

THE ROLE OF ESTROGEN IN THE INDUCTION AND MODULATION OF
SYSTEMIC LUPUS ERYTHROMATOSUS (SLE)

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Systemic lupus erythematosus (SLE) is a systemic autoimmune disease, characterized by circulating auto-antibodies to nuclear and cytoplasmic self antigens. A striking female predominance has been documented in SLE, with a female to male occurrence ratio of 9:1. Although the sex discrepancy in SLE has been attributed to sex hormones especially the female sex steroid estrogen, its importance is still controversial and other mechanisms are proposed, such as X inactivation, imprinting, differential exposures, etc. The present studies were undertaken to investigate the role of estrogen in inducing and accelerating lupus in lupus-prone and non-autoimmune mice and identify the possible mechanisms involved.

We first ovariectomized or castrated female or male lupus-prone SNF₁ mice in order to determine the impact that removal of physiological levels of sex hormones would have on the pathogenesis of lupus nephritis; exogenous 17 β -estradiol (E-2) at a dose of 1mg/kg was also administered to male mice. The results suggested that E-2 accelerated and exacerbated SNF₁ lupus nephritis by inducing pathogenic idiotypic-reactive T cell populations that led to increased production of Id^{LN}F₁⁺ IgG which was deposited in the kidneys, resulting in nephritis. In contrast, the removal of physiological level of testosterone had no effect. E-2 was also shown to induce the lupus phenotype and disease in the non-autoimmune mice, DBF₁ mice. Further, the development of lupus nephritis in bone marrow chimeras derived from SNF₁ mice of different genders or between SNF₁ mice and the nonautoimmune-prone DBF₁ cross, required either a female host and/or the addition of exogenous E-2.

The possible mechanism(s) underlying the effect of estrogen were also investigated. Thymectomy of SNF₁ mice (at 30 days of age) delayed disease onset; however E-2 treatment induced disease in thymectomized mice, suggesting that the thymus was not required for E-2 induced upregulation of pathogenic idiotype-reactive T cell populations. Lastly, our data suggest that estrogen's effects on lupus nephritis were ER- α dependent; E-2 exposure led to decreased survival and nephritis in WT (ER α ^{+/+}) but not ER α ^{-/-}, and SNF₁ immune cells constitutively expressed more ER α compared to DBF₁, which was upregulated after E-2 exposure.

BIOGRAPHICAL SKETCH

Feng Feng earned his Bachelor of Medicine and Master of Science degree from Beijing Medical University (now Health Science Center, Peking University, China) in 1996 and 1999. In 1999 he started his graduate study in the field of Immunology at Cornell University. He earned his Master of Engineering in Computer Science in 2004.

To My Family

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LIST OF ABBREVIATIONS

Ab, antibody

APC, antigen presenting cell

Cast, castration

Con A, concanavalin A

DBF₁, (DBA x BALB/c)F₁

dsDNA, double-stranded deoxyribonucleic acid (DNA)

ER, estrogen receptor

E-2, 17 β -estradiol

ELISA, Enzyme-linked immunosorbent assay

ELISPOT, Enzyme-linked immunosorbent spot

H-2, refers to murine MHC molecules; e.g., H-2^d indicates the d haplotype at the MHC locus

Id^{LN}F₁, CRI family of nephritogenic idiotypes in SNF₁

Ig, immunoglobulin

Ovx, Ovariectomy

SNF₁, (SWR x NZB)F₁

SLE, Systemic Lupus Erythromatosus

ssDNA, single-stranded deoxyribonucleic acid (DNA)

Thx, thymectomy

T-1, testosterone

CHAPTER 1

Introduction

1.1 General

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease, characterized by circulating auto-antibodies to nuclear and cytoplasmic self antigens. It has a chronic disease course marked by remissions and relapses [1, 2]. Nearly every organ system of the body can be affected by SLE. The clinical manifestations vary according to organs attacked. Arthritis, skin rash, central nervous system dysfunction, cardiovascular disorder, and renal disease are very common to lupus patients [3-5]. The kidney is the most frequently involved organ in SLE; up to 80% of patients have clinically apparent glomerulonephritis and 10-20% of patients develop end-stage renal diseases [6, 7]. The overall incidence of SLE is 40-50 cases per 100,000. Women, in particular African-Americans, African-Caribbeans, Hispanic-Americans and Asians, at their child-bearing age, are at higher risk [8].

The recognition of SLE as an autoimmune disease evolved over many years. The earliest description and discussion of this disease has been credited to Hippocrates (460-370 BC), who used the term *herpes esthiomenos* at that time [9]. *Lupus* was first used to describe this disease by Rogerius in 1230 after finding that the facial skin lesions resembled those of a wolf bite [10]. The first description of the erythema as a butterfly rash and the erosive nature of the lesion came in 1845 by Herdinand von Hebra [9]. Pierre Louis Cazenave renamed the disease lupus erythemateaux and noted the female predominance of the disease and onset in young adults [10, 11]. By the early 1900's, William Osler recognized that this disease was not restricted to lesions of the skin, but also involved the central nervous system and a non-progressive arthritis in many patients. As the first step in understanding the systemic nature of this

disease, he proposed that some SLE patients actually had visceral lesions before or without ever displaying the classic skin lesions [12-14]. In 1920s, changes in renal function and cardiovascular system were associated with this disease. Hargrave's observation of the Lupus Erythematosus Cell or the LE cell in 1948 was a very important turning point in the diagnosis and understanding of SLE [15]. The LE cells were the end product of a phenomenon in which only the nucleus from a polymorphonuclear leukocyte was engulfed by a phagocyte [15]. This observation guided SLE research toward the recognition of lupus as an autoimmune disease. In 1959, investigations demonstrated that the LE phenomenon was induced by an IgG anti-nucleoprotein autoantibody [16]. Later, sera from SL patients were found to contain antibodies reactive with whole cells such as neurons, erythrocytes and platelets [17-19]. However, antibodies specific for nuclear antigens, including dsDNA, ssDNA, histones and other nuclear antigens, were the most prevalent autoantibodies found in the sera of lupus patients [3]. The role of immune complexes in the pathogenesis of SLE became apparent in the 1970's after the demonstration of the correlation between immune complex deposition and SLE pathology [20-22]. With the better understanding of the systemic and autoimmune nature of lupus, diagnosis criteria of SLE were determined by the American College of Rheumatology, revised in 1982 and updated in 1997 (Table 1.1) [23, 24]. While DNA was thought likely to be the inciting autoantigen after Holman and Kunkel's study [16, 25] showing the presence of DNA binding antibodies in the LE factors, recent evidence suggests that lupus glomerulonephritis can occur in the absence of those autoantibodies and breaking tolerance to nuclear Ags and dsDNA need not be the initial or necessary step in the pathogenesis of lupus nephritis [26, 27]. Abs to renal Ag, both crossreactive and direct-binding, have been postulated to be an important mechanism for IgG deposition, as shown by our and other laboratories [28-33].

SLE once was a rapidly fatal disease with a 5-year survival rate of only 50% in 1950 [34]. With the recent improvement in the understanding and treatment of this disease, there has been an substantial increase in the long-term survival of patients, with the 5-year survival rate exceeding 90% [34, 35] and 15-year survival rates approaching 80% [35]. In the 1900s, renal disease was the most common cause of mortality of SLE patients [36], and recently infection and cardiovascular diseases have been added as the major causes of death [4, 36, 37].

Genetic susceptibility plays an important role in the development of SLE. In monozygotic twins, the concordance rate for both twins developing disease is between 25% and 57%, while the frequency in dizygotic twins is only 1-2% [36, 38, 39]. The frequency of the disease in first-degree relatives of affected individuals is 10-20 fold increased compared with the prevalence of the population at large [40]. Genes in the Major Histocompatibility Complex (MHC) clearly are important in the development of disease, with class II genes as possible candidates. Susceptibility for developing anti-Ro and anti-La antibodies is associated with both the HLA_DR and DQ genes. MHC class I molecule B8 and the class II proteins DR2, DR3 and DRB1 (in Blacks) have been associated with an increased relative risk of acquiring lupus [41]. Further, more than 10 non-MHC loci from NZB, NZW or NZM mice have also been linked to various lupus traits, such as nephritis and autoantibody production. For example, the *Nba1* locus on chromosome 4 and *Nba2* on chromosome 1 have been associated with nephritis. The *Sle1* gene on chromosome 1 has been associated with both autoantibody production and nephritis. *Cgnz1* on chromosome 1 and *Adnz1* on chromosome 4 were linked to nephritis and antinuclear antibody, respectively [27]. Decreased levels of complement also influence the development of SLE, with C2, C5 and C8 deficiencies being the most harmful, as well as the C4 null allele [3].

Table 1.1. The 1982 revised criteria for classification of SLE, updated in 1997 [24]. Diagnosis of SLE requires that 4 of these 11 criteria be found either serially or simultaneously during any period of patient observation.

Criterion	Definition	Lay definition
1. malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds	Rash over the cheeks
2. discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions	Red raised patches
3. photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation	Reaction to sunlight, resulting in the development or increase in skin rash
4. oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician	Ulcers in the nose or mouth, usually painless
5. arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion	Arthritis in which the bones around the joints do not become destroyed, involving 2 or more joints.
6. serositis	a) pleuritis – convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusions OR b) pericarditis – documented by ECG or rub or evidence of pericardial effusion	Inflammation of the membranes surrounding the lungs. Inflammation of the sac that surrounds the heart.
7. renal disorder	a) persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed OR b) cellular casts – may be red cell, hemoglobin, granular, tubular, or mixed	Excessive protein in the urine and/or abnormal elements in the urine, derived from red and/or white blood cells and/or kidney tubule cells
8. neurologic disorder	a) seizures – in the absence of offending drugs or known metabolic derangements; e.g.: uremia, ketoacidosis, or electrolyte imbalance OR b) psychosis – in the absence of offending drugs or known metabolic derangements; e.g.: uremia, ketoacidosis, or electrolyte imbalance	Seizures (convulsions) and/or psychosis in the absence of drugs or metabolic disturbances which are known to cause such effects

Table 1.1 (Continued)

Criterion	Definition	Lay definition
9. hematologic disorder	a) hemolytic anemia – with reticulocytosis OR b) leukopenia – less than 4,000/mm ³ total on 2 or more occasions OR c) lymphopenia – less than 1,500/mm ³ on 2 or more occasions OR d) thrombocytopenia – less than 100,000/mm ³ in the absence of offending drugs	Hemolytic anemia or leukopenia (white blood count below 4,000 cells per cubic millimeter) or lymphopenia (less than 1,500 lymphocytes per cubic millimeter) or thrombocytopenia (less than 100,000 platelets per cubic millimeter)
10. immunologic disorder	a) anti-DNA: antibody to native DNA in abnormal titer OR b) anti-Sm: presence of antibody to Sm nuclear antigen OR c) positive finding of antiphospholipid antibodies based on: <ol style="list-style-type: none"> 1) an abnormal serum level of IgG or IgM anticardiolipin antibodies 2) a positive test result for lupus anticoagulant using a standard method 3) false positive serologic test for syphilis known to be positive for at least 6 months and confirmed by <i>Treponema pallidum</i> immobilization or fluorescent treponemal antibody absorption test 	Positive LE prep test, positive anti-DNA test, positive anti-Sm test or false positive syphilis test (VDRL)
11. antinuclear antibody	An abnormal titer for antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with “drug-induced lupus” syndrome	Positive test for antinuclear antibodies (ANA) in the absence of drugs known to induce it

Apoptosis is a highly regulated process by which cells can be destroyed. In normal immune circumstances, activity against self antigens is prevented by several mechanisms, including the *Apo-1/Fas* pathway of apoptosis, which has been shown to be involved in the process of immune tolerance by deletion of autoreactive T cells and B cells [42]. In animal models of SLE, aberrant expression of apoptosis-related genes, such as overexpression of *Bcl-2* or defects in *Fas* as in MRL-lpr/lpr mice have been associated with the development of a lupus-like autoimmune disorder [43, 44]. However, no defect in expression or function of the Fas receptor in humans with SLE has been found [45].

On the other hand, apoptosis promotes the release of normally sequestered autoantigen and results in prolonged exposure of the immune system to nuclear and cytoplasmic components on the surface of apoptotic blebs, leading to increased autoantibody production. Normal mice injected with apoptotic thymocytes developed antinuclear antibodies, anticardiolipin and anti-ssDNA antibodies, along with IgG deposition in the glomeruli [46]. Increased apoptosis of macrophages in lupus-prone SNF₁ mice led to accelerated glomerular nephritis and autoantibody production [47]. However, other studies have shown that the generalized immune activation seen in SLE is accompanied by a decreased rate of apoptosis. Increased levels of soluble *Fas* are often seen in SLE, inhibiting *Fas* mediated apoptosis of lymphocytes [48]. Lymphadenopathy is seen in approximately 70% of lupus patients, resulting in an accumulation of lymphocytes and a persistence of autoreactive cells [49]. SLE patients have many T cells with increased levels of *Bcl-2* [39]. All of these results suggest that there are significant alterations in the apoptotic mechanisms of patients with lupus.

1.2 Gender bias and autoimmune diseases

Autoimmune diseases comprise nearly 70 distinct clinical entities that affect about 5% of the US population. Among the earliest and most interesting observations was the gender bias in the incidence of autoimmune disease between men and women. Some of the more common autoimmune disorders with their sex distribution are shown in Figure 1.1. The most apparent gender bias is observed in SLE, Sjogren's syndrome, autoimmune thyroid disease and scleroderma; in these diseases more than 80% of the patient population is female. In rheumatoid arthritis, multiple sclerosis, and myasthenia gravis, 60-75% of the patients are women. In diseases such as sarcoid, the inflammatory bowel diseases and immune-mediated diabetes, the female to male ratio

of disease occurrence is about 1:1 [50-52], while B27 spondyloarthropathy and Goodpasture's disease are slightly male predominant [50].

Gender bias has also been observed in experimental animal models of autoimmune disease. The (NZB×NZW)_{F1} mouse model of lupus shows high female incidence and severity (severe lupus nephritis and early death) [53]. In another lupus-prone murine model, (SWR×NZB)_{F1}, female mice spontaneously develop early disease and almost all of the mice die by 12 months of age from chronic glomerulonephritis, while the male mice develop milder lupus nephritis later in their lives and can live up to 18-20 months of age [54]. However, the MRL lpr/lpr model is sex neutral, and the BXSb model is male predominant [55]. Sex differences in disease incidence and severity have also been noted in animal model of autoimmune thyroiditis and spondylophathy [56, 57].

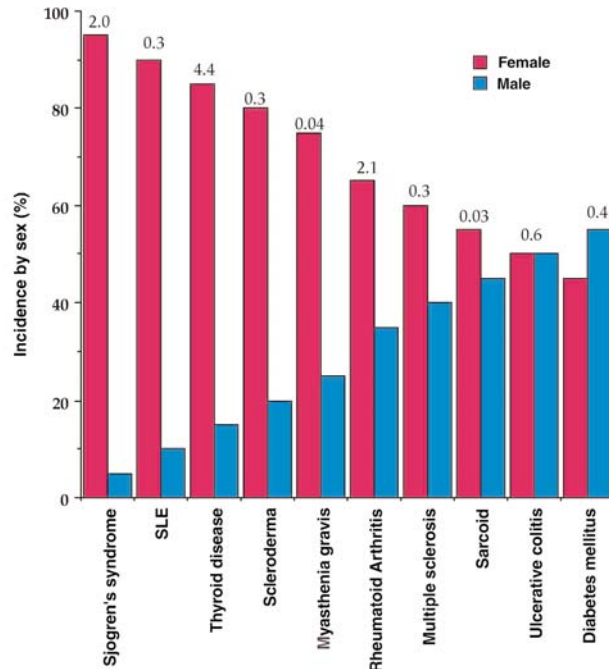


Figure 1.1 The sex distribution of the major autoimmune diseases [58]. The numbers above the bars refer to the total number of disease cases (x1,000,000) in the USA.

Although sex differences are well documented in many autoimmune diseases and some experimental animal models, the mechanism(s) are not well understood. The high female predilection of SLE, along with the significant increase in disease incidence in females after puberty, the reversal of this phenomenon after menopause, and the fluctuation in disease severity throughout the menstrual cycle and pregnancy, suggest the important role of sex hormones (especially female sex hormone, estrogen) in gender bias of autoimmune diseases [59-61]. However, non-hormonal mechanisms, such as skewed X chromosome inactivation, differential exposure, behavior effects, etc., have also been suggested to contribute to loss of immune tolerance and therefore, the increased incidence of autoimmune diseases in females (Table 1.2) [62]. The most striking differences in incidence occur when exposures to infectious agents or toxins are different between the sexes. Therefore, differences in exposure to infectious agents have been proposed to be the plausible explanation for the sex differences by Lockshin [62] rather than sex hormone influences. A better understanding of the immunomodulatory effects of sex hormones will be required to reconcile their role in autoimmune disease.

1.3 Pathogenesis of SLE in SNF1 murine model

Systemic lupus erythematosus (SLE) is a heterogenous autoimmune disease characterized by the production of high titers of immune complexes, which are deposited in multiple target tissues, particularly the kidney [3]. The deposition of immune complexes subsequently results in inflammatory responses through a multitude of events or cascades within the complement or coagulation pathway and leads to damage of target tissue [3, 63]. The pathogenesis of SLE arises from defective

Table 1.2 Nonimmunological mechanisms by which men and women differ in nonautoimmune disease incidence [62].

Level of Study	Mechanism	Example
In utero effect	In utero nutrition, hormone exposure determine adult phenotype	Sexual behavior, prostate size, adult onset diabetes can be influenced by prenatal exposures in animals; girls with prenatal growth restriction have insulin resistance, ovarian hyporesponsiveness later in life
Imprinted gene	Maternal or paternal origin of a gene influences phenotype differently in men and women	Turner syndrome patients whose X chromosome is of paternal origin are more aggressive than are those whose X chromosome is of maternal origin
X inactivation	Because of incomplete inactivation, XX cells may produce higher levels of an X-chromosome gene product than do XY cells	Gastrin-releasing peptide receptor is higher in women, causing increased risk of lung cancer in women smokers compared with men
X-chromosome mosaicism	In the presence of a mutated X-chromosome gene, XX individuals have one healthy allele, but an XY or XO individual does not	Female patients survive incontinentia pigmenti because unaffected X chromosomes exist in mosaic with affected; male patients have only affected X and die
Hormone	Estrogen affects nonhormone cell receptors	Women have a longer QTc interval than men; cardiac ion channel sensitivity renders women more susceptible to drug-induced arrhythmia
Organ difference	Organ function differs between the sexes	Men and women use different parts of the brain in language; gastrointestinal transit times differ in men and women
Exposure	Sexes encounter exogenous substances at different rates	Toxic-oil scleroderma affects women; procainamide-induced lupus affects men
Exogenous chemical processing	Exogenous substances are handled differently by the sexes	κ - and μ -Opioid drugs are more effective in young adult women than in men
Life event	Effects of pregnancy	Fetal cells circulate longer and at higher quantity in scleroderma patients than controls; rheumatoid arthritis remits during pregnancy, related to HLA mismatch between mother and fetus
Behavior	Social activities have different effects in the sexes	Athleticism, diet lead to amenorrhea and osteoporosis in women but not men

central or peripheral tolerance mechanisms, an unusually high activation of T and B lymphocytes including an alteration in cytokine levels and antibody production, and/or insufficient suppression of autoreactive lymphocytes [48]. T cells have definitively been shown to be critical to the development of disease, with both CD4⁺CD8⁻ and CD4⁻CD8⁺ involved [64]. T cell lines with these phenotypes derived from mice and humans with lupus induce the production of anti-DNA antibodies by SNF₁ B cells [65]. Contact-dependent cognate interaction between pathogenic T helper and B cells in SLE most likely occurs. In mice and humans, class switching from IgM to IgG occurs concomitantly with the progression of autoimmune disease [3, 54, 66]. Neutrophils and monocytes as well as the complement system were all shown to be involved in the pathogenesis of lupus nephritis [3].

Study of a systemic autoimmune disease such SLE in humans is difficult due to the numerous stochastic influences. The disease itself or certain treatments can alter endocrine or immune system function [67], so that many murine models are more routinely used to study SLE, such as, (NZB x SWR)F₁, MRL lpr/lpr, NZM and BXSB mouse strains. The (NZB x NZW)F₁ (SNF₁) mouse, used in our laboratory, develops glomerulonephritis with striking similarities to human SLE, such as the presence of anti-nuclear antibodies (ANAs), extensive renal impairment, and more severe disease in females [28, 29, 54, 66]. The NZB mouse develops autoimmune hemolytic anemia, along with antinuclear antibodies and certain other immunologic similarities with SLE patients later in life [68, 69]. Mild glomerulonephritis and autoantibodies against erythrocytes and ds and ss-DNA occur [68], usually resulting in death within 1.5 years [29]. The SWR mouse is a non-autoimmune strain; however, lupus nephritis in the F₁ offspring of the cross between the NZB and the non-autoimmune SWR strains was first noted by Syamal Datta [70]. As with human lupus, disease in female mice of this model was characterized by high titered anti-DNA antibodies, cationic immune

complex glomerulonephritis within 6-9 months and early death by one year, while male mice have milder disease and longer survival. These immune complexes bind to the anionic basement membrane of the kidney [29, 30]. The charge, size, and antigenic characteristics of autoantibodies are important in determining which antibodies can accelerate glomerulonephritis [3]. In 1985, Gavalchin and co-workers studied the anti-DNA antibodies produced by the SNF₁ in order to understand the SWR's contribution to the pathogenesis of lupus [71]. Half of the monoclonal anti-DNA antibodies from the SNF₁ were found to be IgG in class, and sixty-five percent of these antibodies were cationic in charge. Further, the most cationic of the anti-DNA antibodies were isotypically IgG2b, an isotype which has the greatest capacity to fix complement and therefore could lead to immune complex deposition and damage of the glomerulus; some of them also had the SWR allotype [71-73]. Taken together, the results suggested that many of the anti-DNA antibodies produced by SNF₁ had an increased potential for deposition in glomeruli and that they were of origin, at least in part, of genes from the non-autoimmune SWR parent [71].

Immunoglobulin deposited in nephritic SNF₁ kidneys share a family of idiotypic markers, known as Id^{LN}F₁ (Idiotypes-lupus nephritis-SNF₁) [72, 73]. Id^{LN}F₁ is a family of cross reactive idiotypes (CRIs) that were identified by their ability to react with an antiserum induced in rabbits immunized with kidney immunoglobulin eluate [29], and Id^{LN}F₁ idiotypic markers were consistently found in the eluted Ig at all stages of nephritis. Further, the Id^{LN}F₁ Ig from SNF₁ renal lesions had both the NZB and the SWR allotypes represented equally, with the majority of the highly cationic Id^{LN}F₁ Ig bearing the SWR allotype [72]. A hypothesis invoking a role for idiotypic regulation in the pathogenesis of SLE in SNF₁ mice is particularly attractive, since in this disease autoantibodies are produced which are reactive with seemingly unrelated antigens, which could, in fact, be related by the idiotypes they express. This

hypothesis also does not require that a specific antigen elicit disease, and indeed, none has been yet identified for SLE. Moreover, the fact that the relevant antigen could be a self antigen, found on immunoglobulin, would explain the chronic nature of SLE, which normally follows a course of flares and remissions. Thus, we hypothesized that idiotypic network interactions most likely regulate the production of autoantibodies in these mice and that disease pathogenesis of SLE in the SNF₁ mouse may be caused primarily by the dysregulation of pathogenic idiotypes, with the production of anti-DNA antibodies having less significance [72].

Studies using the SNF₁ model have found that the production of Id^{LN}F₁ IgG is increased at 20-24 weeks of age due to an increase in CD4⁺ Id^{LN}F₁-reactive T cells and a concomitant decrease in CD8⁺ Id^{LN}F₁-reactive T cells[54]. As the ratio of total CD4⁺ to CD8⁺ T cells increased, a class switch from IgM to IgG occurred within the Id^{LN}F₁ expressing antibodies. The Id^{LN}F₁ deposition in the kidneys began at 20 weeks, increased three fold by 24 weeks, and continued to increase linearly until the age of 36 weeks, when it plateaued [28, 54, 66]. These observations led to the hypothesis that the Id^{LN}F₁- reactive T cells promoted the class switch to the more pathogenic IgG subclass, thus accelerating disease [54]. In support of this mechanism, Id^{LN}F₁-reactive CD4⁺ T cells induced Id^{LN}F₁⁺ Ig production *in vitro* and accelerated disease when adoptively transferred into young pre-nephritic SNF₁ mice [28]. Furthermore, treatment with anti- Id^{LN}F₁ antibody inhibited the production of Id^{LN}F₁⁺ Ig and delayed the onset of nephritis. Interestingly, autoreactive T cells, including those found in SNF₁ mice, often possess forbidden self-reactive T-cell receptors that were not eliminated during thymic selection. Forbidden TcRs expressed by SNF₁ T cells can include Vβ6, Vβ8, Vβ11, and Vβ17a [66].

Antibody 540, which expressed Id^{LN}F₁ and is deposited in the glomeruli of affected mice [72, 73] had previously been shown to induce the proliferation of the

pathogenic T-cell clones B6 and D2. Peptides from the heavy chain of 540 were isolated and tested for the ability to stimulate T cells from nephritic mice and the T cell clones. Overlapping peptides 54-66 (KDGTKYNEKFKGK) and 62-73 (KFKGKATLTSDK) stimulated these T cells to a greater extent than other peptides, and were nearly as effective as 540 itself. The triple basic amino acid motif BX(X)BXB contained in the overlapping region has been found in autoantibodies more often than would be expected by chance alone [74]. Interestingly, recent work in our laboratory has found that immunization of pre-nephritic female SNF1 mice with this peptide has a protective effect, with prolonged survival and reduced kidney damage (Stoll *et al*, in preparation).

1.4 Sex hormonal influences on the immune functions

Sex hormones clearly have a major impact on both the innate and the adaptive immune responses. The humoral immune response of females, in general, is more vigorous than that of males, with females usually producing higher levels of total immunoglobulin in response to infection or immunization. This difference first becomes apparent during puberty and persists throughout the female reproductive years [60, 75, 76]. Females also reject allografts more rapidly, are relatively resistant to the induction of tolerance and have more effective anti-tumor immunity [77-81]. The higher immune responsiveness of women has also been proposed to contribute to their increased susceptibility to some autoimmune diseases, including SLE, which has a prevalence that is 9-10 times higher in women than men. Indeed, modulation of the clinical manifestations of SLE correlates with alterations in steroid hormone levels, and may be exacerbated during pregnancy, postpartum and, in some cases, with oral contraceptive use [82, 83]. Male and female patients with SLE are often hyperestrogenic and/or hypoandrogenic [84-86], and SLE is found at a higher

incidence in patients with Klinefelter's syndrome [87, 88], a genetic disease of males that is characterized by a variety of sex hormone abnormalities, including increased production of estrogen. These data, therefore, point to a potential significant role for estrogen in the gender-specific modulation of immune responses.

