HORMONES AND DENDRITIC CELLS: INFLUENCES ON THE INITIATION OF THE AUTOIMMUNE DISEASE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

DISSERTATION

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

By

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ABSTRACT

Sex hormones can dramatically modulate immune responses and influence both susceptibility and clinical disease course in autoimmune disease. In multiple sclerosis (MS), sex determines susceptibility and clinical presentation, while pregnancy has profound therapeutic actions over currently approved MS treatments. The objectives of this thesis were to determine the influences of sex hormones on the induction of an immune response using experimental autoimmune encephalomyelitis (EAE).

The influence of sex hormones and genetics on EAE was evaluated using multiple mouse strains with different MHC class II haplotypes. We identified a new model of sexually dimorphic EAE and a model for primary progressive multiple sclerosis. Male B10.PL mice had increased clinical severity, mortality and histopathological infiltrates associated with an increased pathogenic Th1 immune response. Additionally, female sex hormones at the time of disease induction are protective, suggesting that sex hormones present at disease induction are important in influencing EAE.

Since DCs are uniquely able to drive naïve Th0 cell differentiation to shape adaptive immune responses, we evaluated the influence of the pregnancy specific hormone estriol (E3) on DCs and EAE induction. DCs were expanded in vivo in the presence of pregnancy levels of E3. E3 DCs had an “activated regulatory” phenotype (i.e. increased CD80, CD86, PD-L1, PD-L2, B7-H3 and B7-H4), decreased mRNA levels of proinflammatory IL-12, TNF-α and NF-κB and increased mRNA levels of immunoregulatory IL-10 and IDO. E3
DCs produced less IL-12 and Th0 cells proliferated less \textit{in vitro} with E3 DCs. Mice receiving E3 DCs were protected from EAE, having decreased severity, decreased CDS and complete resolution of clinical signs compared to mice receiving Pb DCs. This effect is dose-dependent and, importantly, E3 DCs maintain their protective phenotype in the face of potent inflammatory stimuli (i.e. \textit{in vitro} LPS and \textit{in vivo} adjuvants). Mice receiving E3 DCs are protected by increased IL-4 and decreased IFN-\(\gamma\) with no contribution from CD4+CD25+FoxP3+ regulatory T cells.

Taken together, these results demonstrate that sex hormones present during disease induction can influence the clinical course of EAE. Sex hormones generate regulatory DCs that may have important therapeutic applications in the treatment of inflammatory and autoimmune disease.
Dedicated to my husband Eric and son Jonah
I would like to thank my advisor, Dr. Caroline Whitacre her guidance and support that allowed me to grow both personally and professionally. I would also like to acknowledge the present and past members of Dr. Whitacre’s lab: Nicole Damico for her friendship, invaluable words of wisdom and scientific excellence, Ingrid Gienapp for her experience and technical expertise, Aaron Kithcart for his humor and interest in biomedical research, Todd Shawler for his flow cytometric expertise, Melanie McClain, Fei Song and NaTosha Gatson for helpful discussions and assistance with experiments and all of the faculty, collaborators, staff, residents, fellow students and mice that facilitated the evolution of this thesis.

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<tr>
<td>Ab</td>
<td>antibody</td>
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<td>APC</td>
<td>antigen presenting cell or allophycocanin-conjugated</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<td>FITC</td>
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<td>FL</td>
<td>Flt3-ligand</td>
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<td>GM-CSF</td>
<td>granulocyte-myeloid colony stimulating factor</td>
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<td>HFL</td>
<td>human FL</td>
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<td>HPA</td>
<td>hypothalamic pituitary adrenal</td>
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<td>HPG</td>
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<td>IFN</td>
<td>interferon</td>
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<td>ITAM</td>
<td>immune-tyrosine activator motif</td>
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<td>immune-tyrosine inhibitor motif</td>
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<td>LN</td>
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<td>mAB</td>
<td>monoclonal antibody</td>
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<td>myelin oligodendrocyte glycoprotein</td>
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<td>MS</td>
<td>multiple sclerosis</td>
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<td>myelin proteolipid protein</td>
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<td>PPMS</td>
<td>primary progressive multiple sclerosis</td>
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<td>RRMS</td>
<td>relapsing remitting multiple sclerosis</td>
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<td>spleen or splenocytes</td>
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<td>SPMS</td>
<td>secondary progressive multiple sclerosis</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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CHAPTER 1
INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that is thought to be autoimmune in nature. MS is thought to be caused by autoreactive T cells that recognize and attack components of the myelin sheath such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and myelin proteolipid protein (PLP). These CD4+ T cells are activated by antigen presenting cells such as macrophages and dendritic cells (DC) and then migrate to the CNS. In the CNS, these CD4+ T cells proliferate, release inflammatory components such as cytokines and chemokines, cause edema and recruit other inflammatory cells (1). All of this inflammation and edema cause multiple foci of CNS demyelination with the periventricular gray-white matter regions, optic nerves, brain stem and spinal cord most commonly affected (2, 3). Additionally, progression of the disease is furthered by the loss of axons around the initial lesion site (2, 4).

Depending on the lesion site, severity and stage of disease progression, individuals with MS have a variable clinical presentation. Clinical signs range from mild signs such as optic neuritis, vertigo, transient fatigue, loss of coordination, and numbness
in the extremities that may lead to more severe signs including muscle and limb weakness/spasms, ataxia, cognitive impairment and varying degrees of paralysis (2). Disease presentations, fluctuations and rate of disease progression have been used to classify MS into four specific forms. Relapsing remitting MS (RRMS) has clearly defined relapses (recurrence of symptoms) followed by full or partial recovery and a period of no disease worsening. RRMS occurs in over 80% of patients with MS, has a female preponderance and affects twice as many women as men. In its true form, RRMS patients have little disease progression while progressive relapsing MS patients (PRMS) have continual worsening of disease between relapses. Interestingly, over 50% of patients with RRMS eventually progress to a secondary progress MS (SPMS) where the remitting and relapsing clinical course is replaced by a progressive disease course (5). Finally, approximately 20% of MS patients demonstrate primary progressive MS (PPMS). PPMS is the predominant form of MS in men and manifests with increased disease severity and unrelenting disease progression (2, 6).

The variability of lesion distribution, disease severity and disease manifestations makes treatment problematic. Most MS therapies are immune-modulating and non-specific in nature. While global immunosuppression with methotrexate and azothioprine were commonly used for treating later stages of MS in the past, they were associated with numerous adverse side effects (7). More recently, more refined immunomodulatory drugs have been utilized in the treatment of MS. Methylprednisolone, a corticosteroid, is used primarily in acute relapses and has been shown to restore the blood brain barrier (BBB), decrease the number of lesions by magnetic resonance imaging (MRI) and cause
decreased expression of pro-inflammatory mediators IFN-γ, TNF-α and IL-2 and adhesion molecules required to cross the BBB (8-10). Currently approved FDA drugs for more long-term MS treatments include interferon-1β (betaseron; a genetically engineered form of human interferon beta) and glatiramer acetate (copaxone). Both of these drugs have efficacy in treating early RRMS and preventing relapses (10). Betaseron and related IFN-β treatments are anti-inflammatory, anti-viral, anti-proliferative and immunomodulatory and act by decreasing T cell activation, limiting migration of pathogenic T cells to the CNS, shifting cytokine profiles away from a pathogenic Th1 response, downregulating IL-2 receptors and upregulating IL-10 and reducing inflammatory mediators TNF-α and IFN-γ (7). Copaxone, a random polymer of the amino acids L-alanine, L-lysine, L-glutamic acids and L-tyrosine cross-reacts with MBP and is thought to potentially competitively inhibit MBP binding, increase regulatory T cell activity or shift from a pathogenic Th1 response to a protective Th2 response (7). Interestingly, even with these therapies available, the most profound modulation in disease activity is seen during the hormonal changes of pregnancy and the post-partum period.

Even with several treatment options for MS, the complex nature and undefined etiology of MS complicates the development of therapies. Although suspected to be autoimmune in nature, there are numerous other factors that influence disease susceptibility, including genetics, ethnicity, geography, environmental factors and hormones. The role of genetics has been suggested from studies demonstrating that monozygotic and dizygotic twins have an increased risk of developing MS (25-35% and
3-5% respective increase) compared to the general population (0.1-0.4%) and relatives of MS patients have an increased risk of developing disease (2). Although disease susceptibility is polygenic in nature, linkage analysis indicate that the HLA DR2 locus within the human MHC class II region is commonly associated with disease susceptibility in MS patients (2). While clustering of individuals with similar genetics may, in part, explain the decreased incidence of MS in tropic or subtropical locations, genetics alone cannot completely explain the geographic influences on MS susceptibility (5). In addition to geographic locale, other environmental factors (e.g. infectious agents and toxins) have long been implicated as contributing factors to explain MS susceptibility. Associations with infectious agents have been seen since the 1940’s with an MS outbreak in the Faroe Islands and more recently, have included associations with *Chlamydia pneumoniae* and human herpevirus 6 (11). The role of microbes initiating the onset of MS has been an active area of research and strongly suggests the role of these environmental factors influencing MS susceptibility (11). Finally, the role of sex hormones in influencing numerous autoimmune diseases and within MS is demonstrated by a consistent increased incidence in women and a sexual dimorphism (RRMS in women versus PPMS in men).

**1.2 Experimental Autoimmune Encephalomyelitis**

EAE is an animal model with clinical, immunological and histopathologic similarities to MS that has been a consistently used as an animal model for MS. EAE can be induced in a variety of species including guinea pigs, rabbits, rats and certain strains of mice with the
majority of recent reports in murine EAE (12-14). Susceptible mouse strains include the B10.PL, SJ/L and more recently, the C57BL/6 strain. EAE results from the immunization of susceptible rodents with myelin antigens including myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) or peptides derived from these proteins emulsified in complete Freund’s adjuvant (CFA) and accompanied by pertussis toxin (PT) injections (15, 16). The MHC haplotype, frequency of autoreactive T cells, differences within antigen processing and presentation and relative responsiveness to environmental influences such as inflammatory mediators and hormones influence the strain-specific ability of different myelin components to induce disease.

Clinical disease course differs between strains and depends on the immunizing neuroantigen and these serve as models for the spectrum of disease seen in MS patients. Specifically, B10.PL and SJL mice exhibit a chronic relapsing clinical disease course while the C57BL/6 strain exhibits optic neuritis and chronic progressive disease with such differences modeling the spectrum of disease seen in MS patients (17). In addition to serving as a model for MS, EAE, as a Th1-mediated disease, is an ideal model for understanding factors that influence helper T cell development.

Although specific strains allow investigators the ability to evaluate different clinical courses, mechanistic studies have been particularly assisted through the use of T cell receptor (TCR) transgenic (Tg) mice. Several TCR Tg mice are available including the V\(\alpha\)4/V\(\beta\)8.2 TCR Tg mice on a B10.PL background that recognize the immunodominant N-terminal 1-11 amino acids of MBP and, more recently, the V\(\alpha\)3.2/V\(\beta\)11 TCR 2D2 Tg mice on a C57Bl/6 background that recognize MOG35-55 (17). Similar to other TCR Tg mice, these mice have
the majority (95%) of their CD4+ cells specific for the immunodominant epitope which allows for investigating the mechanisms of antigen restricted DC-T cell interactions and basic immunobiology of autoimmune diseases both in vitro and in vivo (18) (Whitacre, unpublished data). Unlike the DO11.10-OVA TCR Tg mouse, the MBP and MOG transgenic mouse models are well-characterized disease models that allow study of the initiation of the Th1-mediated autoimmune disease EAE.

EAE is mediated by activated CD4+ Th1 cells which recognize neuroantigen in the context of MHC class II and can result from either active immunization or passive transfer of encephalitogenic CD4+ T cells (12-14). The immunodominant encephalitogenic epitope is strain-specific with MOG35-55 (in C57BL/6), PLP139-151 (in SJL) and the N-acetylated region of MBP Ac1-11 (in B10.PL) used as both immunizing antigen and in vitro stimulant. Clinical disease using both active immunization and passive transfer, characterization of helper T cell populations and the effects of Th1 and Th2 cells on the induction, pathogenesis and amelioration of EAE have been well-characterized in the B10.PL, SJL and C57BL/6 strains (19-22). Both active immunization and passive transfer have been shown to generate CD4+ T cells that secrete pro-inflammatory cytokines (e.g. IFN-γ, TNF-α, and, more recently, IL-17) thought to initiate and propagate the inflammation mediating CNS pathology (3, 23). Thus, in general, Th1 cells are immunopathogenic and exacerbate EAE while Th2 cells are protective and decrease/ameliorate clinical disease (19). This divergence in disease progression based on Th1 or Th2 effector cell phenotype and the active immunization and passive transfer models allow the functional assessment of both the induction and effector phase of the adaptive immune (helper T cell) response.
Clinically, actively immunized mice develop ataxia that progresses to hind limb (occasionally front limb) paralysis during the acute phase of disease. Mice recover from the acute phase and then progress to a chronic phase with a variable clinical course. Depending on the mouse strain, immunizing neuroantigen and degree of neurological damage, mice develop a relapsing disease course (e.g. female SJL mice) or a more chronic progressive course (e.g. C57BL/6). Differences in chronic disease course have been attributed to the degree of neurological damage in that mice. Mice with a relapsing disease and clinical recover between relapses have relatively limited neurological damage and lesions limited to perivascular inflammatory infiltrates, edema and demyelination plaques (16, 24). Mice with chronic progressive disease courses are thought to have more early and extensive damage to myelin, neurons and neuropil from inflammation, edema and axonal severing (4, 16, 24, 25).

In most active immunization EAE models, disease severity and exacerbations are linked to the degree of inflammation and pro-inflammatory environment seen with Th1 cytokines (i.e. IL-2, IFN-\(\gamma\), IL-1, IL-6, TNF-\(\alpha\)) whereas suppression of disease is seen with high levels of regulatory cytokines such as IL-4, IL-10 and TGF-\(\beta\) (16, 24, 26). Often, early events in the acute phase of disease have a profound influence on more chronic phases of disease and the initiation of inflammation in the acute phase of disease is critical to polarize T cells to establish EAE. This polarization of T cells is depending on numerous factors that hinge directly on the interaction of dendritic cells (DCs) and naïve helper T cells. Traditionally, two signals; interactions with the MHC class II and the T cell receptor (TCR) (signal 1) and costimulatory molecules such as CD80/CD86 interaction with CD28 and CTLA-4 (signal 2) are critical to the development of helper T cell
responses. Recently, the influences of the local environment on this DC-T cell interaction have been shown to play an important role and such environmental factors act as a third signal to influence the development of an adaptive immune response. Sex hormones are one such factor that can influence the interaction of DCs and T cells and influence the development of the adaptive immune response (27).

1.3 Effect of sex hormones on the immune response and autoimmune disease

Similar to many other autoimmune diseases, women have an increased incidence of MS and are most likely to develop MS in the reproductive years (ages 25-55), a period of hormonal fluctuation (28, 29). Hormones have been known to influence the immune system for over 30 years and have a complex interplay with cells of the immune system. Females of many species (including humans) demonstrate elevated immune responses of both the humoral (antibody) and cell-mediated arms of the immune response (30). Females have higher overall immunoglobulin levels, higher antibody responses to bacteria and viruses, and increased resistance to bacterial, viral and parasitic infections (30-32). Additionally, females have higher CD4+/CD8+ ratios, higher levels of IL-4, IL-1 and IFN-γ and more efficient APC’s than males (31, 33, 34). While this increased immune responsiveness in females may have some benefit in combating infectious diseases, this increased immunological reactivity has been proposed to explain the increased incidence of many autoimmune disease in females. Such gender dimorphism in disease is seen in both MS and EAE. In MS, women tend to have an increased incidence, earlier onset, increased severity and develop RRMS compared to men that are
less often afflicted but develop the more severe and unrelenting PPMS. Within EAE, the
SJL and, more recently, our results with the B10.PL, demonstrate a similar sexual
dimorphism has been demonstrated with female SJL’s modeling the increased severity
and relapsing disease in women and male B10.PL’s modeling the increased severity and
progressive course of men. The MHC haplotype differs between SJL and B10.PL mice
(H-2^s versus H-2^u) and these genetics likely contribute to the sexual dimorphism seen.

While there are a few examples of increased susceptibility to autoimmune
diseases linked to the X or Y chromosomes, the majority of sex hormone influences on
the immune response remains unexplained (35). Genetic mechanisms that may play a
role in influencing susceptibility to autoimmune diseases include co-expression of sex-
related and MHC genes, genetic triggers, chromosomal modulation, strain differences,
altered receptor expression, in utero developmental alterations or dysregulation of the
hypothalamic pituitary adrenal (HPA) and hypothalamic pituitary gonadal (HPG) axes
(28, 32, 36-46). Although the specific role of sexual dimorphism in susceptibility genetic
loci has not been investigated, there are suggestions that a genetic basis underlies some of
the sexual dimorphism seen in MS and EAE. Recent findings suggest that the Y
chromosome plays an important role in susceptibility to autoimmune diseases such as
EAE and that sex is a more important determinant than age or season at time of
immunization(35). In EAE, specific genetic loci (eae4, eae5, eae11 on chromosomes 7,
17 and 16, respectively) have recently been associated with increased susceptibility to
autoimmune disease such as EAE or affect the clinical subtype of EAE (eae3, eae12,
eae7 and eae13 on chromosome 3, 7, 11 and 13, respectively) (39). While this study
suggested sex-specific effects at both *eae4* and *eae11*, the precise influence of sex hormones was not investigated (39). Within MS, the association of HLA DR2 within the human MHC class II region with disease susceptibility may be explained by reported hyperresponsivity of APCs seen in females but these studies have not been undertaken (2, 47). Thus, although the interplay between genetics, environmental factors and increased immunoreactivity in females likely influence an individual’s susceptibility to developing MS or other autoimmune disease the specific effects of sex hormones on disease susceptibility have yet to be thoroughly evaluated. What has been explored in more detail are the influences of sex hormones on the induction and effector phases of autoimmune disease.

Interestingly, although there is an increased susceptibility to autoimmunity in females, elevated female sex hormones such as estrogens can modulate clinical disease in these same individuals. In both MS and EAE, the influence of sex hormones on decreasing clinical disease has been profound and the subject of much interest in the development of therapies. A general paradigm put forth to explain the complex role of hormones acting on the adaptive immune response is that low estrogen levels increase Th1 responses while high estrogen and progesterone increase Th2 responses (6, 48, 49). The elevated estrogen and progesterone concentrations seen during pregnancy are thought to be the primary mediator of the Th2 bias during pregnancy and result in the protection seen in MS and EAE (50, 51), (McClain, manuscript provisionally accepted). Additionally, placental products such as α-fetoprotein, pregnancy-associated plasma
protein A, pregnancy associated α-2 glycoprotein, human chorionic gonadotropin and human placental lactogen may also contribute further by suppressing Th1 responses.

1.4 Pregnancy and the immune response

A Th2 response (IL-4, IL-5, IL-10 and IL-13) is considered necessary for pregnancy maintenance and a strong systemic Th1 immune response increases the risk of resorption and abortion (50, 51). Evidence to support this is that IL-4, IL-5 and IL-10 are secreted by the placenta throughout pregnancy and injection of these cytokines have no ill effects on pregnancy while injection of Th1 cytokines TNF-α and IFN-γ cause abortions and may be required to initiate parturition (52-54). Studies in humans have shown that women with normal pregnancies produce more Th2 cytokines and have increased decidual T cell specific IL-4 and IL-10 production compared to women with recurrent abortions (53, 55). Infectious models have helped substantiate the importance of Th1/Th2 balance in controlling infection versus maintaining pregnancy. Infection of C57BL/6 mice with Leishmania major results in increased pregnancy failures and resorptions due to an increase in Th1 cytokines (IFN-γ and TNF-α) and decrease in Th2 cytokines (IL-4 and IL-10) (56, 57). L. major itself is not causing the resorptions since infection of BALB/c mice (that produce a Th2 response following infection) maintain pregnancies to term (56, 57). Neosporum caninum infection generates a Th2 response and infection with this parasite fails to cause the loss of pregnancy and a Th2 cytokine profile is maintained (58, 59).
Therefore, pregnancy or high (pregnancy) levels of estrogens and progesterone and the associated Th2 bias explain many of the observed hormonal effects on autoimmune diseases. In support of this, Th1 diseases such as MS and EAE are decreased while Th2 mediated SLE is exacerbated during pregnancy or treatment with high hormone levels (49, 60-63). Following parturition, disease severity for both MS and SLE usually returns to pre-pregnancy levels, purportedly because of a return to a non-pregnant Th1-biased immune response. The Th1/Th2 balance is thought to be a primary mechanism by which sex hormones influence the outcome of autoimmunity but recent reports indicate that sex hormones may have additional effects on the immune system such as through the generation of regulatory cell populations (e.g. regulatory T cells). Since high levels of female sex hormones such as seen during pregnancy are known to have profound effects on the immune response, they have been a focus of investigations in both MS and EAE studies.

