy suppression. A placental derived exosome could dually package anti-inflammatory protein as well as antigens specific to the maternal-fetal interface. When presented to a T cell, the idea is that the T cell is reprogrammed to be tolerant of the antigen contained in the exosome derived from the placenta; thereby reducing the hostility of the pregnancy microenvironment toward the fetal allograft. This same suppression is likely the cause of the observed reduction in clinical disease in EAE and MS during pregnancy.
Our studies have shown that pregnancy is protective in EAE models of PPMS and is consistent with our previous findings that the post partum period does not offer protection against increasing disease activity (McClain et al. 2007 J Immunol. in press). These results show that the pregnancy phenomenon is pervasive in that it is not strain specific and may not apply to multiple clinical forms of MS. However, PPMS has the unfortunate characteristic of severe progression.

Using C57Bl/6 mice in progressive EAE, which do not demonstrate a cumulative sex difference, we investigated mechanisms to limit disease progression. We found that blockade or deficiency of PI3Kγ protein resulted in a dramatic decrease in severity and progression of disease. In fact, male mice deficient for the gene were not susceptible to disease induction at all. The novel finding that C57Bl/6 PI3Kγ gene deficient (ko) mice have a clear sex dimorphism in response to EAE immunization was an interesting outcome of these studies as it offered a new avenue for studying sex differences in autoimmunity. We designed multiple experiments to define the mechanisms that allow total protection in ko males and to uncover mechanisms that guide the sex dimorphism observed within the PI3Kγ ko groups.

Gene ko studies established the importance of the PI3Kγ gene in the development of clinical signs. We found that while the PI3Kγ -/- females were susceptible to disease, we could reduce the disease severity through use of the male hormone, testosterone. This is another important consideration related, however, to the male sex hormone testosterone and its use in combination with PI3K inhibitors. A novel role for combination testosterone and PI3Kγ inhibitor therapies is indicated based on these results. The fact that many autoimmune disorders are sex-discrepant in their onset and severity, we feel that this new finding in a model for MS
should add significantly to the research related to sex and autoimmunity. We were able to significantly recapitulate the above findings observed in the gene ko model when moving our investigations into a more clinically applicable model using a PI3K\(\gamma\) specific pharmacologic inhibitor. Using a reversible inhibitor of the gene product also clears any uncertainty of the phenotype being the result of developmental consequences of the gene deficiency. Our goals are to continue to investigate these findings in sex differences and the role for PI3K\(\gamma\) in EAE disease with the objective to aid in the development of therapies and contribute to the ongoing discussion in mitigating autoimmunity.

**Pregnancy and PI3Ks in Future therapies: Advantages and disadvantages.** We report a profound suppression of clinical disease along with decreased inflammatory cytokine production in EAE immunized mice during late pregnancy. As mentioned, pregnancy can be truthfully touted as the best, albeit temporary, therapy for multiple sclerosis. Understanding suppressive factors in reproductive immunology has fuelled debates about which components of the pregnancy state can be mimicked in the design of new therapies. Multiple forms of estrogens have been tested in EAE studies and human clinical trials. As with many of the current therapies, these estrogens show only partial suppression of disease and are most beneficial in the relapsing EAE and MS variants. Our studies looked at the response to pregnancy in both relapsing and progressing EAE models and found that the pregnancy suppressive effect is pervasive.

In all of its therapeutic glory, pregnancy has yet one major caveat. One has to become pregnant! This is a highly sex-discrepant and age-dependent state that is influenced by events
These events certainly have some impact on the immune system, and likely influence clinical outcomes in established immune-mediated diseases. Although there are no studies that directly report on these issues, it is very likely that MS and pregnancy studies (ie Confavreux et al. 1998) included women who experienced such unreported events. In EAE studies, different animal facilities with varying health barrier systems may represent environmental variations of bacterial exposure. Also, there are known stress inducing events between caged mice when establishing a hierarchy; this can be said to serve as variable social stressors. In either case, both MS and EAE studies have still successfully demonstrated disease suppression in response to pregnancy or pregnancy hormones. These findings support the existence of powerful immunosuppressants and protective factors that are released into the host during pregnancy.