How sex hormones modulate immune cells remains a fundamental unanswered question in immunology. Estrogens generally have been implicated as enhancers of the immune response (at least the humoral immune responses), while androgens and progesterone are considered natural immune suppressors [89]. A variety of alterations in immune responses have been observed after estrogen exposure including decreased cell-mediated immune responses and increased humoral immune responses, including decreased responsiveness to mitogens [90], suppressed delayed type hypersensitivity [91], decreased peripheral T cells and decreased NK cell activity [92], and many others. On the other hand, generation of autoreactive T cells [93] and elevated cytokine production [94], have been noted.

Estrogen stimulates CD4⁺ T_H2 lymphocytes and promotes interactions between T and B cells. Estrogen led to increased secretion of IL-4, IL-5, IL-6 and IL-10 by T_H2 lymphocytes [60, 75, 76, 95]. These cytokines are potent stimulators of B-cell proliferation, maturation into plasma cells, and synthesis of antibody. SLE patients have increased levels of IL-6 and IL-10, which can be directly correlated with clinical disease severity [76, 95]. Increased levels of IL-4 in NZB/W and MRL/lpr mice are also evident; IL-4 transgenic mice develop some SLE symptoms, along with hemolytic anemia and immune-mediated renal disease [3]. IL-4 stimulates the activation of antigen-primed B cells, as well as the proliferation and differentiation of activated B and T lymphocytes, and induces a class switch within antibody production from IgM to IgG [63]. In a healthy control, increased estrogen leads to an increase in IFN- γ concentration, presumably as a mechanism of feedback inhibition to prevent

unwanted stimulation of T_H2 cells. In lupus patients, the estrogen-induced increase in IFN- γ secretion is absent [76]. IFN- γ is also found to be important in the initiation of autoimmune disease, but not at later stages [96]. As B/W mice age, the levels of IFN- γ decrease [97]. The administration of IFN- γ to Balb/C mice injected with the 16/6 idiotypic or to B/W mice leads to an increase in lupus symptoms and manifestations, and a decrease in lifespan [97, 98].

IL-1, an acute phase reactant secreted by macrophages that enhances clonal T-cell proliferation on antigen encounter, increases in a dose-dependent manner with macrophage exposure to estrogen [60]. Pro-inflammatory TNF- α , secreted by activated macrophages and monocytes that promotes activation of T cells, is increased after physiologic estradiol administration. The increased production of IL-1 and TNF- α by macrophages leads to persistence of the disease [99]. IL-1 can stimulate activation of helper T cells and promote maturation and clonal expansion of B cells. TNF- α can upregulate the production of other inflammatory cytokines [63], induces apoptosis [63, 100], and has been associated with weight loss [63]. TNF- α can be elevated in SLE patients [39], and in NZB/W and MRL/lpr murine models for lupus [3], while TNF- α has also been shown to lead to increased survival in B/W mice [97].

Estrogen can also modulate IL-2 and TGF- β production [101]. Further, estrogen upregulates expression of the anti-apoptotic *Bcl-2* gene in naive B cells [102, 103]. It has been demonstrated that *Bcl-2* expression blocks tolerance induction, potentiating the survival of autoreactive T and B cell clones. T cells from lupus patients express higher levels of *Bcl-2* than do those of healthy controls [102].

Androgens are also important in development, and are considered natural immune suppressors [89]. It is found that androgens downregulate T and B cell interactions and promote cell-mediated cytotoxic processes through activation of CD4⁺ T_H1 and CD8⁺ lymphocytes [60, 61, 104]. The administration of testosterone

and dihydrotestosterone both result in decreased thymic mass and a decrease in B cell numbers in the bone marrow. Androgen removal by castration leads to an expanded and more diverse B cell repertoire, with a concomitantly greater likelihood that autoreactive cells will be present. Enhanced autoantibody production by splenocytes from castrated male mice supports this theory [105]. Patients with Klinefelter syndrome (androgen-deficiency) have a higher incidence of autoimmune diseases, including SLE compared to the normal population [60, 106].

1.5 Thymus and autoimmunity

The thymus is the primary lymphoid organ in the body responsible for T lymphocyte development. This bilobed organ located above the heart reaches maximal size at puberty, and then continues to undergo atrophy with aging [63]. During T lymphopoiesis, lymphocytes with TCRs that can bind antigens with adequate affinity in the context of self-MHC on thymic epithelial cells are positively selected [107]. Next, autoreactive T cells that bind the MHC/self-antigen with very high affinity are negatively selected by apoptosis [63, 107]. The remaining cells, only about 2% of the thymocytes generated during development, are exported to the periphery. The thymus also generates an important population of regulatory T cells that are able to regulate autoreactive cells that have escaped selection [108].

One of the consequences of estrogen treatment is thymic atrophy, which has led us to hypothesize that sex steroids may contribute to the development of autoimmunity during the development and maturation of immune effector cells in the thymus. The decrease in thymic mass begins at 6 weeks in mice (the age of sexual maturity in mice), and the decrease in thymic function begins essentially at birth [109]. Further, thymic atrophy is delayed following gonadectomy, which is related to the loss of hormones associated with the removal of the sex organs. In contrast, administration

of either androgen or estrogen induced involution of the thymus. However, although the atrophic effect of estrogen on thymus has been known since 1950s [110], the mechanism(s) is still not known.

Estrogen has long been found to affect the thymocyte development. It was determined that E-2 treatment led to a significant reduction in double positive T cells and an increase in immature double negative T cells and CD4⁺ or CD8⁺ single positive T cells [111]. Later, E-2 treatment was found to result in a significant increase in the percentage of CD44⁺CD25⁻CD4⁻CD8⁻CD3⁻ cells compared to other populations [112-114]. Although all thymocyte populations are decreased in number by E-2 treatment, the increase in CD44⁺CD25⁻CD4⁻CD8⁻CD3⁻ cells suggests that some immature thymocyte populations may be more vulnerable to E-2. It was also hypothesized that E-2-induced thymic atrophy occurred as a result of a proliferative block in early T cell development in the thymus [112-114]. There are controversies concerning the potential role of apoptosis in E-2 induced thymic atrophy. It has been shown that an increase in apoptosis *in vivo* can not be detected after E-2 treatment [114-117] and over expression of *Bcl-2* within thymocytes did not prevent E-2 induced thymic atrophy [117]. On the other hand, other groups found that increased apoptosis can be detected *in vitro* after E-2 treatment [112, 115, 118]. Considering the rapid clearance of apoptotic cells by phagocytic cells, better detection techniques for *in vivo* apoptosis must be developed before we can conclude whether or not E-2 induced thymic involution is through apoptosis.

When the thymus is removed in newborn mice, defects in development and selection are noted [119]. These effects are similar to those in mice with a severely aged thymus, and include a decrease in the total number of peripheral T cells is observed, with a concomitant increase in the number of potentially autoreactive T cells [107]. Thymectomy has been proven to modulate the course of disease in organ-

specific autoimmunity; however, conflicting results have been obtained for murine models of SLE. The dependence on the thymus for disease development varies between strains, with acceleration, protection, or no effect observed after thymectomy. Neonatal thymectomy (before day 3 after birth) in non-autoimmune mice leads to the development of organ-specific autoimmune disease [119-123]. Which organs are affected depends on the strain of mouse [124], but may include prostate, testis, ovaries, thyroid and stomach. The underlying pathogenesis mechanism is thought to be related to the loss of regulatory T cells (naturally arising CD25⁺CD4⁺ T cells) in the thymectomized mice and the consequent activation of the neonatal T cell repertoire. The results of thymectomy on systemic autoimmune diseases, such as SLE, differ depending on the strain of mice and the age at thymectomy [121, 122, 125, 126]. No effect, protection and aggravation by thymectomy have all been reported in different mouse strains [125, 126]. Steinberg *et al* reported that neonatal thymectomy of MRL/lpr mice led to a marked reduction in their characteristic massive lymphadenopathy, as well as significantly reduced antibodies to native DNA and prolonged survival [126]; in contrast, neonatal thymectomy of (NZB x NZW)F₁ mice led to accelerated disease [126]. Further, the protective effect of thymectomy in MRL/lpr mice was diminished when thymectomy was delayed beyond 3 weeks post-natally [126]. Similar results have been obtained in another study using additional mouse strains [125]. Recently, Bagavant *et al.* reported a differential effect of neonatal thymectomy on systemic and organ-specific autoimmune disease, in which thymectomy paradoxically protected SNF₁ mice from genetically prone lupus-like glomerulonephritis, yet promoted *de novo* organ-specific autoimmunity [121]. However, in this case, the mechanism of protection was not identified, and recently was proved to not be related to the suppressive effect of regulatory CD25⁺CD4⁺ T cells [122]. Taken together, the variability in the responses of the mice after

thymectomy suggests that the pathogenic and protective mechanisms might vary among different lupus mouse models.

Although the thymus is necessary for T cell development, extrathymic pathways for T cell generation are possible and important in situations when a thymus is not present, or is severely involuted. There is evidence that the development of a functional T cell repertoire can occur without thymic microenvironment [107], both in the spleen and in peripheral lymph nodes [127, 128]. Further, both negative and positive selection have been found during the extrathymic development of T lymphocytes [107]. However, cells produced through extrathymic pathways have an increased proportion of autoreactive cells.

1.6 Estrogen receptor (ER) and ER knock out mice

Estrogens exert their functions through interactions with estrogen receptors, estrogen receptor- α (ER α) and estrogen receptor- β (ER β) [129-134]. Estrogen receptors are ligand-activated transcription factors that bind to estrogen response elements (specific DNA sequences) of target genes and alter their transcription rates. These receptors are encoded by distinct genes, ESR1 and ESR2, found on different chromosomes, and several mRNA splice variants exist for both receptors [135]. The ERs belong to the nuclear hormone receptor super gene family that has a DNA-binding domain and separate protein domains that are responsible for transcriptional activation. While ER α and ER β have a considerable degree of similarity, the amino terminus of the two proteins is poorly conserved [132]. This variation between the two receptors explains why ER α and ER β differ in their capacity to bind coregulators and essentially have different functions [136]. For example, in cell lines and in some tissue, E-2 in the presence of ER α induces proliferation, but in the presence of ER β it inhibits proliferation [137-139]. Both ER α and ER β are found in many tissues in the body,

including ovaries, testes, prostate, and hypothalamus, and immune cells [140].

However ER α is the dominant receptor in the adult uterus, and oviduct, pituitary, heart and aorta are found to only express ER α [141].

Estrogens induce cellular changes through different mechanisms (Figure 1.2). In the classical mechanism of estrogen action, estrogens enter the cell by diffusion and bind to ERs. Prior to ligand binding, the ERs are in a monomeric inactive state in the cytoplasm complexed with heat shock proteins (hsp70 and hsp90) [142-145]. After binding, the receptor becomes phosphorylated and disassociates from HSPs. Then the receptor/estrogen complex in dimeric form (either homodimer or heterdimer) moves to the nucleus [146-148], and binds to estrogen response element sequences directly or indirectly through protein-protein interactions with activator protein 1(AP1) or SP1 sites in the promoter region of target genes, resulting in the recruitment of coregulator proteins and changes in the transcriptional rate of target genes. This classical mechanism, also known as the genomic mechanism, occurs after several hours. Alternatively, estrogen can act more quickly (within seconds or minutes). This non-genomic mechanism is thought to involve the membrane-bound ER or membrane linked estrogen-binding proteins, leading to responses such as increased levels of intracellular Ca²⁺ or NO, and activation of certain kinases [149, 150].

Both ER α and ER β [129-134] have been found to be expressed on many immune cells constitutively and at increased levels after administration of E-2. Estrogen has also been shown to modulate the numbers and functions of peripheral immune cells, as well as the density of some of the cell surface antigens they express, including activation and adhesion molecules. A recent study that investigated ER α and ER β expression among resting and activated PBMC subsets found the ER α was

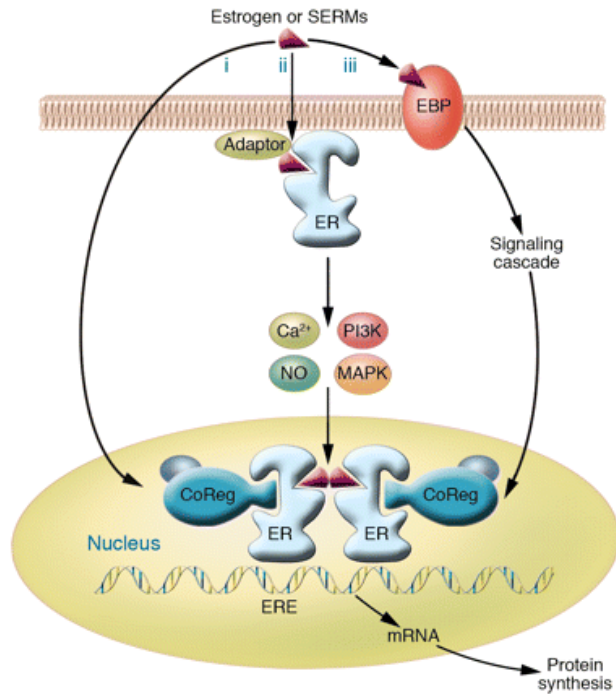


Figure 1.2 Models of estrogen action [151]. In the "classical" pathway of estrogen action (i), estrogen or other selective estrogen receptor modulators (SERMs) bind to the estrogen receptor (ER), a ligand-activated transcription factor that regulates transcription of target genes in the nucleus by binding to estrogen response element (ERE) regulatory sequences in target genes and recruiting coregulatory proteins (CoRegs) such as coactivators. Rapid or "nongenomic" effects of estrogen may also occur through the ER located in or adjacent to the plasma membrane (ii), and may require the presence of "adaptor" proteins, which target the ER to the membrane. Activation of the membrane ER leads to a rapid change in cellular signaling molecules and stimulation of kinase activity, which in turn may affect transcription. Lastly, other non-ER membrane-associated estrogen-binding proteins (EBPs) may also trigger an intracellular response (iii).

expressed at higher levels than ER β in CD4⁺ T cells, while levels were lower and similar in CD8⁺ T cells and monocytes. B cells, on the other hand, expressed higher ER β . Further, gender differences in ER expression were noted [152]. Administration of low doses of E-2 resulted in a striking increase in antigen-specific CD4⁺ T cell responses, that was ER α -dependent [153], and in another study it was shown that

estrogen exposure directly activated T cells, via cell membrane ER [154], suggesting the possibility that differences in the numbers and types of ER expressed on the immune cells of some individuals could explain the differences in their T cell responses to antigen. This hypothesis was further supported by the observation that calcineurin, CD40L expression and PP2B phosphatase activity were increased when SLE T cells, but not T cells from normal women, were cultured with E-2 [149, 155]. Since these responses could be specifically inhibited by the ER antagonist, ICI 182 780, it appeared that estrogen activation of these T cells was ER-dependent [156]. B cell development in the marrow may also be modified by exposure to estrogen [157], resulting in ER-dependent upregulation of several genes involved in B cell activation and survival [44, 102, 158]. This could result in the modulation of B cell populations including autoreactive B cells, as suggested by the observation that autoantibody was produced when non-autoimmune mice were treated with estrogen [159, 160]. NK cells may also be modulated by estrogen and were found in one study to be increased [161], or in another decreased [162] after estrogen exposure in two separate studies, presumably through ER [163]. Estrogen treatment was found to promote the differentiation of a CD11c⁺, CD11b (int) DC population that displayed high levels of MHC Class II and CD86 (B7-2), while in another study, anti-estrogen treatment resulted in mature dendritic cells (DC) that were less effective than immature DC in presenting antigen to allogeneic T cells [164], possibly by altering cytokine production in these cells [165]. This suggested that E-2 could augment the numbers of potent APCs [166], as well as modulate cytokine and chemokine expression. Estrogen induced T_H2-type cytokine response and gender differences in cytokine secretion [167-173], and this has been postulated as an important mechanism in E-2-induced modulation of T cells. Not unexpectedly, estrogen has been shown to modulate autoimmune diseases such as multiple sclerosis (MS), possibly by altering cytokines

as suggested in a study showing that CD4⁺ myelin-basic protein specific T cells clones derived from patients with MS [174], showed changes in the levels of IL-10, IFN- γ and TNF- α production after culture with estrogen, and this effect was ER α dependent [175]. Estrogen also increases the expression of adhesion molecules including E-selectin and VCAM-I [176].

The first ER knockout mouse (ER α KO) was constructed in 1993 [177], at which time only ER α had been identified. This ER α KO mouse was made by the insertional disruption of the first translated exon (exon 1) with a neomycin resistance cassette. The insertion affected the AF-1 domain of the ER protein and altered the reading frame of ER α transcript. The ER α KO was unresponsive to estrogens in uterine weight and vaginal physiology following E-2 treatment [177]. However, the mouse displayed 5% E-2 binding in uterine preparations, which was attributed to non-active splice variants and another unknown estrogen-binding protein [177]. Later, several additional splice variants were reported; some of them could be translated into truncated proteins that were shown to express estrogen dependent activity, but to a significantly decreased extent compared to wild type ER α [178-182]. A second ER α knockout mouse was generated by Dupont and coworkers [183], and this mouse was constructed by disruption of the second translated ER α exon, which resulted in a more complete ER α null strain than ER α KO mouse. No detectable ER α transcripts or truncated protein was found in this second knockout mouse [180, 183].

1.7 Specific Aims of research

The gender bias in the prevalence of autoimmune diseases has been well documented since the earliest descriptions of those diseases. However, the underlying mechanisms are not well understood. Immunomodulatory effects of sex hormones, specifically estrogens and androgens, have been thought to be critical. In this work, we

sought to identify the role of estrogen in the gender bias and pathogenesis of lupus nephritis in the lupus-prone SNF₁ mice.

1) In this study, we first identified the effect of natural levels of estrogen and androgen in the pathogenesis of lupus nephritis in SNF₁ mice using a castration model. Since sex steroids are synthesized mainly in testes of male and ovaries of females, castration will significantly reduce the serum levels of those sex hormones in both male and female mice [184]. By comparing the kinetics of disease development in castrated versus sham-castrated SNF₁ mice, we will discover how normal levels of androgen and estrogen act to affect the pathogenesis of lupus nephritis in the SNF₁ disease model.

2) In the next study, exogenous estrogen was also administered to male SNF₁ mice in order to investigate whether estrogen is responsible for the accelerated pathogenesis of nephritis seen in female mice. The effect of E-2 treatment of non-lupus-prone DBF₁ mice was also investigated with the aim to understand if genetic predisposition towards autoimmunity was required for the pathogenic effects of estrogen, or whether non-autoimmune susceptible mice could be affected.

3) Irradiated bone marrow chimeras between different genders and strains were also generated in order to further identify the role of estrogen in the pathogenesis of lupus nephritis. Bone marrow transplantation can be used experimentally to examine the development of lymphocytes, as opposed to their effector functions. The chimeric model can be used to further understand the roles of both genetic factors and sex hormones in the development of glomerular nephritis, and identify the targets of estrogen effects.

4) One of the consequences of estrogen treatment is thymus atrophy. To identify whether the effect of estrogen on lupus pathogenesis involves modulation of T

cell development in the thymus, we examine disease kinetics of thymectomized mice with or without E-2 administration.

5) Estrogens exert their function through interactions with estrogen receptors. Therefore, the estrogen receptor- α (ER α) effect on estrogen-induced pathogenesis of nephritis is examined using ER α -deficient mice. Lastly, estrogen receptor- α and β expressions on immune cells are also analyzed for both SNF₁ mice and DBF₁ mice after estrogen treatment in order to identify the targets of estrogen effects.

CHAPTER 2

The impact of ovariectomy and castration on the pathogenesis of systemic lupus erythematosus in (SWR x NZB)F₁ mice

Abstract

Systemic lupus erythematosus (SLE) is a complex multi-systemic autoimmune disease, characterized by circulating auto-antibodies to nuclear and cytoplasmic self antigens. Similar to many other autoimmune diseases, the incidence of SLE is higher (9 to 10 times) in females compared with males. Sex hormones, estrogens and androgens, are thought to be potential mediators of the gender bias in this disease, but a direct relationship and the underlying mechanism(s) are not well understood as yet. In the present study, we castrated female and male SNF₁ mice in order to determine the impact of physiological serum levels of sex hormones on the pathogenesis of lupus nephritis in this lupus-prone mouse model. Exogenous β -estradiol 17-valerate (E-2) was also administered to castrated and intact male mice. We found that ovariectomy in female mice led to significantly decreased serum levels of E-2, and prolonged survival, as well as reduced kidney damage compared to the intact controls. The immune responses of ovariectomized mice were also affected with reduced proliferation to pathogenic peptide aa62-73, modulated cytokine production, and decreased idiotypic IgG production by B cells. In contrast, castration had little effect on the course of disease in male SNF₁ mice indicating that the natural level of androgen does not antagonize the response to estrogen in SNF₁ mice. These results suggested that estrogen contributes to the pathogenesis of lupus in SNF₁ mice, and may accelerate lupus nephritis.

2.1 Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterized by an excessive immune and inflammatory response targeting multiple tissues, organs, and systems of the body. Periods of flares and remission are associated with this disease, with common clinical manifestations such as arthritis, skin rash, central nervous system dysfunction, cardiovascular and renal diseases. Similar to other autoimmune diseases, SLE shows a striking gender bias and principally affects women in the childbearing age group [185]. The occurrence of SLE in females is 9 times higher than in males [1, 2, 186, 187]. Although the gender bias has been well documented in many autoimmune diseases, the underlying mechanisms and exact mediators are not known.

Sex hormones, mainly estrogens and androgens, are thought to contribute significantly to the gender dimorphism in immune responses. In general, female mice produce more antibodies and show more vigorous T cell activation than male mice [188, 189]. Similar effects have been observed in humans [190, 191]; for example, it is found that women have more CD4⁺ lymphocytes compared to men, which likely contributes to their stronger immune responses. The male sex hormones, androgens, exert suppressive effects on both humoral and cellular immune responses, and may represent natural anti-inflammatory hormones. *In vitro* studies have shown that androgens decrease the secretion of IL-4, IL-5 and IFN- γ cytokines by T lymphocytes [192], and possibly inhibit antibody production by B cells [193]. In contrast, estrogens exert immunoenhancing activities, at least on humoral immune responses. Cytokine secretion is generally enhanced *in vitro* in the presence of estrogen, especially for IFN- γ , IL-1, IL-10, IL-4 and IL-5 [170, 174, 192]. Furthermore, estrogens induce earlier and sustained expression of IgG anti-dsDNA antibodies [159]. Despite the extensive data supporting the impact of sex hormones on immune

responses, non-hormonal mechanisms such as X chromosome inactivation and differential exposures of pathogens have also been suggested to contribute to loss of immune tolerance and therefore, increased frequencies of autoimmune diseases in females [62].

The SNF₁ mouse, the F₁ cross between NZB and SWR (SNF₁), is an autoimmune murine model of human lupus nephritis. In this model, the mice spontaneously develop a disease characterized by the production of high level anti-nuclear auto-antibodies and a fatal lupus nephritis with a notable gender bias. The female SNF₁ mice spontaneously develop early disease, while the male mice develop milder lupus nephritis later in their lives. Despite many years of research, the pathogenesis of SLE in this model has still not been well characterized, but is thought to arise from defective central or peripheral tolerance mechanisms resulting in an unusually high activation of T and B lymphocytes including an alteration in cytokine levels and antibody production, and/or insufficient suppression of autoreactive lymphocytes [48]. Our laboratory has hypothesized that defective regulation of certain pathogenic cross-reactive idiotypes, known as Id^{LNF₁} [72, 73], found on Ig eluted from nephritic kidneys, might be the primary trigger for the nephritis in SNF₁ mice, with anti-DNA antibodies, although an important diagnostic marker, playing a less important role in the pathogenesis of SLE [27].

In the present study, we investigated whether the physiological levels of the sex hormones, estrogen and testosterone, played a role in the gender bias of lupus nephritis in SNF₁ mice by surgically castrating female and male mice in order to alter the serum levels of these hormones. In another study, exogenous 17 β -estradiol (E-2) at a dose of 1mg/kg was also administered to castrated male mice monthly starting at 7 weeks of age. The results suggested that E-2 levels contribute to and/or accelerate the lupus nephritis in SNF₁ mice, with natural levels of androgens having little or no effect.

2.2 Materials and methods

2.2.1 Mice

Six to eight week old female SWR and male NZB mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The (SWR x NZB) F_1 progeny (SNF $_1$) mice were bred and maintained in the Cornell University College of Veterinary Medicine, Center for Animal Resource and Education (CARE). Mouse body weights were recorded monthly. Mice were castrated at 6 weeks of age.

2.2.2 Castration

Orchidectomy was conducted under aseptic conditions with isoflurane administered by face mask. The scrotum was prepped with betadine and draped. A longitudinal incision, approximately 1 cm in length, was made over the median septum dividing the right and left cavities. The left testicular capsule was incised and the testicle was exteriorized. The spermatic cord was cauterized approximately 1 cm above the head of the epididymis and amputated. The stump and all vessels were cauterized, and then the stump was replaced into the testicular cavity. The right testicle was excised similarly. The wound was closed with surgical glue.

2.2.3 Ovariectomy

Anesthesia was induced by isoflurane administered by face mask, then the exposed skin prepared for aseptic surgery with a betadine scrub followed by a 70% alcohol wipe. A 3/4 cm dorsal flank incision penetrating the abdominal cavity was made for each ovary. The parovarian fatty tissue was identified and retracted. The exposed ovary and associated oviduct were severed and removed, and hemostasis was achieved by hemostat pressure for 1-2 minutes. The incision was closed using a 5-0 nonabsorbable suture in an interrupted pattern or surgical staples and surgical glue.

2.2.4 Estrogen treatment and serum collection

Male mice (castrated or sham-castrated) received β -estradiol 17-valerate (E-2) at 1mg/kg (Sigma) in olive oil vehicle subcutaneously once every month starting at 7 weeks of age; control mice were injected with oil vehicle alone. Body weight was measured monthly. Three mice from each group were sacrificed at the age of 24 weeks. The thymus, spleen and kidney were harvested and single cell suspensions were prepared from individual spleens and thymuses. Kidneys were harvested and fixed, and sections were stained with hematoxylin and eosin (H&E) for glomerular histology analysis.

Retro-orbital eye bleeds on mice were performed monthly under isoflurane anesthesia beginning two weeks after the first E-2 treatment. For terminal bleeds, the mice were euthanized by CO₂ asphyxiation, and cardiac puncture was used to obtain whole blood samples. Sera were collected and then frozen at -20°C until further use.

2.2.5 Determination of serum E-2 and testosterone levels

Five to six mice were bled from the retro-orbital plexus one month after castration and the serum was collected. The E-2 and testosterone (T-1) levels in the serum were detected using EIA kits (Diagnostic Systems Laboratories, Inc) according to the manufacturer's instruction with slight modification.

To determine E-2 levels, the serum sample diluted in PBS-0.1% Tween (1:3 or 1:4) and each standard included in the EIA kit were added in duplicates. The estradiol-biotin conjugate was also added. The samples were incubated with shaking (500-700 rpm) at room temperature for an hour. After washing, streptavidin-enzyme conjugate was added to the wells. The plates were incubated and shaken at room temperature for 30 min. Then, the plates were washed again and TMB chromogen solution was added. After 30 min, the reaction was stopped by the addition of 1M H₃PO₄ and then the developed color was read using a microplate reader (Biorad) at 450nm. The

concentration of hormone in each sample was calculated from the standard curve. The data were expressed as pg/ml.