1.5 Pregnancy hormones: Effects on MS and EAE

Both estrogens and progesterones are immunomodulatory and influence Th1/Th2 balance and regulatory T cell populations (22, 51, 55, 63-67). Pregnancy represents the most profound state of immunomodulation by sex hormones. During pregnancy, sex hormones can be increased 1000-fold over non-pregnancy levels which are necessary to maintain the fetal allograft. As a state of immunomodulation, pregnancy can have dramatic effects on autoimmune (e.g. MS, EAE) and infectious diseases (e.g. Leishmania infection) largely through the Th2 bias during pregnancy (50, 55-57, 68). The levels of
estrogens increase throughout gestation with the ovaries secreting estrogens early and the majority of estrogens secreted by the fetal-placental unit later in gestation. The three active forms are all increased during gestation and include estrone (E1), 17-beta estradiol (E2) and estriol (E3). While E1 and E2 can be found in normal cycling women and are increased to supra-physiological concentrations during pregnancy (approximately 1 to $\geq 10$ ng/ml), E3 is considered a hormone of pregnancy since it is minimally present in non-pregnant women and is produced predominantly by the placenta. Progesterone, as well as many other hormones (e.g. ACTH, cortisol) and fetal-placental factors (e.g. alpha-fetal protein) also increase throughout gestation with the highest levels seen in the third trimester of pregnancy. Besides the immunomodulatory effects of sex hormones, factors from the fetus or fetal-placental unit may additionally be immunoregulatory. One such example is early pregnancy factor (EPF), which has been shown to suppress clinical signs of EAE (69, 70).

While many hormones characteristic of pregnancy have been shown to influence the course of autoimmune diseases, it is noteworthy that pregnancy levels of the female hormone progesterone alone appear not to affect EAE and are minimally synergistic with E2 in CIA models (67, 71-73). Previous evidence demonstrated that progesterone increased IL-4 (a Th2 cytokine) and it was thought to mediate a shift to Th2 (49, 55). However, pregnancy-levels of progesterone (via subcutaneous sustained release hormone) had no effect on EAE or collagen-induced arthritis (CIA) (67, 73). While many factors have the potential to influence the immune response, the estrogens have
been the predominant focus of many studies due to their efficacy in both MS patients and EAE studies (74-76).

Within MS, pregnancy decreases the risk of relapse, disease onset, disease severity and MRI lesions during pregnancy (5, 62, 77-82). Decreased disease is most pronounced during the third trimester and is often followed by an increased relapse rate, disease severity and MRI lesions in the first 3-6 months following parturition and a return to pre-pregnancy rates of relapse approximately 6 months post-partum (5, 62, 77-83). Further evidence for the hormone-immune interactions is that dramatic decreases of these hormones results in an abrupt shift to a Th1 environment which coincides with post-partum exacerbations in MS (5, 62, 77-83).

Protective effects of pregnancy are also seen in EAE and have been known for nearly 30 years. Pregnancy delayed the onset of EAE in guinea pigs and rats and completely prevented disease in rabbits (84-86). The physiological state of pregnancy appears necessary for protection since pregnancy loss (i.e. resorption or abortion) resulted in disease onset in both rabbits and guinea pigs (84-86). Rats did not terminate pregnancies early and exhibited decreased disease during pregnancy and lactation but did have smaller pups than non-immunized rats (87, 88). These results can be explained by the fact that Th1 cytokines have been shown to result in fetal resorption, abortion and decreased viability of offspring (50, 51). Studies in the Lewis rat and SJL mice indicate that the greatest protection from disease development and on pre-existing disease occurs during late pregnancy, a time when sex hormones are at their highest (68, 87) (McClain, manuscript provisionally accepted). Work within our lab and others, demonstrated a
marked effect of pregnancy on the development and progression of EAE (68) (McClain, manuscript, provisionally accepted), (Gatson, unpublished data). Specifically, induction of EAE during high estrogen environment of late pregnancy has a profound and long lasting protective effect on immunized mice. These mice have a delayed onset of disease, mild clinical signs and complete recovery. Additionally, the hormonal environment of late pregnancy protects mice from ongoing EAE in that these mice have complete resolution of disease during the pregnancy period (Gatson N, unpublished data). Taken together, these results indicate that hormones of pregnancy are potent influences on both disease induction and ongoing clinical disease.

Experiments with pregnancy levels of E2, E3 and progesterone suggest that estrogens play the most predominant immunomodulatory role (22, 51, 55, 63-69, 84-86, 89, 90). Within MS, the potential for estrogens or selective estrogen receptor modifiers (SERMs) is an active area of research and a small clinical trial recently demonstrated that estriol (E3) treatment resulted in protection similar to that found in pregnancy women (91, 92). In this study, gadolinium enhancing MRI lesions were significantly decreased in E3 treated patients and the protective effect was directly associated with the E3 in that cessation of E3 resulted in absence of protection and resumption of treatment resulted in rapid return of protection. This protective effect was associated with a decreased Th1-associated responses (e.g. delayed type hypersensitivity (DTH), TNF-α, IFN-γ) and increased Th2 associated cytokines IL-5 and IL-10 (91, 93). While E2 is thought to act through reduced activation of effector T cells, generation of T regulatory cells and enhanced expression of the PD-1 inhibitory costimulatory pathway, the specific mechanism by which
E3 is protective in vivo has not been explored (94). Within EAE, both E2 and E3 are protective at pregnancy levels and E2 has also been shown to be protective at lower (estrus) levels making estrogens of particular interest as therapies (67, 73, 95-98). Oral or parenteral administration of estradiol (E2) or estriol (E3) has been demonstrated to decrease clinical EAE in several murine strains including the SJL, B10.PL and B10.RIII (66, 75, 96, 98). Both E2 and E3 lowers EAE incidence when given prior to immunization (67, 74). Mice treated with estrogen that did develop EAE had delayed onset and decreased peak and cumulative disease score (67, 74). Although most effective at higher estrogen dosages, the effects of E2 were also seen at low (diestrus) concentrations of estrogen (74). E3 treatment prevents the development of EAE and oral administration protects both male and female mice from EAE (67, 97). While there are studies investigating both E2 and E3 effects on EAE, the different clinical effects of these two estrogens have not been thoroughly investigated.

The immunomodulatory abilities of estrogens appear to be relatively global and holds true across species as evidenced by the protective effect in numerous mouse strains (i.e. SJL, B10.PL and C57BL/6 strains), Lewis rats and humans with MS (74-76, 91, 99). Unfortunately, all estrogens are often assumed to mediate similar influences on the immune response. However, recent findings suggest that estrogens and SERMs have profound differences in their mechanism of action and subsequent effects with the end result being similar; immunomodulation. By far, the vast majority of mechanistic studies have investigated E2 and recent evidence also indicates that in addition to the Th1/Th2 shift, E2 have other effects on the T cell compartment such as expansion of regulatory
CD4+CD25+ T cells, induction of T cell trafficking (via expression of adhesion molecules) or sensitivity of lymphocytes to apoptosis (6, 30, 31, 100-103). E2 may also influence DC-T cell interactions by influencing costimulatory pathways (e.g. PD-1) and soluble mediators (e.g. indoleamine 2,3-deoxygenase; IDO) (94, 104). Recent work suggests that the T cells, long thought to be the primary mechanism by which E2 mediates protection in EAE, are not the primary cells mediating protection from EAE with E2 treatment. Rather, an antigen presenting cell (APC) such as the DCs were suggested to play the predominant role in protection but there has been limited work investigating the influence of hormones on DCs within EAE (94, 104, 105). E3 has been much less studied and are less well understood although E3 has been shown to have regulatory effects on T cell migration, cytokines and the transcription factor NFkB (67, 97, 98, 106). The mechanisms by which different estrogens modulate the immune response are still being explored but one important difference in how different estrogens act is through binding different isoforms of the estrogen receptor.

1.6 Estrogen Receptors

Estrogens, like other steroid and sex hormones, are derivatives of cholesterol and mediate their effects primarily by binding a cytosolic estrogen receptor (ER; ER-α and ER-β) and regulating gene transcription (99). Estrogens have two primary receptors; 1) estrogen receptor alpha and 2) estrogen receptor beta (99). Other ERs such as recently described membrane ERs (e.g. ER-X) may account for some of the rapid effects of E2 on T cells or the differing effects of specific estrogens or SERMs but little work has been done on these orphan receptors (107, 108). Knockout mice have demonstrated that ESR-
1 (ER-α) is necessary for estrogen to suppress EAE while the absence of ESR2 (ER-β) has no effect on disease (66, 109-111). Studies using bone marrow chimera mice have determined that the beneficial effect of E2 on EAE does not involve ER-α signaling in blood-derived inflammatory cells such as T cells but ER-α expression in other tissues likely mediated this effect (109). Taken together with the findings by Offner and colleagues, the role of ER-α in antigen presenting cells (APCs) such as DCs play a more important role than T cells in explaining estrogen-mediated protection in EAE (105). Interestingly, both T cells and DCs are known to express ERs and the development of bone marrow DCs has been shown to depend on estrogens for growth and differentiation (66, 112). However, little is known concerning the basic mechanism of how different estrogens influence and signal DCs and the effects that estrogens have on DC biology.

Both the mechanisms by which estrogens influence immune cells and the effects these estrogens have on the subsequent immune response are important aspects of understanding hormone-immune interactions. Estrogens are not simply shifting the immune response from a Th1 to a Th2 response but may be influencing numerous aspects of the immune response. While the exact mechanism of how estrogens influence the disease course are still under investigation, the fact that estrogens are much more efficacious when given prior to disease induction suggests that estrogens play an important role during the induction of an immune response. This data, coupled with the fact that estrogen-mediated protection in EAE appears to not be mediated through T cells but through APCs such as DCs argues for the need to investigate the effects of estrogens on DCs and the DC-T cell interface (105). The fact that estrogens may mediate much of
their action through DCs does not negate the importance of T cells since the development of a Th1 or Th2 response depends on a complex interaction between DCs and naïve helper T cells. By investigating estrogen-mediating signaling on DCs, we will further our understanding of how hormones impact immune function in health and disease.

1.7 Initiating an autoimmune response: Dendritic cell:T cell interaction

DCs bridge the innate and adaptive immune response and play a critical role in the initiation and regulation of inflammatory processes such as autoimmune disease. DCs act as sentinels of the immune system and are often the first cells to encounter an antigen. As APCs, DCs are unique in their ability to prime naïve T lymphocytes and play a critical role in influencing the nature of the adaptive immune response (113-115). DCs present antigen in an MHC class II-restricted manner (signal 1), and through the interaction of costimulatory molecules and cytokine secretion (signal 2), drive T cell differentiation (e.g. Th1, Th2, Th3 or other reg-T cells). These populations are distinct functionally with Th1 cells producing IL-2, IFN-γ and lymphotoxin (LT), Th2 cells producing IL-4, IL-5, IL-6, IL-10 and IL-13, Th17 cells producing IL-17 and regulatory T cells producing TGF-β and IL-10) (14, 23, 116). Lineage specific transcription factors for Th1 Th2, and CD4+CD25+ T cells (i.e. T-bet, GATA-3 and Foxp3, respectively) can be used to phenotypically identify these cells (117-120). While many factors contribute to Th1/Th2/T-reg polarization (e.g. TCR-MHC-peptide complex, costimulatory molecules, adhesion molecules and cytokine/chemokine/IDO release), the most important factors are soluble factors (i.e. cytokines and IDO) and costimulatory molecules (118, 121-124).
Cytokines are a primary mechanism by which DCs can influence T cell differentiation and function. DCs are known to produce IL-12, IL-23, IL-10 and TNF-α and may utilize such cytokines to influence helper T cell differentiation. Cytokines such as IL-12, IL-23, IL-10 and TNF-α play critical roles in the pathogenesis of EAE (3, 116, 125-128). Both reg-DCs and regulatory T cells (i.e. Tr1) have been shown to utilize IL-10 to regulate immune responses (129-132). IL-12 and IL-23 are the predominant cytokine demonstrated to drive the production of IFN-γ producing Th1 cells and IL-17 producing Th17 cells, respectively, while IL-10 inhibits Th1 development and cooperates with IL-4 in the production of Th2 cells (116, 125). IL-10 appears to play a more important role than IL-4 in EAE as shown in IL-10 and IL-4 knock-out mice and IL-10 is necessary to prevent severe EAE and promote recovery (126, 133, 134). TNF-α plays a dualistic role in autoimmune disease since TNF-α mediates both CNS damage and plays a role in protection from relapses and autoimmune disease (3, 128). Although DCs can produce TNF-α and have the potential to contribute to a Th2 response, paradoxically, exposure of DCs to TNF-α generates semi-mature DCs that produce mice from developing EAE (135). Therefore, the definitive role for TNF-α is unclear. One recently described mechanism by which DCs influence T cell function is through increased indoleamine 2,3-deoxygenase (IDO) production, resulting in a suppressive effect on T cell responses (136, 137). The regulation of such proinflammatory factors such as IL-12 and TNF-α or immunoregulatory molecules such as IL-10 or IDO are some of the mechanisms by which DCs can influence T cell development and modulate EAE.
Costimulatory molecule interactions between DCs and T cells also play an important role in influencing T cell responses. Recently it has been recognized that there are both stimulatory and inhibitory costimulatory molecules and expression of certain inhibitory molecules or the overall balance of these signals dictate the resultant immune response (138). Regulation of the autoimmune response has been seen through the increased expression of inhibitory molecules such as CTLA-4, PD-L1, PD-L2 and ILT3/4 and may be one mechanism by which DCs have regulatory function (138-142). DCs may modulate immune function by altering the relative balance of inhibitory and stimulatory costimulatory molecules (i.e. signal 2) on their surface. Indeed, CTLA-4 has been shown to be important in the generation of regulatory DCs and PD-1/PD-L1/PD-L2 may be promising markers of protection in EAE (139, 143, 144). There has been much recent interest in the PD-1/PD-L1/PD-L2 interactions and it has been shown that these interactions play an important role in regulating susceptibility and disease severity in EAE (145, 146). Furthermore, the PD-1 costimulatory pathway has also been shown to be involved in the estrogen-mediated immunomodulation in EAE and is likely mediated through DCs (94).

While inhibitory costimulatory molecules may be increased, stimulatory costimulatory molecules may be increased, decreased or unchanged in response to estrogen. In EAE, E2 treatment of DCs results in increased CD86 and CD40 (and MBP specific IL-10 production) and presumed to be the mechanism producing a Th2 response (147). However, E3 or progesterone treatment of human MS PBMCs did not alter CD40L, CD80 or CD86 expression (148). Therefore, although costimulatory molecule
interaction is a potent mechanism by which DCs influence T cell differentiation, limited studies have investigated patterns of costimulatory molecule expression on different DC subsets or how hormone treatment may alter the costimulatory molecule expression.

Much of the current knowledge regarding DC biology has been derived from studies investigating immunogenic DCs, or those DCs that stimulate an immune response. Studies investigating such immunogenic DCs in EAE have been limited to identifying their role in EAE immunopathogenesis and with experimental proof of principal studies demonstrating that pulsed DCs could induce EAE (149, 150). What is of more interest in autoimmune diseases is the development of therapeutic possibilities and the induction of tolerance in an overly active immune response. Recent studies have demonstrated the critical role of DCs in regulating the immune response and DC-based immunotherapy has become a rapidly growing area of investigation.

1.8 Regulatory DCs (Reg-DCs)

Once thought to serve only in an immunostimulatory role, DCs have recently been shown to negatively regulate immune responses as well and have potent regulatory (or tolerogenic) functions (131, 151, 152). Such DCs are termed tolerogenic or regulatory (reg-DCs). Reg-DCs have been generated using a variety of pharmacological, physiological and microbial-associated factors both in vitro and in vivo. Factors including hormones (e.g. estrogens, vitamin D), cytokines (e.g. TNF-α, IL-10), infectious agents (e.g. LPS, Cholera toxin) and pharmacologic/chemotherapeutic agents have all
been used to generate reg-DCs (131, 153-164). Regardless of the method used for generation of reg-DCs, all such cells, by definition, “regulate” the immune system.

While all the mechanisms by which reg-DCs regulate the immune system are not known, some reg-DCs mediate their effects through production of IL-10 or indoleamine 2,3-dioxygenase (IDO) or through the expression of inhibitory costimulatory molecules (130, 136-139). A newly described mechanism by which reg-DCs are thought to regulate T cells to promote tolerance is through production of IDO. IDO is an enzyme produced by reg-DCs that degrades tryptophan which suppresses T cell responses (136, 137). Indeed, IDO has been suggested as one mechanism by which estrogen-derived reg-DCs mediate their protective effect (104, 136). Reg-DCs can also exert profound influences on T cells through expression of specific costimulatory molecules. Increased expression or a shift in the balance of stimulatory versus inhibitory costimulatory molecules may result in distinct influences on helper T cell differentiation (138-142). However, at present, hormonally-derived reg-DC costimulatory molecule expression and IL-10/IDO production have not been extensively characterized.

Regardless of the mechanism used to generate reg-DCs, much of their regulatory function is through to be through the generation of either a Th2 response (such as would be protective in the Th1-mediated disease EAE), or regulatory T cell (T-reg) populations. T-reg cells are known to play important roles in regulating self-tolerance and ongoing immune responses (120, 165, 166). Both natural and adaptive T-reg cells have been described. Natural T-reg cells are defined as resident, originating from the thymus, CD4+CD25+, costimulation dependent (i.e. CD28, CD80/CD86) and specific for self-
antigens in a cell-contact/cytokine-independent manner (120). Conversely, adaptive T-reg (i.e. Th3, Th2 and Tr1), develop in the periphery, have variable CD25 expression, are costimulation independent and recognize tissue-specific and foreign antigens through a cell-contact independent, cytokine dependent mechanism (120). Each of the adaptive T-reg cells has associated cytokine secretion profiles defined as follows: Th2 cells produce IL-4/IL-13, Th3 cells produce TGF-β and Tr1 cells produce IL-10 (120, 129, 167-170). It appears that many (but not all) regulatory T cell populations express the transcription factor foxP3 (171). Regardless of how these cells are defined, they are potent regulators of the immune system and have been shown to play important roles in controlling autoimmune diseases such as EAE (167, 170, 172-174). In particular, hormones have been shown to have profound effects on regulatory T cells with E2 inducing and expanding regulatory T cells (66, 103, 175).

1.9 Sex hormones and DCs

As described, sex hormones can be profoundly immunomodulatory (31, 176). Both pregnancy and individual sex hormones have demonstrated effects on DCs, T cells, granulocytes, macrophages and NK cells (104, 111, 132, 135, 177-184). The sex hormones of pregnancy (estrogens), long presumed to act only on T cells, are now thought to mediate actions through DCs (105, 185). In fact, one study has shown that E2 may be necessary for the differentiation of functional DCs from murine bone marrow precursors (186). However, the influence of sex hormones on reg-DC generation, DC function and the
downstream effects on subsequent helper T cell differentiation has not been evaluated in detail.

There are only a handful of studies investigating the effects of estrogens on DCs in the context of the disease model EAE with the majority of these studies limited to E2 both with \textit{in vitro} rat DCs and \textit{ex vivo} splenic mouse DCs \cite{104, 132, 135, 147}. Studies with mouse DCs demonstrate that E2 results in decreased TNF-\(\alpha\), IFN-\(\gamma\) and IL-12 production in mature DCs and MBP-specific T cells cocultured with E2 pretreated splenic DCs expanded \textit{ex vivo} with GM-CSF and IL-4 demonstrate a shift towards production of Th2 cytokines IL-4, IL-10 and a concomitant decrease in production of Th1 cytokines TNF-\(\alpha\) and IFN-\(\gamma\) \cite{132, 142}. E2-exposed DCs prevented the expansion of CD4\(^+\) T cells and increased proportions of regulatory T cells producing IL-10 and CD4\(^+\)CD28\(^-\) suppressor T cells, \cite{135}. This was accompanied by an increased IL-10 and reduced TNF-\(\alpha\) production. Infiltrates of CD68\(^+\) macrophages within the central nervous system and MBP 68-86-induced T cell proliferation were inhibited in rats injected with estrogen-exposed DC compared to rats injected with naive DC. In these studies, E2 did not affect the expression of MHC class II, CD80 and CD86 by DCs but did inhibit the ability of these DCs to stimulate T cell proliferation, produce Th1 and Th2 cytokines and increased T cell apoptosis \cite{104}. In a separate studies, it was demonstrated that E2 can affect the differentiation, maturation and function of DCs from EAE rats \cite{147}. E2 activated DCs had upregulated MHC II and costimulatory molecule expression CD80, CD86 and CD40. These E2 DCs secreted more nitric oxide (NO) \textit{in vitro} but showed decreased antigen presentation ability and IFN-\(\gamma\) secretion and no effect on IL-10 secretion when cocultured
with T cells. Additionally, E2 up-regulated the expression of IDO, which promotes tolerogenic properties of DC (104). These effects of E2-exposed DCs on T cell proliferation and apoptosis were partially abolished by the addition of an IDO inhibitor (1-methyl-dl-tryptophan, 1-MT), indicating that estrogen-exposed DC induced IDO-dependent T cell suppression (104).

These limited studies suggest numerous possible mechanisms by which estrogens influence DC function and subsequent DC-T cell interactions. Altered cytokine production, costimulatory molecule expression and IDO production may all contribute to estrogen-mediated immunomodulation. However, all of these studies have been limited to the effects of E2 and did not investigate the pregnancy-specific immunoregulatory estrogen E3.