There is not believed to be a “magic bullet” that can be isolated from the serum that will reproduce the full suppression induced by pregnancy. Our reports in R-EAE using SJL mice show a complete clinical recovery during late pregnancy; however, there remained elevations in IFNγ and increased T cell proliferative responses. These lingering inflammatory mediators are likely to be the major players in the postpartum exacerbation of EAE disease. This postpartum flare is also influenced by the combined reduction of protective placental derived factors such as steroid hormones, protein hormones or placental exosomes. Estrogens and corticosteroids have been directly linked to the regulation of the transcription factor NFkB. Interestingly, estrogens
have been linked to that same inflammatory pathway that leads to NFkB through the PI3K Class IA signaling cascade. Understanding how these factors are involved in inflammatory signaling cascades will be useful in developing new targets for therapies.

As mentioned above, therapies have not been studied or developed to address possible sex-dependent variations in response to treatment. We have the benefit of exploiting the animal models and PI3Kγ pharmacologic blockade to determine important dose modifications and to consider combination therapies in MS. Our studies did not evaluate the use of PI3Kγ-inhibitor in females C57Bl/6 mice. However, our collaborators in Geneva, Switzerland at Merck Serono International, developers of the AS-605240 PI3Kγ inhibitor, completed multiple experiments using C57Bl/6 females immunized for EAE and the PI3Kγ inhibitor. Their studies revealed only a mild suppression of clinical signs and a modest delay in clinical onset of disease (personal communication with Dr. Christian Rommel, Principal Investigator). Results of the use of testosterone pellets in PI3Kγ null female mice in EAE implicate the use of testosterone as an adjunct to PI3Kγ inhibition. Although most men with MS have the progressive type, it is known that within the PPMS group, both women and men are represented equally. This is accounted for by the ~3 fold increase in female MS patients as compared to males world-wide. These findings help to support a need to further investigate the role for certain drug combinations and dose specification based on sex differences.

Another advantage of these studies is the potential applicability in treating other autoimmune disease states based on reported increased statistical clustering of autoimmune diseases within families. More importantly, there are reports of patients with two or more concurrent Th1 mediated autoimmune diseases. As mentioned above, PI3Kγ inhibitor therapies
have been shown to be effective in Th1 and Th2 mediated diseases such as RA (Camps 2005, Rommel 2007) and SLE (Barber 2005). While it is uncommon to have both a Th1 and Th2 autoimmune disease, it is likely that one autoimmune disease can cause cytokine shifts and other derivations in the host microenvironment that makes them more susceptible to similar autoimmune states. For example there are several reported cases of patients with MS who also suffered from psoriasis, (Th1 mediated skin disease), uveitis, rheumatoid arthritis and autoimmune thyroiditis (Ramagopalan 2007). It would be interesting to compare the effectiveness of PI3Kγ-inhibitors to other known treatments for MS in reducing the clinical reports of combination autoimmune diseases in treated patients.

With respect to PI3Ks and pregnancy investigations, PI3Ks have been reported to be upregulated in response to estrogen receptor-α stimulation (Bryant et al. 2006). There are no reports by our lab or others that investigate PI3K activity in postpartum period. However, it can be argued that rapid postpartum decreases in estrogen lead to decreased Class IA PI3K activity and loss of neural protection. Class IA PI3Ks are expressed on most tissues in the body and likely have variable expression of PI3K receptors on different tissues. These kinases may regulate structural and parenchymal protective or inflammatory changes. The known class IB PI3K (PI3Kγ) is exclusively expressed on hematopoetic cells and regulate cellular responses to hormonal postpartum changes in the microenvironment. As mentioned above, PI3Kγ is reported to limit APC migration and possibly limits activated T cells trafficking into site of inflammation.

The PI3K signaling cascade is depicted below (Fig. 30) and includes Class IA stimulation by estrogen. Mannella and Brinton (2006) proposed that 17β-estradiol (E2) induced neuroprotection was due to an upregulation of PI3K signaling cascades; however, their studies
did not include any measure of neuronal survivability in PI3K inhibition experiments. Their work did, however establish a clear protein-protein interaction between estrogen receptors and PI3K regulatory subunit p85 (Class IA). Mannella and Brinton (2006) cited well established actions downstream of MAPK and pERK 1/2 pathways leading to the inactivation of pro-apoptotic proteins such as Bcl-II associated death protein (BAD) and Bcl-x after estrogen exposure. Other investigators have shown that E$_2$ activation of the PI3K–Akt pathway protected against glutamate and β-amyloid induced neurotoxicity (Honda 2000; Singh 2001). However these studies did not evaluate impacts on CNS relative to class IB PI3K influenced peripheral and CNS cellular responses. It is likely that estrogen positively stimulates the class IA pathway and inhibits class IB to provide maximal neuronal protection through the actions of PI3K$_\gamma$ on inflammatory cell migration.