To determine T-1 levels, the serum sample diluted in PBS-0.1% Tween (1:3 or 1:4), and each standard included in the EIA kit were added in duplicates. Testosterone-enzyme conjugate solution was also added, followed by addition of Testosterone antiserum. The plates were incubated with shaking (500-700 rpm) at room temperature for one hour. After washing 2 times, TMB chromogen solution was added to wells. The plates were incubated and shaken at room temperature for 30 min. The reaction was stopped by the addition of 1M H₃PO₄ and then the developed color was read using a microplate reader (Biorad) at 450nm and the concentration for each sample was calculated from the standard curve. The data were expressed as pg/ml.

2.2.6 Cytokine production

Splenic lymphocytes were cultured in triplicate at a concentration of 1×10^6 cells per well with Concanavalin A (ConA) at 2µg/ml, aa62-73 300µg/ml or anti-CD3 mAb (40µg/ml) for three days at 37°C under 5% CO₂. Supernatants were harvested and analyzed by ELISA (eBioscience) for IL-2, IL-4, IL-5, IL-6, IL-12, TNF-α and IFN-γ according to the manufacturer's instruction. The data were expressed as pg/ml.

2.2.7 T cell proliferative responses

Five hundred thousand splenocytes/well were mixed with aa62-73 peptide at 100ug/ml or ConA at 2µg/ml. After incubation at 37°C with 5% CO₂ for three days, 1mCi/well of [³H]-thymidine (Amersham, Arlington Heights, IL) was added, and incubation continued overnight. The cells were then lysed, collected onto filter mats and incorporated radioactivity measured using a scintillation counter (Packard, Meriden, CT). The data were expressed as the proliferation index (cpm of sample versus ConA treated control).

2.2.8 ELISPOT determination of B cell frequencies

For determination of the frequency of Id^{LN}F₁-producing B cells, 96-well microtiter plates with nitrocellulose bottoms (Millipore) were coated with rabbit anti-Id^{LN}F₁ Ab, aa62-73, control peptide Mal, anti-540 Ab, or normal rabbit Ig overnight at 4°C. The plates were washed with PBS-0.1% Tween and blocked with PBS-3% BSA, then washed again. Splenic lymphocytes diluted in culture medium were added at a concentration of 5 x 10⁶ mononuclear cells/well in triplicate. The plates were incubated overnight at 37°C with 5% CO₂. Specific Igs secreted by B cells bound to the plate-bound antibody were detected by biotin-conjugated anti-mouse IgG, followed by SA-HRP (streptavidin conjugated to horseradish peroxidase, Amersham Life Sciences) and 3-amino-9-diethylcarbazole and H₂O₂ as substrate. Brown spots corresponding to cells secreting specific Ig were counted, and the data were expressed as the frequency of splenic B cells per 10⁵ cells.

2.2.9 Antibody production

In triplicate, 5x10⁵ Ig⁺ splenocytes purified by Ig panning were cultured with 1x10⁵ affinity-purified T cells in various combinations with aa62-73 (300µg/ml). The cultures were incubated for seven days at 37°C with 5% CO₂. The supernatants were then harvested and frozen at -20°C until analyzed by ELISA for total anti-Id^{LN}F₁ IgG, as previously described [29]. Briefly, Immulon 1B 96-well plates (Krackeler Scientific, Albany, NY, USA) were coated with prepared rabbit anti-Id^{LN}F₁ Ig. Then the diluted sample was added in triplicate. A standard curve was generated using duplicates of eight, two-fold serial dilutions of affinity-purified specific IgG starting at 1µg/ml in PBS/0.1% Tween for total IgG /IgM levels. A standard curve for anti-dsDNA, anti-ssDNA and anti-Id^{LN}F₁+IgG were composed of eight serial dilutions of pooled serum from old (at least 24 weeks old) female SNF₁ mice starting at 1:100 in PBS/0.1% Tween. Then goat anti-mouse IgG or IgM-horseradish peroxidase (HRP) conjugate

(Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) was added per well and the incubation was continued overnight. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was added to each well. Color development proceeded for five minutes to one hour at room temperature (RT) until significant blue color developed. The stop solution (1M phosphoric acid) was added and the O.D. was read at 450nm. The Ig concentrations were determined from the corresponding linear standard curves. The data were calculated and represented as fold over procedure control.

2.2.10 Flow cytometric analysis of splenic lymphocytes

The splenocyte suspension was treated with tris-ammonium chloride (TAC) and then enhanced for T cells by affinity chromatography (T cell Recovery Column, Biotex), and 1×10^5 cells were stained with the appropriate dilution of biotin-, fitc-, and phycoerythrin-conjugated antibodies and streptavidin-red670 (PharMingen). The following antibodies were used, anti-CD44-PE, anti-CD4-PE, anti-B220-PE, anti-CD45RB-Bio, anti-CD8a-Bio, anti-CD3-Bio, anti-CD25-Bio, anti-CD4-Fitc, and Id^{LN}F₁-Fitc (PharMingen, with the exception of Id^{LN}F₁⁺ Fitc-conjugated antibody, which was derived from monoclonal 540 antibody-producing hybridoma cell culture supernatants and labeled using a fitc-conjugation kit [54]). The stained cells were fixed in 1% paraformaldehyde and flow cytometry was carried out on a Becton-Dickinson FACSCalibur[®] flow cytometer and analyzed with the WinMDI program.

2.2.11 Determination of serum immunoglobulin (Ig) levels

A direct binding ELISA was used to determine the levels of total serum IgG, anti-dsDNA IgG, anti-ssDNA IgG, and Id^{LN}F₁⁺ IgG as previously described [29]. Immulon 1B 96-well plates (Krackeler Scientific, Albany, NY, USA) were coated with goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN, USA), calf-thymus dsDNA (Sigma), or rabbit anti-Id^{LN}F₁ Ig respectively. Then the diluted serum

was added in triplicate. A standard curve was generated using duplicates of eight, two-fold serial dilutions of affinity-purified specific IgG starting at 1ug/ml in PBS/0.1% Tween for serum IgG level. A standard curve for anti-dsDNA and anti-Id^{LN}F₁+IgG were composed of eight serial dilutions of pooled serum from old (at least 24 weeks old) female SNF₁ mice starting at 1:100 in PBS/0.1% Tween. Incubation continued overnight at 4°C. Then the plates were washed with PBS/2%Tween and goat anti-mouse IgG or IgM-horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) was added per well. Incubation was continued overnight. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was added to each well. Color development proceeded for five minutes to one hour at room temperature (RT) until significant blue color developed, then the stop solution (1M phosphoric acid) was added and the O.D. was read at 450nm. Serum total IgG concentrations were determined from the corresponding linear standard curves, and were expressed as ug/ml for total IgG. The Id^{LN}F₁ IgG and anti-dsDNA IgG concentrations were determined from the corresponding linear standard curves and data expressed as standard units, with one standard unit equivalent to a 1:1 dilution of the standard sera sample.

2.2.12 Determination of serum cytokine levels

Mouse serum was diluted 1:3 or 1:4 and analyzed by ELISA (eBioscience) for IL-2, IL-4, IL-5, IL-6 and IFN-gamma, according to manufacturer's instruction. The data were expressed as pg/ml.

2.2.13 Renal histology

Individual glomeruli from each kidney were blindly evaluated and graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis.

2.2.14 Survival

At least 6 mice from each treatment group were kept for the survival. The mice were euthanized when moribund as determined by CARE veterinarians. The data were expressed as the percentage of surviving mice.

2.2.15 Statistics

Statistical significance of treatment on survival was determined by the Logrank test. Glomerular damage grades were analyzed by nonparametric Mann-Whitney test. Other data were analyzed by the Student's t-test or one-way ANOVA test to compare means from two treatment groups. One-way ANOVA followed by Fisher LSD test was used for multiple comparisons of means. Statistical significance was ascribed when p values were less than 0.05.

2.3 Results

2.3.1 The impact of ovariectomy on survival, body weight and serum estradiol levels in female SNF₁ mice

Serum E-2 levels were measured one month after ovariectomy of mice (Fig 2.1A). We found that after ovariectomy, the serum E-2 levels in female SNF₁ mice decreased significantly ($p < 0.01$), from about 150pg/ml to about 1 ~ 2 pg/ml, which is similar to the serum E-2 levels normally found in male SNF₁ mouse. Body weights were also recorded monthly, and ovariectomized female mice showed significantly increased body weights starting at 3 months of age ($p < 0.05$, Fig 2.1B). At least 6 mice from each group were kept for survival analysis (Fig 2.1C). Median survivals were 44.1 weeks and 62.1 weeks for sham-ovariectomized and ovariectomized female mice respectively, suggesting that ovariectomy significantly prolonged the survival of female SNF₁ mice ($p < 0.001$). Male SNF₁ mice had a median survival greater than that

of ovariectomized female SNF₁ mice (69.1 weeks, $p > 0.05$ compared with ovariectomized mice, $p < 0.001$ compared with sham-ovariectomized female mice).

2.3.2 Ovariectomy accelerates lupus nephritis in female mice

Hematoxylin&Eosin stained kidney sections from female ovariectomized and intact mice obtained at 24 weeks of age were blindly evaluated and graded for severity of damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. A decrease ($p = 0.16$) in damaged glomeruli was seen in ovariectomized mice (Table 2.1). The mean grade of damaged glomeruli for ovariectomized mice was 3.3 ± 0.33 , while the mean grade for intact mice was 4.0 ± 0.0 .

2.3.3 The effect of ovariectomy on splenic T lymphocyte functions

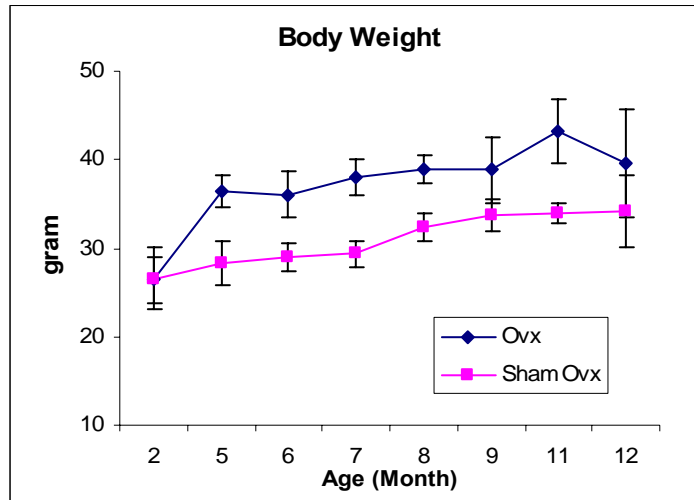
T lymphocyte functions were measured in order to determine the effects of ovariectomy on the pathogenic immune cells of female SNF₁ mice. At 24 weeks of age, splenocytes from ovariectomized and sham-ovariectomized female mice were isolated, and *in vitro* proliferative responses and cytokine production were measured. Removal of estrogen by ovariectomy resulted in increased mitogen-induced proliferation and decreased proliferation to pathogenic peptide aa62-73 (Fig 2.2A), although statistical significance was not reached. The proliferation index (PI) calculated as the ratio of aa62-73 proliferation to ConA proliferation showed that the aa62-73 response was significantly decreased in ovariectomized mice compared to intact female mice ($p < 0.05$).

Figure 2.1 The effect of ovariectomy on serum estradiol (E-2) levels, body weight and survival of female SNF₁ mice. A. Ovariectomy reduces serum E-2 level. Serum E-2 level was determined by EIA one month following the surgery (10 weeks of age). Ovariectomy of female mice significantly ($P < 0.01$) reduced the serum E-2 level. B. Ovariectomy leads to increased body weights. Mouse body weight was recorded monthly, and ovariectomy of female mice led to increased body weight beginning at 3 months of age. C. Survival of female SNF₁ mice is prolonged by ovariectomy. Female SNF₁ mice were ovariectomized at 6 weeks of age (n=6). Female ovariectomized SNF₁ (F+Ovx) and male SNF₁ mice (M) had similar median survivals, which were significantly ($p < 0.01$ for a Logrank Test) prolonged compared to control intact female mice (F).

A. Serum E-2 Levels

	Mean(pg/ml)	Std Err
Female+Ovx	1.018	1.183
Male	1.802	1.802
Female	157.300	2.590

B.



C.

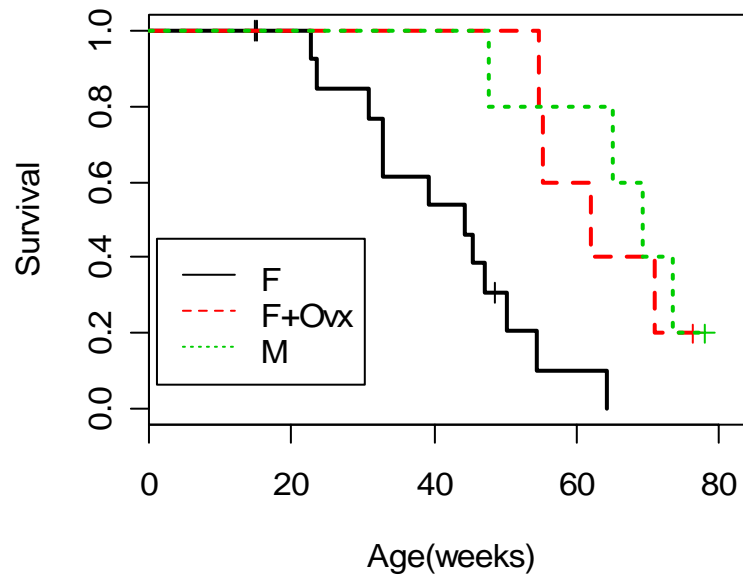


Table 2.1 Ovariectomy leads to decreased kidney damage in SNF₁ mice. Female mice were ovariectomized at 6 weeks of age. Kidney sections from 24-week old mice in each treatment group were stained with hematoxylin and eosin (H&E) for pathological examination. Individual glomeruli from each kidney were graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. The data were expressed as mean grade \pm SE for each treatment group.

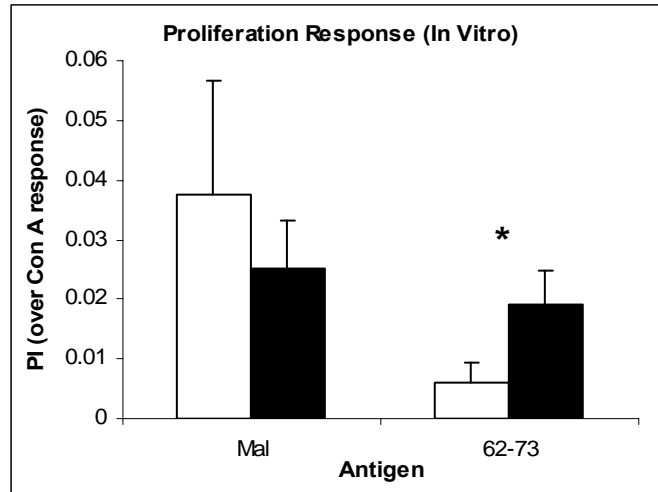
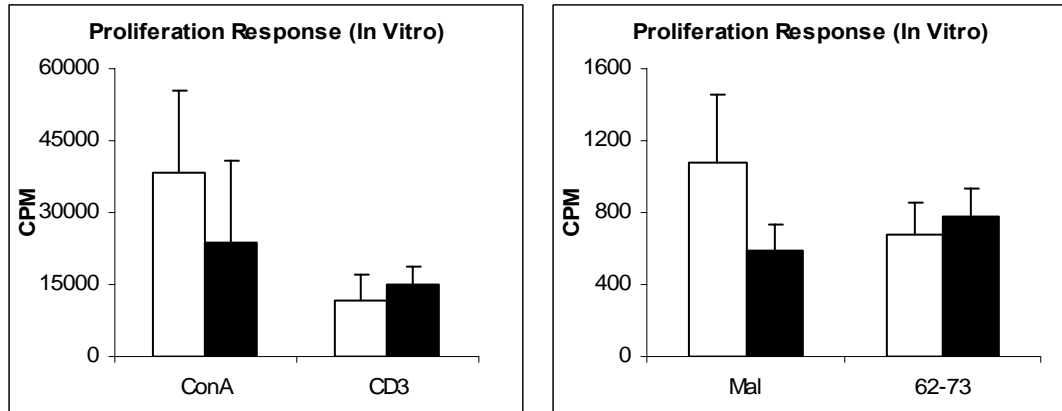
	Glomerular damage
Ovx	3.3 \pm 0.33
Intact	4.0 \pm 0.0

Note: n=3 for each treatment group

Cytokine production after mitogen stimulation by splenocytes from ovariectomized female mice was increased including IFN- γ , IL-12, IL-4, IL-5 and IL-6 (Fig 2.2B). However, in the presence of pathogenic aa62-73, the cytokine production by cells from these from ovariectomized mice was decreased for IL-5 and IL-6 and significantly increased for IL-12. The production of the pro-inflammatory cytokine TNF- α was also decreased for ovariectomized mice. This pattern of altered cytokine production has been shown to be associated with protection in lupus nephritis in other studies and usually is the reverse effect of E-2 treatment [3, 39, 49, 99, 100, 194].

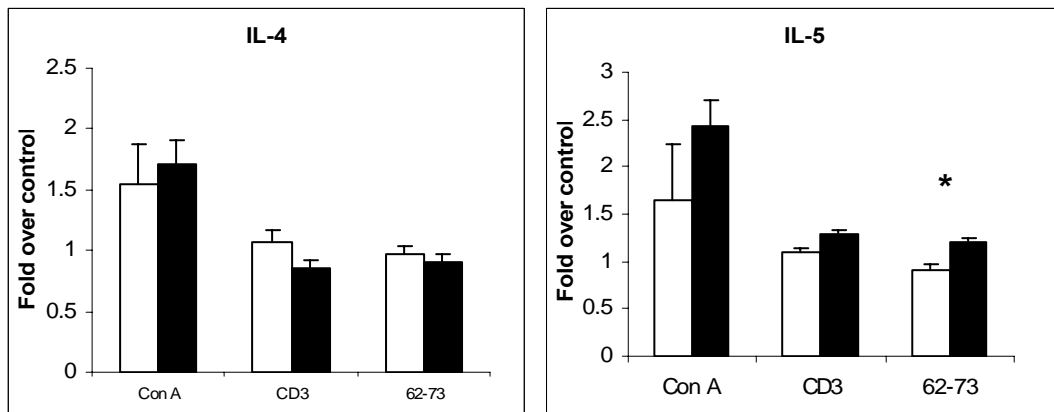
Figure 2.2 Modulation of T cell functions after ovariectomy of female SNF₁ mice. Splenocytes from ovariectomized female and control mice were isolated at 24 weeks of age. The cells were cultured with ConA, anti-CD3 mAb, aa62-73 peptide or Mal peptide (a non-relevant control peptide), respectively. A. Proliferative responses were determined by [³H] thymidine incorporation, and were found to be increased for ovariectomized mice with mitogen (ConA) stimulation but decreased in response to the pathogenic peptide aa62-73, although statistical significance was not reached. However, the proliferation index (PI) showed that the proliferative responses to aa62-73 were reduced significantly ($p < 0.05$). B. Cytokine levels in the supernatant were detected by ELISA. IFN- γ and IL-12 production were decreased significantly in ovariectomized mice after ConA stimulation ($p < 0.05$). In the presence of aa62-73, IL-12 production was increased, and IL-5 and TNF- α production were significantly decreased in ovariectomized mice. Results are expressed as (mean fold over peptide control) \pm SE. (*, $p < 0.05$, compared with sham-ovariectomized mice).

A. Proliferative responses (*in vitro*)



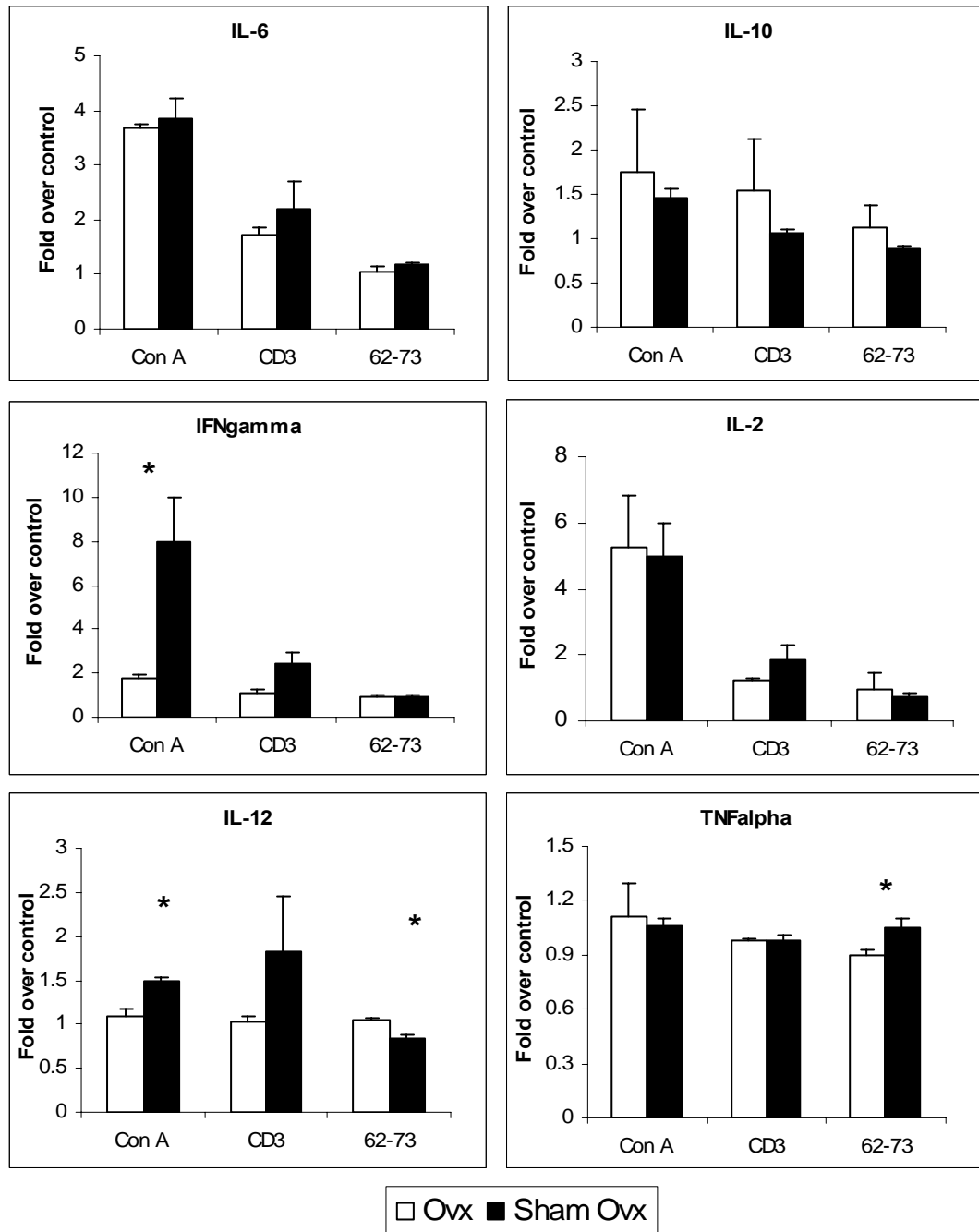
□ Ovx ■ Sham Ovx

B. Cytokine Production (*in vitro*)



□ Ovx ■ Sham Ovx

Figure 2.2 (Continued)



2.3.4 The effect of ovariectomy on B cell functions

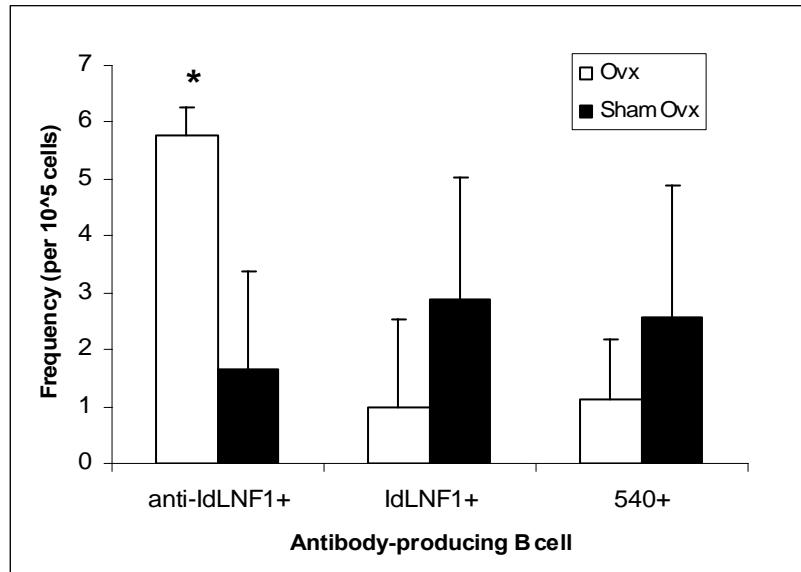
B cell functions of female mice were also affected by ovariectomy, as determined by ELISPOT and *in vitro* immunoglobulin (Ig) production performed at 24 weeks using total splenocytes and Ig⁺ cells from both groups of mice. We found that the frequency of protective anti-Id^{LN}F₁⁺ antibody forming cells was increased significantly ($p < 0.05$) in ovariectomized mice ($5.8 \pm 0.48 / 10^5$ cells vs. $1.7 \pm 1.5 / 10^5$ cells in sham-ovariectomized controls), while the frequency of pathogenic Id^{LN}F₁⁺ antibody forming cells was decreased in ovariectomized mice (Fig 2.3A), although statistical significance was not reached. Ig assays also supported a protective role for ovariectomy (Fig 2.3B), since, in general, the levels of Igs detected in this study were decreased in ovariectomized female mice either in the presence of pathogenic aa62-73 or without stimulation (blank). Most importantly, the pathogenic Id^{LN}F₁⁺IgG level ($1.26 \pm 0.15 / 10^5$ cells) in ovariectomized mice was significantly decreased ($p < 0.01$) after aa62-73 stimulation compared to the level in control mice (2.04 ± 0.02).

2.3.5 Immune cell phenotypes in ovariectomized and intact female SNF₁ mice

Phenotypic analysis was performed on splenocytes or splenic T cells from 24 weeks old mice in order to identify the effects of ovariectomy on different immune cell populations. One major difference between ovariectomized and sham-ovariectomized mice was that there were significantly ($p < 0.05$) lower percentages of pathogenic Id^{LN}F₁⁺ T cells in ovariectomized mice (Table 2.2), specifically Id^{LN}F₁⁺CD3⁺ ($2.19 \pm 0.13\%$ vs. $3.62 \pm 0.63\%$), Id^{LN}F₁⁺CD4⁺CD3⁺ ($1.32 \pm 0.02\%$ vs. $2.58 \pm 0.41\%$) total splenocytes and Id^{LN}F₁⁺CD4⁺ splenic T cells ($6.44 \pm 1.33\%$ vs. $19.25 \pm 6.86\%$). Although the Id^{LN}F₁⁺CD8⁺ splenic T cells, which potentially contain Id^{LN}F₁-specific regulatory cells [195], were also decreased in ovariectomized mice, the ratio of Id^{LN}F₁⁺CD4⁺ to Id^{LN}F₁⁺CD8⁺ cells was decreased as well, suggesting a overall

Figure 2.3 Changes in B cell functions induced by ovariectomy of female SNF₁ mice. Splenocytes were isolated from ovariectomized SNF₁ female and control mice at 24 weeks of age. A. Antibody-forming cells were detected by ELISPOT. Ovariectomy led to an increased frequency of anti-idiotypic antibody forming cells compared to control mice (p<0.05). B. Ig⁺ splenocytes and affinity-purified T cells were isolated from ovariectomized and intact control mice, and then the cells were mixed and cultured in the presence of aa62-73 or PBS vehicle (blank) for 7 days. The supernatants were then analyzed by ELISA for total IgG, Id^{LN}F₁⁺ IgG, anti-ssDNA IgG, and anti-dsDNA IgG. The results were expressed as the fold of the antibody level over control. (*, p<0.05, compared with intact control mice).