1.10 Objectives

Sex hormones have a dramatic ability to influence the immune response and are capable of influencing both susceptibility to autoimmune disease, as well as, influence already established disease. Within MS, gender is a determinant of susceptibility and clinical presentation while pregnancy and its associated female sex hormones have some of the most profound therapeutic actions, surpassing the efficacy of currently approved MS treatments. Thus, understanding how sex hormones influence the development (i.e. induction) of an immune response is an important step to uncovering hormone-related therapeutic possibilities. The objectives of this thesis were to determine the influence of sex hormones on the induction of EAE. Chapter 3 evaluates the influence of genetics and
sex hormones on the induction and development of EAE to identify new models for gender-related EAE studies. Since DCs are a critical component in the development of a helper T cell response, the remainder of this thesis looks at the influence of estriol, a pregnancy specific hormone, on DCs. Chapter 4 explored the methodology necessary to evaluate different *in vitro* and *in vivo* DC populations while Chapter 5 looked at the ability of estriol to generate regulatory DCs (reg-DCs). These reg-DCs were characterized and their ability to influence helper T cells were further explored using both *in vitro* methods and the *in vivo* disease model EAE.
2.1 Mice

Age-matched male and female SJL (H-2s), ASW (H-2s), PL/J (H-2u), B10.PL (H-2u), NZW/LAC (H-2d), NOD/Lt (H-2g7) and C57BL/6 (H-2b) were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed 4-5/cage. Mice were used at 6-8 weeks of age except NZW/LACJ that were 12-16 weeks old. Mice were maintained on a 12-hour light/dark cycle with lights on at 0600 h and lights off at 1800 h and given food and water ad libitum. Upon arrival, mice were randomly assigned to experimental groups, followed by an acclimation period of at least 3 days prior to any experimental manipulation. \( V^{\alpha 3.2/V^\beta 11MOG TCR Tg mice} \): \( V^{\alpha 3.2/V^\beta 11} \) MOG TCR transgenic mice were obtained from Dr. V.J. Kuchroo (Harvard, MA) and then maintained in our breeding colony maintained at the Ohio State University animal facility. Progeny were screened by flow cytometry for expression of either \( V^{\alpha 3.2} \) or \( V^\beta 11 \) transgene on CD4+ peripheral blood lymphocytes. Transgene positive animals were used at 6-10 weeks of age. All mice were maintained on a 12-hour light/dark cycle and given food and water ad libitum.
2.3 Antigens

Peptides were purchased from Sigma-Genosys (The Woodlands, Texas), Princeton Biomolecules Corporation (Langhorne, PA) or purified in the lab. All peptides were purified by HPLC and had a purity of >90%. The following were from Sigma-Genosys: PLP 139-151 (HCLGKWLGHPDKF), PLP 178-191 (NTWTTCQSIAFPSK), PLP 258-273 (IAATVNFAVLKMGRG), MBP 87-99 (VHFFKNIVTPRTP) and MBP 84-104 (VHFFKNIVTPRTPPSQGKGR) and Nac 1-11 (ASQKRPSQRHG). Myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (MEGVYRSPSRVHYLRNGK) and MBP peptides NAc1–11 (Ac-ASQKRPSQRHG-COOH) were synthesized by Princeton Biomolecules Corporation (Langhorne, PA) and was purified by HPLC. Guinea pig (GP) MBP was extracted from spinal cords (Rockland, Gilbertsville, PA) by the method of and MBP was further purified on a Sephadex G-50 column eluted with 0.01 N HCl (187). Individual fractions were analyzed by SDS-PAGE, and fractions containing a single band of the appropriate m.w. were pooled. The purified protein was dialyzed against water and lyophilized.

2.3 Induction of EAE

Mice were immunized subcutaneously in four sites over the back (left and right shoulder, left and right flank) with neuroantigen emulsified in equal volumes of complete Freund’s adjuvant (CFA; containing 200 μg heat-killed Mycobacterium tuberculosis Jamaica strain). Guinea pig myelin basic protein was extracted from guinea pig spinal cords (Harlan Sprague-Dawley, Indianapolis, Indiana, USA) using the method of
Swanborg et al. (187) while peptides were synthesized by Sigma-Genosys (The Woodlands, TX, USA). Mice received 200 ng pertussis toxin (PT) in 0.2 ml PBS (List Biological Laboratories, Campbell, CA, USA) intraperitoneally (IP) at the time of immunization and 48 hours later except where indicated. SJL mice were immunized with 150 μg of PLP139-151 (one of two peptide sequences differing at position 140). The peptides were designated PLPc (HCLGKWLGHPDKF) and PLPs (HSLGKWLGHPDKF) and were administered with adjuvants, except for PLPc which did not require administration of PT. ASW, PLJ and SJL mice were immunized with 100 μg MOG92-106 (DEGGYTCFFRDHSYQ) plus adjuvants. C57BL/6 and NOD mice were immunized with 100 μg of MOG35-55 (MEVGWYRSPFSRVVHLRNGK) (or C57Bl/6 mice immunized with 400 μg of MOG35-55 for Chapters 4 and 5) combined with adjuvants and NZW/LAC, B10.PL and SJL mice were immunized with MBP (200 μg/each) plus adjuvant.

2.4 Clinical scoring of EAE

Mice were evaluated daily for clinical signs of EAE for at least 35 days and scored as follows: 0, no clinical signs; +1, limp tail or waddling gait with tail tonicity; +2, ataxia or waddling gait with tail limpness; +3, partial hindlimb paralysis; +4, total hindlimb paralysis; and +5 moribund/death. Several parameters were reported for each strain including incidence (percentage of animals exhibiting signs of EAE), day of onset (day of first signs of EAE), mean cumulative disease score (i.e. the mean of the sum of individual daily scores for animals in each group), mean clinical score (mean clinical

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score of all animals (i.e. both clinically affected and unaffected) on evaluated day), mean highest clinical score (mean of highest clinical score of animals in each group) and mortality (percentage of animal death attributed to EAE). Mice that have died from EAE were given a score of 5 on the day of death and removed from further evaluation and calculations.

2.5 Surgical Procedures: Gonadectomies and Hormone Pellet Administration

2.5.1 Gonadectomies: Male and female B10.PL mice were anesthetized and gonadectomies were performed. Briefly, testes were removed by making a small (5mm) median incision through the scrotum followed by a small (2.5 mm) incision into the scrotal sacs. The spermatic blood vessels were ligated and testes excised. Ovaries were removed by making a small (5mm) transverse midlumbar skin incision followed by blunt dissection of underlying musculature. The ovarian fat pad was exteriorized, ovarian vessels ligated and ovaries excised. A ligature was placed within the lumbar musculature and surgical staples were used to close the skin. Animals were allowed to recover for 7-10 days before immunization.

2.5.2 Hormone Pellet Administration: Mice were anesthetized and 60-day release estriol (E3) or placebo (Pb) pellets were implanted subcutaneously over the shoulder blades. Mice were allowed to recover for at least 4 days.
2.6 Histopathology

At varying times after immunization (days 5, 10, 14, 21 and 30), brains and spinal cords were evaluated by histopathology. Spinal columns and brains were either manually removed or were sectioned \textit{in situ} with vertebral bodies following 24 hours of TBD-2 decalcification solution (Thermo-Shandon, Pittsburgh, PA). Tissues were fixed in 10\% phosphate buffered formalin and then dissected and embedded in paraffin. Sections were then processed for hematoxylin and eosin staining. Sections were scored for degree of inflammation on a scale of 0 (no inflammation) to 3 (widespread and diffuse parenchymal infiltration) and number of neutrophils on a scale of 0 (no neutrophils) to 3 (predominance (>75\%) in many lesions).

2.7 Proliferation assays and Cytokine Determination (Supernatant)

Peripheral lymph nodes (inguinal, axillary, brachial, cervical, popliteal and periaortic) and spleens were removed from mice on days 10-12 post immunization. Single cell suspensions were prepared and suspended in RPMI 1640 containing 10\% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin and 5 \times 10^{-5} \text{ M} 2-ME in round-bottom 96-well plates (4 \times 10^5 cells/well). Alternatively, purified CD11c+ and CD4+ cells were cocultured at a ratio of 1:5 and 1:10 with a total of 100x10^6 cells/well. Cells were cultured with medium alone or with immunizing or related antigen (whole MBP (40 \mu g/ml), MBP Ac1-11 (10 \mu g/ml), MOG 35-55 (10 \mu g/ml)), LPS (1 \mu g/ml), conA (1 \mu g/ml) or anti-CD3 (2 \mu g/ml). Cultures were
incubated for 24-72 hours at 37°C and 7%CO₂, including an 18 hour pulse with [³H] thymidine (1 μCi per well) for cell proliferation assays. Supernatents were collected, stored at –20 °C and evaluated by standard ELISA. For cell proliferation assays, 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) or were harvested onto a 96 well microfilter plates with bonded GF/C filter and counted on a Perkin Elmer Top Count NXT with Top Count NXT software. The means of triplicate wells were determined, and results are expressed as the total counts per minute (CPM) (mean counts per minute of cultures with Ag/mean counts per minute of cultures with medium alone) ± SEM for all animals in the group.

2.8 Analysis of secreted cytokines by ELISA

Supernatants were harvested at 24, 48, and 72 and 96 hours from 24-well plate cultures of spleen and lymph node cells (4 X 10⁵ cells/well) following stimulation with neuroantigen, LPS, conA or anti-CD3 (as described in 2.6). OPT-EIA Sandwich ELISA kits (Pharmingen, San Diego, CA) were used to determine the levels of IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6 and IL-10. Briefly, flat bottom polystyrene microtiter plates were coated with 100 μl of anti-cytokine antibody and diluted in carbonate or phosphate buffer. After overnight incubation at 4°C, unbound antibody was washed away and the plates were blocked for 2 hours with PBS/BSA to inhibit any non-specific binding. Blocking buffer was then rinsed away and 200 μl of sample is added to each well. After an overnight incubation period at 4°C, plates were washed with PBS/Tween 20 (0.05%).
Biotinylated antibody (0.2 μg/ml) was then added to each well and allowed to incubate for 1 hour at room temperature. Av-HRP conjugate was diluted in blocking buffer and 100 μl per well was added and allowed to incubate at room temperature for 30 minutes. After washing 5 times with PBS/Tween 20, 100 μl ABTS substrate solution was added. Color development occurred within 5-80 minutes of incubation at room temperature in the dark. The optical density was then be read with a SpectraMax Plus high throughput microplate spectrophotometer and analyzed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

2.9 Frequency of cytokine production by ELISPOT analysis

Frequencies of cytokine secreting cells were determined for IFN-γ, IL-2, IL-4, IL-5, IL-10 and TNF-α. Microtiter plates with nitrocellulose bottoms (Millipore, Bedford, MA) were coated overnight at 4°C with anti-IFN-γ, anti-IL-2, anti-IL4, anti-IL-10, anti-IL-12 or anti-TNF-α capture antibody diluted 1:60 in sterile 1x PBS (Antibody specific ELISPOT Development modules, R&D systems, Minneapolis, MN). Plates were washed with sterile 1x PBS and blocked with 1% BSA (Sigma, St. Louis, MO) for 1-2 hours at room temperature. Spleen cells or lymph node cells (4X10^5 cells/100 μl) were resuspended in HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 1% L-glutamine and 1/1000 gentamicin and added to the plates in duplicate or triplicate with medium alone or with the following peptides (10 μg/ml): PLP 139-151, MBP Ac1-11, MOG 35-55 or MBP (40 μg/ml), or activating agents; LPS (1 μg/ml), conA (1 μg/ml) or anti-CD3
Cultures were maintained at 37°C for 24-96 hours (IFN-γ, IL-2, IL-4, IL-5, TNF-α, IL-12 and IL-10. Plates were washed three times with 0.05% Tween 20 in 1x PBS and 100 μl appropriate corresponding capture antibodies (diluted 1:60 in filtered 1% BSA in 1x PBS) were incubated overnight at 2-8°C. After overnight incubation, plates were washed 3x with 0.05% Tween 20 in 1x PBS and an ELISPOT blue color module was used to develop the plates. Briefly, 100 μl of a 1:60 Strep-avidin in 1x PBS solution was added to each well and plates were incubated at room temperature for two hours. After a final three washes, plates were developed in the dark with a BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate p-toluidine saturated and nitro blue tetrazololium chloride in organic solvent) for 5-10 minutes until spots were apparent. Plates were allowed to dry overnight and frequencies of TNF-α and IL-10 secreting cells were determined using cytokine specific kits from R and D systems. Image analysis of ELISPOT plates was performed by computer-assisted image analysis using KS ELISPOT software and microscope control processor MCP4 (Carl Zeiss Vision GmbH, Thornwood, NY). Data are expressed as the number of cytokine-producing cells per million +/- SEM for all animals in a group.

2.10 Flow Cytometric Analysis

Single cell suspensions of lymphoid cells derived from lymphoid and spleen tissue were stained for CD4, CD8, CD11b, CD11c, CD25, CD28, CD44, CD62L, CD69, CTLA-4, GITR, CB80/B7-1, CD86/B7-2, PD-1, PD-L1/B7-H1, PD-L2/B7-DC, B7-H3, B7-H4, CD40, ICOS, ICOS-L with various combinations of fluorescein isothiocyanate
(FITC)-conjugated, phycoerythrin (PE)-conjugated or Allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) (BD Pharmingen, Franklin Lakes, NJ) and analyzed using two or three color flow cytometry. Suspensions of $10^6$ cells were incubated with labeled antibodies diluted in PBS plus azide with 2% mouse serum. After 30 minutes cells were washed and fixed with 1% paraformaldehyde. Isotype control mAbs (Pharmingen) were matched for fluorochrome and used for cursor placement. Lymphocytes were gated based on forward versus side scatter and a total of 10,000 events were analyzed on a FACs Calibur flow cytometer using cell quest and cell quest pro analysis software (Becton-Dickerson, San Jose, CA).

2.11 Intracellular Cytokine and foxp3 Measurement

Suspensions of $10^6$ cells from spleens and lymph nodes were stained for the intracellular cytokine IL-10, intracellular cell surface marker CLTA-4 and intracellular transcription factor foxP3. **Intracellular IL-10 staining:** Single cell suspensions of spleens and LNC 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 mg/ml streptomycin and 5 X $10^{-5}$ M 2-ME in round-bottom 6-well plates (20 X$10^6$ cells/well). Cells were cultured with MOG35-55 (10 μg/ml) for 48 hours, including a pulse with the Golgi inhibitor, monensin for the last five hours. Intracellular cytokine staining was performed following manufacturer’s instructions (IL-10 intracellular cytokine staining protocol ebioscience). Briefly, cells were fixed with fixation buffer (ebioscience) and then permeabilized. Cells were then stained with anti-IL-10 mAb and analyzed.
**Intracellular CTLA-4 staining:** Approximately 1x10^6 cells were added to flow tubes and blocked for 15 minutes at 4°C. Surface CD4 (FITC-conjugated) was stained and cells incubated for 30 minutes at 4°C in the dark. After two washes with staining buffer (1x PBS with 1% FCB and 0.09% sodium azide), extracellular CTLA-4 sites were saturated with an excess (2-4 μl CTLA-4 antibody) of antibody and cells washed once with staining buffer. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) for 20 minutes at 4°C in the dark. Cells were washed twice in 1x Permeabilization/Wash solution and PE-conjugated CTLA-4 or isotype control were incubated at 4°C for 30 minutes in the dark. Cells were washed twice in x Permeabilization/Wash solution and resuspended in 1% paraformaldehyde for flow cytometric analysis.

**Intracellular foxP3 staining:** Approximately 100 μl of prepared cells (1-2x10^6) to each tube. Surface molecules CD4 were stained using regular surface protocols as previously described. Cells were washed in cold PBS, resuspended and 1 ml of freshly prepared Fixation/Permeabilization working solution added to each sample after a second vortexing. Cells were incubated for 0.5-4 hours at 4°C and washed once with 2 ml of 1X Permeabilization Buffer followed by centrifugation and decanting of supernatant. A second wash with 1X permeabilization buffer was followed by Fc blocking (100 μl volume containing Fc block in 1x permeabilization buffer) and incubation at 4°C for 15 minutes. Without washing, fluorochrome-conjugated anti-Foxp3 antibody or isotype control in 1X permeabilization buffer were allowed to incubate at 4°C for at least 30 minutes in the dark. Cells were washed twice with 2 ml 1X
permeabilization buffer, centrifuged and resuspended in 300-500 μl flow cytometry staining buffer and analyzed on a flow cytometer.

### 2.12 Magnetic labeling and separation of CD11c+ and CD4+ cells

Single cell suspensions of spleen cells (CD11c+) or spleen and lymph node (CD4+) cells were centrifuged at 300xg for 8 minutes, erythrocytes lysed with lysis buffer and cells washed twice in cold MACs buffer containing PBS, 0.5% BSA and 2mM EDTA. Following centrifugation, the cell pellet was resuspended in 90 μl MACs buffer per 10 X 10^6 total cells. 10 μl anti-CD11c or anti-CD4 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 500μl of MACs buffer. Then labeled cells were then positively 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 CD11c- or CD4- cells were 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. Purity of the negative and positive fraction was assessed by flow cytometry analysis and purity >95% cell populations used for further experimentation.
2.13 Reverse transcriptase PCR (RT-PCR) evaluation of purified CD11c+ DCs

**RT-PCR:** Purified CD11c+ DCs were pelleted, resuspended in 0.5-1.0 ml of Trizol, vortexed vigorously for at least 30 seconds and frozen at –70 °C for later RT-PCR evaluation. Samples were thawed and the RT-PCR protocol began with removing the pellet (cell debris) and adding 1 volume of 70% ethanol to the supernatant (lysate) and mixing by pipetting. Up to 700 μl of sample to RNeasy mini column was placed into 2 ml collection tube and centrifuged at >10,000 rpm for 15 seconds. The flow throw was discarded. 700 μl of Buffer RW1 was added to the RNeasy column, tube closed and centrifuged at >10,000 rpm for 15 seconds. RNeasy column was washed twice with 500 μl of Buffer RPE followed by centrifugation for 15 seconds at >10,000 rpm and flow-through discarded. The RNeasy silica gel membrane was dried by 2 minutes centrifugation at >10,000 rpm and RNA eluted with 30-50 μl of RNase-free water. Optical density and the 260/280 ratio determined with samples >1.8 used for further evaluation by 1.5% agarose-formaldehyde gel and digital images taken. DC production of IL-12, IL-23, TNF-α, NF-κB, TGF-β, IL-10 and IDO mRNA were evaluated using RT-PCR.

**cDNA Synthesis:** cDNA was synthesized using SuperScript First Strand Synthesis System (Invitrogen) from 1-5 ug of total RNA using oligo(dT) (Invitrogen) and cycling conditions 35 cycles-PCR 94° (3 min); 94° (30 sec), 57° (30 sec), 72° (45 sec); 72° (5 min); 4° hold. Primers developed in our laboratory are as follows (Forward-Reverse): IL-12 (FAGGTGCCTTCCTCGTAGAGA-RAAAGCCACCAAGCAGAAGA), NF-kB (FCTGACCTGAGCCTTCTGGAGCAGGCTATTGCTCATCACA), TNF-α
(FCTGGGACAGTGACCTGGACT-RGCACCTCAGGGAAGAGTCTG), TGF-β (FAGCCCGAAGCGGACTACTAT-RTCCACATGTTGCTCCACACT), IL-10 (FCCAACGCTTTATCGAATGA-RTTTTCACAGGGGAGAAATCG), IDO (FAGCTCCGAGAAGAAGTCGAGAAA-RTGTAAACCTGTGTCCCTCAGTTTC), GAPDH (FAACTTTGCGACTTGGGAACG-GGAGGGTAGGAACA) and Beta actin (FTGCTGACACGATGCAGAAAGA-RCGCTCAGGAGGAACATGAT).

2.14 Differentiation and maturation of bone marrow DCs

Male and female 6-12 week old C57BL/6 or B10.PL mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used as a source for bone marrow precursor cells. Bone marrow precursors were flushed from femurs, tibias and humeri of naïve C57BL/6 mice and cultured in the presence of supplemented RPMI with 20% mFL-containing supernatant, 200 ng/ml hFL or 20 ng/ml GM-CSF (Peprotech Inc., Rocky Hill, NJ). Cells were plated at a concentration of 3-4x10⁶ cells/ml in 6 well tissue culture plates and 80% of the medium was replaced every 48-72 hours. Cells were removed from culture at days 7, 9, 11, 14 and 16, washed in cold PBS and labeled using the antibodies described below. Day 10-12 BM-DCs were used for DC maturation and EAE studies. To mature BM-DCs, immature BM-DCs (derived after 10-12 days of culture) were washed, re-plated at a concentration of 4-6 x10⁶ cells/ml and cultured with or without myelin oligodendrocyte glycoprotein peptide (MOG35-55 (MEVGWYRSPFSRVVHLRNGK)) with medium alone or matured with TNF-α (5 μg/ml, Peprotech, Inc. Rocky Hill, NJ), LPS (1 μg/ml, (Sigma-Aldrich, St. Louis, MO) or
a combination of LPS + TNF-α. Cells were removed from culture after 24 hours, washed in cold PBS and evaluated phenotypically (flow cytometric labeling for CD40, CD80, CD86 and MHC Class II) and functionally in vivo into naïve mice prior to EAE induction.