There are several plausible explanations that address differences in protective responses to activation of the two arms of the Class I signaling cascade and reconcile differences between our findings and those reported by Mannella et al. (2006). Briefly, Mannella et al. (2006) reports that upregulation of PI3K pathways are neural protective. Our studies in PI3K$_\gamma$ inhibition demonstrate neural protective phenotype in CNS autoimmune injury:

1) Mannella and Brinton (2006) did not investigate protective or damaging effects of specific PI3K inhibitors, but used the broad inhibitor wortmanin to examine production of apoptotic factors. Blockade of the PI3K Class IB arm of the PI3K signaling cascade, as demonstrated in our studies, could homeostatically drive class IA responses and lead to increased cell survival (refer to the PI3K signaling cascade figure 28 below). Inhibition of PI3K$_\gamma$ in our studies resulted in decreased clinical severity and CNS pathology likely
related to effects on inflammatory cells; however, we did not specifically look at serum levels of apoptotic factors.

2) Mannella et al. (2006) evaluated protein-protein interactions in primary cortical cell cultures treated with doses of estrogen. There are several caveats to this technique in comparison with our studies. a) it is not certain that CNS physiologic levels of estrogen were pre-determined for cell cultures, b) interactions were measured at 10 minutes and 30 minutes which is not suitable to study chronic CNS injurious states, and c) cortical neurons may express different levels of estrogen receptors or PI3K receptors as compared to cerebellar, hippocampal or spinal cord neurons found to be damaged in EAE. While cortical lesions are common in MS, these areas have not been found to be the predominate focus of inflammation in EAE (Brown et al. 2007).

3) Work done by Mannella et al. (2006) was useful to confirm the link between estrogen and PI3K signaling. It is likely that early pregnancy progesterone, late pregnancy estrogen and postpartum prolactin levels influence survival outcomes variably in the CNS.

With respect to this third comparison, a study published in *The Journal of Neuroscience* (Gregg 2007) showed enhanced remyelination was observed in the “maternal CNS” in connection with elevated prolactin levels. This study claimed to measure remyelination of CNS area including the hippocampus and olfactory areas using a triple labeled for BrdU (FITC), GST_\pi (rhodamine – oligodendrocyte marker), and MBP (Cy5). While this study is useful to implicate a postpartum hormone in CNS protection, the use of BrdU cannot be said to identify new
remyelinating oligodendroglia, as BrdU is taken up in the nucleus of oligodendrocytes and remyelination of CNS neurons does not involve the nucleus of these myelinating cells. Our studies were not aimed at evaluating remyelination, rather assessing level of demyelination at specific time points in disease or therapy. It would be interesting to study the regulation of PI3Ks in response to a more broad range of hormones specific to the pregnancy and post pregnancy periods.

As with any new targeted therapy, there is the concern of non-specific cross-reactivity or other long term exposure effects related to immunosuppression. Our studies did not evaluate the susceptibility of PI3K null mice to other infectious agents or do exhaustive studies of changes in immune response to immunization in aged mice. It is important to determine these factors especially since it is well established that hormone levels change in response to aging, weight
gain, weight loss and pregnancy. Females are known to have increasing levels of testosterone with aging and males, conversely, produce less. Further investigations are needed with respect to these changes and PI3K signaling.

A quite interesting caveat, however, with PI3Kγ interruption is the novel report by our lab that PI3Kγ null males undergo a dramatic increase in body size, primarily increased adipocyte deposition, as they increase in age. Also, pronounced decreases in renal and hepatic parenchymal integrity (Fig. 31). These changes are not observed in their female counterparts. Young PI3Kγ ko males maintain similar weights as wt males. WT males increase in size about 3-6 grams as they age from 2 months to 8 months; however, ko males were observed to increase in size 21-26 grams over the same time period. So, if a doubling body size is the major side-effect of PI3Kγ inhibition – then I suspect the volunteer lines for clinical trials will be short. In defense of the therapeutic potential for pharmacologic blockade of PI3Kγ, it is likely that the findings in the gene-ko model are the result of other developmental changes linked to complete removal of the PI3Kγ gene. Effects of the PI3Kγ-inhibitor are reversible. We did not evaluate decreases in life-span of these males, as after about 10 months the mice were severely limited in mobility in the cages and mice were euthanized. It is important to note that the increase in size did not appear to be linked to an increase in feeding behavior; however, more strict dietary studies would have to be completed to confirm this finding.