A. Ig-specific B cell frequency



B. Ig Production (*in vitro*)

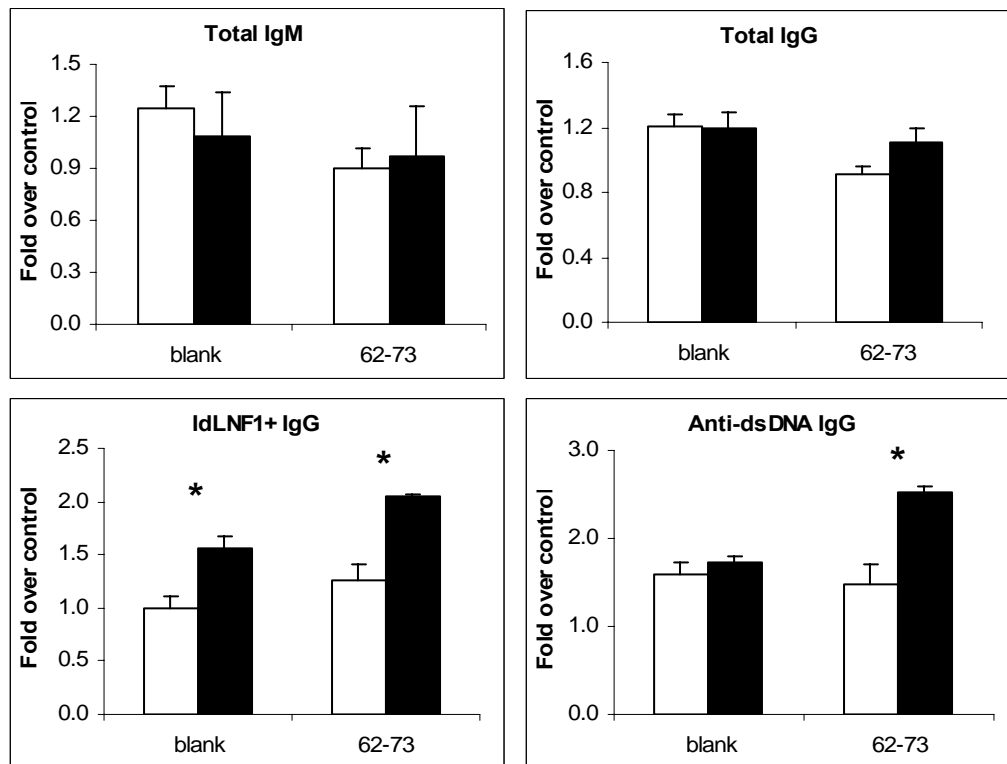
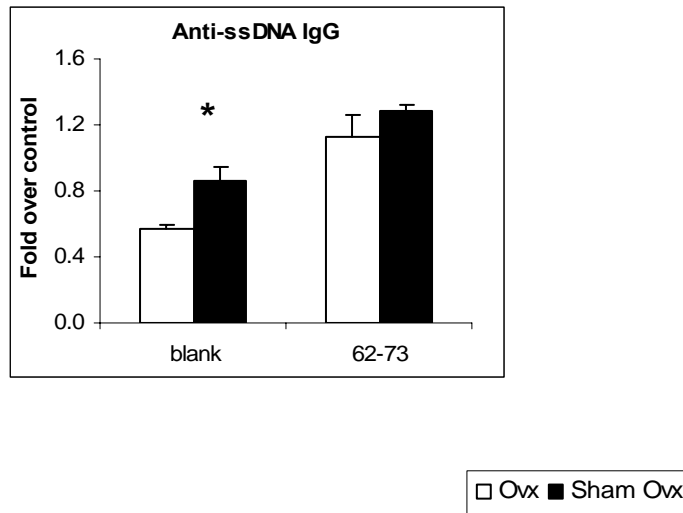


Figure 2.3 (Continued)



protective role of ovariectomy [54]. Other changes included significantly ($p < 0.05$) fewer $CD44^+CD45^-CD4^+$ T cells (memory phenotype) ($30.02 \pm 1.21\%$ vs. $37.59 \pm 2.02\%$) and fewer $CD44^+Id^+CD4^+$ T cells (activated cells) ($6.82 \pm 1.87\%$ vs. $18.72 \pm 3.67\%$) in ovariectomized female mice compared to intact mice.

2.3.6 The impact of castration on serum testosterone levels, body weight and survival of male SNF_1 mice

Male SNF_1 mice were castrated or sham-castrated at 6 weeks old. Serum testosterone (T-1) levels were measured one month following castration (Fig 2.4A), and the results showed a significant reduction in the serum T-1 level in castrated male (5.28 ± 1.76 pg/ml) compared to that in intact controls (16.34 ± 2.00 pg/ml), as expected ($p < 0.05$). Mice were treated with 1mg/kg E-2 or oil monthly beginning 2 weeks post-surgery. Treatment groups included intact mice treated with oil

Table 2.2 Phenotypic analysis of ovariectomized and control sham-ovariectomized SNF₁ mice. Splenocytes (A) or affinity-purified splenic T cells (B) from 24 week old mice were stained with the appropriate antibodies, and analyzed with flow cytometry. Data were expressed as the mean percentage \pm SD (*, p<0.05; **, p<0.01 compared to intact control mice).

A. Total Splenocytes						
	Ovariectomized			Sham Ovariectomized		
B220+	75.90	\pm	6.28	75.41	\pm	2.62
CD3+	21.92	\pm	6.05	24.89	\pm	6.55
Id ^{LN} F ₁ +CD3+	2.19	\pm	0.13	3.62	\pm	0.63*
Id ^{LN} F ₁ +CD4+CD3+	1.32	\pm	0.02	2.58	\pm	0.41**

B. Affinity-purified T cells						
	Ovariectomized			Sham Ovariectomized		
Id ^{LN} F ₁ +	6.44	\pm	1.33	19.25	\pm	6.86**
CD4+	60.02	\pm	5.39	57.46	\pm	3.00
CD8+	25.48	\pm	2.07	22.00	\pm	6.65
Id ^{LN} F ₁ +CD4+	8.48	\pm	1.46	26.08	\pm	11.10**
Id ^{LN} F ₁ +CD8+	6.60	\pm	2.43	14.84	\pm	6.19*
Id ^{LN} F ₁ +CD4+/ Id ^{LN} F ₁ +CD8+	1.38	\pm	0.43	1.83	\pm	0.54
CD44+CD45-CD4+	30.02	\pm	1.21	37.59	\pm	2.02*
CD44+ Id ^{LN} F ₁ +CD4+/ CD4+	6.82	\pm	1.87	18.72	\pm	3.67*

(intact&oil), intact mice treated with E-2 (intact&E2), castrated mice treated with oil (cast&oil) and castrated mice treated with E-2 (cast&E-2). At least 6 mice for each treatment group were kept for survival analysis (Fig 2.4C). The median survivals were

69.3, 58.4, 79.7 and 53.0 weeks for intact&oil, intact E2, castr&oil, and castr&E2 group mice, respectively. Logrank analysis indicated that E-2 treatment significantly decreased survival ($p<0.05$), regardless of whether the mice were castrated or intact. In contrast, castration did not affect survival significantly ($p>0.05$) with or without E-2 administration. E-2 treated mice had significantly lower body weights compared to that of oil treated controls starting at 3 month of age, and castration of mice did not affect the body weights significantly (Fig 2.4B).

2.3.7 Histology examination of kidneys from castrated and intact male mice with E-2 treatment

Hematoxylin&Eosin stained kidney sections from male castrated and intact mice obtained at 24 weeks of age were blindly evaluated and graded for order of severity of damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. E-2 treatment led to increased kidney damage in both castrated and intact mice compared to oil controls ($p<0.16$) (Table 2.3). The mean grades of damaged glomeruli for E-2 treated mice (castrated or intact) were both 3.7 ± 0.33 , and the grades for both oil treated groups were 3.0 ± 0.0 .

2.3.8 The effect of castration and E-2 treatment on splenic T lymphocyte functions

Two weeks following the 4th dose of E-2 or oil (about 25 weeks of age), splenocytes from all of the treatment groups were harvested, and the proliferative response and *in vitro* cytokine production were measured as described for the ovariectomy study above. Repeated E-2 administration led to significantly decreased

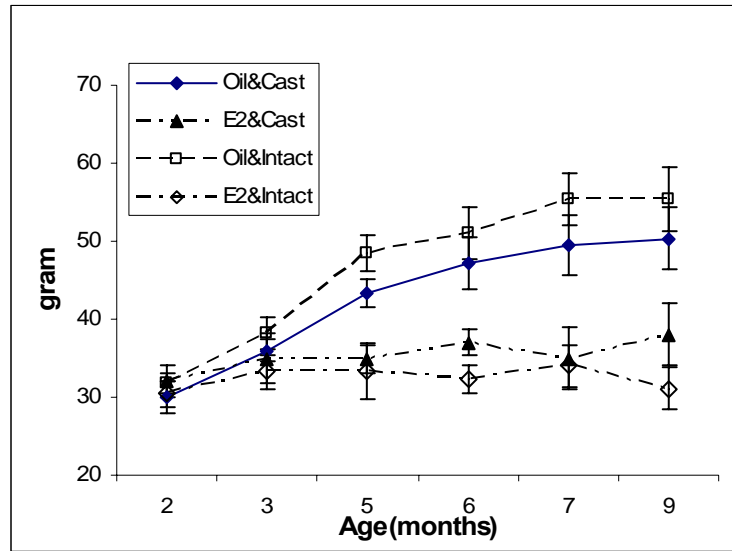
Figure 2.4 The effect of castration on serum testosterone (T-1) levels, body weight and survival of male SNF₁ mice. A. Castration reduced serum T-1 level. Serum T-1 levels were determined by EIA one month following castration surgery (at 10 weeks). Castration of male mice significantly ($P < 0.01$) reduced the serum T-1 level. B. E-2 treatment leads to decreased body weight. Body weights were recorded monthly and E-2 administration led to decreased body weights starting at 3 months of age. C. Survival of male SNF₁ mice after castration and E-2 administration. Male SNF₁ mice were castrated or sham-castrated at 6 weeks of age and then E-2 at a dose of 1mg/kg or oil was injected s.c. monthly starting at 8 weeks of age. Treatment groups included: male castrated SNF₁ mice with oil (cast&oil) or E-2 (cast&E2) and male sham-castrated mice with oil (intact&oil) or E-2 (intact&E2) (n=6 for all groups). E-2 treated mice showed significantly decreased survival compared to oil treated mice ($p < 0.01$), while castration did not affect survival significantly, regardless of oil or E-2 treatment.

A Serum T-1 level

	Mean(pg/ml)	Std Err
Castrated Male	5.28	1.76
Intact Male	16.34	2.00
Female	3.5 ^a	1.7 ^a

Note: a, the female serum T-1 level is the expected value from EIA kits manufacturer DSL.

B. Body weight



C. Effect of castration and E-2 treatment on survival

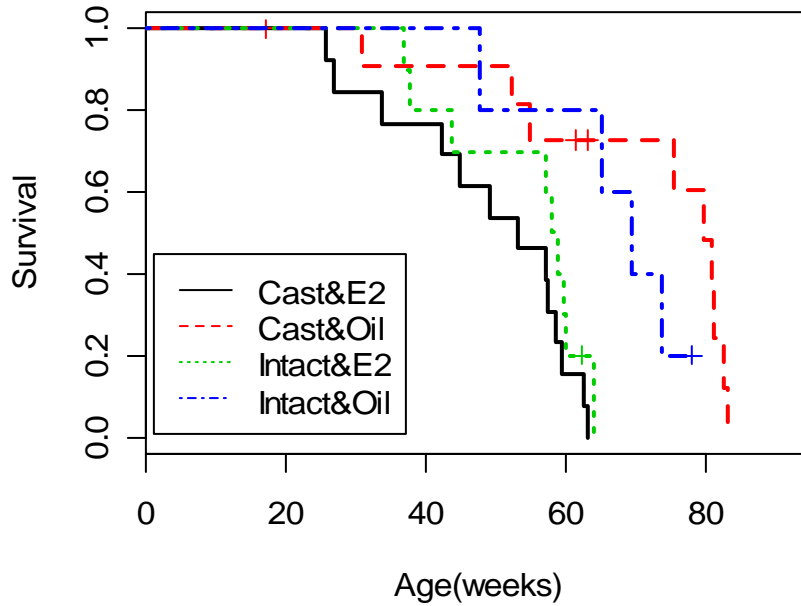


Table 2.3 Effect of castration on kidney damage in male SNF₁ mice. Male mice were castrated at 6 weeks of age. Kidney sections from 24-week old mice in each treatment group were stained with hematoxylin and eosin (H&E) for pathological examination. Individual glomeruli from each kidney were graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. The data were expressed as mean grade \pm SE for each treatment group.

Glomerular damage	
Cast + Oil	3.0 \pm 0.0
Cast + E-2	3.7 \pm 0.33
Intact + Oil	3.0 \pm 0.0
Intact + E-2	3.7 \pm 0.33

Note: n=3 for each treatment group

mitogen-induced proliferation in sham-castrated male mice ($p < 0.05$), but not in castrated mice. E-2 administration increased proliferation to aa62-73 in both intact and castrated mice (Fig 2.5A), but the increase was greater in intact mice ($p < 0.05$).

Castration had little effect on both mitogen-induced and peptide-specific proliferation.

E-2 treatment of male mice enhanced mitogen-induced cytokine production, specifically IL-5 and IFN- γ , and decreased the production of IL-2 significantly ($p < 0.05$) (Fig 2.5B). However, in the presence of pathogenic aa62-73, the cytokine profile was biased toward T_H2 with a notable increase in IL-4, IL-5 and IL-6, and decrease in IL-12 and IL-2 ($p < 0.05$). These changes were similar to those we found in ovariectomized SNF₁ mice.

2.3.9 E-2 administration but not the castration increases the frequency of Id^{LN}SNF₁⁺ antibody-producing cells in male SNF₁ mice

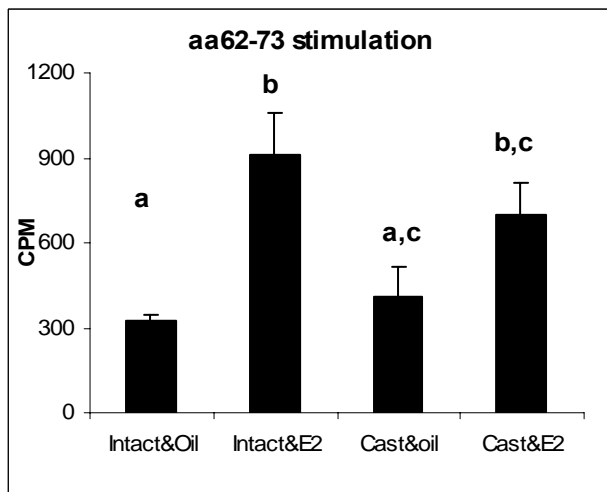
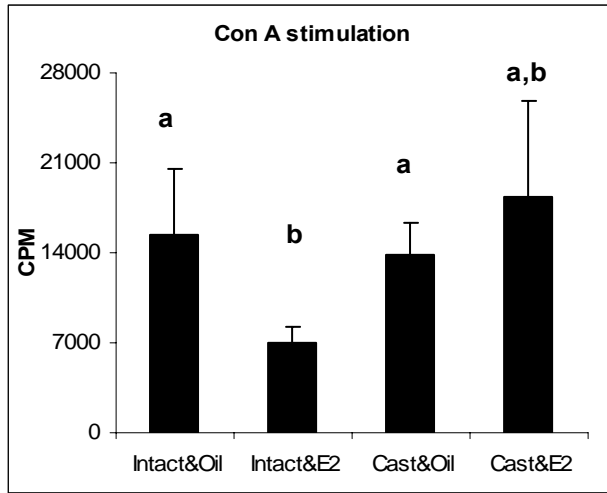
The frequency of specific antibody producing B cells was determined by ELISPOT assay 2 weeks after the 4th injection of E-2 or oil (about 25 weeks old). The frequencies of anti-Id^{LN}F₁⁺ (anti-aa62-73) antibody-producing B cells were comparable in all 4 treatment groups (Fig 2.6); however, the frequencies of Id^{LN}SNF₁⁺ antibody-producing cells were significantly increased in both E-2 treated groups compared to both oil treated groups respectively (Fig 2.6). Castration did not have a significant impact on the frequency of Id^{LN}F₁⁺ antibody producing cells in oil treated mice.

2.3.10 Immune cell phenotypes in castrated and E-2 treated male SNF₁ mice

Phenotypic analysis was performed on cells from mice 2 weeks after 4th injection of E-2 or oil (about 25 weeks old). E-2 treatment led to significantly ($p < 0.05$) more Id^{LN}F₁-reactive CD3⁺ ($3.29 \pm 0.26\%$ vs. $6.35 \pm 0.55\%$ in intact mice; $4.66 \pm 0.84\%$ vs. $5.73 \pm 0.91\%$ in castrated mice) and CD4⁺CD3⁺ cells, increased ratio of Id^{LN}F₁⁺CD4⁺ to Id^{LN}F₁⁺CD8⁺ ($0.88 \pm 0.2\%$ vs. $2.11 \pm 0.45\%$ in intact mice; $1.91 \pm 0.41\%$ vs. $2.00 \pm 0.37\%$ in castrated mice) and greater numbers of CD44⁺ Id^{LN}F₁⁺CD4⁺ cells ($4.96 \pm 0.77\%$ vs. $9.27 \pm 1.25\%$ in intact mice; $2.50 \pm 0.59\%$ vs. $3.99 \pm 0.78\%$ in castrated mice) (Table 2.4). These changes indicated an expansion of pathogenic cell populations by E-2. In contrast, castration did not have any notable effect.

Figure 2.5 Modulation of T cell functions after castration and E-2 administration in male SNF₁ mice. Splenocytes were isolated at 25 weeks of age. The cells were cultured in the presence of ConA or aa62-73 peptide respectively. A. Cells from E-2 treated intact mice show significantly decreased proliferation to mitogen (ConA) stimulation compared with cells from oil treated controls. Cells from E-2 treated intact and castrated mice show increased proliferation to pathogenic peptide aa62-73 compared with cells from their respective oil treated controls. B. *In vitro* cytokine production was modulated after E-2 treatment. IL4, IL-5 and IL-6 levels were increased after aa62-73 stimulation in E-2 treated castrated and sham-castrated mice ($p < 0.05$), and IL-2 was decreased. Results are expressed as Mean \pm SE. Nonoverlapping letters (a, b, c) indicate significant difference ($p < 0.05$).

A. Proliferative responses (*in vitro*)



B. Cytokine production (*in vitro*)

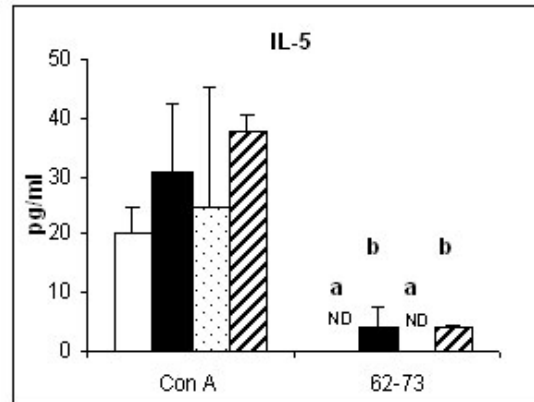
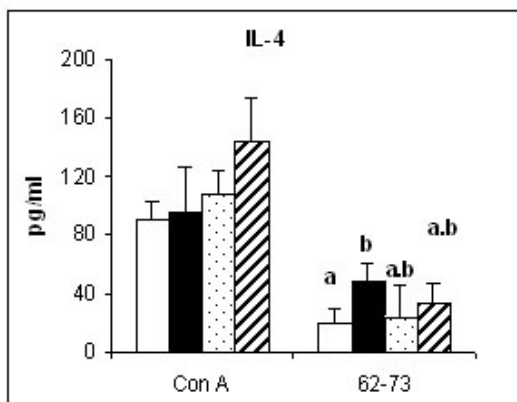
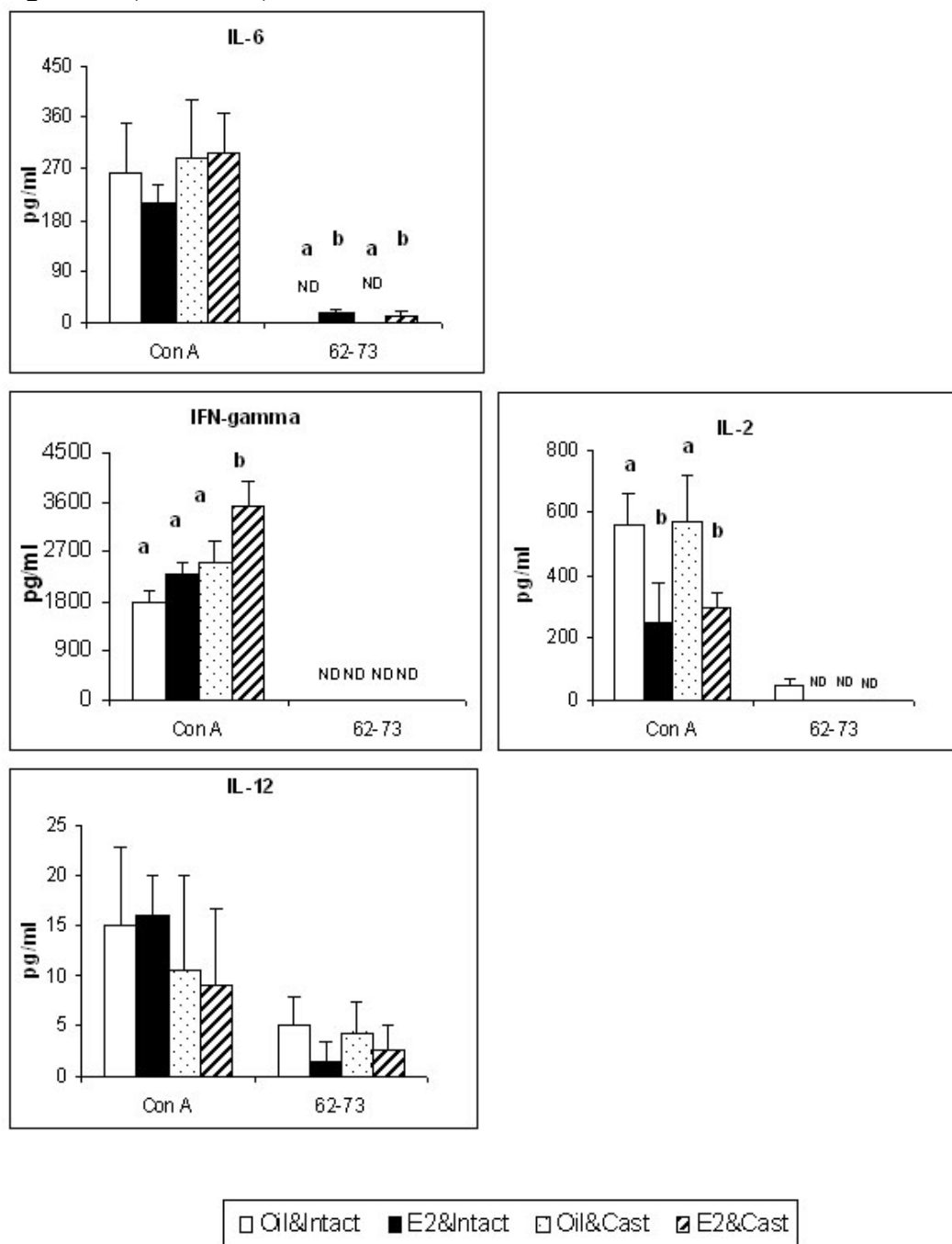


Figure 2.5 (Continued)



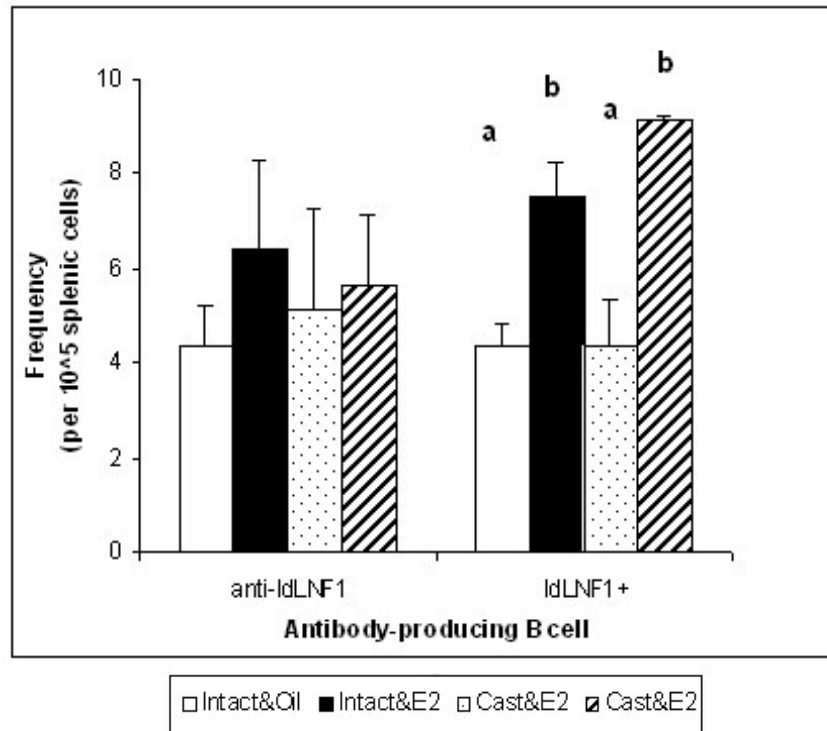


Figure 2.6 E-2 treatment leads to a decreased frequency of B cells producing pathogenic antibody. Splenic lymphocytes were isolated from castrated or intact mice treated with E-2 or oil at 2 weeks after 4th treatment, and antibody-producing cells were detected by ELISPOT. E-2 treated mice, both castrated and intact, had a significantly ($p < 0.05$) higher frequency of pathogenic antibody-forming cells compared to oil treated mice. Castration did not have any significant effect. Nonoverlapping letters (a, b, c) mean significant difference ($p < 0.05$).