2.15 *In vivo* DC expansion Methods

Male and female 6-12 week old C57BL/6 or B10.PL mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used for *in vivo* DC expansion. *In vivo* DC expansion was carried out using i) administration of recombinant hFL, ii) continuous delivery of mFL using a melanoma delivery system and iii) injection of both hFL and mFL plasmid by hydrodynamic injection. Phenotypic, functional and comparative aspects of these different derivation methods were evaluated.

2.15.1 *Daily injection of human recombinant (hFL):* hFL (kindly provided by Amgen Thousand Oaks, CA) was used to expand DCs *in vivo* and consisted of daily administration for 9 days of hFL (200 μl containing 200 ng/ml in 0.1% mouse serum albumin) subcutaneously in the nape of the neck. Control mice were given vehicle alone (200 μl of 0.1% mouse serum albumin).

2.15.2 *Continuous tumor-derived mFL administration:* Approximately 1-3 x 10⁶ B16 melanoma cells (C57BL/6 background) transfected with murine FL cDNA using a retroviral vector MFG (kindly provided by Dr. Paula Bryant, Ohio State University), were injected subcutaneously divided into two sites over the flanks. Mice were sacrificed when tumor size measured 1-1.5 cm in diameter as previously described (188).
2.15.3 Preparation of mFL and hFL plasmid DNA: Plasmid DNA (both mFL and hFL) was purchased from Aldevron (Fargo, ND, developed by University of Michigan Vector Core (UMVC)). Plasmid transformation, harvesting and DNA isolation were performed. Briefly, 1 μl of plasmid at center bottom of a Falcon tube and 40 μl of ice-thawed Library Efficiency DH5 competent cells, (Life Technologies of Invitrogen) were gently added to the plasmid. Cells were incubated on ice for 30 minutes, heat shocked for 40 seconds in 42° C waterbath, placed on ice for 2 minutes. Approximately 360 ml of room temperature S.O.C. medium (Invitrogen) were added and the mixture was shaken at 225 rpm at 37° C for 1 hour. Approximately 10 μl of solution was added to agar plates and sterile glass beads used to streak entire plate. Agar plates were inverted overnight at 37° C and colonies harvested the following day. Individual colonies were added to 1X LB broth (containing 10 μg/ml kanamycin) in a 15 ml Falcon tube and placed into a shaker for 6-7 hours at 37° C. Tubes with significant cell growth were added to 250 Erylenmeyer flasks containing 200 ml of 1x kanamycin-LB broth which were then placed on a shaker at 37° C overnight. Samples were removed, with the majority pelleted at 5100 rpm for 10 minutes and either stored at –80° C or used for DNA isolation following one freeze-thaw cycle. A small amount of samples were used to make glycerol stocks containing 150 ul sterile filtered glycerol + 850 ul sample on wet ice (70% ethanol and dry ice) and stored at –80° C. DNA isolation was performed using Qiagen Midiprep protocol in concert kit (Valencia, CA). Approximately 10 ml of equilibration buffer were used to wash columns and suspended cells filtered. Cells were lysed and incubated at room temperature for 5 minutes, followed by the addition of 4 ml of neutralization buffer.
Samples were filtered using high purity filters and loaded onto columns. Flow through was discarded and the column washed 2x with Wash buffer. Plasmid DNA was precipitated by adding 5 ml of elution buffer and 500 ul of isopropanol added to each 1.5 ml of eluate. Samples were then placed at –20° C for 1.5 hours, centrifuged for 30 minutes at 4° C (14,000 rpm), isopropanol discarded and samples washed with 350 ul of 70% ethanol. This was followed by a second wash for 10 minutes at 14,000 rpm at 4° C, the ethanol removed and the pellet air-dried for 10 minutes and resuspended to a total volume of 50 μl of 1X TE. Restriction enzyme assay utilized enzymes Pst I, Sal I and BamH I (Invitrogen) and were run on a 0.8T agarose gel for 1 hour.

2.15.4 Hydronamic injection of mFL and hFL plasmid DNA: Plasmid DNA was rapidly injected IV to induce transient transfection of tissue cells. Specifically, the volume of DNA solution is based on 0.08-0.12ml/ gram of body weight for a mouse (i.e. 1.6 mls for an 18-20 g mouse) containing 5-20 μg of plasmid injected rapidly over 5-6 seconds. Both 10 and 20 μg of mFL and hFL plasmid DNA were injected over different IV injection times and relative expansion of DC populations was evaluated.

2.16 Estriol DC Protocol and Experiments

Age-matched 6-8 week old female C57BL/6 (H-2b) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed 4-5/cage. Mice were anesthetized and 60-day release estriol (E3) or placebo (Pb) pellets were implanted subcutaneously over the shoulder blades. Mice were allowed to recover for at least 4 days and hFL was administered as described above over 9 days to expand DCs in vivo. Splenic DCs were
purified (positive selection of CD11c+ DCs using magnetic bead separation) and pulsed with MOG35-55 for 2 hours. Cells were washed and then administered IV to naïve recipients (range of 1-2x10^6 to 8-10x10^6 DCs). Subsets of washed cells were used for in vitro phenotypic and functional evaluation of purified CD11c+ cells (flow cytometry, ELISPOT and RT-PCR) and CD11c+:CD4+ co-cultures (proliferation assays, ELISA, ELISPOT and flow cytometry). Maturation of ex vivo DCs was accomplished by culturing SPL DCs for 18-24 with maturation stimuli; 5 μg/ml TNF-α (Peprotech, Inc. Rocky Hill, NJ) or a combination of 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) and 5 μg/ml TNF-α. One day after IV injection of DCs, recipients were then immunized with MOG35-55 with adjuvants and monitored for the development of clinical disease or sacrificed at days 10-12 for evaluation of cell proliferation, cytokine production and expansion of regulatory T cells (CD4+CD25+foxp3+) cells.

2.17 Statistical analyses

The results pertaining to clinical scores and day of disease onset were analyzed using the Student’s t-test, the Wilcoxon Rank Sum test or the Kruskal-Wallis nonparametric test with multiple comparison of mean. Mortality and incidence of disease was compared using the Chi-square or Fischer’s exact test. Kruskall-Wallis test was used to determine whether there was a significant overall group effect for the four groups (using alpha value of 0.008). Significant group effects were further compared using the Wilcoxon Ranks sum test. Proliferation, CBA, ELISPOT, ELISA and EIA data were statistically analyzed using a non-parametric ANOVA with Tukey’s post-hoc test to
determine differences between groups or a Student’s T test. Groups were considered significantly different at p<0.05.
3.1 Sex differences in EAE in the SJL mouse and other mouse strains

Sex differences in disease course of EAE were first reported by Keith (1978). Female Lewis rats immunized with whole nervous tissue exhibited clinical relapses compared to a monophasic disease course observed for male rats. These results were explained by hormones of the estrus cycle or adrenal glucocorticoid secretion (189). More recently, sex differences in EAE have been studied in mice, particularly in the SJL strain. The female SJL shows an increased incidence and greater severity of adoptively transferred EAE than does the male SJL (65, 190-192). In addition, SJL females show multiple relapses of EAE while males do not. This sexual dimorphism is largely attributed to sex hormones and their influence on the development of Th1 or Th2 responses (Whitacre 1999). While the SJL has been the predominant strain utilized in studies of sex differences in EAE, comparative responses to neuroantigen immunization between the sexes in other strains are less well studied. The purpose of this study was to evaluate whether hormones influenced the induction and disease course of EAE in different inbred mouse strains utilized in EAE research.
We chose seven different mouse strains based on their known susceptibility to EAE (based on MHC Class II haplotype) and use in other autoimmune disease models. The SJL strain was evaluated for sex differences in EAE using 3 different peptide sequences for immunization (PLPs 139-151 + CFA + PT; PLPc 139-151 + CFA and MOG 92-106 + CFA + PT). PLPs contains serine at position 140, is the sequence found within native PLP and the most commonly used PLP peptide for EAE immunization while PLPc contains cysteine at position 140 and allows for immunization without PT (98, 193). Table 3.1 and Figure 3.1 show that with all three immunization regimens, female SJLs demonstrate increased clinical scores during both the acute disease period as well as the chronic relapsing phase. There was no mortality from EAE observed in this strain (Table 3.1). Female SJL mice showed increased disease regardless of the presence of PT in the immunization mixture (Figures 3.1A and 3.1B). The pattern of clinical disease was also noteworthy as it differed between the sexes even when different neuroantigens were used for immunization. Female SJLs immunized with PLPs had increased acute disease (days 10-13) (Figure 3.1A), while female SJLs immunized with PLPc showed markedly increased disease at later time points (days 22-42) (Figure 3.1B) and increased cumulative disease scores (Table 3.1). Males immunized with either of the PLP peptides showed no relapses as previously reported by Bebo et al. (191). Immunization of SJL mice with MOG92-106 induced acute and relapsing EAE in both sexes, with progressive worsening and more severe clinical scores in the females (Figure 3.1C). This data demonstrates that the female SJLs show an increased incidence of EAE and severity of clinical signs compared to males, using myelin-associated
immunodominant peptides derived from two different neuroantigens (i.e. PLP and MOG).

We tested other murine strains to determine whether there was a sex difference in disease incidence or severity. Only the B10.PL strain showed greater mortality in males with no difference in mortality seen in other strains. The ASW and NZW/LACJ strains demonstrated increased disease in females compared to males (Figures 3.2A, 3.3A) with increased incidence in female NZW/LACJ mice and increased mean clinical score in female ASW mice (Table 3.1). The C57BL/6 and NOD showed no significant differences between males and females (Figure 3.2B, 3.2D) while the PL/J immunized with MOG92-106 showed higher mean clinical scores for males (Figure 3.2C, Table 3.1). It is interesting to note that although there is no statistical difference in overall clinical disease in male versus female C57BL/6 mice, a periodicity is observed in the clinical scores of females that may reflect hormonal fluctuation in the murine estrus cycle (Figure 3.2B). Immunization of the NZW/LACJ strain with MBP revealed a striking difference in disease incidence (25% for males, 85% for females; significant at p<0.05). However, disease severity differed between the sexes. Those males who did show signs of disease had more severe disease. Figure 3.3 demonstrates the varying disease patterns when all NZW males and females are included in the score calculation (Figure 3.3A) versus only those showing disease (Figure 3.3B).
3.2 Sex Differences in EAE in the B10.PL Mouse

Male B10.PL mice immunized with MBP show significantly increased disease during the acute phase of EAE compared to female B10.PL mice, with no differences observed during the relapsing phase (Figure 3.4). Specifically, males have a slightly earlier onset and significantly increased disease at days 5-15 post immunization compared to females (Wilcoxon p<0.05). Additionally, the increased severity in males is accompanied by increased mortality ($X^2$; p<0.05) and an increased cumulative disease score both between days 0-30 (acute EAE) and days 0-50 (overall EAE disease course) (Table 3.2). A neutrophilic meningomyelitis was seen in both males and females but males had an overall increased inflammation in both the brain and spinal cord and an increase in the number of neutrophils within the spinal cord compared to females (Figure 3.5). Interestingly, males often had an earlier onset of inflammation in addition to the increased severity (Figure 3.6A) compared to females (Figure 3.6B) and, as expected, inflammation was limited to the CNS (Figure 3.7).

Since increased disease in the B10.PL male represented a novel observation, we examined sex differences in the immune response in the B10.PL strain. Lymph node cells from male B10.PL mice in response to immunizing antigen (MBP) and an increase in the production of Th1 cytokines compared to females (Figure 3.8). IFN-$\gamma$, IL-2 and IL-4 responses were observed to be significantly increased in males relative to females, whether stimulated with MBP or the immunodominant peptide NAc1-11. Females are reported to exhibit a more robust Th1 response but these findings have only been validated in the SJL mouse model of EAE. However, in the B10.PL strain, there may be
an overall increased responsiveness in male immune cells since males have increased production of IL-4 irrespective of the presence of neuroantigen (Figure 3.8D). These findings suggest that male B10.PL mice may be inherently hyperresponsive to neuroantigen and, thus predisposed to developing severe EAE following immunization. Therefore, the B10.PL strain may represent a model for men with primary progressive MS, since both severity and mortality are increased in these men compared to women with a relapsing remitting course of MS.

3.3 Female sex hormones protect B10.PL mice from acute EAE

In order to determine whether the sex differences observed in B10.PL mice was hormone-mediated, we performed gonadectomy experiments in both males and females. Gonadectomized males, gonadectomized females and intact males all demonstrated similar disease patterns with increased clinical signs during the acute disease period of EAE relative to intact females (Figure 3.9). In other words, removal of the gonads of females converted their disease pattern to that of the male. No differences were observed in animals whose gonads had been removed (i.e. castrated males versus ovariectomized females). Kruskall-Wallis test was used to determine whether there was a significant overall group effect for the four groups. Significant group effects were seen on days 5, 8-12 and 20-24 which were further compared using the Wilcoxon Ranks sum test. Castrated males were different from intact males at days 8 and 9, from females at day 23 ovariectomized females different from males on day 8 and males and females differed on day 8 and 12. (p<0.008). Thus, these results suggest that the increased acute disease in
male B10.PLs is hormone responsive and that the presence of ovarian-derived sex hormones in the female serves a protective effect (Figure 3.9).

3.4 Summary: Hormonal environment influences induction of EAE.

The data presented in this chapter support the concept that sex hormones play an important role in influencing the clinical course of EAE. In addition to confirming the reported sexual dimorphism in SJL mice, the results show a sexual dimorphism in B10.PL, another widely used strain in EAE research. Male B10.PL mice demonstrate an increased severity, mortality and histopathologic lesions limited to acute EAE, suggesting either that male B10.PLs are predisposed to increased severity or that female sex hormones in B10.PL mice are protective. Gonadectomy experiments would suggest the female sex hormones are playing a protective role. Specifically, compared to all other groups (gonadectomized males and females and intact males), only intact female mice showed protection against acute EAE (Figure 3.9). However, genetic strain is an obvious important factor in determining EAE susceptibility and male B10.PL may have an increased susceptibility to developing EAE. Our results show that B10.PL mice with EAE have an increased Th1 response and T cell proliferation compared to females and that female hormones are protective in B10.PL. This is in sharp contrast to SJL mice where females have increased EAE, a Th1 response and male hormones are protective. Thus, while genetic strain may influence the relative susceptibility of a given mouse strain, sex hormones have a further modulating effect. Our data in both Chapter 3 and a pregnancy model (McClain, manuscript accepted, pending revisions) and reports from the
literature would suggest that sex hormones or the hormonal environment present at the time of disease initiation is important in determining disease outcome.

Since the induction of an immune response depends on a complex interaction between DCs and naïve helper T cells and hormones appear to be influencing the induction of EAE, the remainder of this thesis explores how DCs can be modulated by hormones to then influence the Th1/Th2 balance and the development of the autoimmune disease EAE.
Table 3.1  EAE Sex Differences in Multiple Murine Strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Neuro-antigen</th>
<th>n</th>
<th>Incidence (%)</th>
<th>Mortality (%)</th>
<th>Day of onset</th>
<th>Mean clinical score</th>
<th>Mean cumulative disease score</th>
<th>Mean high score</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJL</td>
<td>M</td>
<td>PLPs</td>
<td>10</td>
<td>30*</td>
<td>0</td>
<td>12.3 ± 1.2</td>
<td>0.0 ± 0.0*</td>
<td>7.2 ± 1.7</td>
<td>0.7 ± 0.45</td>
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<tr>
<td>F</td>
<td>10</td>
<td>70*</td>
<td>0</td>
<td>9.9 ± 0.4</td>
<td>0.3 ± 0.0*</td>
<td>19.3 ± 7.52</td>
<td>1.9 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJL</td>
<td>M</td>
<td>PLPc</td>
<td>15</td>
<td>33</td>
<td>0</td>
<td>14.2 ± 1.0</td>
<td>0.1 ± 0.0*</td>
<td>8.8 ± 4.2*</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>F</td>
<td>15</td>
<td>47</td>
<td>0</td>
<td>18.0 ± 2.5</td>
<td>0.5 ± 0.0*</td>
<td>35.3 ± 9.7*</td>
<td>1.8 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJL</td>
<td>M</td>
<td>MOG92-106</td>
<td>10</td>
<td>80</td>
<td>0</td>
<td>21.5 ± 4.7*</td>
<td>0.6 ± 0.0*</td>
<td>33.4 ± 13.0</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>100</td>
<td>0</td>
<td>12.6 ± 1.9*</td>
<td>1.2 ± 0.1*</td>
<td>59.4 ± 4.8</td>
<td>3.5 ± 0.4</td>
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</tr>
<tr>
<td>B10.PL</td>
<td>M</td>
<td>MBP</td>
<td>50</td>
<td>100</td>
<td>24*</td>
<td>6.8 ± 0.2</td>
<td>1.6 ± 0.0</td>
<td>64.0 ± 2.7</td>
<td>3.4 ± 0.2</td>
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<tr>
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<td>100</td>
<td>4*</td>
<td>7.7 ± 0.3</td>
<td>1.6 ± 0.0</td>
<td>59.4 ± 4.0</td>
<td>3.0 ± 0.2</td>
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<tr>
<td>ASW</td>
<td>M</td>
<td>MOG92-106</td>
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<td>80</td>
<td>20</td>
<td>22.3 ± 2.4</td>
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<td>44.5 ± 6.5</td>
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<tr>
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<td>MOG92-106</td>
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<td>100</td>
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<td>1.4 ± 0.3</td>
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<tr>
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<td>MOG35-55</td>
<td>12</td>
<td>100</td>
<td>8</td>
<td>14.8 ± 0.8</td>
<td>1.3 ± 0.0</td>
<td>96.0 ± 5.0</td>
<td>2.4 ± 0.5</td>
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<tr>
<td>F</td>
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<td>100</td>
<td>33</td>
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<td>1.4 ± 0.1</td>
<td>95.5 ± 10.1</td>
<td>3.8 ± 0.3</td>
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<td></td>
</tr>
<tr>
<td>NZW</td>
<td>M</td>
<td>MBP</td>
<td>8</td>
<td>25*</td>
<td>13</td>
<td>20.5 ± 1.3</td>
<td>0.3 ± 0.2</td>
<td>6.6 ± 3.3</td>
<td>1.1 ± 1.3</td>
</tr>
<tr>
<td>F</td>
<td>7</td>
<td>85*</td>
<td>0</td>
<td>18.7 ± 1.1</td>
<td>1.2 ± 0.4</td>
<td>28.3 ± 4.7</td>
<td>2.6 ± 0.7</td>
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</tr>
<tr>
<td>NOD</td>
<td>M</td>
<td>MOG35-55</td>
<td>15</td>
<td>87</td>
<td>0</td>
<td>18.7 ± 2.8</td>
<td>0.5 ± 0.1</td>
<td>21.9 ± 5.1</td>
<td>2.4 ± 0.4</td>
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<tr>
<td>F</td>
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<td>93</td>
<td>0</td>
<td>15.2 ± 1.4</td>
<td>0.7 ± 0.1</td>
<td>32.2 ± 5.9</td>
<td>2.8 ± 0.3</td>
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</tbody>
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* Statistically significant difference between males and females (p<0.05).
Figure 3.1. Female SJL have increased severity and greater incidence of EAE compared to males regardless of immunizing neuroantigen. Male and female SJL mice were with immunized with A. PLPs139-151 (HSLGKWLGHPDKF), B. PLPc139-151 (HCLGKWLGHPDKF) and C. MOG92-106 (DEGGYTCFFRDHSYQ).
Figure 3.2: Minimal sex differences in clinical scores of immunized ASW, C57BL/6, PL/J and NOD strains. Male and female ASW, C57BL/6, PL/J and NOD strains were immunized with commonly used neuroantigen (plus adjuvants) for each representative strain. A. ASW with MOG92-106, B. C57BL/6 with MOG35-55 (MEVGWYRSPFSRVVHLRYRGK), C. PL/J with MBP and D. NOD with MOG35-55.
Figure 3.3: Disease scores are influenced by incidence. Apparent increased disease in females when mean clinical score was plotted for all (i.e. clinically affected and unaffected) mice, but increased disease in males when only the clinically affected mice were plotted. Male and female NZW/LACJ were immunized with guinea pig MBP + PT and clinical scores were calculated for A. all immunized animals and B. only animals manifesting clinical disease. Incidence was 25% and 85% for males and females, respectively.
Figure 3.4: Male B10.PL mice have earlier onset and increased disease in early EAE compared to females. Male and female B10.PL mice were immunized with MBP (plus adjuvants) and clinical scores ± S.E.M. were calculated. * indicates significant differences between males and females (p<0.05).

Table 3.2: Morbidity, mortality and cumulative disease score of male and female B10.PL mice. Male and female B10.PL mice were immunized with MBP and monitored for the development of EAE. Mice that died (score of 5) or were humanely euthanized with a score of 4.5 were included in the mortality calculations. Male B10.PL mice demonstrated a significantly increased cumulative mortality over the course of EAE compared to females (p<0.05 by Chi-squared test) following immunization with MBP. The majority of death occurred within the first 15-18 days following immunization. Data represent summarized results from 5 separate experiments. In all individual experiments, increased mortality was seen in male mice and male mice demonstrated increased CDS in both the acute phase of EAE or over the entire duration of EAE. Values are statistically different at $^A$p<0.05, $^B$p<0.01, $^C$p<0.001.
Figure 3.5 Increased neuroinflammation in male B10.PL mice. Spinal columns and brains were removed from male and female B10.PL mice at day 9-15 after MBP immunization were evaluated for degree of inflammation and number of neutrophils. Sections were scored for degree of inflammation on a scale of 0 (no inflammation) to 3 (widespread and diffuse parenchymal infiltration) and number of neutrophils on a scale of 0 (no neutrophils) to 3 (predominance in many lesions). Males had statistically significant increased CNS inflammation in both the A. brain and B. spinal cord (p<0.05) compared to females with the neutrophil component increased within the brain of male mice. Inflammation was characterized as neutrophilic meningomyelitis with males demonstrating an increased neutrophil component within brain lesions compared to females. Values are statistically different at * = p<0.05.
Figure 3.6. Histopathology of male and female B10.PL mice. Histological (H&E) staining of thoraco-lumbar junction of unaffected female (A) and affected male (B) B10.PL mice immunized with MBP (plus adjuvant). Inflammatory cell infiltrates (neutrophils and mononuclear cells) are prominent in ventral spinal cord.