Recent reports help in the targeting of specific organs in response to changes in PI3K signaling. Price (2007) reported renal cell damage in response to increase IA class PI3K signaling. We found that mice deficient in the Class IB PI3K had increased renal cell damage. Two reports tie a normal to decreased levels of PI3K-Akt associated pathways and insulin
resistance (Shao 2000; Nadler 2001). There are several reports on the function of PI3Ks role in
the development of diabetes that make the above kidney and liver damage findings intriguing in
our ko model as it relates to our finding during pregnancy. PI3Ks interact with the insulin
receptor substrate (IRS) to regulate glucose uptake through a series of phosphorylation events.
Inhibition of PI3Ks induce insulin resistance by reducing the expression of an important glucose
transporter. This leads to excessively high blood sugars and a resultant insulin insensitivity,
diabetes mellitus. Increased adipocyte formation happens down the line causing suppression of
leptin responses and other changes seen in diabetics (Terauchi 1999; Pirola 2003). Leptin is a
protein hormone that is produced by adipocytes that results in decreased appetite and increased
metabolism. Patients that are insensitive to insulin, usually have low leptin levels as well. Low
leptin levels result in increased appetite and slower metabolism, leading to weight gain.
Incidentally, leptin is also an inflammatory molecule. This is likely the cause for the excessive
weight gain in the PI3Kγ null males. However, it does not fully explain why females do not
experience this weight gain. Ideas discussed below about estrogens and their induction of some
PI3K responses are a plausible explanation as to why ko females do not gain weight as they age –
and possibly have increased levels of leptin contributing to the increased susceptibility to EAE
immunization. Another idea is that expression of PI3Ks on muscle tissues, important for making
glucose, may be variable between males and females or may be upregulated in muscle during
pregnancy period offering another explanation for ties between gestational diabetes and
pregnancy in the face of overall immunosuppression.

Interestingly, several reports discuss the role of PI3Ks in angiotensin II (AII) regulation
resulting in vasoconstriction and hypertension with concomitant insulin resistance [diabetes-like
symptoms] (Isenovic 2004; Marrero 2004). With the known link between pregnancy increases of estrogens, and estrogen induced PI3K signaling, it is likely that answers as to causes for gestational diabetes and hypertension-eclampsia can be elucidated in continued investigations. This is an interesting connection as it applies to our studies done in pregnancy suppression of disease. Pregnancy increases in estrogen drive PI3Ks to induce a protective vasoconstriction. The placental microenvironment must be well regulated as well as that of the CNS. It is possible that the role for differentially expressed PI3Ks on the endothelial surfaces of these two organs during inflammation could be that of a “gate keeper”. That is to say, PI3K action on free radical production and subsequent increases in AII, which leads to vasoconstriction, may be quite suitable as one line of defense against foreign invaders of the brain and the placenta.

It would be of great benefit to complete a series of studies that evaluate changes in the PI3K signaling cascade during different time points during pregnancy. It would be important in these studies to evaluate changes at the level of the CNS, placenta, spleen and muscle tissue. It is also likely that hepatic and renal PI3K and estrogen response elements are regulated during pregnancy. Late pregnant mice are maximally suppressed and PI3Kγ null mice are protected. Further investigation into the link between the two molecules based on the information discussed in this document related to NFkB transcription, estrogen hormone influences and the effects of PI3Ks on vascular tissues will help to drive these new experiments. The PI3Kγ specific inhibitor has only recently become available for use in research (Pomel 2006), however, with restricted privileges and permission from the developers. Access to this specific inhibitor will allow for more clinically relevant studies in evaluating the variable tissue expression of Class IB PI3Ks during Th1, pregnancy or other Th2 states.
Figure 31. PI3Kγ null males are twice as large as their wt counterparts and have increased kidney and liver damage at age 8 months. (photo) Average weight of ko males at 8 months was ~50.8g while age matched controls were ~19.5g (largest of each group photographed). (top) KO males have increased glomerular damage relative to wt controls (Congo red stain of kidney). (bottom) KO males have increased fatty change of the liver relative to wt controls (H&E stain of liver) [n=6].
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115


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