2.3.11 Effect of castration and E-2 treatment on serum immunoglobulin levels of male *SNF₁* mice

Castrated and treated mice were bled monthly beginning 2 weeks after the first E-2 or oil dose. Serum Ig levels were measured by ELISA. The total serum IgG levels (in Fig 2.7A) were significantly increased after 4 doses of E-2 ($p < 0.05$), with the levels in castrated mice only slightly lower than those for intact mice ($p > 0.05$). When anti-dsDNA IgG was measured (Fig 2.7B), only E-2 treated castrated mice developed significantly higher levels, after the 6th injection ($p < 0.05$). Pathogenic antibody $\text{Id}^{\text{LN}}\text{F}_1^+$ IgG levels were also elevated by E-2 in both intact and castrated mice

compared to oil control mice (Fig 2.7C); interestingly the levels of this antibody in castrated mice treated with E-2 appeared at later time points compared to intact mice, which produced higher Id^{LN}F₁⁺ IgG levels much earlier. However, this difference was not statistically significant.

2.3.12 Effect of castration and E-2 treatment on serum cytokine levels of male SNF₁ mice.

Serum cytokine levels of castrated and E-2 or oil treated male SNF₁ mice were measured monthly. The results (Fig 2.8) showed that E-2 administration led to significantly ($p < 0.05$) higher levels of IL-5, IL-6, TNF- α and IFN- γ in both castrated and sham-castrated male mice at multiple time points; however, IL-12 serum levels were decreased by E-2 treatment in both groups. On the other hand, the castration alone did not have any notable effect on serum cytokine levels of interest.

2.4 Discussion

Autoimmune diseases, in general, are diseases in which the adaptive immune system initiates an injurious attack on normal tissues of self. More than 70 chronic disorders, which afflict ~5% of the US population, are classified as autoimmune diseases, either systemic (involving many body organs) or organ-specific. Sex differences were noted in the earliest descriptions of Systemic Lupus Erythematosus (SLE) and multiple sclerosis (MS). The most apparent gender bias is observed in SLE, Sjogren's syndrome, autoimmune thyroid disease and scleroderma; in these diseases more than 80% of the patient populations are female. In rheumatoid arthritis, multiple sclerosis, and myasthenia gravis, 60-75% of the patients are women. In diseases such as sarcoid, the inflammatory bowel diseases and immune-mediated diabetes, the

Table 2.4 Phenotype analysis. Castrated and sham-castrated control SNF₁ mice treated with E-2 or oil were sacrificed 2 weeks after the 4th injection of E-2. Splenocytes (A) or affinity-purified splenic T cells (B) (1×10^5 cells) were stained with the appropriate antibodies, and analyzed by flow cytometry. Data was expressed as the mean percentage \pm SD (*, p<0.05; **, p<0.01 compared to intact control mice).

A. Total splenocytes

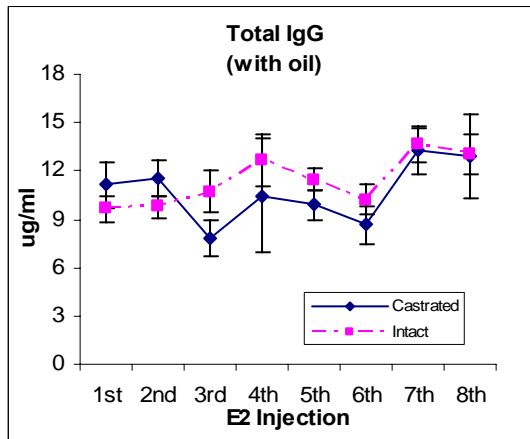
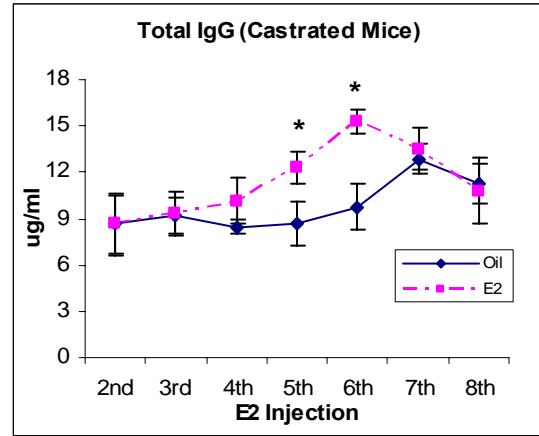
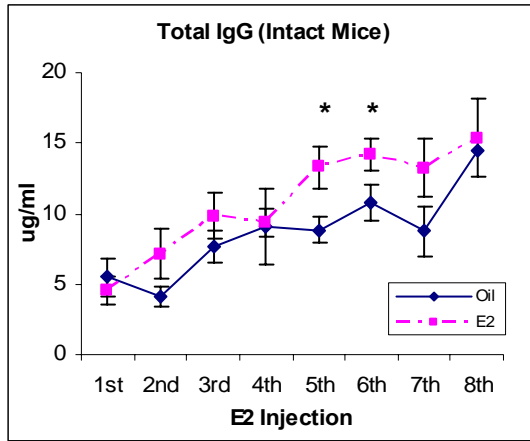
	Oil Intact		E2 Intact		Oil Castrated		E2 Castrated	
B220+	65.20	\pm 4.14	38.16	\pm 0.88*	63.69	\pm 8.45	28.15	\pm 4.32*
CD3+	21.48	\pm 4.58	54.06	\pm 2.03*	37.18	\pm 11.46	50.66	\pm 3.54*
Id ^{LN} F ₁ +CD3+	3.29	\pm 0.26	6.35	\pm 0.55*	4.66	\pm 0.84	5.73	\pm 0.91*

B. Affinity-purified T cells

	Oil Intact		E2 Intact		Oil Castrated		E2 Castrated	
Id ^{LN} F ₁ +	4.39	\pm 1.09	9.37	\pm 1.18*	3.41	\pm 0.48	5.37	\pm 0.23*
Id ^{LN} F ₁ +CD3+	3.20	\pm 0.66	9.09	\pm 0.76*	2.66	\pm 0.69	4.55	\pm 0.52*
CD4+	70.74	\pm 2.21	63.80	\pm 1.11	67.44	\pm 3.26	62.80	\pm 2.33
CD8+	9.59	\pm 1.54	24.22	\pm 3.88	15.48	\pm 2.94	21.22	\pm 0.78
Id ^{LN} F ₁ +CD4+/ Id ^{LN} F ₁ +CD8+	0.88	\pm 0.20	2.11	\pm 0.45*	1.91	\pm 0.41	2.00	\pm 0.37
CD44+CD4+ Id ^{LN} F ₁ +/CD4+	4.96	\pm 0.77	9.27	\pm 1.25*	2.50	\pm 0.59	3.99	\pm 0.78

Figure 2.7 Serum Immunoglobulin levels in castrated or intact male SNF₁ mice. The mice were bled from the retro-orbital plexus monthly beginning two weeks after the first injection of E-2 or oil. Serum total IgG, anti-dsDNA IgG and Id^{LN}F₁⁺ IgG were detected using ELISA. For each Ig, 3 plots were generated, intact mice with E-2 v. oil, castrated mice with E-2 vs. oil and intact vs. castrated mice with oil. (*, p<0.05, compared to intact control or oil control mice).

A. Total IgG



B. anti-dsDNA IgG:

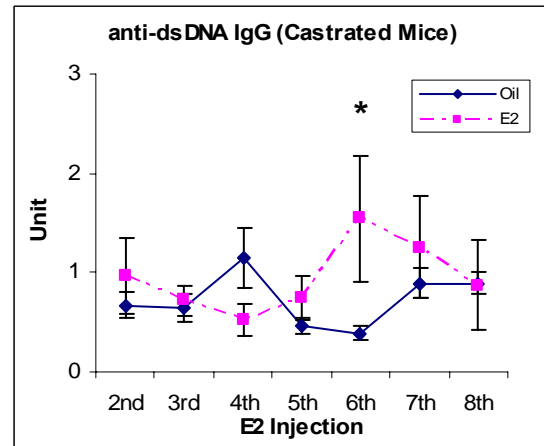
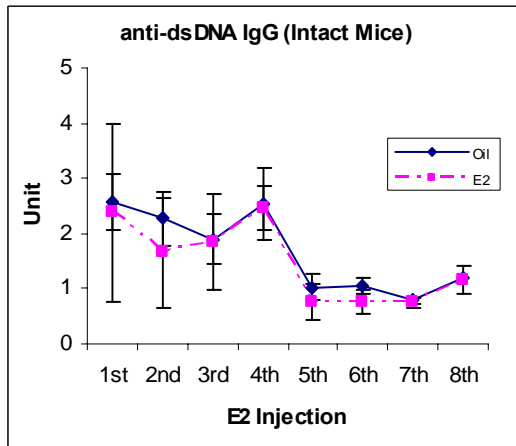
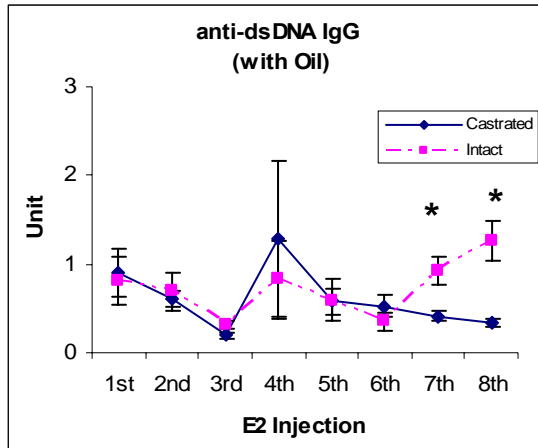


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C. Serum Id^{LN}F₁⁺ IgG level

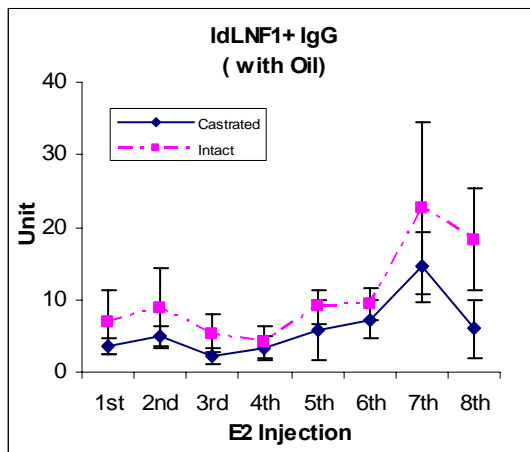
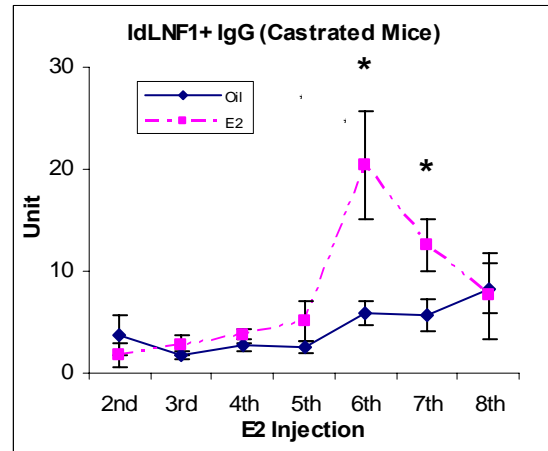
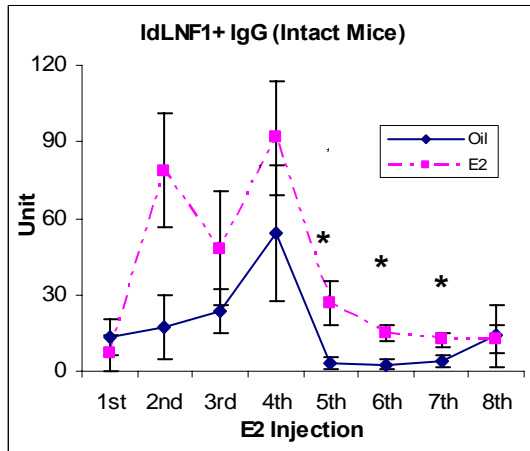
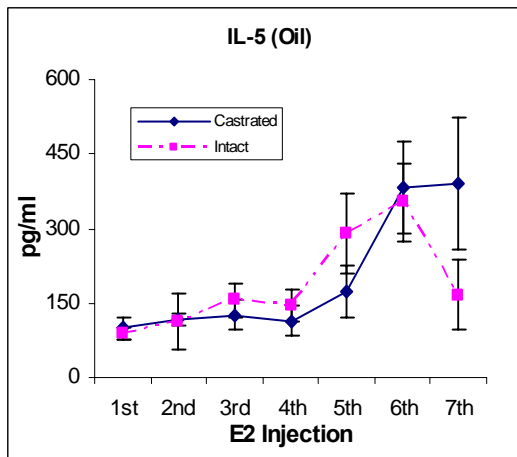
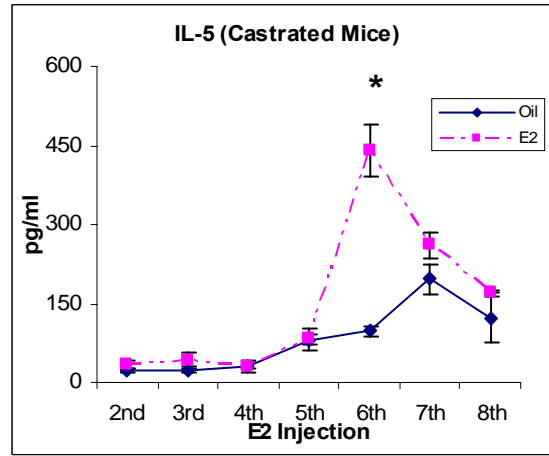
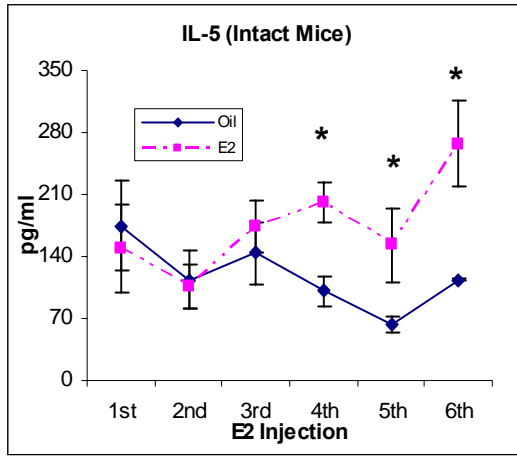


Figure 2.8 Serum cytokine levels in castrated or intact male SNF₁ mice treated with or without E-2. Mice were bled from the retro-orbital plexus monthly beginning two weeks after the first injection of E-2 or oil. Serum was collected and the IL-2, IL-12, IFN- γ , IL-4, IL-5, IL-6, IL-10 and TNF- α level in the serum were detected using ELISA. For each cytokine, 3 plots were generated, intact mice with E-2 v. oil, castrated mice with E-2 vs. oil and intact vs. castrated mice with oil. (*, $p < 0.05$, compared to intact control or oil control mice).

A. IL-5:



B.

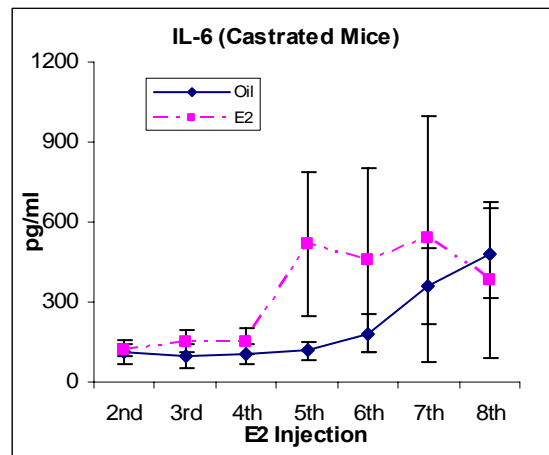
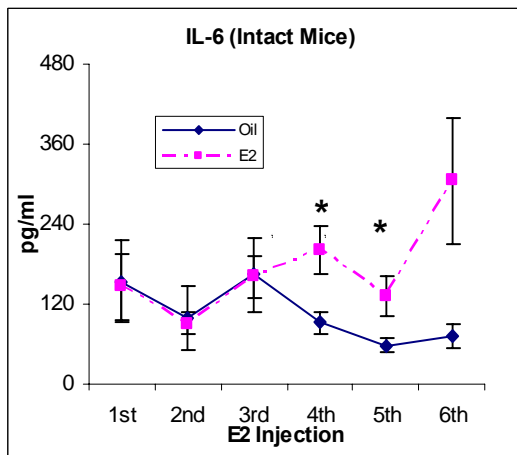
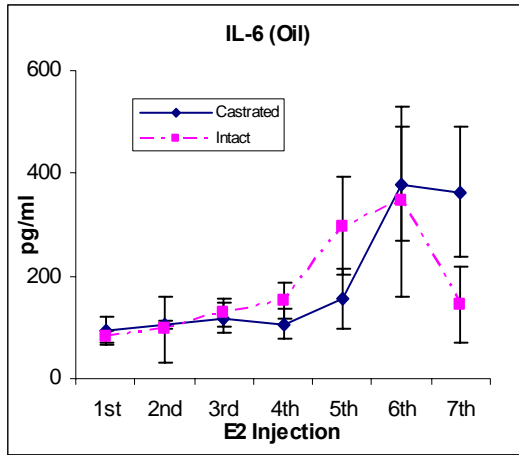


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C.

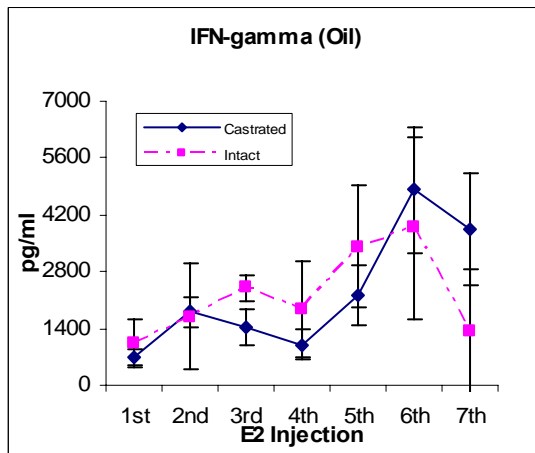
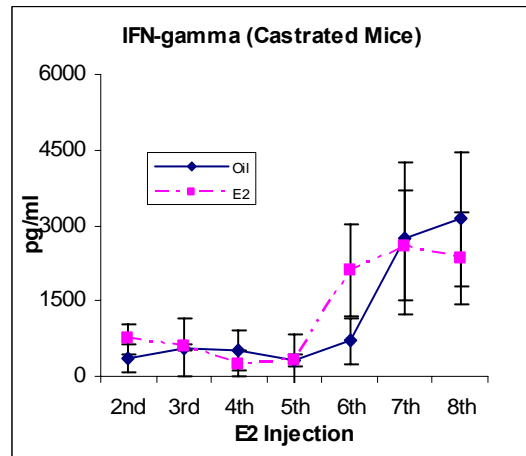
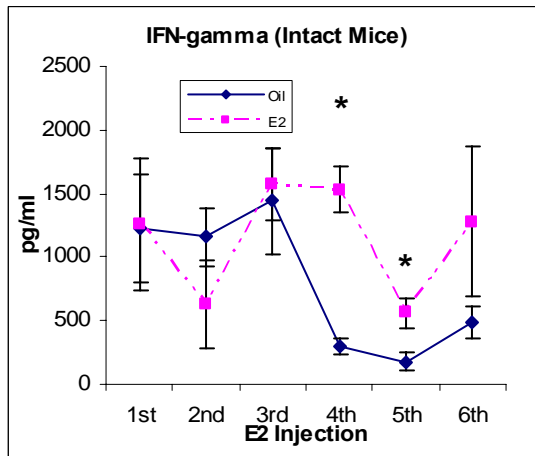
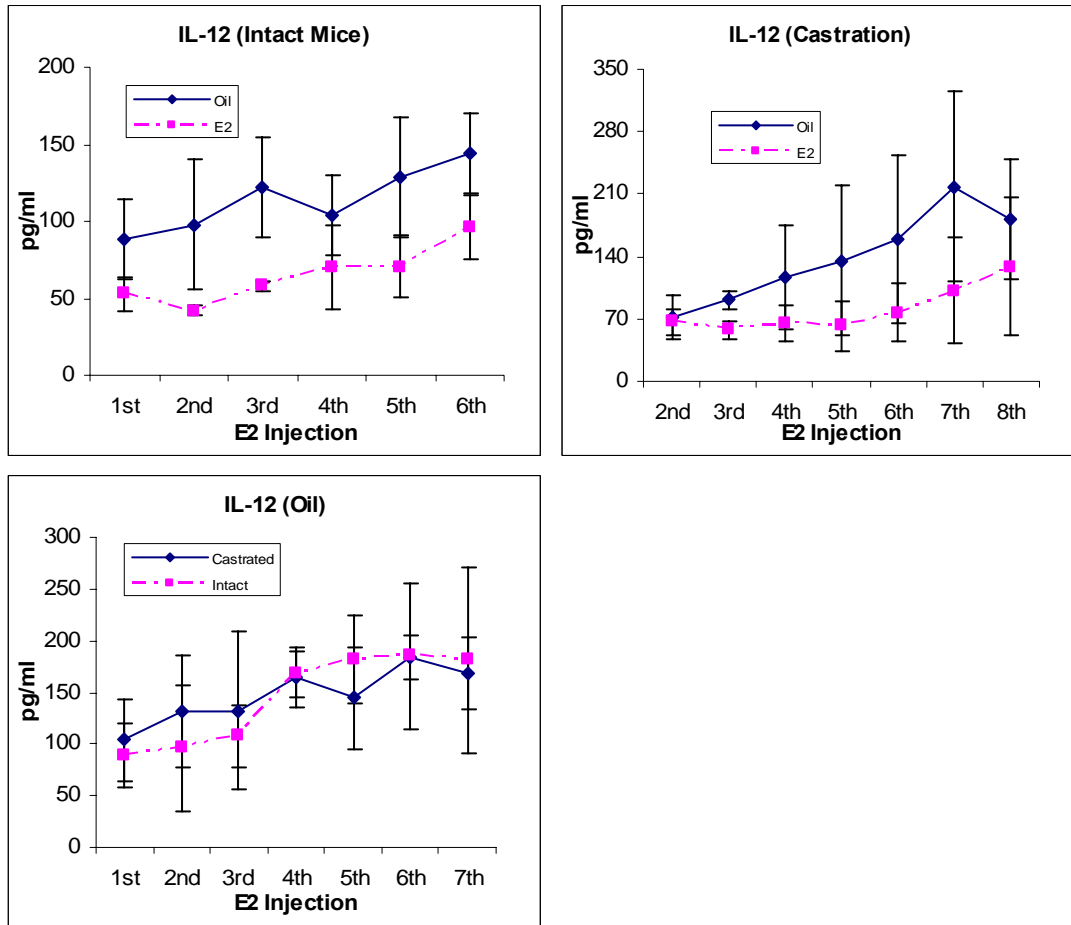


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D.



E.

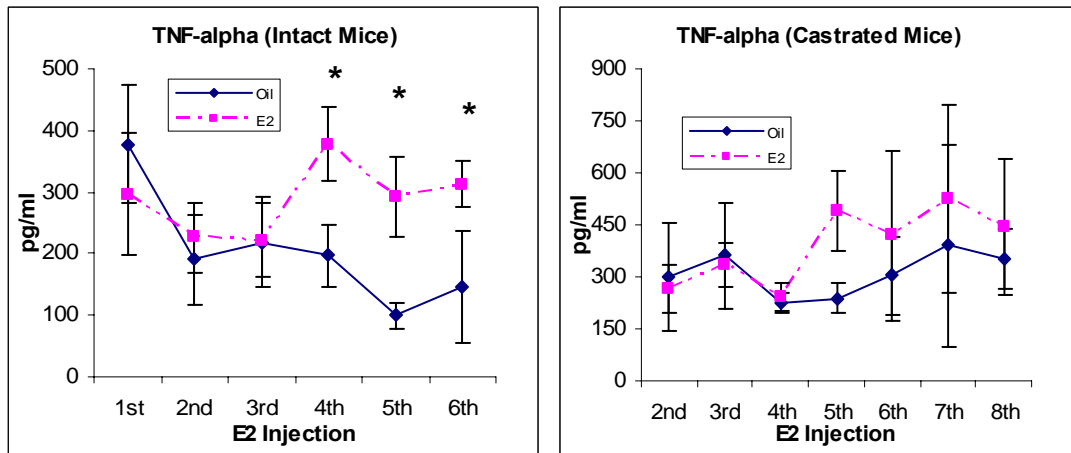
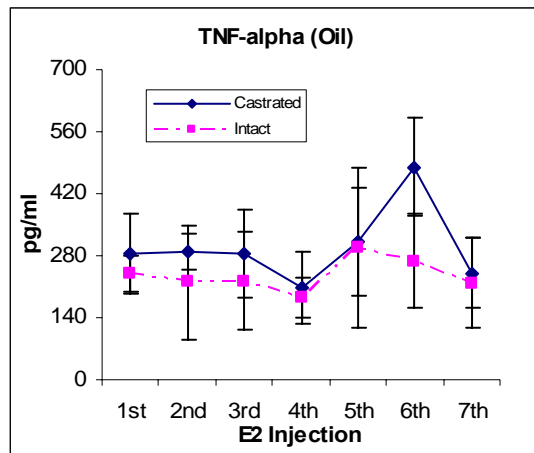


Figure 2.8 (Continued)



female to male ratio of occurrence is about 1:1 [50-52]. In addition, B27 spondyloarthropathy and Goodpasture's disease are male predominant [50]. Although the sex differences are well documented in many autoimmune diseases and some experimental animal models, the mechanism(s) is not fully understood.

Systemic lupus erythematosus is a multi-systemic autoimmune disease with a striking sex difference and female to male ratio of 9:1 [1, 2, 50, 51, 185-187]. The F_1 cross between NZB and SWR, (SWRXNZB) F_1 (SNF $_1$), is an experimental model for SLE. In this model, the mice spontaneously develop a disease resembling human lupus, which is characterized by the production of high levels of anti-nuclear auto-antibodies and fatal lupus nephritis. However, there is a notable gender bias in terms of disease onset and severity. Female SNF $_1$ mice spontaneously develop early disease and almost all of the mice die by 12 months of age from chronic glomerulonephritis, while the male mice develop mild lupus nephritis later in their lives and can live up to 18-20 months of age [54]. Sex differences are also documented in other mouse models of lupus, such as (NZB X NZW) F_1 mice and MRL *lpr/lpr* mice, both of which show high

female incidence and severity. On the other hand, the BXS_B model shows male predominance.

Sex hormone influences on the immune system are thought to be involved in gender bias in autoimmune diseases, including SLE [59, 60]. However, non-hormonal mechanisms, such as skewed X chromosome inactivation, exposure, behavior, etc, have been suggested to contribute to the loss of immune tolerance and therefore, increased incidence of autoimmune diseases in females (reviewed in [62]). A better understanding of the immunomodulatory effects of sex hormones will be required to reconcile some of the contradictions observed. In the present study, the impact of sex hormones (estradiol and testosterone) on the course of lupus was investigated by ovariectomy or castration as well as E-2 treatment of male and female SNF₁ mice in order to further understand the role of sex hormones in the pathogenesis of SLE.