Figure 3.7. Inflammatory infiltrate is limited to the central nervous system (CNS). Peripheral nerve roots are unaffected. Shown is hematoxalin and eosin (H&E) staining of distal spinal cord (cauda equina). Infiltrate consists predominantly of neutrophils with some mononuclear cells and disruption of CNS white matter (insert).
Figure 3.8. Male B10.PL lymph node cells (LNC) proliferate more and secrete increased cytokines. Male and female B10.PL mice were immunization with MBP. LNC were removed 10 days later and evaluated for proliferation and cell-specific cytokine secretion. A. Male LNC proliferated more robustly to MBP than female LNC. Cell specific cytokine secretion (ELISPOT) for Th1 and Th2 cytokines were evaluated and male mice produced increased Th1 cytokines B. IFN-γ and C. IL-2 compared to females in response to both immunizing (MBP) antigen and immunodominant epitope Ac1-11. D. Male SPL also had an increased ability to produce the Th2 cytokine IL-4 irrespective of the presence of neuroantigen, suggesting an overall increased responsiveness in male SPL immune cells. Values are statistically different from corresponding media controls at * p<0.05.
Figure 3.9: Female hormones protect against more severe acute EAE. Removal of the ovaries converts a female B10.PL disease pattern to the male pattern while removal of the testes had no effect. Ovariectomized female, orchiectomized male and sham-surgery treated male and female B10.PL mice were immunized with MBP + adjuvants and clinical score ± S.E.M. were calculated. Significant group effects (*) were seen on days 5, 8-12 and 20-24 (p < 0.008).
4.1 Background and biology of dendritic cells (DCs)

DCs are unique among antigen presenting cells (APCs) in their ability to prime naïve T lymphocytes. By bridging innate and adaptive immunity, DCs are positioned to influence the character of the immune response and impact inflammatory and immune-mediated diseases such as EAE. Importantly, DC-based immunotherapy has shown promise in the treatment of transplantation, allergic and autoimmune diseases and the ability to influence DCs has direct implications in numerous aspects of T cell function (162, 194-196). One primary limitation in studying DCs is the fact that they represent a trace population in vivo and are difficult to isolate in large numbers. One approach has been to expand resident DCs in vivo with the growth and differentiation factor Flt3-ligand (FL) while a second approach is to expand bone marrow precursors with growth factors (primarily GM-CSF) in vitro to drive differentiation of BM-DCs. While human recombinant FL (hFL) has been used to expand DCs in vivo for nearly 10 years, the quantity of hFL required for DC expansion limits the usefulness of this method. Thus, this chapter will focus on alternative methods to expand DCs in vivo and in vitro. The remainder of this chapter will compare these newly developed methodologies to generate
DCs and evaluate the biological interactions of these DCs with T cells both in vitro and in vivo.

FL is a growth factor that binds to receptor tyrosine kinase fms-like tyrosine kinase 3 (flt, flk-2 or STK-1), highly expressed on hematopoietic progenitor cells. Ligation of FL with its receptor results in expansion of DCs in vivo in mice, humans and macaques (197-205). FL has been shown to expand myeloid, lymphoid and plasmacytoid DC populations and is the primary method to expand DCs in vivo (198, 206-208).

Techniques for FL administration to expand DCs in vivo include daily subcutaneous injections of human or murine recombinant FL, FL-secreting tumors, FL-expressing adenoviruses and, most recently, hydrodynamic-based gene delivery of FL plasmid DNA (188, 199, 201, 209-213). The most widely used and well-characterized method for in vivo DC expansion is daily injection of hFL into mice (199). However, this method has limitations requiring: requires large quantities of hFL for in vivo DC expansion, nine day injection regime and administration of xenogeneic compound in a mouse model system. Sections 4.1 and 4.2 demonstrate alternative methods to expand DCs in vivo that would address these limitations.

Initial studies were undertaken to develop methods using mFL, the mouse homologue of hFL, that would more accurately represent biologically relevant expanded DCs. The reason for utilizing mFL stems from the fact that there are differences reported between hFL and mFL sequences, form and function that influence resultant DC populations. While mFL and hFL both share an overall amino acid sequence homology of 72%, there are differences in the cytoplasmic domains, biologically active isoforms
and overall potency (197, 211, 214-216). Additionally, mFL expanded DCs have increased B220 expression (seen on regulatory plasmacytoid DCs) and decreased production of IL-6, IL-10, IFN-γ and TNF-α (217). Such differences in DC populations can result in profoundly divergent biological effects that may have implications in the development of DC-based immunotherapy. For these reasons, initial studies were employed to evaluate the efficacy of utilizing mFL continuously delivered by a tumor-based system (tFL) compared to hFL expanded DCs.

4.2 Tumor-derived FL (tFL) DC expansion and comparison to hFL expanded DCs

An alternative method for in vivo expansion of DCs in the use of transfected B16 melanoma lines that either overexpress FL or GM-CSF(188). FL-secreting B16 cells which are poorly immunogenic, elicit comparable effects on hematopoietic populations as injections of recombinant FL (199, 201).

Using a melanoma line overexpressing mFL, we found that mice given 1-2 million melanoma cells had a detectable expansion of splenic DCs within two weeks post-injection. Expansion of DCs in the spleen continued to increased to 50% CD11c+ (approximately 241x10^6) DCs by the end of the experiment at week 4 (Figure 4.1). In contrast, expansion of DCs in lymph nodes (LN) plateaued with the number of CD11c+ cells by flow cytometry averaging 16% (Figure 4.1). Interestingly, LN of mice with large tumors (weeks 3-4) occasionally had small black foci primarily affecting the LN. These foci were presumed to be metasases that limited the use of LN at later dates following
injection. However, no metastases were observed grossly in the spleen, lungs or other organs and splenic scatter plots were similar to control mice (data not shown).

These findings demonstrate that a melanoma based delivery system of mFL is a viable method to generate large numbers of expanded DCs in peripheral lymphoid organs in mice and is particularly useful at generating large numbers of splenic DCs. The next series of experiments compared DCs generated with the continuous mFL tumor-based delivery system to daily injections of recombinant hFL.

Human FL has been used to expand DCs in vivo in a variety of studies, but mFL expanded DCs have been less thoroughly explored (199-205, 207, 209, 210, 212, 218-220). Experiments were performed to compare mFL and hFL expanded DCs. SPL and LN from mice treated with tumor-derived mFL, daily hFL injections or a vehicle control (0.1% mouse serum albumin in PBS) were obtained and evaluated by flow cytometry. Mice receiving mFL or hFL showed an expanded number of CD11c+ cells within the spleen compared to vehicle-treated controls (Figure 4.2A). Similar findings were seen in the lymph nodes (data not shown). Interestingly, the phenotype of mFL and hFL DC populations differed. HFL-expanded DCs showed a significant decrease in expression of CD11b, CD80 and CD86 compared to control mice, while the phenotype of mFL DCs did not differ from controls (Figure 4.2B). These data suggest that although hFL and mFL similarly expand overall DC numbers, the resultant DCs differ in phenotype and activation status. Specifically, mFL DCs more closely resemble the phenotype of normal (non-expanded) splenic DCs. Taken together, these data demonstrate that mFL is equally
capable of expanding DCs \textit{in vivo} as hFL and results in an expanded resting (immature) DC population that more closely resembles normal splenic DCs (vehicle control).

Although the tumor-based delivery of mFL consistently expanded DCs and appears to be a viable method to generate mFL-expanded DCs, there are obvious limitations to a tumor-based delivery system. Specifically, there was variability in DC phenotype and function depending on relative tumor burden and time post-injection of tumor, inconsistent growth of tumors in recipient mice, and other tumor-related side effects on the immune system and general mouse physiology that are difficult to quantitate. For all of these reasons, other methodologies to expand DCs \textit{in vivo} were investigated.

\subsection*{4.3 Hydrodynamic injection of hFlt3L and mFlt3L to expand DCs}

Hydrodynamic based transfection with a DNA plasmid containing hFL has successfully been used to expand DCs \textit{in vivo} (Song YK 2002, Liu 1999, He 2000). This method involves rapid intravenous (IV) injection of large volumes containing plasmid DNA that exceeds cardiac output, resulting in accumulation of DNA in the inferior vena cava and higher pressure within the venous circulation. This results in a reflux of DNA solution into the liver and kidneys and the transient increased hydrostatic pressure in these organs allows DNA to enter cells (i.e. transient transfection). The high volume and rapid infusion rate is key to successful transfection of cells. Specifically, the volume of DNA solution is based on 0.08-0.12ml/ gram of body weight for a mouse (i.e. 1.6 mls for an 18-20 g mouse) containing 5-20 ug of plasmid injected rapidly over 5-6 seconds.
Such rapid injection of large volumes results in plasmid DNA distribution throughout the body and plasmid quantities greater than 5 μg reported to reach saturation levels (221, 222). Studies to explore the use of mFL plasmid began by evaluating both 10 and 20 μg of mFL plasmid DNA injected IV using different injection times.

Results demonstrate that hydrodynamic injection of either 10 μg or 20 μg mFL plasmid into mice resulted in expansion of splenic CD11c+MHCII+ DCs (14.8% and 11.3%, respectively) compared to control mice not receiving plasmid (3.1%) (Figure 4.3). Interestingly, increased plasmid amount (i.e. 20 μg versus 10 μg plasmid) did not result in an increased expansion of splenic DCs. In contrast to the minimal effects of increasing plasmid concentration, the duration of the plasmid injection was critical. IV injection exceeding 7 seconds resulted in a dramatic decrease of mFL expanded DCs. Specifically, 20 μg of plasmid injected within 7 seconds yielded 11.3% CD11c+ cells while 20 μg of plasmid injected in more than 7 seconds yielded 6.6% CD11c+ cells (data not shown). Thus, plasmid concentration was less important than rapid IV injection of the plasmid. These studies are the first to utilize mFL plasmid injection to expand DCs in vivo in mice.

Although mFL introduced into mice represents a homologous system, the fact that the vast majority of studies evaluating in vivo expanded DCs utilized hFL necessitated the investigation of hFL plasmid injection. Results demonstrated that hFL is similarly capable of expanding DCs after a single hFL injection and yields 6.9 ± 3.4 % CD11c+ DCs in the LN and 19.5 ± 9.8 % CD11c+ DCs in the SPL. Interestingly, injection duration appears to be most important in LN with hFL and has a minimal but variable effect on the expansion of CD11c+ cells in the SPL (Table 4.1). Interestingly, hFL
plasmid appears to more effectively expand SPL DCs with relative yields ranging from 12-44% (as shown in Table 4.1) compared to mFL expanded SPL DCs ranging from 11-18% (Figure 4.3 and data not shown). The reason for this is unknown but may be due to species-specific differences resulting in additional immune stimulation with hFL plasmid injection. Reports indicate that yield may be further increased by administering a second plasmid injection 5-7 days after the first injection. Data in Figure 4.3 demonstrate that a second hydrodynamic injection of plasmid hFL DNA results in a further expansion of DCs compared to a single injection alone, with the most dramatic expansion seen in the spleen. Mice injected with a second injection of plasmid 5-7 days after the initial injection had 9.5 ± 5.5% CD11c+ DCs in the LN and 43.9 ± 25.4 % CD11c+ DCs (compared to 6.9 ± 3.4 % CD11c+ DCs in the LN and 19.5 ± 9.8 % CD11c+ DCs in the SPL with a single injection).

4.4 In vitro DC expansion methods

An alternative to in vivo expansion of existing DC populations is the generation of DCs from bone marrow precursor populations. Bone marrow cells have the ability to differentiate into a variety of cell populations depending upon the environmental milieu of cytokines and growth factors during differentiation. While the generation of bone-marrow derived macrophages using L929 fibroblast supernatant containing M-CSF has been employed for over 20 years, the ability to reliably generate large numbers of DCs from bone marrow precursors occurred relatively recently (223, 224).
The majority of studies utilize both GM-CSF+IL-4 and recent reports have shown that GM-CSF alone or other growth factors such as FL can be utilized to generate BM-DCs (199, 207, 220, 223, 225, 226). GM-CSF (with or without IL-4) is an extensively used method to generate in vitro DCs (223, 224). While GM-CSF+IL-4 is the most commonly used method to generate BM-DC, it has been shown that resultant BM-DC populations differ in form and function depending upon the method of generation. GM-CSF restricts differentiation toward a myelomonocytic lineage and the addition of IL-4 helps to inhibit the development of macrophages (227). The addition of IL-4 is also thought to enrich DC populations, maintain DCs in a more immature state, capable of antigen capture and processing. IL-4 is thought to also influence important functions of DCs such as activation, migration and the ability of DCs to induce a Th2 response (228-232). GM-CSF derived DCs are predominantly of the myeloid (CD8α-) phenotype with the addition of IL-7 necessary to produce thymic or lymphoid (CD8α+) DCs (233).

Although GM-CSF is commonly used to generate BM-DCs in vitro, it is thought that GM-CSF is not essential for DC development in vivo since DCs develop independently in GM-CSF-/- mice and mice over-expressing GM-CSF (234). Additionally, GM-CSF appears to play a more important role in the expansion of myeloid DCs since transgenic mice overexpressing GM-CSF fail to increase the numbers of DCs in peripheral lymphoid organs and systemic administration of GM-CSF produces only minimal effects on splenic DC populations (199, 235). Taken together, a current hypothesis is that the primary role of GM-CSF in acquired immunity may lie at the level of DC activation, survival and/or migration to lymphoid tissues, rather than DC
development (236). Data demonstrating that the maturation state of GM-CSF+IL-4 differentiated DCs influences helper T cell differentiation and can influence the clinical course of EAE supports the concept that DC maturation status plays an important role in GM-CSF DC function. However, GM-CSF BM-DCs are not known to have subpopulations such as plasmacytoid DCs with potential regulatory function compared to other BM-DC culture methods.

Recently, it has been reported that bone marrow precursors cultured in the presence of recombinant hFL and mFL produce myeloid (CD8α-), lymphoid (CD8α+) and plasmacytoid (CD11c+B220+Gr-1+CD45RB+Ly6c+CD11b+) DCs and that hFL BM-DCs have demonstrated splenic DC counterparts (207, 237, 238). However, the studies evaluating the effects of hFL and mFL on BM-DC differentiation and subsequent biological effect of these populations are limited. For all of these reasons, alternatives to GM-CSF in the generation of BM-DCs were explored.

4.5 Development, characterization and comparison of mFL BM-DC to other BM-DC derivation methods.

Limited reports describe that FL (hFL and mFL) can be used to generate BM-DCs that are potentially more regulatory than GM-CSF-derived BM-DCs (198, 206-208, 237). We were interested in adapting the mFL utilized for in vivo expansion to an in vitro system. We observed that over 95% CD11c+ cells were generated from bone marrow precursor cells after 14 days of culture with mFL-containing supernatant (Figure 4.5A), with a dramatic shift in CD11c expression as early as day 11 of culture (Figure 4.5B). A
comparison of phenotypic properties showed that, similar to hFL derived BM-DCs, mFL BM-DCs were smaller, more round and demonstrated fewer dendrites compared to GM-CSF BM-DCs (data not shown). Interestingly, CD11c+ expression differed between mFL, hFL and GM-CSF DCs, with the lowest level of expression in mFL and the highest in GM-CSF treated cells, suggesting that BM-DCs expanded in the presence of mFL are relatively less mature (Figure 4.6A). Comparison of phenotypic markers between these three methods of BM-DC generation revealed many phenotypic similarities between mFL and hFL compared to GM-CSF BM-DCs. Specifically, levels of CD11b, MHC II and CD86 expression were quite similar between mFL and hFL DCs, showing markedly less expression of CD11b and greater expression of MHC II than GM-CSF treated cells. Interestingly, expression of B220 and CD8α was markedly decreased in mFL BM-DCs relative to GM-CSF BM-DCs and higher on a per cell basis compared with hFL BM-DCS (Figure 4.6B). CD80 expression in mFL BM-DCs was intermediate between hFL and GM-CSF DCs. Based on CD11c and costimulatory molecule expression, it appears that mFL BM-DCs may lie between hFL-derived BM-DC and a more mature or “inflammatory” GM-CSF-derived BM-DC. Importantly, fundamental differences in the BM-DC populations generated with mFL versus hFL (based on B220 and CD8α expression) indicate that mFL and hFL may differ in their ability to generate regulatory DC populations. Based on these findings, mFL BM-DCs may represent an appropriate in vitro correlate to in vivo mFL expanded DCs described in Section 4.1.
4.6 Summary of *in vivo* and *in vitro* derivation methods.

The ability to expand DC populations is necessary in order to evaluate DC function; the methodologies developed in this chapter allowed the reliable expansion of DCs both *in vivo* and *in vitro*. Specifically, these results characterized *in vivo* expansion methods (daily hFL injection, melanoma-based mFL delivery and hydrodynamic injection of both mFL and hFL plasmid DNA) and a newly developed *in vitro* method to generate mFL-derived BM-DCs.

Our results show that a melanoma-based mFL expression system generated up to 50% CD11c+ cells within the spleen and 16% in the LN (Figure 4.1) and that the ability of mFL to expand DCs *in vivo* was similar to daily injections of hFL. Phenotypically, mFL splenic DCs were phenotypically more similar to resting (control) splenic DCs than hFL splenic DCs that had increased expression of CD11b, CD80 and CD86. We also explored the hydronamic injection of plasmid DNA for both hFL and mRL. Our results show that both mFL plasmid injection expanded CD11c+ DCs from 3.1% in the controls to 11.3-14.8% (20 μg versus 10 μg mFL plasmid) and that increased plasmid dose (20 μg) did not increase DC yield. Evaluations of injection duration indicated that injections < 7 seconds resulted in the highest DC expansion within the lymph nodes with a injection duration being less important in splenic DC expansion. A second injection of plasmid DNA (hFL) is capable of additionally expanding CD11c+ DCs from 6.9 ± 3.4 % CD11c+ DCs in the LN and 19.5 ± 9.8 % CD11c+ DCs in the SPL after a single injection to 9.5 ± 5.5% CD11c+ DCs and 43.9 ± 25.4 % CD11c+ DCs in the LN and SPL, respectively. These results demonstrate that a melanoma-based mFL expression system, daily injection
of hFL and plasmid injection of either mFL or hFL are all viable methods for *in vivo* DC expansion.

The ability to develop BM-DCs is also important in the investigation into DC biology and we successfully adapted an *in vitro* method to generate mFL BM-DCs. Using mFL-containing supernatant, we generated CD11c+ cells *in vitro* from bone marrow precursor cells and found that these mFL BM-DCs were more similar to hFL derived BM-DCs than GM-CSF BM-DCs. Phenotypic markers indicated that mFL BM-DCs were less mature than GM-CSF based on lower MHC II and CD86 expression. These results indicate that mFL BM-DCs were less mature than GM-CSF BM-DCs and we further explored their response to maturation stimuli and influence on the development in EAE in Chapter 5.
Figure 4.1. Expansion of DCs in vivo with mFL secreting melanoma. Both lymph nodes (LN) and splenic (SPL) DCs by two weeks post-injection of mFL-secreting melanoma cells. A. The percent of CD11c+ cells plateaued by week 3 for LN DCs but continued to increase in SPL DCs until the termination of the experiment at week 4. The total number of DCs obtained from LN was approximately $16 \times 10^6$ CD11c+ cells while there were over $240 \times 10^6$ CD11c+ cells in SPL by week 4.
Figure 4.2. *In vivo* expansion of splenic CD11c+ cells following mFL or hFL treatment or in vehicle controls (VEH). Phenotype of CD11c+ cells in SPL following *in vivo* expansion using continuous mFL administration or daily hFL injection. Single cell suspensions of splenocytes were gated on CD11c+ and levels of CD11b, CD80 and CD86 were evaluated. Results are expressed in percent positive cells +/- SE with * indicating p<0.05. hFL expanded DCs showed decreased expression of CD11b, CD80 and CD86 compared to age-matched controls. Data are representative of three experiments.
Figure 4.3: Flow cytometry of splenocytes from mice following hydrodynamic injection of either 10 μg or 20 μg mFL plasmid. Mice that received 10 μg and 20 μg mFL plasmid had a relative expansion of CD11c+MHCII+ DCs (14.8% and 11.3%, respectively) compared to control mice not receiving plasmid (3.1%), indicating that 10 μg plasmid is sufficient to result in DC expansion.
Table 4.1. Duration of hFL injection (in seconds) is important in the expansion of CD11c+ DCs in the LN but has a variable effect on the % CD11c+ DCs within the SPL. Injection duration < 7 seconds consistently expanding LN DCs versus injections > 7 seconds having decreased or variable % CD11c+ recovery. The duration of injection had variable effects on recovery or % CD11c+ cells.