As expected, ovariectomy of female SNF₁ mice effectively reduced the serum E-2 concentration to a level nearly identical to that of the male mouse. Body weight was increased in ovariectomized mice compared to intact control mice, and the median survival of ovariectomized mice was also increased, from 44.1 weeks to 62.1 weeks. This was accompanied with a notable reduction in glomerular damage in kidneys compared to sham-operated control mice. Also as expected, castration of male SNF₁ mice led to decreased serum T-1 levels. In contrast to ovariectomy, castration had no effect on survival compared to intact mice, and histological examination showed comparable immunopathology between castrated and intact control mice. However, the administration of E-2 to male SNF₁ mice, either castrated or intact, led to significantly reduced survival (55 weeks vs. 70 weeks) and more severe glomerulonephritis. To summarize, in general, the reduction in E-2 levels in female mice appeared to be protective, and exogenous E-2 administration to male mice

induced lupus, while the reduction in T-1 levels in male mouse had little effect on SNF₁ disease.

The effects of sex hormones on survival and renal histopathology were accompanied by the changes in the immune responses of the treated mice. In the female mouse, the reduction in serum E-2 levels by ovariectomy led to decreased proliferative responses to pathogenic peptide aa62-73 and a shift in the *in vitro* cytokine profile toward T_H1, with more IL-12 and IL-2 produced and less IL-5, IL-6, and TNF- α . This was in agreement with the observations that, in general, SLE shows a T_H2 bias, with skewing to T_H1 beneficial or protective [39, 49]. We also found that ovariectomized mice produced more anti-Id^{LN}F₁⁺ antibody (anti-aa62-73) producing cells and fewer Id^{LN}F₁⁺ antibody producing cells. Further, the splenocytes from intact female mice produced more total IgG, anti-dsDNA IgG, anti-ssDNA and Id^{LN}F₁⁺ IgG. Of a critical importance to disease pathogenesis, pathogenic T cell populations, Id^{LN}F₁⁺CD3⁺ and Id^{LN}F₁⁺CD4⁺CD3⁺ [28, 54], were decreased significantly by ovariectomy. Conversely, in male mice, the administration of E-2 had the opposite effect and exacerbated disease. E-2 treatment of both castrated and sham-castrated mice led to increased proliferation to pathogenic peptide aa62-73. We also noted shifted cytokine production to T_H2 cytokines and increased numbers of Id^{LN}F₁⁺ antibody-producing cells in E-2 treated mice compared to oil-treated controls. However, castration alone of mice did not lead to modulation of lupus-related changes in either the T or B cell compartments, which is consistent with its lack of effect on survival and renal histology. The changes in serum levels of autoantibodies and cytokines after E-2 treatment of male SNF₁ mice also confirmed that E-2 administration accelerated and exacerbated the disease, with natural levels of androgens having little or no effect on disease pathogenesis.

The action of sex hormones is thought to be largely mediated through specific receptors for estrogen and androgen. To date, two estrogen receptors has been identified with both nuclear and membrane forms [129-131, 133, 134]. Various immune cells have also been found to express ER, such as T lymphocytes, B lymphocytes, macrophages, dendritic cells and NK cells [169, 196]. After binding to ER, estrogen is translocated to the nucleus as a homodimeric steroid receptor, and binds to specific response elements (ERE's or estrogen response elements) in the promoter regions of a variety of genes, modulating their transcription [197]. There is also evidence supporting the existence of quick E-2 actions via membrane forms of ERs, which leads to rapidly increased levels of Ca^{2+} or NO, calcineurin and activation of kinases [149, 154]. Indeed, gender differences as well as E-2 induced differences in the expression of ER isoforms in some lymphocyte subsets have been noted [169, 176], which could be responsible for the gender difference in immune responses, and the bias towards females in the incidence of autoimmune diseases. Expression of the androgen receptor has been documented in lymphoid and nonlymphoid cells of thymus and bone marrow, but not in mature peripheral lymphocytes [198]. This expression pattern indicates that the major impact of androgens is likely to be on the developmental maturation of T and B lymphocytes rather than on mature effector cells. Based on the findings in this study and the literature, one hypothesis to explain the role of estrogen in SLE is that E-2 might act directly on the pathogenic T cell populations stimulating their expansion. These cells then provide help to B cells to produce pathogenic autoantibody. Another possibility is that E-2 may act directly on B cells to initiate or exacerbate disease development.

It should be mentioned that in one study, the castration of mice from a different lupus mouse strain, (NZB X NZW) F_1 , yielded opposing results as to current study, that is castration prolonged the survival while ovariectomy had little effect [199].

Exogenous testosterone treatment has been shown to protect lupus development in another study [193], while we found that castration had no effect. The variable responses suggest that the pathogenic mechanisms involved in lupus nephritis and/or the role of estrogen in disease may vary among different lupus mouse strains and patients. Another factor that may have limited the impact of castration could have been the timing of surgery, particularly since the expression pattern of androgen receptor suggest that androgen may act on immature lymphocytes during development [198].

CHAPTER 3

Estrogen is responsible for the sex difference in the development of autoimmune lupus-like nephritis in mouse model: evidence from a bone marrow transplantation model

Abstract

A very striking female predominance has been documented in the autoimmune disease systemic lupus erythematosus (SLE), which has a female to male occurrence ratio of 9:1. Many mechanisms are proposed to explain the sex discrepancy of incidence among SLE and other autoimmune diseases, such as sex hormones (especially the female sex steroid, estrogen), X inactivation, gene imprinting, differential exposures to antigens and pathogens, etc. In this study, we first treated male SNF₁ mice, which develop later onset disease, with estrogen to determine estrogen's effects on disease kinetics and immunological events involved. As expected, β -estradiol 17-valerate (E-2) treatment at 1mg/kg monthly accelerated lupus nephritis and shortened the life span of male SNF₁ mice, so that disease kinetics were exactly similar to female SNF₁ mice, accompanied with augmentation of pathogenic T and B cell responses. Next, we constructed bone marrow chimeric mice using both autoimmune and non-autoimmune mice of different genders with or without estrogen treatment to identify the effects of estrogen on the development and pathogenesis of lupus disease. Studies of chimeric mice derived from male and female SNF₁ mice suggested the male and female SNF₁ immune systems were equally pre-disposed to develop lupus if estrogens were present. Only female mice reconstituted with male or female bone marrow developed early disease, while E-2 treatment was required to accelerate disease in male mice after reconstitution with either male or female marrow. These results suggested the important role of E-2 in lupus pathogenesis. Chimeras

derived from DBF₁ and SNF₁ also suggested the role of E-2 in the pathogenesis of lupus, in that DBF₁ chimeras reconstituted with SNF₁ bone marrow developed lupus disease in the presence of E-2 naturally (in female mice) or exogenously (in E-2 treated male mice). Taken together, these results support strongly a critical role for estrogen in determining sexual dimorphism in SLE.

3.1 Introduction

Human diseases affect males and females differently. In some disorders such as those that affect sex organs or result from X or Y chromosome dysfunctions, the reasons are obvious; however in others the reasons are not apparent. Autoimmune diseases comprise more than 70 distinct clinical entities affecting about 5% of the US population. Among the earliest and most intriguing observations on autoimmune diseases is the dimorphism in the prevalence of disease between men and women. A very striking sex discrepancy has been documented in systemic lupus erythematosus (SLE), which has a female to male occurrence ratio of 9:1 [60, 61, 158, 200]. Similar to observations in human patients, lupus in the majority of mice mouse models, such as the (NZB x NZW)F₁ mouse, (SWR x NZB)F₁ mouse and MRL *lpr/lpr* mouse, shows high female incidence and severity (earlier onset, more severe disease and shorter lifespan).

The sex dimorphism of SLE, along with the dramatic increase in disease incidence in females after puberty, the decreased occurrence after menopause, and the variation in disease severity correlating with the menstrual cycle and pregnancy, suggests a role for sex hormones in this autoimmune disease, SLE [185, 200, 201]. Besides effects on sex differentiation and reproduction, sex hormones have been appreciated for their role in immunophysiology [202]. The female sex steroid,

estrogen, is found to affect both adaptive and innate immune responses, such as the promotion of T_H2 cytokine production by lymphoid and dendritic cells, suppression of antigen presentation by antigen-presenting cells and decreased responses of natural killer cells [2, 76, 203, 204]. Despite these and many other observations in support of the immune-regulating effects of estrogens, the actual role of estrogen in the gender bias of SLE and the possible mechanisms involved are still uncertain. X inactivation, imprinting, X or Y chromosome genetic modulators and intrauterine influences have also been proposed as the alternate explanation for sex-based differences in incidence [205]. Furthermore, differential exposure to pathogens and vulnerable periods might also contribute to the sex discrepancy [205]. Lastly, influences by sex hormones in nonimmunologic ways, such as hormone effects on endothelium, may also be critical in disease initiation.

Bone marrow transplantation is used experimentally to examine the development of lymphocytes, as opposed to their effector functions. It involves destroying the immune system of a host mouse (usually by a lethal dose of irradiation) and replacing it with hematopoietic stem cells from a donor mouse's bone marrow, such that the host develops the immune system of the donor. In the present study, male (NZB x SWR)F₁ (SNF₁) mice, an autoimmune murine model of lupus that exhibits gender bias in disease onset [72, 73], were treated with β -estradiol 17-valerate (1mg/kg) to examine its effects on disease and immune responses. Then male/female chimeric mice from autoimmune SNF₁ and non-autoimmune DBF₁ mice were derived in order to identify the role of estrogen in the development of immune responses and the lupus disease in the host mouse. To our knowledge, this study is the first using bone marrow transplantation techniques to identify the role of estrogen in the pathogenesis of lupus.

The results establish a clear relationship between estrogen and the development of autoimmune disease.

3.2 Materials and methods

3.2.1 Mice and E-2 treatment

Six to eight week old female SWR/J, male NZB/BLNJ mice, male DBA/1 mice and female BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The (SWR x NZB) F_1 progeny (SNF $_1$) and (DBA x BALB/c) F_1 progeny (DBF $_1$), both H-2^{d/q}, were bred and maintained in the Cornell University College of Veterinary Medicine, Center for Research Animal Resources and Education (CARE). Mice received β -estradiol 17-valerate (1mg/kg, Sigma) in olive oil vehicle subcutaneously once every month starting at 7 weeks of age for some groups; control mice were injected with vehicle alone. Body weights were recorded monthly. Groups of 3-4 mice were sacrificed at 2 weeks after 4th injection. The thymus, spleen and kidney were harvested and cell suspensions prepared.

3.2.2 Serum collection

Blood was obtained from the retro-orbital sinus under isoflurane anesthesia monthly to determine the kinetics of disease progression, beginning two weeks after the first E-2 treatment. For terminal bleeds, mice were euthanized via CO₂ asphyxiation, and blood was obtained by cardiac puncture. Sera was collected by centrifugation and then frozen at -20°C until use.

3.2.3 Bone marrow reconstitution

To prepare donor cells, six-week old SNF $_1$ or DBF $_1$ mice (n=3) were sacrificed, and a bone marrow cell suspension was made from femurs. One million cells in PBS containing 2% fetal bovine serum were injected intravenously by tail vein into age-

matched recipient mice that had been X-irradiated by split dose (1100 rads total) 1 day earlier. The reconstituted mice were maintained on acid water (pH of 2) for at least a month.

3.2.4 T cell proliferative response

Splenic T cells (2.5×10^5) and APCs (2.5×10^5 , gamma-irradiated at 3000 rads) were combined to form the different treatment groups for cell mixing responses. For total splenocyte responses, 5×10^5 splenocytes per well were cultured. aa62-73 peptide or Mal control peptide at $300 \mu\text{g/ml}$, anti-CD3 mAb (Sigma) at $40 \mu\text{g/ml}$, or ConA (Boehringer Mannheim) at $2 \mu\text{g/ml}$ were added to the cells. After incubation at 37°C with 5% CO_2 for three days, 1mCi/well of [^3H]-thymidine (Amersham, Arlington Heights, IL) was added, and incubation continued overnight. Then, the cells were lysed, collected onto filter mats and incorporated radioactivity measured by a scintillation counter (Packard, Meriden, CT).

3.2.5 Cytokine production

Splenic lymphocytes were cultured in triplicate at a concentration of 1×10^6 per well with ConA ($2 \mu\text{g/ml}$) or aa62-73 ($300 \mu\text{g/ml}$) for three days at 37°C under 5% CO_2 . Supernatants were harvested and stored at -70°C until analysis by ELISA (eBioscience) for IL-2, IL-4, IL-5, IL-6 and IFN- γ according to the manufacturer's procedure. The data were expressed as pg/ml .

3.2.6 ELISPOT determination of B cell frequencies.

For determination of the frequency of Id^{LN}F₁-producing B cells, 96-well microtiter plates with nitrocellulose bottoms (Millipore) were coated with rabbit anti-Id^{LN}F₁ Ab, aa62-73, control peptide Mal, anti-540 Ab, or normal rabbit Ig overnight at 4°C . The plates were washed with PBS-0.1% Tween and blocked with PBS-3% BSA for 1 hour, then washed again. Splenic lymphocytes diluted in culture medium were added at a concentration of 5×10^6 cells/well in triplicate. The plates were

incubated overnight at 37°C with 5% CO₂. Specific Igs secreted by B cells bound to the plate-bound antibody were detected by biotin-conjugated anti-mouse IgG, followed by SA-HRP (streptavidin conjugated to horseradish peroxidase, Amersham Life Sciences) and 3-amino-9-diethylcarbazole and H₂O₂ as substrate. Brown spots corresponding to cells secreting specific Ig were counted, and the data were expressed as the frequency of B cells per 10⁵ cells.

3.2.7 Antibody production

In triplicate, 5x10⁵ Ig⁺ splenocytes purified by Ig panning were cultured with 1x10⁵ affinity-purified T cells from different treatment groups in the combinations with 50µl aa62-73. The cultures were incubated for seven days at 37°C with 5% CO₂. The supernatants were then harvested and frozen at -20°C until analyzed by ELISA for total anti-Id^{LN}F₁ IgG, as previously described [29]. Briefly, Immulon 1B 96-well plates (Krackeler Scientific, Albany, NY, USA) were coated with prepared rabbit anti-Id^{LN}F₁ Ig. Then the diluted sample was added in triplicate. A standard curve was generated using duplicates of eight, two-fold serial dilutions of affinity-purified specific IgG starting at 1µg/ml in PBS/0.1% Tween. Then goat anti-mouse IgG or IgM-horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) was added per well and the incubation was continued overnight. The plates were then washed and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was added to each well. Color development proceeded for five minutes to one hour at room temperature (RT) until significant blue color developed. The reaction was stopped by the addition of 1M phosphoric acid and the O.D. was read at 450nm (Biorad). Ig concentrations were determined from the corresponding linear standard curves, and were calculated as µg/ml.

3.2.8 Flow cytometric analysis of splenic lymphocytes

The splenocyte suspension was treated with tris-ammonium chloride (TAC) and then enhanced for T cells by affinity chromatography (T cell Recovery Column, Biotex). 1×10^5 cells were stained with the appropriate dilution of biotin-, fitc-, and phycoerythrin-conjugated antibodies and streptavidin-red670 (Pharmingen). The following antibodies were used, anti-CD44-PE, anti-CD4-PE, anti-B220-PE, anti-CD45RB-Bio, anti-CD8a-Bio, anti-CD3-Bio, anti-CD25-Bio, anti-CD4-Fitc, and Id^{LN}F₁-Fitc (PharMingen, with the exception of Id^{LN}F₁⁺ fitc-conjugated antibody which was derived from monoclonal 540 antibody-producing hybridoma cell culture supernatants and labeled using a fitc-conjugation kit). The stained cells were fixed in 1% paraformaldehyde and flow cytometry was carried out on a Becton-Dickinson FACSCalibur[®] flow cytometer. The data were analyzed with WinMDI program.

3.2.9 Determination of serum Ig levels

ELISA was used to determine the levels of serum total IgG, anti-dsDNA IgG, anti-ssDNA IgG, and Id⁺ IgG as previously described [29]. Immulon 1B 96-well plates (Krackeler Scientific, Albany, NY, USA) were coated with goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN, USA), or prepared calf-thymus dsDNA (Sigma). Then the diluted serum was added in triplicate. A standard curve was generated using duplicates of eight, two-fold serial dilutions of affinity-purified specific IgG starting at 1ug/ml in PBS/0.1% Tween. Then goat anti-mouse IgG or IgM-horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) was added to each well and the incubation was continued overnight. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was added to each well. Color development proceeded for five minutes to one hour at room temperature (RT) until significant blue color developed. The stop solution (1M phosphoric acid)

was added and the O.D. was read. Serum Ig concentrations were determined from the corresponding linear standard curves.

3.2.10 Determination of serum cytokine levels

Mouse serum was diluted 1:3 or 1:4 in PBS/0.1%Tween and analyzed by ELISA (eBioscience) for IL-2, IL-4, IL-5, IL-6 and IFN- γ as per the manufacturer's instructions. The data were expressed as pg/ml.

3.2.11 Renal histology

Renal histology examination was performed either quantitatively or qualitatively.

Quantitatively, one hundred glomeruli from hematoxylin and eosin (H&E)-stained sections of kidneys were blindly evaluated and graded as either normal, with no or only segmental lymphocyte infiltration or damaged, with globular infiltration, and/or sclerosis. The data were expressed as the percent damaged glomeruli from each treatment group.

Qualitatively, individual glomeruli from each kidney were blindly evaluated and graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis.

3.2.12 Survival

At least 5 mice for each group were kept for the survival, and were euthanized when moribund, as determined by CRAR veterinarians. The data were expressed as the percentage of surviving mice.

3.2.13 Statistics

Statistical significance of treatment on survival was determined by the Logrank test. Glomerular damage grades were analyzed by nonparametric Mann-Whitney test. Cell mixing proliferation data were analyzed by two-way ANOVA. Other data were

analyzed by the Student's t-test or one-way ANOVA test to compare means from two treatment groups. One-way ANOVA followed by Fisher LSD test was used for multiple comparisons of means. Statistical significance was ascribed when p values were less than 0.05.

3.3 Results

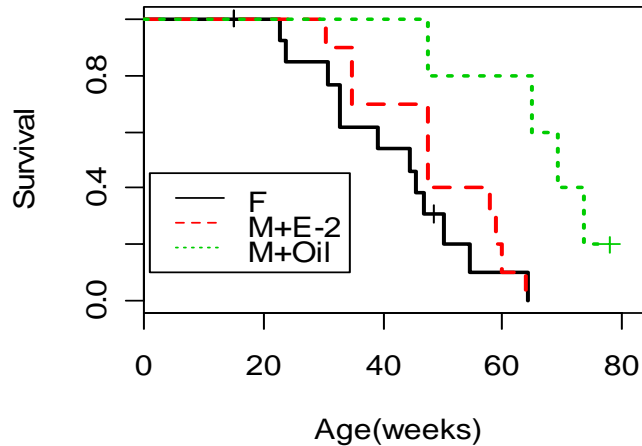
3.3.1 Repeated E-2 administration significantly decreases the survival of male SNF₁ mice

Male SNF₁ mice were injected with 1mg/kg E-2 subcutaneously every month starting at 7 weeks of age. This dosing regimen elevates mouse serum E-2 level to 150~200pg/ml, which is the peak physiological serum E-2 level in female mice [206]. The level of E-2 is maintained for about 7-10 days and decreases to normal levels found in male mice (<10pg/ml) 2 weeks after treatment. We found that survival of E-2 treated male SNF₁ mice was significantly shortened (mean survival of 47.7 weeks) compared to oil treated mice (mean survival of 69.3 weeks) (p<0.05) (Fig. 3.1A). Body weight was also decreased significantly (p<0.05), as early as after the second E-2 exposure (Fig. 3.1B). While the mean body weight of E-2 treated mice did not increase much over time, the mean body weight of the control group went up significantly, so that at 7 months (after the 5th E-2 injection), the mean body weight of oil control group (55.4g) was nearly twice that of E-2 treated group (30.8g).

3.3.2 E-2 administration of SNF₁ male mice leads to increased glomerular damage.

Hematoxylin & Eosin stained kidney sections from SNF₁ male mice obtained two weeks subsequent to the 4th dose were blindly evaluated and graded for order of severity of damage as being normal, with no lymphocytic infiltration, or damaged, having segmental and/or globular infiltration, or sclerosis. A significant increase

A.



B.

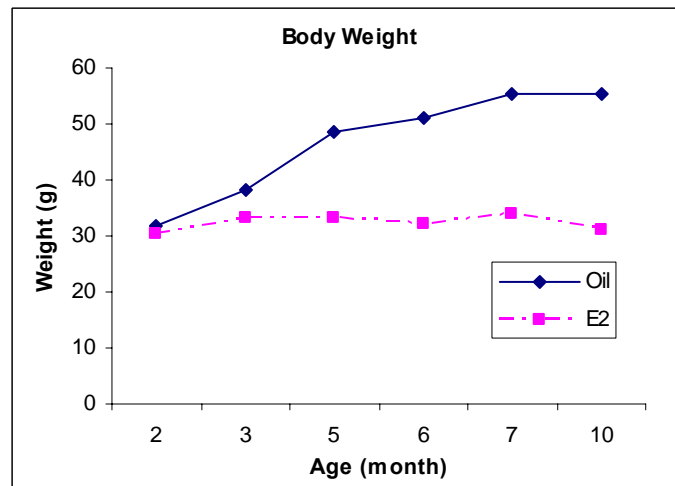


Figure 3.1 E-2 administration leads to decreased survival of male SNF₁ mice. A. Male SNF₁ mice were injected with E-2 (1mg/kg) or oil subcutaneously every month starting at age of 7 weeks. The survival curve for male SNF₁ mice with E-2 (M+E2), male SNF₁ mice with oil (M+Oil) and female SNF₁ mice (n=6 for each treatment group) were plotted for comparison. E-2 exposure significantly reduced survival of male SNF₁ mice (mean survival = 47.7 weeks) compared to controls (mean survival = 69.3 weeks); survival for E-2 treated male mice was similar to females (mean survival = 45.3 weeks). B. Body weight is decreased by E-2. Male mice body weights were recorded monthly and E-2 treatment in male mice led to the decreased body weights starting at 3 months of age.

($p < 0.05$) in damaged glomeruli ($38.3 \pm 5.8\%$ in E-2 treated mice vs. $15.4 \pm 5.4\%$ in oil-treated control) with a concomitant significant decrease in normal glomeruli (not shown) was seen in SNF₁ mice after 1mg/kg E-2 treatment (Fig 3.2).

3.3.3 E-2 treatment leads to increased proliferation to pathogenic aa62-73 peptide.

The proliferative responses of T cells were measured to determine the effect of estrogen treatment on cell function. At 24 weeks (2 weeks after 4th E-2 treatment), splenocytes from treated and control male mice were harvested and isolated, and the proliferative responses to ConA, anti-CD3 mAb, aa62-73 or irrelevant Mal peptide of total splenocytes or affinity-purified T cells from each treatment group were measured. As shown in Fig 3.3A, E-2 treatment led to decreased mitogen-induced proliferation in splenocytes (6941 ± 1375 cpm vs. 15467 ± 5049 cpm), but proliferation was increased in the presence of anti-CD3 mAb (20418 ± 1327 cpm vs. 7768 ± 3807 cpm, $p < 0.05$) and the pathogenic aa62-73 peptide (4317 ± 127 cpm vs. 3224 ± 199 cpm, $p < 0.05$).

To further identify whether the effect of estrogen was on T cells and/or APCs, proliferative responses were measured in mixed cell cultures. Affinity-purified splenic T cells were used as responder cells and APCs were either irradiated splenocytes (Fig 3.3B) or irradiated Ig⁺ cells (Fig 3.3C). The former population contained all possible antigen-presentation cells, such as B cells, dendritic cell, macrophages, and the latter is using primarily B cells. Cell cultures were set up as follows: oil treated APCs plus oil treated T cells, E-2 treated APCs plus oil treated T cells, oil treated APC plus E-2 treated T cells, and E-2 treated APCs plus E-2 treated T cells. The results were analyzed using two-way ANOVA with T cell effect and APC effect as 2 factors. We found that E-2 treatment could lead to significantly altered proliferation due to affected T cell function, with decreased T cell proliferation to Con A ($p < 0.05$) and increased T cell proliferation to anti-CD3 mAb ($p < 0.01$) and pathogenic aa62-73 ($p < 0.001$).

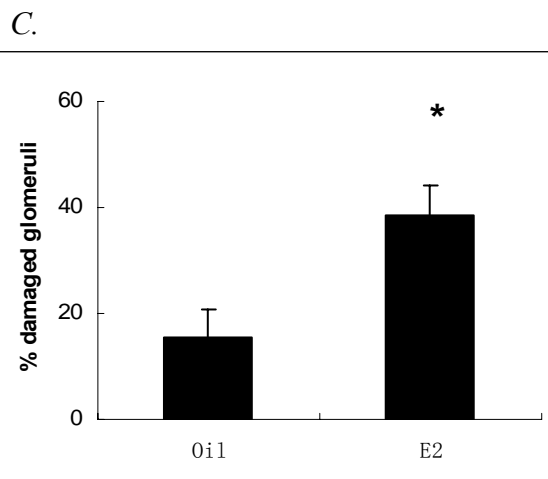
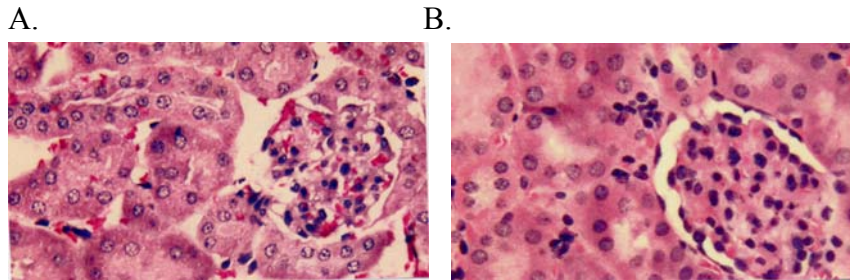


Figure 3.2 E-2 treatment leads to increased kidney damage. Male SNF1 mice were injected subcutaneously with E-2 (1mg/kg) in olive oil monthly, and the control mice were treated with oil vehicle alone. Kidney sections from 24-week old mice in each treatment group were stained with H&E for pathological grading. A, Oil treated SNF₁. Capillary loops were blood filled and there were a small number of cells in the mesangium; B, E-2 1mg/kg treated SNF₁. E-2 treatment led to an increase in mesangial cells and a reduction in blood capillaries. In these glomeruli, the cellular number was increased and the glomeruli were enlarged. There was also obliteration of much of the capillary structure in the glomeruli; C, Summary of glomerular changes caused by E-2 treatment. One hundred glomeruli from hematoxylin and eosin (H&E)-stained sections of kidneys were graded as normal (with no or only segmental lymphocyte infiltration), or damaged (with globular infiltration and/or sclerosis). The data were expressed as percent damaged glomeruli from each treatment group. (*, $p < 0.05$ compared with oil control group).

Moreover, E-2 treatment also led to increased antigen-presentation of aa62-73 peptide by Ig⁺ cells (in Fig 3.3C), and increased proliferation of responder cells to that peptide ($p < 0.05$). Other APCs were not found affected by E-2 treatment (in Fig 3.3B).

3.3.4 E-2 administration increases the frequency of Id^{LN}F₁⁺ antibody producing cells in male SNF₁ mice.

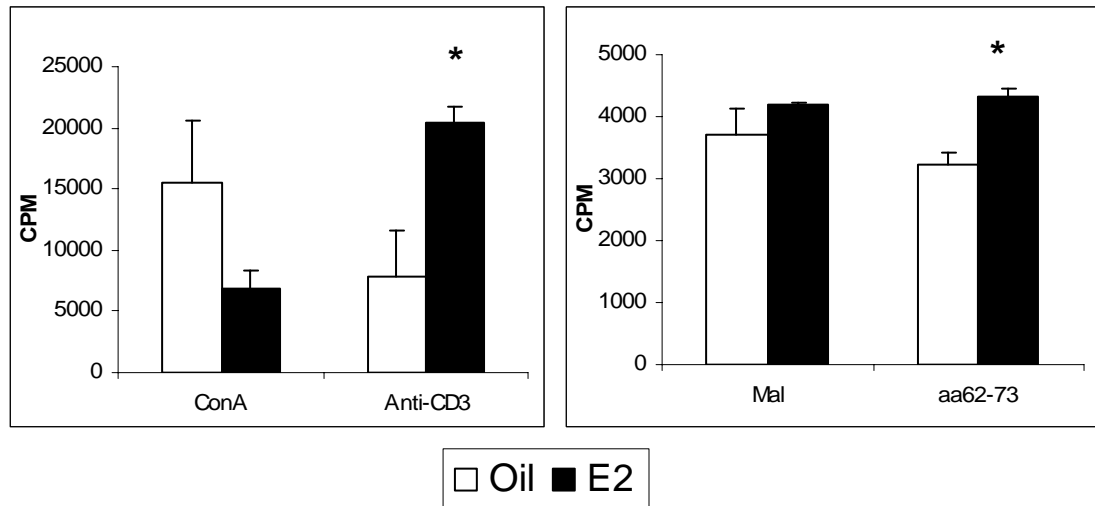
The frequency of antibody-producing B cells in E-2 treated male mice were determined 2 weeks after 4th injection of E-2 or oil (24 weeks of age) by ELISPOT. The results (in Fig 3.4) showed that the frequency of the Id^{LN}F₁⁺ antibody producing cells were significantly increased ($p < 0.05$) in E-2 treated mice ($5.6 \pm 0.5 / 10^5$ cells vs. $8.1 \pm 0.2 / 10^5$ cells), while the frequencies of anti-Id^{LN}F₁ antibody-forming B cells were similar between E-2 ($4.3 \pm 0.9 / 10^5$ cells) and oil treated mice ($5.6 \pm 1.3 / 10^5$ cells) ($p > 0.05$).

3.3.5 Modulation of immune cell phenotypes by E-2 exposure.

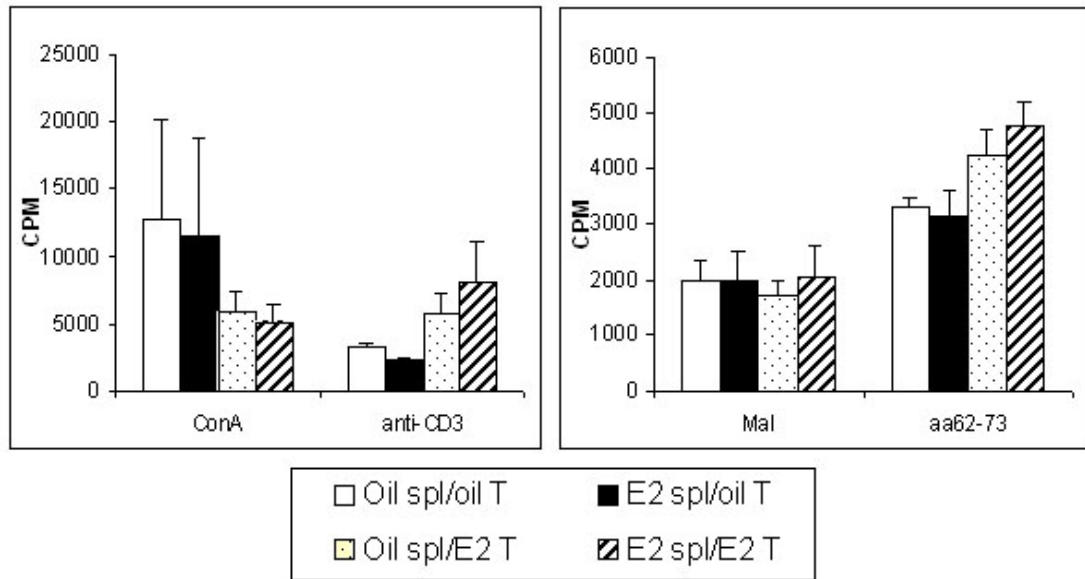
Lymphocyte phenotypes were analyzed 2 weeks after 4th injection of E-2 or oil (24 weeks of age). Both total splenocytes and affinity-purified splenic T cells were analyzed. The results showed that E-2 treatment led to significantly more pathogenic Id^{LN}F₁+CD3⁺ ($7.63 \pm .81\%$ in E-2 treated mice vs. $6.93 \pm 0.62\%$ in controls) and Id^{LN}F₁+CD4+CD3⁺ cells ($4.57 \pm 0.48\%$ in E-2 treated mice vs. $3.86 \pm 0.24\%$ in controls), an increased ratio of Id^{LN}F₁+CD4⁺ to Id^{LN}F₁+CD8⁺ and more CD44+ Id^{LN}F₁⁺ and CD44+CD45- Id^{LN}F₁⁺ (memory phenotype) cells (Table 3.1). These changes indicated the pathogenic cell population involved in SNF₁ nephritis was up-regulated by E-2. We also found that there were significantly more CD25+Id^{LN}F₁⁺ T cells in E-2 treatment mice ($1.86 \pm 0.28\%$) compared to oil controls ($3.35 \pm 0.29\%$) ($p < 0.05$), which is indicating there were more activated pathogenic Id^{LN}F₁-reactive T

Figure 3.3 E-2 administration decreases mitogen-induced proliferation and increases the pathogenic peptide-induced proliferation. Splenocytes were isolated from E-2 treated male mice and control mice two weeks after the 4th injection. A. Splenocytes were cultured in the presence of ConA, anti-CD3 mAb, 62-73 peptide or Mal peptide (a non-relevant control peptide) respectively, and then after 72 hrs the proliferation responses were measured. The splenocytes from E-2 treated mice (E2) had decreased responses in the presence of Con A ($p=0.09$), increased responses in the presence of anti-CD3 antibody and pathogenic aa62-73 ($p<0.05$ and $p<0.01$, respectively). (*, $p<0.05$, compared with oil treated mice). B. Splenic T cells were cultured with irradiated splenocytes as APCs in four combinations: 1)oil treated splenic T cells with oil treated splenocyte APCs (oil Spl/oil T); 2)E-2 treated splenic T cells with oil treated splenocyte APCs (E2 Spl/oil T); 3)oil treated splenic T cells with E-2 treated splenocyte APCs (oil Spl/E2 T); 4)E-2 treated splenic T cells with E-2 treated splenocyte APCs (E-2 Spl/E-2 T). The results were analyzed by two-way ANOVA procedure to identify the effect of E-2 on both T cells and APCs. E-2 treatment led to significantly decreased mitogen induced responses by T cells, and increased anti-CD3 and aa62-73 induced responses by T cells. C. Splenic T cells and irradiated Ig⁺ cells as APCs were cultured in the same combinations as for (B) above. Results suggested that E-2 treatment led to significantly decreased mitogen induced responses by T cells, but increased anti-CD3 and aa62-73 induced responses. Furthermore, the results also showed that E-2 treatment led to enhanced function of Ig⁺ as APCs in the presence of aa62-73, which contributed to the increased proliferative responses of splenocytes from E-2 treated mice to this pathogenic peptide.

A. Total splenocyte



B. Affinity-purified splenic T cells with irradiated splenocytes as APCs.

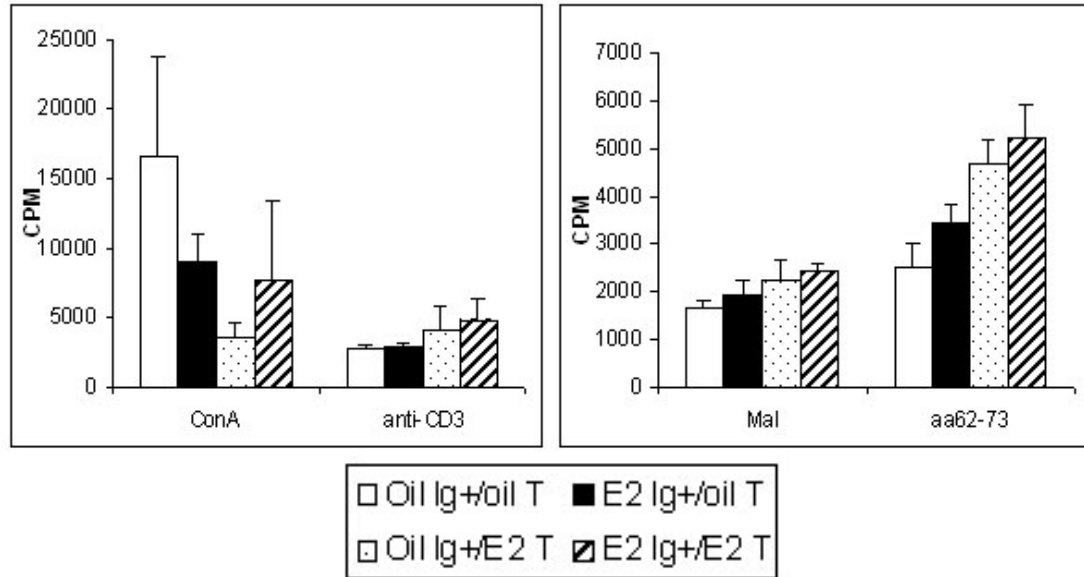


Summary of 2-way ANOVA of E-2 effect

Source of E-2 effect	Stimulus	Effect	P-value
T cells	Con A	Decreased	0.05
T cells	anti-CD3	Increased	0.004
T cells	aa62-73	Increased	0.0006

Figure 3.3 (Continued)

C. Affinity-purified splenic T cells with irradiated Ig+ cells as APCs



Summary of 2-way ANOVA of E-2 effect

Source of E-2 effect	Stimulus	Effect	P-value
T cells	Con A	Decreased	0.05
T cells	anti-CD3	Increased	0.04
T cells	aa62-73	Increased	<0.0001
Ig+ Cells	aa62-73	Increased	0.03

cells in E-2 treated mice. E-2 treated mice also had a lower percentage of B220+ cells ($p < 0.05$) and slightly higher percentage of CD3+ cells.

3.3.6 Effect of E-2 exposure on serum immunoglobulin (Ig) and cytokine levels.

Mice were bled 2 weeks after E-2 or oil administration every month. The serum Ig levels were measured by ELISA. The total IgG level (in Fig 3.5A) was

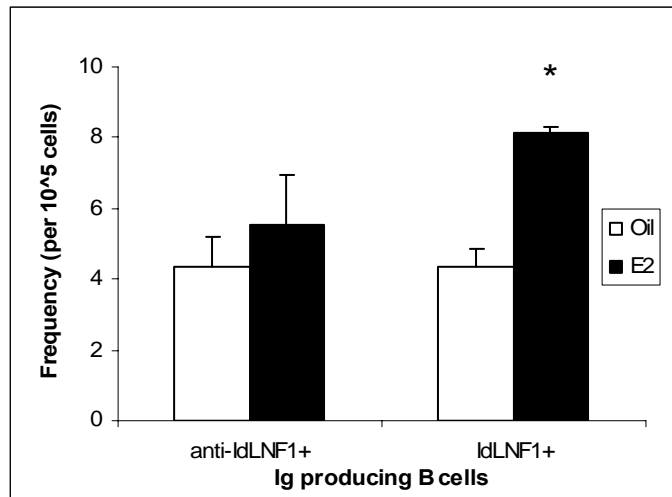


Figure 3.4 B cells producing pathogenic Id^{LN}F₁+ antibody were increased in E-2 treated mice. Splenic lymphocytes were isolated 2 weeks after the 4th E-2 treatment from mice treated with E-2 or oil, and then antibody-producing cells were detected by ELISPOT. E-2 treated mice had a significantly ($p < 0.05$) higher frequency of pathogenic Id^{LN}F₁+ antibody-forming cells compared to oil treated mice (*, $p < 0.05$, compared with oil treated mice).

significantly increased after 4 administrations of E-2 ($p < 0.05$), and was maintained at a higher level throughout the study. Anti-ssDNA IgG levels were significantly increased after the 5th E-2 treatment ($p < 0.05$). E-2 treatment did not lead to increase in anti-dsDNA IgG level; moreover the levels of that antibody in E-2 exposed mice (0.0021 ± 0.00071 mg/ml) were even lower compared to that of oil treated mice (0.005 ± 0.00095 mg/ml) after the 5th E-2 treatment ($p < 0.05$). The pathogenic Id^{LN}F₁+ IgM and IgG levels were elevated by E-2 exposure; Id^{LN}F₁+ IgM increased to a significant level as early as after second injection, while the increase in Id^{LN}F₁+ IgG levels did not achieve significance until the 4th injection ($p < 0.05$).

Table 3.1 Effect of E-2 treatment on lymphocyte phenotypes. E-2 treated and control male SNF₁ mice were sacrificed at 2 weeks after 4th injection of E-2. Splenocytes (a) or affinity-purified splenic T cells (b) (1×10^5 cells) were stained with the appropriate antibodies subjected to four-color flow cytometry. Data were expressed as mean percentage \pm SD (*, $p < 0.05$; **, $p < 0.01$ compared to intact control mice).

(a) Splenocytes						
	Oil			E-2		
B220+	71.36	\pm	6.56	55.47	\pm	3.99*
CD3+	28.33	\pm	5.37	35.93	\pm	2.19
Id ^{LN} F ₁ +CD3+	6.93	\pm	0.62	7.63	\pm	0.81
Id ^{LN} F ₁ +CD4+CD3+	3.86	\pm	0.24	4.57	\pm	0.48
(b) Affinity-purified T cells						
	Oil			E2		
CD4+	63.91	\pm	5.34	57.29	\pm	2.27
CD8+	22.00	\pm	3.88	21.00	\pm	1.38
CD44+CD45-	45.60	\pm	3.68	57.34	\pm	6.26*
Id ^{LN} F ₁ +CD8+/CD3+	0.60	\pm	0.04	0.71	\pm	0.19
Id ^{LN} F ₁ +CD4+/CD3+	2.57	\pm	0.55	2.81	\pm	0.34
Ratio Id ^{LN} F ₁ +CD4+/ Id ^{LN} F ₁ +CD8+	4.29	\pm	0.89	5.20	\pm	2.41
Id ^{LN} F ₁ +CD25+	1.86	\pm	0.28	3.35	\pm	0.29*
Mean Fluorescence of CD25	355.30	\pm	5.43	651.30	\pm	11.90**
Id ^{LN} F ₁ +CD44+	7.96	\pm	2.38	9.38	\pm	0.51
Id ^{LN} F ₁ +CD44+CD45-	1.44	\pm	0.41	2.69	\pm	0.49*

Serum cytokine levels were skewed after E-2 treatment (Fig 3.5B). Specifically, IL-5 and IL-6 serum levels were greatly increased after the 4th injection in E-2 treated mice, and IL-10 levels were decreased after the 3rd dose of E-2 although no statistical significance was reached. T_H2 cytokines including IL-5, IL6 and IL-10

are generally thought to play a role in pathogenesis of SLE [39, 49]. Serum IFN- γ was also increased after the 4th treatment of E-2 (823.3 ± 267 pg/ml vs. 154.7 ± 101 pg/ml in oil controls), and IFN- γ is thought to be important in the initiation of disease, but not in the later stages [96]. The pro-inflammatory cytokine, TNF- α , was also increased by E-2 administration, and has been reported to mediate the pathology of lupus nephritis [3, 39, 99, 194]

3.3.7 Survival of bone marrow chimeras.

Gender bias is striking in SLE with a female predominance in both human patients and experimental mouse models. There are still controversies as to whether sex hormones play a direct role in disease pathogenesis [62]. X-chromosome inactivation, differential exposure to exogenous antigens and other non-hormonal factors have also been proposed as alternative mechanisms to sex hormones. In order to determine whether estrogen can influence the development of disease irregardless of genetic background, we derived chimeras from male and female SNF₁ or DBF₁ mice with or without E-2 treatment. Survival is plotted in Figure 3.6. The first experiment (Fig 3.6A) examined the role of gender and compared the survival for SNF₁ mice reconstituted with bone marrow derived from the different genders. The results suggested that female recipients have significantly shorter life spans whether receiving male (mean survival of 49.6 weeks) or female (mean survival of 47.4 weeks) donor cells. Furthermore, the similarly shortened life spans of the female SNF₁ mice that received either male bone marrows or female bone marrows suggested that both male and female immune cells were equivalent in terms of SLE pathogenesis. To further define a direct role for E-2, reconstituted male SNF₁ mice were treated with E-2 or oil (in Fig 3.6 B). There was no difference in survival of male SNF₁ mice reconstituted with either male (mean survival > 60 weeks) or female (mean survival >

Figure 3.5 The effect of E-2 exposure on serum immunoglobulin and cytokine levels in male SNF1 mice. Serum was collected monthly. The total IgG, anti-ssDNA IgG, anti-dsDNA IgG, Id^{LN}F₁⁺ IgM and Id^{LN}F₁⁺ IgG in the serum were detected using ELISA (A). IL-12, IFN-gamma, IL-4, IL-5, IL-6, TNF-alpha and IL-10 were also detected by ELISA (B). (*, p<0.05, compared to intact control or oil control mice).

A. Serum Ig Levels

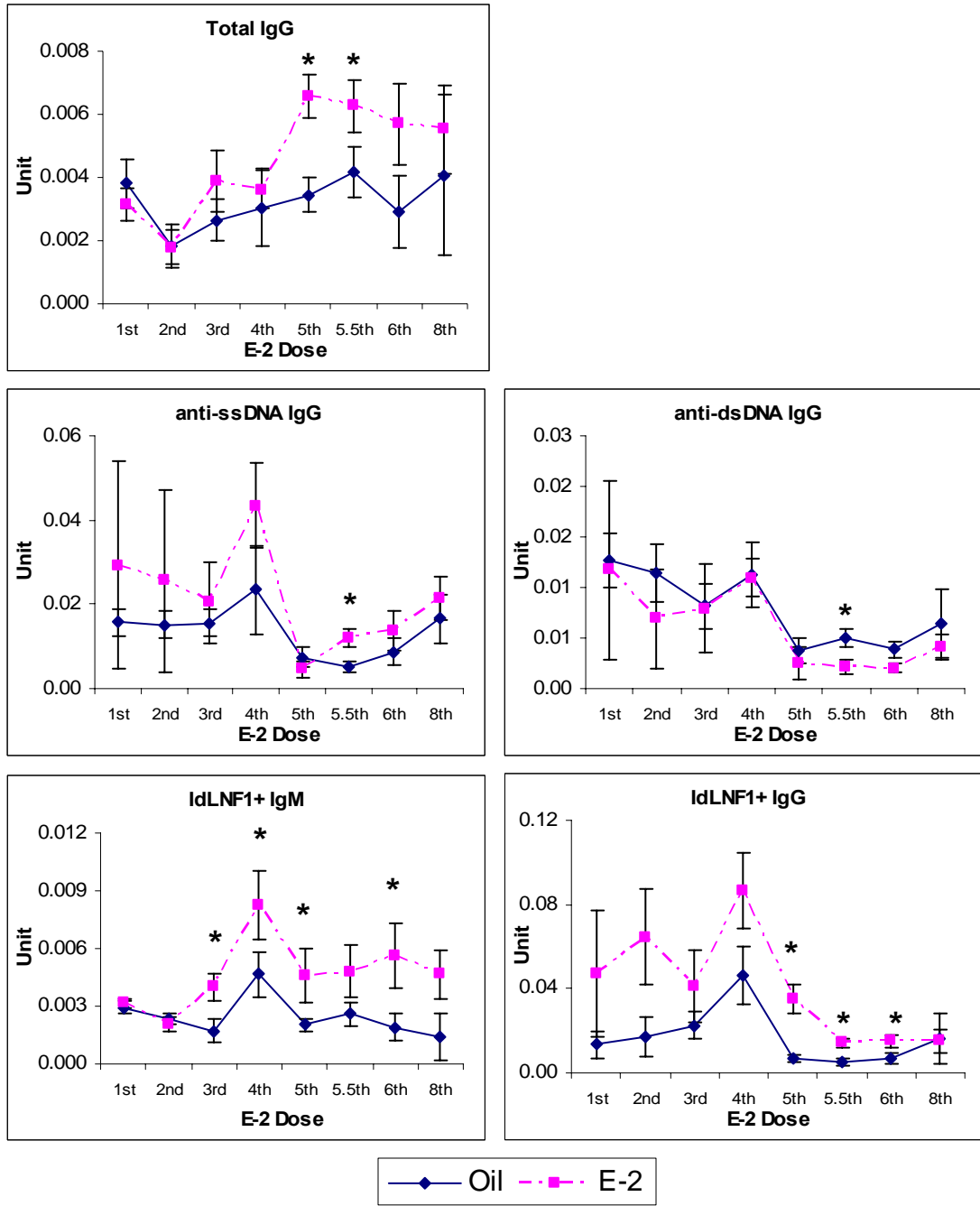


Figure 3.5 (Continued)
 B. Serum Cytokine Levels

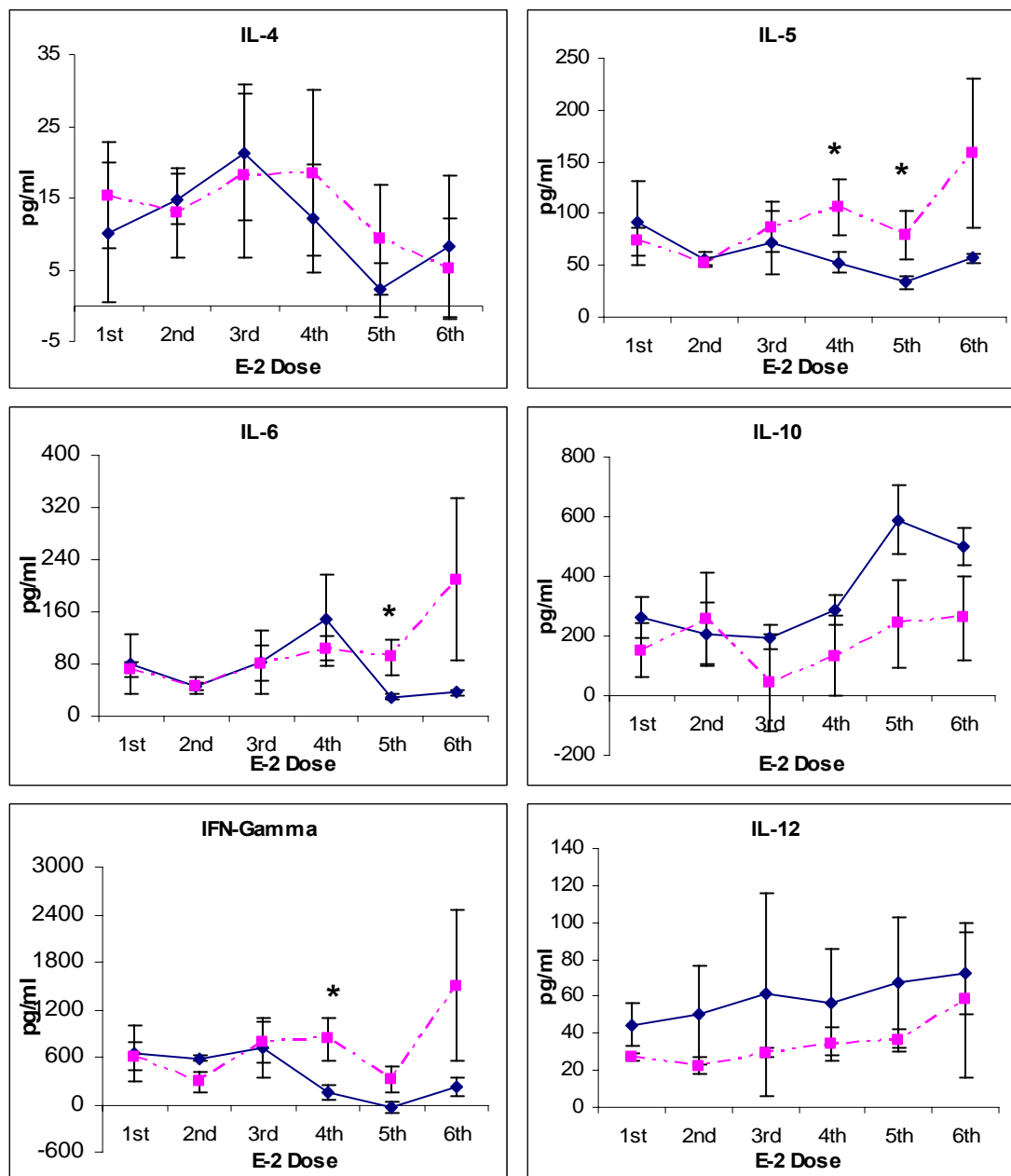
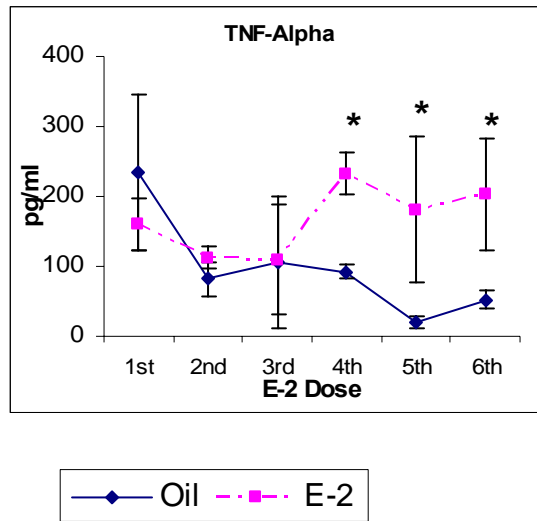


Figure 3.5 (Continued)

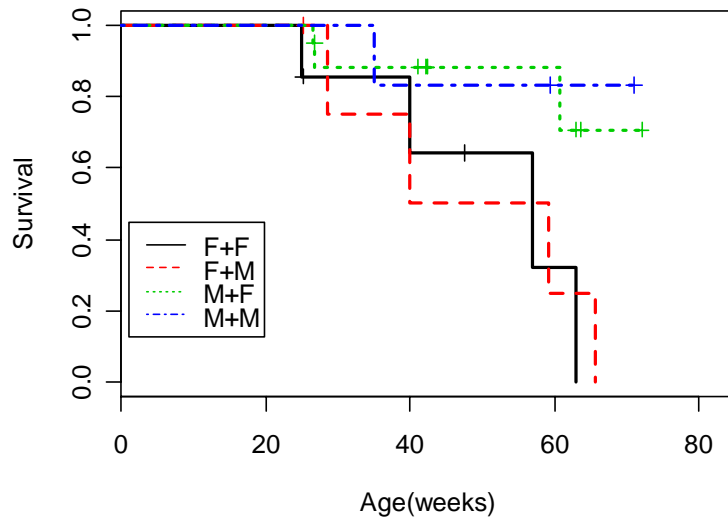


60 weeks) bone marrow and treated with oil. However, treatment with E-2 led to decreased survival for male chimeras reconstituted with both male (mean survival of 35.7 weeks) and female (mean survival of 32.1 weeks) marrow, suggesting a critical role for E-2 in SNF₁ disease pathogenesis. In another experiment, haplotype-matched non-autoimmune DBF₁ mice were used as donors or recipients in bone marrow transplantation (Fig 3.6C). Survival of DBF₁ female mice reconstituted with SNF₁ female mouse bone marrows (mean survival of 42.1 weeks) was significantly reduced ($p < 0.001$) compared to DBF₁ female mice receiving DBF₁ marrow (mean survival >70 weeks), which indicated that female SNF₁ immune cells could transfer disease. Finally (Fig 3.6D), when E-2 was administered to male DBF₁ mice reconstituted with bone marrow from SNF₁ female mice, survival of these mice (mean survival of 30.0 weeks) was decreased significantly ($p < 0.05$), and similar to that of E-2 treated SNF₁ male mice (mean survival of 32 weeks). This result again suggested a critical role of E-2 in disease pathogenesis.