<table>
<thead>
<tr>
<th>hFL Time injxn (s)</th>
<th>LN % CD11c+</th>
<th>SPL % CD11c+</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>8.15</td>
<td>41.98</td>
</tr>
<tr>
<td>6</td>
<td>7.09</td>
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<tr>
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<td>11.96</td>
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<td>2.6</td>
<td>36.12</td>
</tr>
</tbody>
</table>

Figure 4.4. Additional expansion of DCs in vivo with second injection of hFL plasmid DNA. Comparison of one versus two hydrodynamic injections of plasmid hFL DNA. Mice that received a single hFL plasmid DNA injection on day 0 had slightly increased percent CD11c+ cells compared to historical controls.
Figure 4.5. Development of *in vitro* BM-DC culture method using mFL-containing supernatant. (A) Kinetics of CD11c+ expression in bone marrow precursor cells following *in vitro* exposure to mFL-containing supernatant for 16 days. (B) BM-DC CD11c+ cells (green thick line) staining at day 11 of culture with mFL compared to isotype controls (black thin line). Data are representative of five individual experiments.
Figure 4.6. Phenotype of mFL, hFL and GM-CSF derived BM-DCs. BM-DC were derived from bone marrow precursors in the presence of either mFL-containing supernatant (thick black line), recombinant hFL (thin blue line) or GM-CSF (red dashed line). Cells were collected on day 11 of culture and compared by flow cytometry. MFL-derived BM-DCs appear intermediate between GM-CSF and hFL derived BM-DCs for CD11C expression (A), showing similarities with hFL for some markers (e.g. CD11b, MHC II) and differences relative to both GM-CSF and hFL (e.g. B220, CD8α) for other markers (B). Data shown are representative of three representative
DCs bridge the innate and adaptive immune response and play a critical role in the initiation and regulation of inflammatory processes. With their unique ability to prime naïve T lymphocytes, DCs play an important role in influencing the development of an adaptive immune response (113-115). Long thought of as predominantly pro-inflammatory, recent evidence demonstrates that DCs are critical in maintaining immunologic homeostasis and are capable of potent regulatory (or tolerogenic) functions (reg-DCs) (131, 151, 152). Investigating the mechanisms by which these reg-DCs can be generated and how they influence T cell development increases our understanding of DC-T cell interactions and our ability to harness these potent cells for application in the treatment of diseases.

There are numerous environmental factors that can influence DC function and impact their role in modulating the adaptive immune response. Two factors evaluated in this chapter are DC maturation status and exposure to pregnancy hormones. Using these two factors, both immunogenic and regulatory DCs were generated that influence the disease course of EAE.
5.1 Differentially matured DCs Influence EAE

Functional activities of DCs are dependent on their state of activation and differentiation (160). In essence, terminally differentiated mature DCs can efficiently induce T cell responses, while immature DCs are involved in the maintenance of peripheral tolerance. However, recent evidence indicates that even semi-mature DCs (matured in the presence of TNF-α) are capable of promoting antigen-specific tolerance in EAE (239). Thus, both immature and semi-mature DCs have been shown to demonstrate regulatory or tolerogenic properties in that they protect against the development of EAE (160, 239). Although not fully mature, these DCs are active and capable of protecting against the development of EAE.

The majority of work investigating immature DCs has utilized GM-CSF BM-DCs, with few studies investigating the maturation status and functional nature of FL DCs. Data presented in Chapter 4 suggests that FL generated DCs (both in vitro and in vivo) have significantly lower levels of maturation markers CD80 and CD86 compared to GM-CSF BM-DCs. These results suggest that mFL BM-DCs are immature and, similar to other immature DCs, may be capable of protecting mice from EAE.

In order to evaluate whether mFL BM-DCs were capable of maturation, we exposed these DCs to known maturation-inducing stimuli, TNF-α or a combination of TNF-α and LPS, for 24 hours. Exposure to either maturation stimulus resulted in increased expression of CD80, CD86, CD40, PD-L1 and ICOSL (Figure 5.1). Exposure to a combination of LPS and TNF-α resulted in additional increases in maturation markers CD80, CD86 and CD40. Based on phenotype, mFL BM-DCs are immature but
capable of maturation following exposure to either TNF-α or LPS+TNF-α, but the most profound maturation stimulus was a combination of LPS+ TNF-α.

In order to evaluate whether mFL BM-DCs behaved similarly to other reported immature DCs, the protective effects of immature and matured mFL BM-DCs were evaluated in vivo in EAE. Both immature and matured mFL BM-DCs (treated with TNF-α + LPS) administered to naïve mice prior to the induction of EAE were capable of influencing the development of disease (Figure 5.2). Specifically, mice receiving immature mFL BM-DCs had less severe disease while those receiving mature mFL BM-DCs had increased disease compared to control mice receiving no DCs. While transfer of immature BM-DCs appeared to have a trend towards protecting against EAE (compared to actively immunized mice receiving no DCs), this did not achieve statistical significance.

Mice receiving matured BM-DCs prior to immunization had significantly increased cumulative disease (26.79 ± 10.12) and peak disease scores (3.07 ± 1.16) compared to immature mFL BM-DCs (20.21 ± 7.64 and 1.86 ± 0.7, respectively). Interestingly, peptide alone appears to partially mature DCs, resulting in EAE severity similar to mice receiving no DCs, while TNF-α or LPS alone did not produce the marked increased severity in acute EAE as seen in LPS+TNF-α matured DCs (data not shown). Therefore, similar to other DC populations, the maturation status of mFL BM-DCs influenced the clinical outcome of EAE.

In summary, mFL DCs are capable of responding to maturation stimuli as assessed phenotypically (i.e. increased costimulatory molecule expression, Figure 5.1)
and functionally (i.e. further exacerbating EAE, Figure 5.2). Taken together, these results suggest that mFL may expand DCs that are immature and capable of regulating immune responses but still maintain the ability to become immunogenic when exposed to maturation stimuli. Thus, similar to immature DCs reported previously in the literature, mFL BM-DCs were capable of modulating the EAE disease course, simply based on their maturation status.

5.2 Estriol derived regulatory DCs

While the maturation status of DCs is considered an important aspect of DC functionality, additional environmental factors can influence DC function. Once thought to serve only in an immunostimulatory role, DCs have recently been shown to negatively regulate immune responses as well and are termed regulatory or tolerogenic DCs (reg-DCs). Potent immunoregulatory abilities of DCs have important implications in proinflammatory conditions such as autoimmune diseases. The generation and characterization of such reg-DCs is the focus of the remainder of this chapter.

Pregnancy and pregnancy levels of estrogens influence helper T cell responses. Recent evidence indicates that sex hormones such as estrogen may mediate some of their actions through DCs. However, the influence of these hormones on reg-DC generation and the downstream effects on subsequent helper T cell differentiation has not been evaluated in detail. Previous work has shown that a pregnancy environment at the time of disease induction, when DCs interact with naïve T cells, results in a delay in onset of EAE, decreased severity, complete resolution of clinical signs and no relapses (McClain,
manuscript provisionally accepted). These findings, along with results in Chapter 3 (Figure 3.9) support the hypothesis that the hormonal environment at the time of disease initiation influences the disease course. Since DCs are critical cells involved in the initiation of the immune response, we explored whether the hormonal environment of pregnancy or of a pregnancy-specific hormone estriol would influence DC phenotype and function.

5.2.1 DC phenotype in mice immunized during pregnancy

To first evaluate whether the hormonal environment altered DCs during the establishment of EAE, we measured DC number and phenotype in pregnant and non-pregnant immunized mice during the initiation (day 4 post-immunization) of the autoimmune response or following the establishment of a helper T cell response (day 14 post-immunization). Using mice that were either in late pregnancy or non-pregnant (controls) during immunization, splenic DCs were evaluated by flow cytometry. We found that the overall numbers of splenic CD11c+ DCs decreased from day 4 to 14 in both control (6.94% to 1.1%) and late pregnant (6.28% to 2.25%) immunized mice (data not shown). However, relative numbers were similar between controls and late pregnant immunized mice. Therefore, a selective depletion of DCs in pregnant versus control mice does not explain the difference in clinical disease.

Next, we evaluated whether DCs differed in phenotype between the two timepoints. We assessed gated CD11c+ cells for expression of stimulatory (maturation) markers (CD80 (B7-1), CD86 (B7-2) and CD40 as well as inhibitory (PD-L1, PD-L2, and
ICOSL) molecules. We hypothesized that mice immunized during late pregnancy would produce DCs that had a more regulatory phenotype of costimulatory molecule expression. Specifically, mice immunized during late pregnancy were expected to show increased inhibitory molecule expression (i.e. PD-L1, PD-L2 and ICOSL). We found elevated expression of the inhibitory molecule PD-L2 both on day 4 and day 14 post-immunization (Table 5.1A and B) and that this elevation continued to 14 days post-immunization, well into the post-partum period for late-pregnant immunized mice (Table 5.1B). Additionally, by day 14 post-immunization, PD-L1 expression was also significantly elevated over controls. Interestingly, we also found increases in CD80, CD86 and CD40, indicating that these DCs were mature. CD80, CD86 and CD40 expression were all elevated 4 days post-immunization while only CD80 continued to be elevated at day 14. Increased MHC II expression was also observed by day 14 post-immunization. Based on phenotype, these results suggested that DCs from a pregnancy environment were activated regulatory (i.e. increased CD80, CD86, MHC II, CD40 and increased PD-L1 and PD-L2, respectively) cells that may be responsible for the protection seen in late pregnancy immunized mice.

Although activation markers such as MHC class II, CD80, CD86 and CD40 in pregnant DCs were increased, we hypothesized that these more activated DCs in the pregnant mice differed in the character of their activation status and that these DCs were activated cells with regulatory function.
5.2.2 Estriol generates DCs with a distinct phenotype

In order to determine whether specific hormones of pregnancy were capable of generating a regulatory DC phenotype, we investigated the effects of the pregnancy-specific estrogen, estriol (E3), on DC phenotype. DCs from mice given either placebo (Pb) or 5 mg (pregnancy levels) E3 pellets prior to in vivo expansion of DCs with hFL were evaluated by flow cytometry for costimulatory marker expression (i.e. CD80, CD86, CD40, PD-L1, PD-L2, B7-H3 and B7-H4). Figure 5.3 demonstrates the phenotype of of DCs derived from either Pb or E3 pelleted mice. Consistently, all inhibitory markers PD-L1, PD-L2, B7H3 and B7H4 were up-regulated (Figure 5.3A) in E3 treated mice. As in late pregnant immunized mice, these DCs appeared phenotypically mature and activated based on elevated CD80 and CD86 expression (Figure 5.3B).

5.2.3 E3 DCs express decreased proinflammatory and increased regulatory mediators

To begin investigating whether these E3 DCs have potentially immunoregulatory function, proinflammatory and regulatory molecules were evaluated. Fewer purified E3 DCs produced IL-12 than Pb DCs both after culture with media and following MOG stimulation as shown by ELISPOT analysis (Figure 5.4A). Additionally, purified E3 DCs have less pro-inflammatory mRNA for IL-12, TNF-α and NF-κB compared to Pb DCs (Figure 5.4B). In contrast, mRNA for immunoregulatory mediators such as IL-10 and IDO are increased (Figure 5.4C) in purified E3 DCs, while TGF-β1 levels did not differ (data not shown). Thus, E3 DCs appear to have the potential to be immunoregulatory through either increased
inhibitory costimulatory molecules (Figure 5.3A), decreased expression of proinflammatory mediators (Figure 5.4A, B) or increased immunoregulatory mediators (Figure 5.4C).

To investigate whether E3 DCs were functionally different from Pb DCs, E3 or Pb DCs were co-cultured with purified CD4+ MOG Tg T(Th0) cells. Similar to E3 DCs in culture alone, E3 DCs co-cocultured with Th0 cells produced decreased IL-12p40 compared to Pb DCs, regardless of in vitro stimulus (MOG, LPS, conA or α-CD3) (Figure 5.5A). Additionally, E3 DCs were less efficient at presenting antigen to Th0 cells when cultured in the presence of medium, MOG or LPS (Figure 5.5B). However, Th0 cells were capable of responding to α-CD3 stimulation in the presence of either E3 or Pb DCs (Figure 5.5B). Therefore, E3 DCs differ from Pb DCs in both phenotype and function. Differences in costimulatory molecule expression, proinflammatory or immunoregulatory mediators all potentially influence initial interactions of DCs with Th0 cells.

5.2.4 E3 DCs protect mice from EAE

We next explored whether E3 DCs could modulate the disease course of EAE. Purified E3 and Pb DCs pulsed with neuroantigen in vitro were transferred IV into naïve mice. These recipient mice were then immunized with MOG35-55 together with adjuvants and monitored for the development of EAE. Approximately 8-10 million DCs from E3 treated mice were capable of protecting mice from EAE (Figure 5.6A). Importantly, these mice completely recover from EAE, a clinical pattern that has not been seen previously with MOG-induced EAE. There were significant differences in disease parameters with E3 DC recipients exhibiting having 1.3 ± 0.8 cumulative disease score (CDS) and 1.2 ± 0.7 peak
disease score compared to Pb DC recipients with 26.3 ± 15.2 and 3.2 ± 1.8, respectively. There were no differences in day of onset between these groups. Similar results were observed in two repeat experiments. Interestingly, this effect appears to be cell dose-dependent since mice receiving 1-2 million E3 DCs demonstrated decreased severity but failed to completely recover from EAE. Further activation of E3 DCs with LPS \textit{in vitro} or exposure to adjuvants \textit{in vivo} during immunization failed to abrogate this protective effect (Figure 5.6B). The CDS of both E3 and E3 plus LPS DC recipients were significantly less (4.8 ± 4.8 and 6.1 ± 3.8, respectively) than Pb or Pb plus LPS DC recipients (21.7 ± 6.2 and 20.1 ± 7.1, respectively) (Figure 5.6B).

5.2.5 E3 DC recipients are protected by a shift to Th2 cytokine production and not generation of regulatory T cells

Protection from EAE has been associated with either a shift to a Th2 response or through the generation of regulatory T cells. E2 has been shown to generate both Th2 responses and regulatory T cells. In order to investigate the relative contributions of a Th2 response or a regulatory T cell response in E3 DC recipients, we analyzed peripheral lymphoid organs at day 10 post-immunization.

Recipients of DCs from E3 treated mice showed an increase in MOG-specific IL-4 secreting cells and a decrease in MOG-specific IFN-γ secreting cells by ELISPOT, (Figure 5.7A). IL-4 production in anti-CD3 stimulated cells did not differ while LPS stimulation increased IL-4 production (Figure 5.7B). IFN-γ production in all other groups did not differ (data not shown).
DC recipients were additionally evaluated for the presence of regulatory T cells. Specifically, relative numbers of CD4+CD25+ T cells were measured and this cell population evaluated for cell surface expression of GITR, CTLA-4, intracellular CTLA-4 and FoxP3. Mice receiving E3 DCs did not show increased numbers of CD4+CD25+ T cells (Figure 5.8A) or increased intracellular Foxp3 expression (Figure 5.7B). CTLA-4 (intracellular and surface) and surface GITR staining were unchanged from controls (data not shown). Thus, it appears that the protection afforded by E3 DCs is due, at least in part, to a shift towards a Th2 response rather than an increase in regulatory T cells.

5.2.6 Summary of E3 Results

Taken together, these data suggest that E3 DCs may protect mice from EAE due to a combination of decreased inflammatory mediators (e.g. IL-12/IL-23 and TNF-α) and increased immuno-regulatory mediators (e.g. IL-10 and IDO) that influence T cell differentiation. Indeed, we have found increased IL-10 and decreased TNF-α in late pregnant immunized mice (McClain, manuscript provisionally accepted). These results demonstrate that the hormonal environment at the time of disease initiation results in a qualitative and long-lasting change to the adaptive immune response and resultant protection from EAE. Additionally, our results demonstrate that hormonal treatment of DCs alone is able to produce protection in actively immunized mice, implicating DCs as a key player in responding to hormones and regulating the immune response. Such potent regulatory cells that are capable of maintaining their protective phenotype in the face of potent inflammatory stimuli (i.e. in vitro LPS and in vivo adjuvants) are likely to have important therapeutic
application in inflammatory and autoimmune diseases. Investigating the mechanisms by which these reg-DCs can be generated and how they influence T cell development increases our understanding and ability to harness these potent cells for application in the treatment of diseases.

5.3 Overall Summary: DCs and Induction of EAE

The ability to influence the adaptive immune response is key to treating numerous inflammatory and immune-mediated diseases. DCs are critical cells that bridge the innate and adaptive immune responses and are well known for their unique ability to influence T cell differentiation. While much work has focused on creation of immunogenic DCs to stimulate an immune response, the role of DCs in regulating the immune response represents an exciting new area of research that has important applications in a variety of diseases such as inflammatory and autoimmune diseases. Understanding how different DCs are generated and the form and function of these DC subsets are likely to shed light on basic immunologic mechanisms of DC-T cell interactions.

In this chapter, we explored the generation and in vivo effect of immunogenic and regulatory DC populations. We characterized the phenotype of immature and matured mFL BM-DCs and found that maturation of these DCs generated immunogenic DCs that, upon transfer, were capable of exacerbating EAE severity (as compared to recipients of immature BM-DC). We then evaluated whether hormones of pregnancy were capable of generating reg-DCs. Based on the finding that DCs from mice immunized during late pregnancy (that were protected from EAE) expressed increased inhibitory costimulatory
markers, we explored the influence of E3, a pregnancy specific hormone, on the generation of reg-DCs. We found that E3 is capable of generating reg-DCs that have an activated regulatory phenotype, express less proinflammatory, and increased immunoregulatory mRNA, appear less immunogenic \textit{in vitro}, and, upon transfer, protect mice from the development of severe EAE. We found that this protection is mediated predominantly by a shift to Th2 cytokines and not through the generation of CD4+CD25+Foxp3+ regulatory T cells. Thus, these findings begin to elucidate the mechanisms by which pregnancy hormones, long known to have protective effects in EAE and MS, influence the autoimmune response. To our knowledge, these findings are the first to investigate the role of estriol in generating reg-DCs and demonstrate \textit{in vivo} protective effects of such hormonally derived reg-DCs.
Figure 5.1. BM-DCs cultured in the presence of different maturation stimuli demonstrate distinct phenotypic differences in costimulatory molecule expression. Immature (10-12 day) BM-DCs were cultured in the presence of 10 μg/ml MOG35-55 and matured in the presence of medium, LPS+TNF-α or TNF-α (1 μg/ml each) for 18-24 hours. Cells were washed and immediately evaluated after culture (gated on CD11c) for the expression of costimulatory molecules CD80, CD86, CD40, PD-L1, PD-L2 and ICOSL. Bold red lines represent LPS+TNF-α, thin blue lines TNF-α alone and shaded areas media-treated cells, respectively. Figures are representative of three independent experiments.
Figure 5.2. *In vivo* protective effect of day 10 mFL expanded and matured BM-DC. BM-DCs were pulsed in the presence of MOG (10 μg/ml) with or without the maturation agents LPS+TNF-α (both at 1 μg/ml) for 18-24 hours. Cells were transferred IV to naïve recipients. Three days later, recipients were immunized with MOG35-55 and adjuvant and monitored for the development of EAE as described in the Materials and Methods. Immature mFL-derived DCs were capable of protecting mice from development of disease while maturation of these DCs produced more severe disease compared to control mice not receiving DCs. Data are representative of three individual experiments.
Table 5.1 Phenotype of DCs immunized late-pregnant and control mice. DC phenotypic differences between pregnant and non-pregnant immunized mice during the A. initiation (day 4 post-immunization) of the autoimmune response or B. following the establishment of a helper T cell response (day 14 post-immunization). Mice were immunized either in late pregnancy or non-pregnant (controls) during immunization and splenic DCs were evaluated by flow cytometry. Gated CD11c+ cells were evaluated for expression of costimulatory (CD80, CD86, CD40, PD-L1, PD-L2 and ICOSL) molecules. Expression of activation markers CD80, CD86 and CD40 were elevated at day 4 post-immunization and CD80 and MHC II were elevated at day 14 post-immunization. Inhibitory costimulatory marker expression was elevated at both day 4 and 14 post-immunization with PD-L2 expression elevated at both days and PD-L1 elevated at day 14.

<table>
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<th>Marker (d4)</th>
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<th>Late Pregnant</th>
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<td>MHC II</td>
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<table>
<thead>
<tr>
<th>Marker (d14)</th>
<th>Control</th>
<th>Late Pregnant</th>
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<tr>
<td>MHC II</td>
<td>22.47 ± 4.89</td>
<td><strong>31.44 ± 1.43</strong></td>
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<tr>
<td>CD80</td>
<td>57.75 ± 3.03</td>
<td><strong>78.37 ± 1.46</strong></td>
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<tr>
<td>CD86</td>
<td>78.52 ± 84.50</td>
<td>84.50 ± 0.83</td>
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<tr>
<td>CD40</td>
<td>60.85 ± 3.41</td>
<td>64.65 ± 1.98</td>
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<tr>
<td>PD-L1</td>
<td>82.01 ± 6.03</td>
<td><strong>90.38 ± 1.43</strong></td>
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<tr>
<td>PD-L2</td>
<td>29.26 ± 5.07</td>
<td><strong>47.08 ± 1.30</strong></td>
</tr>
<tr>
<td>ICOSL</td>
<td>82.34 ± 0.79</td>
<td>90.15 ± 0.45</td>
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*P < 0.05 compared to control.
Figure 5.3. Activated regulatory phenotype on E3 DCs. Splenic DCs from E3 and placebo pelleted animals show increased expression of A) inhibitory costimulatory molecules PDL1, PDL2, B7H3 and B7H4 and B) markers of DC activation CD80, CD86 and CD40.
Figure 5.4: E3 DCs produce less inflammatory mediators and increased regulatory mediators. E3 DCs produce
A. less baseline IL-12 (ELISPOT), B. less mRNA for proinflammatory signals IL-12, TNF-α and NFκB and C.
increased mRNA for IL-10 and indoleamine 2,3 deoxegenase compared to placebo (Pb) DCs.
Figure 5.5 E3 DCs produce less IL-12 and cause less Th0 proliferation. E3 DCs were co-cultured with naive MOG transgenic CD4+ T cells. E3 DCs produce A. less IL-12p40 protein (ELISA) when cultured with naïve MOG Tg CD4+ T cells and B. Th0 cells proliferate less when cultured with E3 DCs.
Figure 5.6  E3 DC recipients have reduced disease and this effect is dependent on number of DCs transferred. Clinical scores of mice actively immunized one day after receiving E3 or Pb DCs (IV) pulsed *in vitro* with MOG35-55. A. 8-10 million E3 or Pb DCs were transferred and recipients had decreased clinical severity and complete resolution of clinical signs. B. 1-2 millions E3 or Pb DCs were transferred after *in vitro* pulse with MOG35-55 with or without LPS prior to IV transfer. Mice had a delay in onset and reduced clinical severity but mice failed to completely recover.
Figure 5.7  E3 DC recipients are protected by increased IL-4 and IFN-γ production (Th2) response. A. Cell-specific cytokine secretion of lymph node cells from mice receiving E3 or Pb DCs. Lymph node cells were obtained 10 days after immunization with MOD and cell cell-specific cytokine secretion was evaluated using ELISPOT. Mice that had received E3 DCs one day prior to EAE immunization had increased IL-4 and decreased IFN-γ cytokine production. B. Lymph node cells from E3 DC recipients had both T cell antigen-specific (i.e. MOG) and antigen non-specific (i.e. LPS stimulated) increases in IL-4 production.
Figure 5.8. E3 DC recipients are not protected by increased numbers of CD4+CD25+FoxP3+ regulatory T cells. Lymph node cells were evaluated by flow cytometry for the presence of phenotypic markers CD4 and CD25 and the intracellular transcription factor *foxp3* at day 10 post-immunization. A. There was no difference in the number of CD4+CD25+ T cells within the lymph nodes at day 10 post-immunization. B. There was no difference in intracellular FoxP3 expression in the CD4+CD25+ T cell population.
CHAPTER 6
DISCUSSION AND CONCLUSIONS

Sex hormones are known to affect the immune system and have been shown to influence both susceptibility and the clinical course of autoimmune disease. Females are at increased risk for developing autoimmune disease in general, but the course of disease can be profoundly modulated during pregnancy where protection can be observed. These apparent dichotomous effects have been explained primarily through the effects of sex hormones on T cell responses, and with recent evidence that sex hormones may influence the Th1/Th2 balance by acting further upstream of the T cells (i.e. at the DC and DC-T cell interface). Previous work from this lab has shown that sex hormones present at the time of disease induction, when DCs first interact with T cells, can dramatically affect the clinical course of EAE. In this thesis, I explored how sex hormones influence the induction of EAE by examining i) the influence of sex and mouse strain (i.e. genetics) on induction of EAE and ii) the influence of a protective pregnancy hormone, E3, directly on DCs and the DC-T cell interaction.

Females have an increased predilection for developing MS that has been attributed to an interplay between sex hormones, genetics and environmental factors. The combination of sex hormones and genetics likely plays an important role in influencing
the different clinical manifestations of MS, seen in men and women. EAE is the most common animal model used for the study of MS with chronic relapsing murine EAE having the greatest similarity to clinical MS. Although several mouse and rat strains are employed in the study of EAE, the SJL mouse was the only reported model demonstrating sexual dimorphism in clinical disease and it has been used extensively as a model for the sex differences seen in MS (65, 66, 190, 240, 241). Most of the studies in the SJL utilized adoptively transferred EAE, with limited investigations exploring the influence of sex hormones on the induction of EAE (i.e. actively induced EAE) in the SJL, C57BL/6 mice and Lewis rats (191, 242, 243).

The utility of the SJL strain as a model for investigation of female prevalence and as a model for RRMS is unquestioned, but results from this one strain should be interpreted with caution. The SJL mouse strain lacks approximately 50% of all T cell receptor (TcR) genes, lacks NK1.1+ T cells and, consequently, produces low amounts of IL-4 (i.e. decreased Th2 response) (244). For these reasons, other mouse strains with complete immunological repertoires may be more useful to investigate the influence of sex hormones on the immune response during EAE. In order to evaluate whether a sexual dimorphism existed in other strains that may be capable of modeling different clinical manifestations of EAE, we explored the influence of sex and genetics (i.e. MHC class II haplotype) on the development of EAE.

As reported previously, the SJL mouse strain (H-2^s^) has increased susceptibility to EAE, increased disease severity and relapsing disease in females (65, 190, 240, 241). This increased severity in acute disease is observed following immunization with MBP,
PLPc, PLPs and MOG peptide. Pertussis toxin (PT) does not have a dramatic influence on disease severity. This consistent sexually dimorphic clinical course is most likely due to a combination of the increased frequency of autoreactive PLP139-151 reactive cells circulating in SJL mice and the absence of a macrophage population (i.e. I-A+Mac-1+, Mac-2-, Mac-3+) in male SJLs which results in a failure of epitope spreading and failure to relapse (245). Based on these observations, female SJLs are inherently more susceptible and exhibit increased EAE severity compared to males.

While MHC haplotype influences EAE susceptibility and may, in part, contribute to sexual dimorphism, sex hormones themselves can have a profound influence on clinical disease. In the SJL, the fact that males have decreased disease and fail to relapse suggests that male hormones such as testosterone may be protective. Indeed, MS in men coincides with decreasing testosterone levels. Moreover, administration of testosterone to female SJLs results in less severe EAE and castration of male SJLs induces relapses (64, 240, 241, 246). This may be due to a direct effect of androgens on T cells resulting in more of a Th2 phenotype (192, 247).

Testosterone does not appear to be uniformly protective, since adoptive transfer of female cells into males resulted in severe signs of EAE, equivalent to that seen in female recipients (190, 192, 240). Additionally, the protective effects of testosterone may be strain specific since there did not appear to be any protective effect of castration in male B10.PL mice (Figure 3.9) or of testosterone administration in male C57BL/6 mice (64). Male SJL mice show a decreased ability to mount a Th1 response (i.e. decreased IL-12 production) and an increased tendency to shift to a Th2 response (i.e. decreased
production of IFN-γ and increased IL-10 production), when compared with the female which may be partly explained by the deficient macrophage populations in male SJLs (191, 245, 248). Thus, some of the gender-specific effects seen in the SJL mice may be due to its unique genetic and immunologic composition with sex hormones playing an additional modulating role.

Unlike the SJL strain, male B10.PLs showed increased clinical signs of EAE during the acute disease period. Similarly, other investigators have demonstrated that in a related (i.e. B10.RIII) strain, male mice also demonstrate increased severity in collagen-induced arthritis (CIA) and EAE, both Th1-mediated diseases (75, 249)(VJ Kuchroo, personal communication). The increased severity of EAE in male B10.PLs, B10.RIIIs and PL/Js may represent an underlying bias towards Th1 responses in male mice of specific haplotypes. Indeed, males with more severe clinical signs of EAE demonstrate an increased Th1 response that is associated with greater inflammatory infiltrates, CDS and increased mortality (Figure Figures 3.4-3.8 and Table 3.2). However, it appears that sex hormones may further influence disease outcome, beyond the MHC-associated influence.

Our results demonstrate that female sex hormones can serve to protect B10.PL mice from the development of severe acute EAE (Figure 3.9). Female hormones have been shown to play a protective role in the B10.PL and B10.RIII strains. Prior reports demonstrated that ovariectomized females had increased EAE and CIA in B10.PL and B10.RIII strains while supplementation with female hormones (e.g. 17-β estradiol and estriol) is protective against EAE in B10.PL, SJL and C57BL/6 mice (75, 96, 102). The
reason for the increased severity in male B10.PLs (or decreased severity in female B10.PLs) is not known but may be due to such factors as Th1/Th2 balance, cellular trafficking, neuroprotection, effects of sex hormones on immune cells or other less direct mechanisms such as increased glucocorticoid levels in females, or through the actions of EAE susceptibility genes (e.g. eae9) that may be sexually dimorphic. Interestingly, the eae9 susceptibility gene has been demonstrated to regulate the infiltration of mononuclear cells into the spinal cord of male C57BL/6 mice with EAE (250). Moreover, mice immunized with PT have EAE susceptibility and severity regulated by eae9. Therefore, it may be argued that the increased disease severity observed in male B10.PL mice during the acute phase of disease may be due to PT.

PT is a major component of adjuvants required for most murine models of EAE induction, and acts by shifting the immune response to Th1 cytokines, enhancing cytokine production by T cells, inducing lymphocytosis/neutrophilia, increasing blood-brain-barrier permeability and influencing genetic susceptibility loci (250, 251). The increased severity observed in male B10.PL mice may reflect a sexually dimorphic response to PT. Indeed, we have found that the increased severity and mortality in male B10.PL mice is associated with marked neutrophilic meningoencephalomyelitis compared to females (Figures 3.5-3.6), suggesting that males may be more susceptible to PT and manifest with a more robust neutrophilic response compared to females. Reports indicate that the Bphs, a gene that controls PT responsiveness, contributes to EAE susceptibility, but its role in sexually dimorphic EAE has not been evaluated (252). These results demonstrate that the increased severity in male B10.PL mice may indicate
an increased sensitivity of male B10.PLs to PT, but PT alone cannot explain the increase since immunization with MBP and CFA without PT still results in increased severity and a chronic disease course in males compared to females (data not shown). Additionally, responsiveness to PT may be strain specific, since PT administration did not influence disease susceptibility in the SJL immunized with PLPc versus PLPs.

Finally, sexual dimorphism seen in the B10.PL strain may simply represent an overall difference in immune response in male versus female B10.PLs. Indeed, male B10.PL mice were more sensitive to tolerance induction to MBP Ac1-11 [4Y], an effect that was obviated by castration (190). Thus, gonadal hormones may influence the development of regulatory T cells involved in tolerance to autoimmune disease. In fact, the sex differences seen in the B10.PL mice parallel differences reported in treatment of MS with oral myelin (253). The cause of the increased severity in EAE seen in male B10.PL mice remains to be determined. However, the sexual dimorphism seen in the B10.PL argues that conclusions regarding gender-specific findings in the SJL should be interpreted with caution. In addition to the fact that the B10.PL strain may represent a novel model to investigate the increased severity seen in men with PPMS, the complete T cell repertoire in B10.PL mice may more accurately represent the effects of sex hormones on immune function.

In the B10.PL strain, decreased acute EAE is seen only when female sex hormones are present at the time of disease induction. Similarly, experiments in our lab with a pregnancy model demonstrate that pregnancy hormones present at the time of disease induction dramatically decrease EAE, even after the protective pregnancy
hormones are no longer present (McClain, manuscript provisionally accepted). Thus, female sex hormones appear to play an important role in the induction of the immune response. While many studies have investigated the effects of sex hormones on the resultant adaptive Th1 or Th2 response, little has been done to determine the mechanism by which sex hormones influence the generation of a helper T cell response. Many studies have concluded that if sex hormones generate a specific T cell response, then sex hormones must act on the T cells.

While T cells do have hormone receptors (i.e. estrogen and progesterone receptors) and are capable of responding to sex hormones, recent evidence demonstrates that the T cells are not the critical cells responsible for estrogen-mediated protection. Rather, APCs such as DCs or microglial cells have been implicated as the critical cell responsible for estrogen’s protective effect in EAE (94, 105). Since the generation of an adaptive helper T cell response is critically dependent upon DC-T cell interactions during the induction phase of the immune response, we turned our attention to the DCs and DC-T cell interactions. The remainder of this thesis evaluated the i) generation methods of DC populations and ii) influence of maturation and estriol, a pregnancy-specific estrogen on DCs and DC-T cell interactions both in vitro and in vivo.

Expansion of DC Populations

DCs are uniquely able to prime naïve T lymphocytes and influence the character of the resultant adaptive immune response. This instruction of T cells by DCs involves a combination of receptor interactions and soluble mediators, all influenced by the
environment. The ability to investigate DC biology and DC-T cell interactions had been relatively limited due to the fact that DCs represent a trace population in vivo. However, recent developments have allowed expansion of large quantities of DCs and have facilitated the study of these cells. Most studies evaluate either in vitro differentiated bone marrow derived DCs (BM-DCs) or in vivo expanded DCs, and methods used to generate these populations vary markedly. At present, BM-DCs are derived in vitro in the presence of cytokine cocktails typically containing granulocyte macrophage colony stimulating factor (GM-CSF) with or without IL-4 while in vivo expansion of DC populations (e.g. myeloid, lymphoid and plasmacytoid DCs) is accomplished through the administration of the growth factor FL (199, 207, 220, 223, 225, 226). The purpose of Chapter 4 was to evaluate various in vitro and in vivo methods to generate DCs.

The most commonly used method for in vivo DC expansion is daily injection of hFL (199). However, the xenogeneic nature (human versus mouse) of hFL used in a mouse system was a potential cause for concern. For these reasons, we initially explored the use of a tumor-based delivery system of mFL for use in expanding DCs. While recent data indicates that differences exist in both the properties of hFL and mFL and the biological activity of resultant DCs, a direct comparison of phenotypic differences between hFL, mFL and resting splenic DCs had not been performed (197, 211, 214-217). Development of a mFL-specific methodology has the advantage of a more biologically relevant system with changes in DC populations more accurately representing normal mouse physiologic and immunologic responses. Our results demonstrate that while both mFL and hFL similarly expand overall DC numbers, the resultant DCs differ in
phenotype and activation status. Importantly, mFL expanded DCs are more similar to resting splenic DCs than hFL expanded DCs and likely represent an expanded resting (immature) DC population (Figure 4.2).

While it can be argued that use of a tumor-based mFL delivery system may influence DC phenotype or function, it has been shown that mFL-secreting B16 melanoma cells elicit comparable effects on hematopoetic populations as injections of recombinant mFL. Resultant mFL-expanded DCs function efficiently as stimulators of mixed lymphocyte reactions and these DCs are capable of migration and T cell priming \textit{in vivo} (188, 199, 201, 254). The possibility exists that the presence of tumor cells could generate activated DCs. However, our data indicates that mFL DCs have a phenotype that is more similar to resting splenic DCs than hFL expanded DCs. Thus, the tumor-based mFL delivery system does not dramatically alter expanded DC phenotype or function and is a viable method for expanding large numbers of DCs \textit{in vivo}. Most importantly, utilizing a murine growth factor may represent a physiologically relevant alternative to xenogeneic hFL when evaluating subtle differences in DC populations within mouse models.

However, due to the potential limitations with a tumor-based delivery system, we explored alternative methods for \textit{in vivo} expansion of DCs. Recent studies demonstrate expansion of DCs \textit{in vivo} with a hydrodynamic based transfection with a DNA plasmid containing hFL (213, 221, 222). Rapid IV injection of large volumes containing plasmid DNA (ranging from 5 μg-20 μg plasmid DNA) were used to successfully expand DCs \textit{in vivo}. These studies demonstrate that the high volume and rapid infusion rate are key to
successful transfection of cells. We explored the feasibility of this methodology as an alternative to daily hFL injection and a tumor-based mFL delivery system to expand DCs \textit{in vivo}.

Our results demonstrate that hydrodynamic injection of both mFL and hFL plasmid were capable of expanding splenic DCs and that increasing mFL plasmid amount (20 $\mu$g versus 10 $\mu$g) did not further increase DC expansion (Figure 4.3). The fact that increasing plasmid concentrations failed to increase expansion suggests that 10 $\mu$g mFL plasmid is sufficient to “saturate” the system and result in maximal transient transfection of organs with mFL. We are further evaluating the influence of lower doses of plasmid DNA.

As an alternative to the commonly used daily hFL injection, we also evaluated hydrodynamic injection of hFL DNA plasmid. Similar to mFL, we found that hFL was capable of expanding DCs after a single injection (6.9 $\pm$ 3.4 % CD11c+ DCs in the LN and 19.5 $\pm$ 9.8 % CD11c+ DCs in the SPL). A study has shown that a second injection 5-7 days after the first will significantly increase DC yield. As reported, we also found that the duration of injection was critical with > 7 second injection time dramatically reducing the number of expanded DCs (Table 4.1). Similar to others, we found that a 2\textsuperscript{nd} injection of hFL plasmid DNA further expanded LN and SPL DCs (Figure 4.4)(213). Specifically, LN DC yield increased from 6.9 $\pm$ 3.4 % CD11c+ DCs to 9.5 $\pm$ 5.5% CD11c+ DCs while splenic DCs increased from 19.5 $\pm$ 9.8 % CD11c+ DCs to 43.9 $\pm$ 25.4 % CD11c+ DCs. Presumably, an increased injection time decreased hydrostatic pressure required to exceed cardiac output. The reflux of plasmid DNA into organs
would result in decreased transfection of these organs and decreased systemic circulating mFL required to expand DCs. Taken together, these findings demonstrate that FL plasmid concentration was less important than rapid IV injection of the plasmid.

One additional interesting finding was that hFL plasmid appears to more effectively expand SPL DCs with relative yields ranging from 12-44% (Table 4.1) compared to mFL expanded SPL DCs ranging from 11-18% (Figure 4.3 and data not shown). While the reason for this is presently unknown, one possibility is that species-specific differences may result in an additional immune stimulation with hFL plasmid injection. To our knowledge, these are the first studies utilizing mFL plasmid injection to expand DCs in vivo in mice, and we are continuing to expand and characterize mFL (and hFL) DCs as an alternative to tumor-based mFL delivery.

The ability to utilize DNA plasmids represents an exciting and novel method for expanding DCs in vivo. Although the rapid IV injection of large volumes of plasmid is technically challenging, hydrodynamic injection of plasmid DNA has several benefits compared to currently available FL delivery systems. The single injection is superior to the daily injection regimen over nine days required for hFL. Moreover, in vivo expression of FL without the need for tumor or adenovirus-mediated delivery eliminates untoward tumor and viral-related peripheral effects. While limitations include both technical difficulty (i.e. time-critical IV delivery) and slightly decreased expansion compared to other methodologies (i.e. tFL or hFL), additional injections of plasmid at 5-7 day intervals can substantially increase DC yield (Figure 4.4). Finally, although not yet evaluated, this technology has the potential to be utilized with other plasmids such as
GM-CSF that can result in in vivo expanded GM-CSF DCs, previously only accomplished through melanoma-based delivery systems (188). Therefore, while introduced only recently, hydrodynamic injection of plasmid DNA is a viable and potentially superior alternative to previously used methodologies. Based on its versatility and utility, injection of plasmid DNA may supercede other in vivo DC expansion methods.

Although FL is the primary factor used to expand DCs in vivo, GM-CSF is the predominant cytokine in cocktails used to generate BM-DCs (197-205, 223, 226). Recent work suggests that the primary role of GM-CSF may lie at the level of DC activation, survival and/or migration to lymphoid tissues, rather than DC development (223, 226, 236, 255). In support of this are the facts that GM-CSF expanded DCs produce a more robust Th1 response (versus mFL expanded DCs) and that levels of GM-CSF increase during inflammation and have a synergistic effect with other maturation stimuli (e.g. LPS, TNF-α or IFN-α) on DC activation (188, 198, 256). Thus, GM-CSF derived BM-DCs may be inherently biased towards a proinflammatory phenotype and response. Our results support this in that GM-CSF BM-DCs demonstrate increased CD80 and CD86 compared to either mFL or hFL BM-DCs (Figure 4.6). The idea that GM-CSF may serve more of an inflammatory role while FL (either human or mouse) may function more in states of homeostasis or immune regulation has important implications in the evaluation of DC populations for inherent immunogenic or regulatory functional capabilities.
In order to explore the influence of mFL, hFL and GM-CSF methods on the generation of DC subsets, we compared the phenotype of bone marrow precursors cultured in the presence of these differentiation factors. In order to generate *in vitro* DCs that were comparable to our established splenic DCs, we developed an *in vitro* correlate to tumor-delivered mFL expanded DCs *in vivo* by using mFL-containing supernatant to generate mFL BM-DCs (Figure 4.5). We found that such mFL DCs appeared phenotypically less inflammatory and more immunoregulatory than GM-CSF or hFL BM-DCs. Specifically, mFL BM-DCs appeared to be smaller with fewer dendrites and had lower levels of CD11c expression (Figure 4.6A). Phenotypically, mFL and hFL were more similar to one another than to GM-CSF with low levels of CD11b and high MHC Class II expression. Additionally, maturation markers CD80 and CD86 were lower in mFL BM-DCs than GM-CSF BM-DCs and mFL BM-DCs increased expression of phenotypic markers (i.e. B220 and CD8α) commonly associated with more regulatory function (Figure 4.6B) (151, 211). Based on the fact that mFL BM-DCs appeared to be less mature than the other BM-DC populations, we hypothesized that these mFL BM-DCs represented an immature DC population that may have protective functions *in vivo*.

To evaluate whether these immature mFL BM-DCs were capable of maturation, we exposed them to known maturation agents LPS and TNF-α. We found that they were capable of upregulating maturation markers CD80, CD86 and CD40 upon exposure to the maturation stimuli TNF-α +/- LPS (Figure 5.1). Furthermore, the maturation status of these mFL BM-DCs influenced the clinical course of EAE when given prior to the onset of EAE (Figure 5.2). Immature DCs are well known to have regulatory or tolerogenic
effects and we found that disease severity differed significantly between mice receiving immature and matured mFL BM-DCs. Both CDS (26.79 ± 10.12) and peak disease scores (3.07 ± 1.16) were significantly increased in mice receiving matured BM-DCs compared to those receiving immature mFL BM-DCs (20.21 ± 7.64 and 1.86 ± 0.7, respectively). If these groups were compared to immunized mice that had received no DCs, there was a trend towards protection against EAE in mice receiving immature mFL BM-DCs and increased disease in mice receiving mature mFL BM-DCs. In summary, mFL DCs are capable of responding to maturation stimuli which then influences the clinical course of EAE. Taken together, these results indicate that mFL BM-DCs are immature and potentially regulatory but maintain the ability to mature and become immunogenic in the disease model EAE. To further explore the generation of regulatory DCs, we investigated the effects of the pregnancy environment on DCs and hormonal modulation of DC function.

**Hormonal Modulation of DC Function**

Prior work within our laboratory had demonstrated that pregnancy hormones present at disease induction can dramatically protect mice from EAE. Pregnancy hormones present at the initial DC-T cell interaction (i.e. mice immunized during late pregnancy) result in delayed onset, decreased severity and complete resolution of clinical signs in EAE. We first evaluated whether DCs differed in phenotype in these mice immunized during late pregnancy. We found that DC numbers did not differ at day 4 and 14 post-immunization but DC phenotype did. Specifically, increased levels of both
stimulatory (i.e. CD80, CD86 and CD40) and inhibitory (i.e. PD-L1, PD-L2 and ICOSL) molecules were increased in DCs from late pregnant immunized mice. These results suggested that hormones of late pregnancy may generate DCs that were activated, but had regulatory capabilities and may represent a novel population of “activated regulatory” DCs. To our knowledge, an activated regulatory DC phenotype is a novel finding since regulatory functions of DCs are thought to be mediated through immature or semi-mature DC populations.

To further explore the possibility that hormones were capable of generating regulatory DCs, we investigated the influence of E3, a pregnancy specific estrogen, on DCs. E3 has profound effects on the development of EAE. E3 given prior to disease induction has been shown to be protective in EAE in both male and female mice. E3 treated mice exhibit delayed onset, decreased incidence, mortality, severity, CNS infiltrates and often complete resolution of disease. These findings are true regardless of sex or strain (i.e. B10.PL and SJL) (67, 74, 97). The clinical observations are associated with decreases in T cell proliferation and Th1 cytokines IFN-γ, TNF-α, IL-2 and IL-6 in male and female mice, as well as, increased IL-5 in male mice (67, 97). A separate study demonstrated increased IL-10 secretion and IgG1 Ab production associated with E3 treatment (67, 97). E3 treated MS patients demonstrated decreased MRI lesions, decreased Th1-associated responses and increased Th2-associated responses (92, 93). These protective effects of E3 coupled with the fact that E3 is an available oral therapy (commonly used for controlling symptoms of menopause) and considered safe with few side-effects support the feasibility of estriol as a therapy in MS patients (257). Indeed, recruitment for a clinical trial utilizing E3
as a treatment for MS patients is currently underway at the Ohio State University (Michael Racke, personal communication).

We first evaluated whether E3 would influence DC phenotype by expanding DCs with hFL in pregnancy levels of E3. Similar to DCs from late pregnancy immunized mice, DCs from E3 treated mice had an activated or mature regulatory phenotype. Specifically, E3 DCs had increased levels of inhibitory markers PD-L1, PD-L2, B7H3 and B7H4 and these DCs were phenotypically mature and activated based on elevated CD80 and CD86 expression (Figures 5.3A and 5.3B). The significance of the inhibitory marker expression on E3 DCs is not known, but recent studies suggest an important role for such molecules in regulating the immune system and modulating the Th1/Th2 balance. (141). Mice deficient in PD-1 have increased autoimmune disease, and it has been proposed that the balance of inhibitory versus stimulatory costimulatory molecules on APCs determine the balance between tolerance and autoimmunity (142). The PD-1/PD-L2 interaction has been shown to inhibit T cell proliferation and cytokine production, and this effect is influenced by antigen concentration. Low antigen concentration decreases both proliferation and cytokine production while high antigen concentration decreases only cytokine production with no effect on proliferation. Additionally, the inhibitory interaction of PD-1 with its ligands leads to cell cycle arrest in G0/G1 but does not increase apoptosis (141). Such studies demonstrate that PD-1 and its ligands likely influence autoimmunity through a variety of mechanisms.

Both PD-1 and its ligands PD-L1 and PD-L2 have been investigated in EAE and MS. PD-1 is expressed by activated T cells (CD4+ and CD8+), B cells and myeloid cells and
interacts with its ligands PD-L1 and PD-L2 (258). PD-L1 expression is on both lymphoid and non-lymphoid tissue (e.g. placenta, heart, kidney, etc.) while PD-L2 is more limited in its expression to DCs and other antigen-presenting cells (258-260). Using knock-out mice, PD-L1/PD-L1 interactions appear to be more crucial in attenuating T cell responses in that elevated proinflammatory cytokines IFN-\(\gamma\), IL-6 and IL-17 were seen in PD-1 and PD-L1 knock-out mice (145). Interestingly, there is likely a reciprocal regulation of PD-L1/PD-L2 expression in the Th1/Th2 balance. Specifically, Th1 or microbial (pro-inflammatory) products can enhance PD-L1 expression while Th2 cells up-regulate PD-L2 suggesting that PD-L1 and PD-L2 might have different functions in regulating Th1 and Th2 responses but detailed investigations are lacking (144).

The influence of strain in EAE demonstrate that the susceptibility and chronic progression of EAE are regulated by PD-L1 and PD-L2 in a strain-specific manner (146). In BALB/c mice, PD-L1 blockade increased EAE incidence while blockade of both PD-L1 and PD-L2 enhanced EAE in B10.S mice. In B6 and NOD mice, PD-L2 blockade exacerbated EAE, while PD-L1 had no effect compared to SJL that only had decreased EAE following PD-L1 blockade. In B6 mice, blockade of PD-1 also results in increased T cell activation, expansion, cytokine production with an increase in IFN-\(\gamma\) producing cells, Th1 response, high anti-MOG antibody and increased leukocyte infiltration in the CNS. The fact that blockade of PD-L2 in both CD28 knock-out and wild type mice result in augmentation of EAE, suggests that PD-L2 is a critical mediator of disease severity in B6 mice and that PD-L2 does not signal through CD28 (143). Interestingly, in B6 mice, the relative expression of PD-L1 and PD-L2 on DCs, macrophages or on CNS
inflammatory cells but were associated with a decrease in the production of protective Th2 cytokines (146). Thus, the associations of PD-1 with its ligands PD-L1 and PD-L2 on DCs are a likely mechanism by which reg-DCs regulate an immune response.

The PD-1 pathway has been associated with estrogen-mediated protection and may play a role in both APCs and CD4+CD25+ regulatory T cells but not via a direct effect on effector T cells (94). This study found that both E2 and pregnancy enhanced PD-1 expression on APC populations, particularly on DCs, and that this effect is mediated by ER-α (94). In this model, PD-L2 appears to play a particularly important role in E2 mediated effects on DCs with further modulation by both DC maturation status and T cell:DC ratio in vitro (94).

In MS, expression of inhibitory markers PD-1/PD-L1/PD-L2 has been associated with disease progression, treatment efficacy and susceptibility. SPMS patients had increased CD80 and decreased PD-L1 on DCs, with associated increased IL-12 and TNF-α production compared to RRMS and control patients. DCs from RR-MS also induced higher Th1 (IFN-γ, TNF-α) and Th2 (IL-4, IL-13) cytokines from controls, suggesting that DC abnormalities in MS may contribute to the different clinical patterns of MS (261). MS patients have increased PD-L1 expression, suggesting that IFN-β may act by negatively influencing APC-T cell interactions (262). The fact that a PD-1 genetic polymorphism (i.e. defective PD-1 gene) influences disease progression in MS suggests a similar immunoregulatory role for the PD-1 family (263). The elevated PD-L1, PD-L2 and other inhibitory costimulatory molecules suggest that E3 DCs may mediate protection, in part, through such a mechanism.
In order to evaluate whether E3 DCs had regulatory functions associated with their regulatory phenotype, we evaluated their production of proinflammatory and immunoregulatory molecules and determined the effects of E3 and Pb DCs on naïve transgenic T cells. Our results show that E3 DCs have less pro-inflammatory levels of IL-12, TNF-α and NFκB mRNA compared to Pb DCs and increased mRNA levels of immunoregulatory mediators such as IL-10 and IDO (Figures 5.4B and C). Although TGF-β has been associated with protection and regulatory function, TGF-β1 levels did not differ. However, different TGF-β isotypes were not evaluated and may still potentially play a role. Additionally, E3 DCs may not utilize TGF-β to influence T cell function but may generate regulatory T cells that produce TGF-β that then subsequently regulates the immune response. Thus, in addition to an activated regulatory phenotype based on cell surface marker expression, E3 DCs may also regulate immune function through decreased expression of proinflammatory mediators (Figure 5.4A, B) or increased immunoregulatory mediators (Figure 5.4C). Indeed, a study by Offner and colleagues investigating the effects of E2 on DCs demonstrated similar findings as our studies in that splenic DCs had a mature phenotype but decreased ability to produce TNF-α, IFN-γ and IL-12 (132).

The role of cytokines in influencing Th1 and Th2 responses is well known. DCs are capable of producing numerous cytokines which are known to influence T cells such as IL-12, IL-23, TNF-α and IL-10 (3, 116, 125-128). IL-12 is the predominant cytokine that drives a Th1 response, IFN-γ production and has long been considered critical to the development of EAE. However, recent findings suggest that IL-23 and the resultant IL-17 production may play an important role in some aspects of EAE pathogenesis. While
IL-23 was not specifically evaluated, the increased mRNA levels of the IL-12p40 subunit may be due to contributions from IL-23, since IL-12 and IL-23 share the p40 subunit. However, ELISPOT data support a role for IL-12 since the ELISPOT IL-12 data at the protein level evaluates IL-12 and not IL-23 (Figure 5.4A). Thus, there may be additional contributions of IL-23 that may influence DC-T cell interactions that were not evaluated in this thesis. Interestingly, Offner has shown that E2 results in decreased APC-specific IL-12 production that may contribute to the protective effects seen in EAE (132). While these studies looked at APCs from the peritoneal cavity which were most likely macrophages, it indicates that E2 may be acting through decreased production of the Th1 promoting cytokine IL-12 (94).

While TNF-α is often considered to be proinflammatory and to facilitate the generation of Th1 responses and CNS pathology in EAE, it has also been associated with protection from relapses (3, 128). Thus, the absolute significance of increased TNF-α mRNA in E3 DCs is not known. To further complicate the role of TNF-α and DCs, TNF-α has also been used to create “semi-mature” DCs which have demonstrated protective effects in EAE (164). The use of TNF-α knock-out mice and neutralizing antibody studies could shed additional light on the relative role of TNF-a production by DCs.

E3 DCs also had definitive increases in both IL-10 and IDO mRNA. IL-10 is a cytokine well known to regulate the immune system by inhibiting Th1 development and cooperating with IL-4 in the production of Th2 cells (116, 125). It is often the balance between IL-10 and IL-12 that influences the Th1/Th2 balance or generation of regulatory
cell populations. In EAE, IL-10 is an immunoregulatory cytokine that has important protective effects in disease development (116, 125). Specifically, IL-12 (and recently IL-23) are disease exacerbating while IL-10 is necessary to prevent severe EAE and promote recovery (126, 133, 134).

The use of IL-10 is also an established method to generate reg-DCs, which have reduced functional ability to generate an immunogenic T cell response(27, 264, 265). Much of this has been attributed to the ability of IL-10 to maintain DCs in a more immature, and thus tolerogenic, state (160). However, there are studies demonstrating that DCs actively produce IL-10 to generate T cells with regulatory capabilities such as Tr1 or other Treg cells (116, 125, 266). Although we have found increased mRNA levels in E3 DCs, we have not detected IL-10 by either ELISA or ELISPOT. However, preliminary studies have found suggestive increases in mean fluorescence intensity (MFI) in both DCs and T cells by intracellular IL-10 cytokine assays (data not shown). These findings suggest that both DCs and T cells are producing increased IL-10 on a per cell basis and the inability to detect larger quantities of IL-10 protein may be due to relatively low IL-10 production or reutilization at the DC-T cell interface. Whether DCs produce enough immunoregulatory IL-10 to mediate protection or whether they amplify a protective response through the generation of IL-10 producing regulatory T cells (e.g. Tr1) is not known at present. Interestingly, some successful treatment modalities in MS may also be mediated, in part, through IL-10 (and TNF-α). Glatiramer acetate treatment has been associated with increased production of IL-10 by both DCs and macrophages and suppressed TNF-α production by macrophages (127).
Therefore, IL-10 production by DCs and its resultant influence on T cell populations is another potential mechanism by which E3 DCs may mediate protective effects on EAE.

One final and recently described mechanism by which reg-DCs influence T cell function is through increased IDO production (136, 137). IDO is an enzyme, produced by DCs, that degrades tryptophan resulting in a subsequent suppression of T cell responses (136, 137). IDO has been implicated in the induction of tolerance and is thought to play an important role in the immunomodulation necessary to maintain pregnancy (267). In EAE, IDO levels contrast with IFN-\(\gamma\) mRNA levels in the spinal cord during the course of EAE, and pharmacological inhibition of IDO results in exacerbation of clinical and histopathological disease (268). The influence of estrogens were evaluated in a separate study which demonstrated that IDO is upregulated following E2 treatment and the decreased T cell proliferation and increased T cell apoptosis seen with E2 treatment was partially abolished by inhibition of IDO (104). Thus, IDO has been proposed to be one mechanism by which estrogen-derived DCs may mediate their regulatory and protective function (104, 136).

The influence of soluble mediators such as proinflammatory cytokines IL-12 and TNF-\(\alpha\) or immunoregulatory factors such as IL-10 and IDO likely contribute to the regulatory capabilities of E3 DCs. Additionally, E3 may have more of a global effect on DC function as demonstrated by differences in NF-\(\kappa\)B expression between E3 and Pb DCs. NF-\(\kappa\)B is one of the pivotal signaling pathways and mechanisms by which proinflammatory responses are propagated. The role of decreased NF-\(\kappa\)B expression in E3 DCs is uncertain. While it can be argued that low NF-\(\kappa\)B may simply represent
immaturity in E3 DCs, these E3 DCs have maturation markers CD80 and CD86 and are capable of producing IL-12 and stimulating Tg T cells to proliferate, albeit at decreased levels. While DC maturation involves expression of genes controlled by NF-κB which facilitates activation of T cells and the generation of Th1 responses, \textit{in vitro} DC activation with LPS did not decrease the ability of E3 DCs to protect mice from EAE (Figure 5.6). This argues that either these E3 DCs were incapable of activation with LPS or were already mature and activated. The CD80 and CD86 expression would argue for the latter possibility.

E3 may have additional effects on NF-κB and act as an NF-κB inhibitor. Pharmacologic agents that interfere with NFκB activation have demonstrated that inhibition of immature DCs enhanced their tolerogenic abilities in EAE (269). Additionally, E2 treatment was associated with decreased LPS-induced NFκB-binding activity that resulted in E2-associated inhibition of cytokine production (270). While these studies suggest that estrogens are somehow influencing NF-κB, detailed mechanistic studies are lacking. Interestingly, there may be an association between NF-κB expression and inhibitory costimulatory molecule expression in that NFκB p50(-/-)p65(+/-) DCs have decreased expression of PD-L2 (271). While our results demonstrate distinct phenotypic and functional differences between E3 and Pb DCs, the relative importance of i) costimulatory molecule, ii) proinflammatory factor and iii) immunoregulatory factor expression in mediating the protection afforded by E3 DCs remains to be fully characterized.
Initial investigations *in vitro* demonstrated that E3 DCs were functionally different from Pb DCs when co-cultured with purified CD4+ MOG Tg T(Th0) cells. E3 DCs co-cultured with Th0 cells produced decreased IL-12 compared to Pb DCs, regardless of *in vitro* stimulus (i.e. MOG, LPS, conA or α-CD3; Figure 5.5A). When evaluating the ability of E3 DCs to stimulate Th0 cells, E3 DCs were less able to cause proliferation in the presence of MOG or LPS (Figure 5.5B). Taken together, these results suggest that E3 DCs were less immunogenic in that they produced less IL-12 regardless of the presence of T cells and were less able to cause T cell proliferation. While these data suggested a relative immaturity of E3 DCs, increased CD80 and CD86 expression and the failure of maturation stimuli to abrogate the protective effects of E3 DCs *in vivo*, argue that these E3 DCs are not simply immature. Offner’s group has found that E2 also results in a similar decreased ability to produce IL-12, yet these DCs were phenotypically mature (132). Although this study employed *ex vivo* expansion of E2 exposed DCs using GM-CSF and IL-4 rather than *in vivo* expansion with hFL, it suggests that E2 and E3 may have similar actions on DC phenotype and function (132). Our results demonstrate that E3 DCs differ in both phenotype and function from Pb DCs, with differences in costimulatory molecule expression, proinflammatory or immunoregulatory mediators all potentially influence initial interactions of DCs with Th0 cells.

To address whether E3 DCs had a biologically relevant effect *in vivo* on disease, we administered E3 DCs prior to the induction of EAE and found that E3 DCs protected mice from EAE. CDS in E3 DC recipients was 1.3 ± 0.8 compared to Pb DC recipients (26.3 ± 15.2) and peak disease score was 1.2 ± 0.7 and 3.2 ± 1.8, for E3 and Pb DC recipients,
respectively. The dose of DCs is important in that administration of 1-2 million E3 DCs was able to decrease clinical disease but did not cause complete resolution of clinical signs (Figures 5.5A, B). Most interestingly, *in vitro* activation with LPS (Figure 5.6B) and *in vivo* activation with adjuvants (Figures 5.6A and B) during EAE immunization failed to influence the regulatory capabilities of E3 DCs. This suggests that concerns of DC plasticity preventing the useful application of DCs for immunotherapy may be unwarranted. To our knowledge, these are the first studies to generate a protective effect of hormonally derived reg-DCs in EAE.

Since EAE is a Th1 disease and protection has been seen through both Th2 responses and the generation of regulatory T cell populations, we evaluated the relative contribution of these factors in the E3 DC mediated protection. We found that increased IL-4 and decreased IFN-γ production underly the protection seen in mice at day 10 post-immunization (Figure 5.7) and that CD4+CD25+FoxP3+GITR+CTLA-4+ T-reg cells do not contribute to the protection. Thus, the protection afforded by E3 DCs is predominantly through a shift towards a Th2 response. This is in contrast to reports by Offner’s group that E2 generates Treg cells that mediate protective effects in EAE, suggesting that E2 and E3 may have differential influences on regulatory T cell populations(94). This may be due to differences in E2 versus E3 signaling on DCs, differences in DC populations between GM-CSF and FL expansion or differences in any combination of costimulatory expression, transcription factor expression or soluble mediator levels. Determining the relative contribution of these factors in how reg-DCs are generated and subsequently influence helper T cell responses will likely
demonstrate important mechanisms that can be utilized for therapeutic purposes in the
treatment of autoimmune diseases.

Taken together, we have shown that sex hormones play an important role in
influencing the induction of an immune response using the autoimmune disease model EAE.
Our findings in Chapter 3 demonstrate that sex hormones and genetics play an important role
on sexual dimorphism seen in EAE with different mouse strains. We report and characterize
a new model of EAE sexual dimorphism demonstrating increased severity in males and a
protective effect of female sex hormones. This data, in conjunction with findings in a late
pregnancy immunization model, suggested that sex hormones present in the environment at
the time of disease induction were critical. Due to the importance of DC-T cell interactions
during the disease induction, we hypothesized that DCs (and therefore the DC-T cell
interactions) were influenced by environmental influences such as maturation stimuli and sex
hormones. Chapters 4 developed methodologies to generate both in vitro and in vivo DCs
while Chapter 5 evaluated the influence of maturation stimuli and the protective pregnancy
hormone estriol on generating immunogenic and regulatory DCs, respectively. All of these
findings demonstrate that the environment plays an important role in shaping the T cell
response and that the immunomodulatory and protective actions of sex hormones may be
mediated primarily through DCs.
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