Figure 3.6 Effect of bone marrow transplantation on survival. At 6 weeks of age, the recipient mice were lethally irradiated at 1100 rads, and then reconstituted with 1×10^6 bone marrow cells from the age-matched donors. After reconstitution, the mice were treated with oil or E-2 monthly. A. Survival of SNF₁ male and female chimeras. Four groups of mice were tested: female SNF₁ recipient with female SNF₁ mouse bone marrow (F+F); female SNF₁ recipient with male SNF₁ mouse bone marrow (F+M); male SNF₁ recipient with female SNF₁ mouse bone marrow (M+F); male SNF₁ recipient with male SNF₁ mouse bone marrow (M+M). The results suggested that female reconstituted SNF₁ mice had shorter life span despite donor's gender ($p < 0.05$). B. Survival for E-2 and oil treated SNF₁ male chimeric mice. E-2 treatment led to decreased survival of male recipient mice reconstituted with either female mice bone marrow (M+F+E2) or male mice bone marrow (M+M+E2) compared to oil treated male recipient reconstituted with either female bone marrow (M+F+oil) or male bone marrow (M+M+oil) ($p < 0.05$). C. Survival of DBF₁ mice reconstituted with female SNF₁ or DBF₁ mouse bone marrow. Female DBF₁ mice receiving female SNF₁ marrow had a significantly shorter life span ($p < 0.05$). D. E-2 treatment of DBF₁ male mice reconstituted with bone marrow from female SNF₁ mice leads to significantly decreased survival ($p < 0.05$).

A.

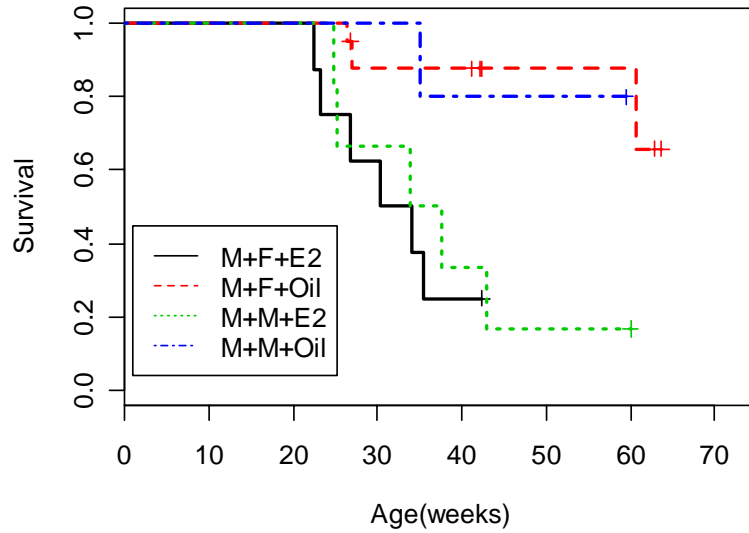
Recipient	Donor	Notation
SNF ₁ Female	SNF ₁ Female	F+F
SNF ₁ Female	SNF ₁ Male	F+M
SNF ₁ Male	SNF ₁ Female	M+F
SNF ₁ Male	SNF ₁ Male	M+M



B.

Recipient	Donor	Treatment	Notation
SNF ₁ Male	SNF ₁ Female	E-2	M+F+E2
SNF ₁ Male	SNF ₁ Female	Oil	M+F+Oil
SNF ₁ Male	SNF ₁ Male	E-2	M+M+E2
SNF ₁ Male	SNF ₁ Male	Oil	M+M+Oil

Figure 3.6 (Continued)



C.

Recipient	Donor	Notation
DBF ₁ Female	DBF ₁ Female	F+DF
DBF ₁ Female	SNF ₁ Female	F+SF
DBF ₁ Male	DBF ₁ Female	M+DF
DBF ₁ Male	SNF ₁ Female	M+SF

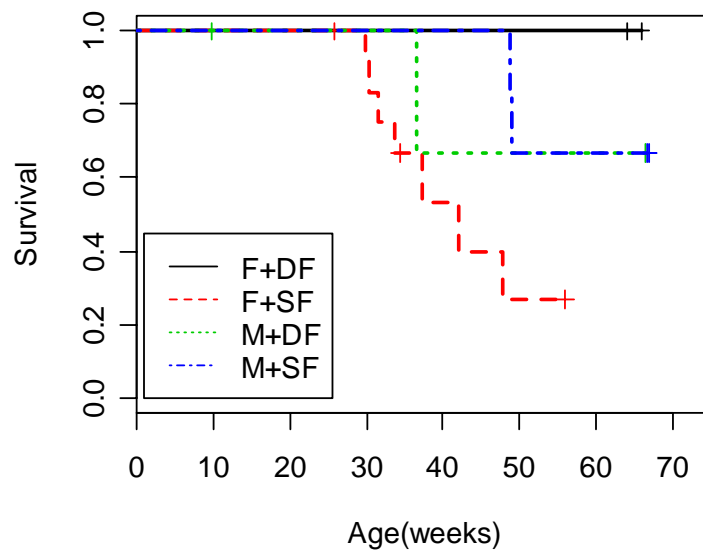
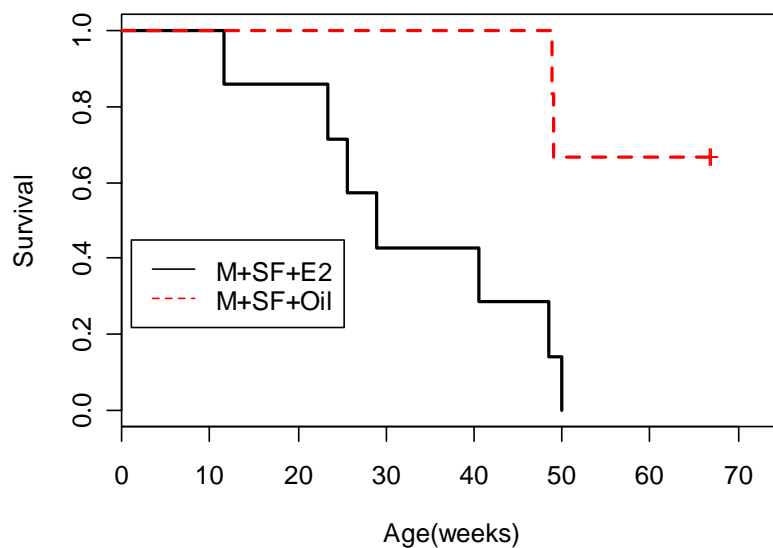


Figure 3.6 (Continued)

D.

Recipient	Donor	Treatment	Notation
DBF ₁ Male	SNF ₁ Female	E-2	M+SF+E2
DBF ₁ Male	SNF ₁ Female	Oil	M+SF+Oil



3.3.8 Kidney Pathology in bone marrow chimeric mice

Hematoxylin&Eosin stained kidney sections from chimeric mice obtained at 24 weeks of age were blindly evaluated and graded for order of severity of damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with

lymphocytic infiltration, and/or sclerosis. We found that glomeruli of female SNF₁ chimeras reconstituted with marrows from either gender (4.3 ± 0.25 and 4.0 ± 0.0), had a higher pathological grade compared to male chimeras (3.1 ± 0.11 and 3.0 ± 0.0) (Table 3.2). When treated with E-2, the damage grades were increased in both SNF₁ and DBF₁ male chimeras. Furthermore, female DBF₁ chimeras reconstituted with SNF₁ bone marrow had increased glomerular damage with higher grades compared to those reconstituted with DBF₁ bone marrow (2.5 ± 0.29 vs. 1.7 ± 0.33), while male DBF₁ chimeras reconstituted with SNF₁ marrow required E-2 treatment for greater pathology (Table 3.2).

3.3.9 Immune modulation in bone marrow chimeric mice.

The immune functions in bone marrow chimeric SNF₁ mice were evaluated. These changes were consistent with the findings in survival and kidney pathology that showed accelerated disease, with pathogenic autoreactive immune cells expanded in mice with decreased lifespan. Splenocytes from female SNF₁ mice receiving either male or female bone marrow or male SNF₁ mice reconstituted with male or female bone marrow and treated with E-2 proliferated more to pathogenic aa62-73 compared to reconstituted male mice treated with oil (Figure 3.7). There were also more Id^{LN}F₁+ antibody forming cells in reconstituted female SNF₁ mice or E-2 treated reconstituted male mice (Fig 3.8); furthermore, there were higher levels of Id^{LN}F₁+ IgG produced by splenocytes from reconstituted female mice or reconstituted male mice with E-2 exposure (>1.5 fold over control) compared to male mice treated with oil (<1 fold over control) (Fig 3.9).

Table 3.2 Kidney damage in bone marrow chimeric mice. At 6 weeks of age, the recipient mice were lethally irradiated at 1100 rads, and then reconstituted with 1×10^6 bone marrow cells from the age-matched donors. After reconstitution, the mice were treated with oil or E-2 monthly. Kidney sections from 24-week old mice in each treatment group were stained with hematoxylin and eosin (H&E) for pathological examination. Individual glomeruli from each kidney were graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. The treatment groups were set up as in the survival study (Figure 3.6). A. Kidney pathology of SNF₁ male and female chimeras. B. Kidney pathology of E-2 and oil treated SNF₁ male chimeric mice. C. Kidney pathology of DBF₁ mice reconstituted with female SNF₁ or DBF₁ mouse bone marrow. D. Kidney pathology of E-2 treated DBF₁ male mice reconstituted with bone marrow from female SNF₁ mice. The data were expressed as mean grade \pm SE for each treatment group.

A. SNF₁ male and female chimeras.

	Glomerular damage
F + F	4.3 ± 0.25
F + M	4.0 ± 0.0
M + F*	3.1 ± 0.11
M+ M	3.0 ± 0.0

Note: n=9 for (8) and n=4 for all other group

B. E-2 treated SNF₁ male chimeras.

	Glomerular damage
M + F + Oil	2.7 ± 0.67
M + F+ E-2	2.3 ± 0.33
M + M + Oil	2.7 ± 0.33
M+ M + E-2	4.0 ± 0.0

Note: n=3 for each treatment group.

Table 3.2 (Continued)

C. DBF₁ male and female chimeras.

	Glomerular damage
F + DF	1.7 ± 0.33
F + SF*	2.5 ± 0.29
M + DF	1.0 ± 0.0
M+ SF	1.3 ± 0.0

Note: n=4 for (*) and n= 3 for all other groups.

D. E-2 treated DBF₁ male chimeras.

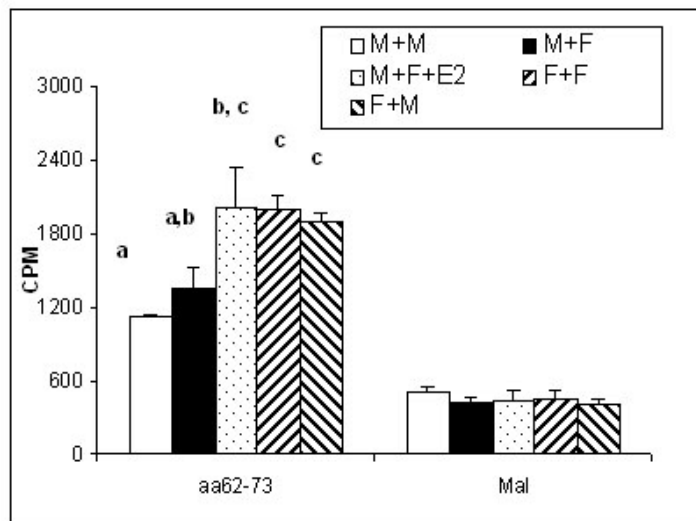
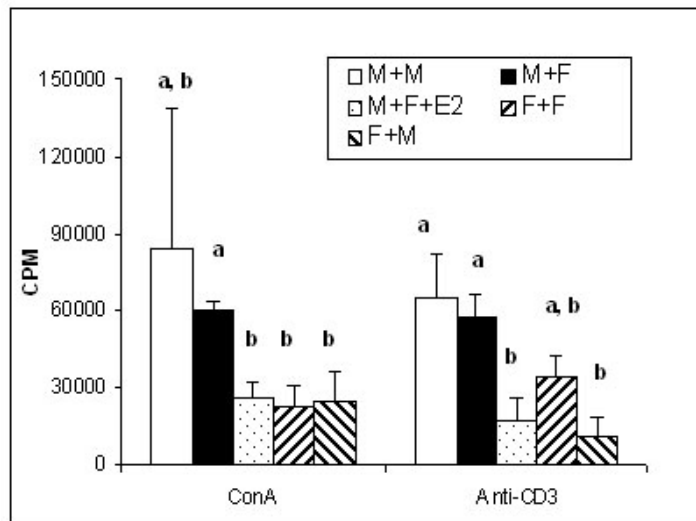
	Glomerular damage
M + SF +Oil	1.7 ± 0.33
M + SF + E-2	2.7 ± 0.33

Note: n= 3 for each treatment group

3.4 Discussion

In this study, we first demonstrated that E-2 could modulate the lupus nephritis in the autoimmune SNF₁ murine model. We found that E-2 treatment of male SNF₁ mice at 1mg/kg monthly led to an increase in serum E-2 level to 150~200pg/ml, which is the peak physiological level of E-2 in female mice [206]. This relatively high level persisted for about 7-10 days and returned to the level normal for male SNF₁ mice (<10pg/ml) by two weeks after the treatment. We further found that this dose of E-2, administered monthly, led to significantly decreased survival of SNF₁ male mice compared to control mice treated with oil vehicle only. In fact, the survival curve for

Figure 3.7 *In vitro* proliferative response of splenocytes from chimeric SNF₁ mice. Splenic lymphocytes were isolated from bone marrow chimeric reconstituted mice 2 weeks after the 4th dose of E-2 at 24 weeks of age, and then the proliferative responses were measured. M+M, male SNF₁ mice reconstituted with male SNF₁ bone marrow; M+F, male SNF₁ mice reconstituted with female SNF₁ bone marrow; M+F+E2, male SNF₁ mice reconstituted with female SNF₁ bone marrow and then treated with E-2 monthly starting at 7 weeks of age; F+F, female SNF₁ mice reconstituted with female SNF₁ bone marrow; F+M, female SNF₁ mice reconstituted with male SNF₁ bone marrow. Non-overlapping letters (a, b, c) indicate significant differences (p<0.05).



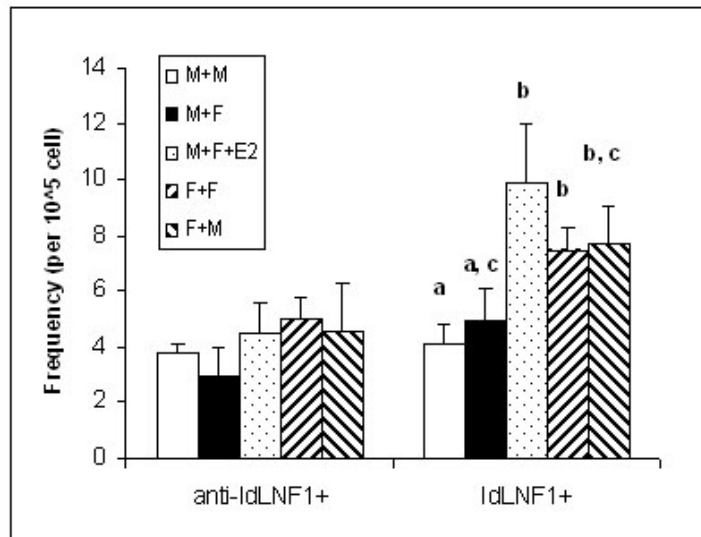


Figure 3.8 Frequency of B cells producing pathogenic antibody in bone marrow reconstituted SNF₁ mice. Splenic lymphocytes were isolated from bone marrow reconstituted mice 2 weeks after 4th E-2 dose, and then antibody-producing cells were detected by ELISPOT. M+M, male SNF₁ mice reconstituted with male SNF₁ bone marrow; M+F, male SNF₁ mice reconstituted with female SNF₁ bone marrow; M+F+E2, male SNF₁ mice reconstituted with female SNF₁ bone marrow and then treated with E-2 monthly; F+F, female SNF₁ mice reconstituted with female SNF₁ bone marrow; F+M, female SNF₁ mice reconstituted with male SNF₁ bone marrow. Non-overlapping letters (a, b, c) indicate significant differences (p<0.05).

E-2 treated male SNF₁ mice was very similar to that of female mice that develop spontaneous disease. At same time, E-2 treated male SNF₁ mice developed more severe lupus nephritis after 4th injection of E-2 compared to controls, at 24 weeks of age. Further, E-2 treated male mice showed significant changes in both T and B lymphocytes, which, as expected, mirrored those found in SNF₁ females with disease. T lymphocytes from E-2 treated mice proliferated more in response to pathogenic aa62-73 peptide *in vitro*, although the response to Con A was suppressed. B cell functions were also affected by E-2 treatment, and induced an increased frequency of auto-reactive Id^{LNF1+} antibody-producing B cells; further, Ig⁺ cells were also more effective as APC presenting aa62-73 after E-2 treatment. Phenotypic analysis showed an up- regulation of the pathogenic populations after E-2 exposure, with increased

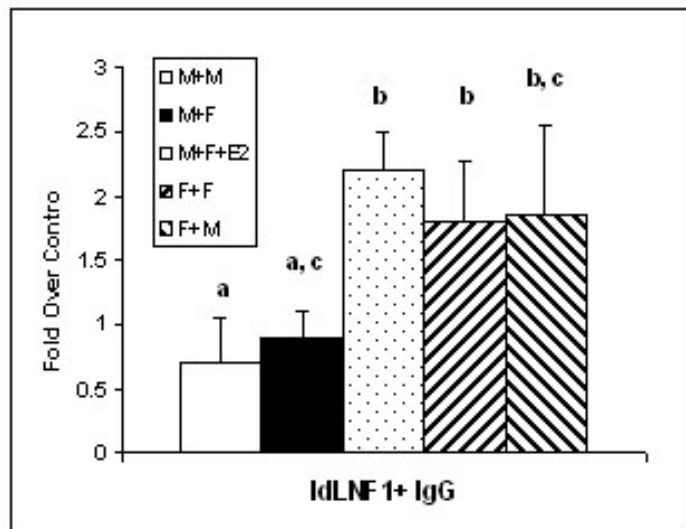


Figure 3.9 Id^{LN}F₁+ IgG production by Ig⁺ splenocytes *in vitro* from bone marrow reconstituted SNF₁ mice. Ig⁺ splenocytes and the affinity-purified T cells were isolated from bone marrow transplanted mice, and then cells were mixed and cultured in various combinations in the presence of aa62-73 for 7 days. The supernatants were then analyzed by ELISA for Id^{LN}F₁+ IgG. The groups were set up as in Fig 3.8. Non-overlapping letters (a, b, c) indicate significant differences (p<0.05).

percentages of Id^{LN}F₁+CD3⁺ splenocytes, Id^{LN}F₁+CD4⁺ T cells, Id^{LN}F₁-reactive memory T cells and an increased Id^{LN}F₁+CD4⁺ to Id^{LN}F₁+CD8⁺ ratio, as is seen in female SNF₁ disease [28, 54]. Serum levels of immunoglobulins and cytokines also confirmed the immunoregulatory effects of E-2, with increased pathogenic antibody levels and a cytokine profile skewed to favor the lupus development in male mice after E-2 exposure, with an increase in IL-5, IL6, TNF- α and IFN- γ and a decrease in IL-12. These observations suggest that E-2 could accelerate lupus in SNF₁ mice.

Additional supporting data were obtained from bone marrow transplantation experiments. In the first group of experiments, autoimmune SNF₁ mice of both genders were reconstituted with bone marrow from mice of either the same or the opposite gender. We found that regardless of the donor marrow, recipients of one

gender tended to have survivals and a degree of glomerulonephritis similar to intact mice of the host gender. This suggested that immune cells from either male or female mice could transfer the autoimmune SNF₁ lupus nephritis. In contrast, host mice of different genders showed a striking gender bias in the development of lupus when reconstituted with either male or female bone marrows. Female mice reconstituted with either male or female marrow developed accelerated nephritis and died earlier than reconstituted male mice reconstituted with marrow of either gender, which suggested that the female hosts provided a critical factor or factors influencing the development of disease. We hypothesized that a possible non-genetic factor might be the sex hormone, estrogens. Data in support of its importance in disease was obtained in bone marrow transplantation experiments using male recipients reconstituted with female or male donor marrow with or without E-2 treatment. E-2 treatment was required to reproduce the female disease course in male mice reconstituted with female bone marrow. Survival of these mice was similar (mean survival of 30 weeks) to that of female reconstituted mice, as was kidney damage. In summary, these studies suggested that E-2, in addition to intrinsic genetic factors, was an important factor in the development and pathogenesis of lupus nephritis.

Furthermore, we found that non-autoimmune female DBF₁ mice reconstituted with bone marrow from female SNF₁ mice developed severe lupus glomerulonephritis and had a life span equal to that of the autoimmune SNF₁ female mice (mean survival of 30 weeks), while the control female DBF₁ mice reconstituted with female DBF₁ bone marrow had a much longer life span (mean survival >60 weeks) and minimum kidney damage. This result strongly suggested that transplanted immune system from the SNF₁ mouse was sufficient to induce the lupus disease even in a non-autoimmune host. In comparison, male DBF₁ mice had normal lifespan (mean survival >70 weeks) when reconstituted with female SNF₁ bone marrow; however, E-2 treatment of these

mice led to disease development with shortened survivals (mean survival of 27 weeks) and kidney damage as we had seen in the previous SNF₁ bone marrow transplantation experiments. Again, these results emphasize the important role of E-2 in lupus development and pathogenesis.

In summary, our studies suggest that E-2 will trigger as well as modulate lupus nephritis irregardless of genetic factors. These data strongly support a critical role for estrogen in mediating the sex difference in lupus disease, as opposed to other postulated mechanisms such as X inactivation, imprinting, differential exposure, etc [205]. Furthermore, our studies in DBF₁ chimeras emphasize the importance of genetic factors since the chimeras reconstituted with SNF₁ marrow developed accelerated disease compared to mice reconstituted with marrow from the non-autoimmune DBF₁ mouse in DBF₁ hosts. This also showed that there were also no differences in the target organs of lupus nephritis; disease was equivalent in both host strains.

As expected, T lymphocytes from reconstituted female SNF₁ mice and E-2 treated reconstituted male SNF₁ mice proliferated more in response to pathogenic aa62-73 peptide. Further, there were more Id^{LN}F₁⁺ antibody producing B cells in female SNF₁ chimeras or E-2 treated male SNF₁ chimeras, and more pathogenic Id^{LN}F₁⁺ antibodies were produced *in vitro* by cells from those mice. These results combined with survival and kidney histology suggest that E-2 directly modulate lupus pathogenesis, possibly through its immunoregulatory effects.

One mechanism by which estrogen may exert its function is through its interaction with estrogen receptors (ERs). Estrogen receptors belong to the nuclear hormone receptor super family [207], and are ligand-activated transcription factors that bind to specific DNA sequences of target genes and then enhance their transcription. Two types of estrogen receptors, α and β , have been cloned so far [133,

196]. Both ERs are found in the thymus [132] and ER α expression was increased with the administration of estrogen following castration [131]. Various immune cells including T cells, B cells and monocytes have also been found to express either ER isoform or both, with the highest levels expressed in macrophages relative to T and B cells [149, 196, 208]. That estrogen binding to immune cells has direct effects on their function has been shown by several groups. Estrogen binding to a cell surface ER has been shown to directly activate T cells, as measured by an increase in [Ca²⁺] within seconds after binding [154], and increased calcineurin mRNA levels and phosphatase activity [200]. Estrogen exposure also may result in the ER-dependent upregulation of several genes involved in B cell activation and survival [44, 102, 158]. This could result in the modulation of B cell populations including autoreactive B cells, as suggested by autoantibody production when non-autoimmune mice were treated with estrogen [159, 160]. NK cells may also be modulated by estrogen and were found to be increased [161], or decreased [162] after estrogen exposure, presumably through ER [163]. All of these findings suggest that through interactions with ERs estrogen exposure could interact directly with immune cells, leading to expansion of autoreactive T cells as well as Id^{LN}F₁+ B cells and autoimmune lupus disease development.

In conclusion, this study provides evidence that E-2 is playing a critical role in triggering the development of autoimmune lupus nephritis, possibly through its immunomodulatory effects including expansion of pathogenic Id^{LN}F₁+ reactive T cells as well as B cells. Results from bone marrow chimeras also provide strong evidence that E-2 is primarily responsible for the sex differences in SLE. These findings are highly significant, given the increasing numbers of people who are using sex hormones as oral contraceptive, replacement therapy and dietary supplements.

CHAPTER 4

Treatment with 17 β -estradiol induces autoimmunity in non-autoimmune mice

Abstract

Previous work has suggested that estrogen accelerates autoimmune diseases in mice that are genetically predisposed to their development, such as the lupus-prone mice (SWR x NZB)F₁ (SNF₁). Female SNF₁ (H-2^{d/q}) mice develop spontaneously accelerated nephritis. Male mice develop later diseases; however, it may be accelerated by the treatment with estrogen. Here, we asked whether treatment of non-autoimmune male mice with estrogen would induce the development of lupus nephritis, by treating non-autoimmune cross between DBA and BALB/c mice (DBF₁) with 17 β -estradiol (E-2). We found that repeated E-2 treatment resulted in a shortened lifespan, and histological examination of kidneys from the E-2 treated mice revealed significant glomerular damage associated with nephritis. Flow cytometric analysis showed that the pathogenic Id^{LN}F₁-reactive T cell population was in a more active state after E-2 treatment expressing higher levels of CD25 and CD44 surface molecules. Furthermore, E-2 treatment led to a significant increase in both Th-1 and Th-2 cytokine production by splenocytes after stimulation with ConA, and IFN- γ production in response to pathogenic peptide aa62-73 was decreased in E-2 treated mice. There was also an increase in the numbers of B cells producing pathogenic Id^{LN}F₁ antibody and protective anti-Id^{LN}F₁ antibody following E-2 treatment. Taken together, these results suggested that E-2 alters immune responses and leads to the induction of autoimmune diseases even in mice not normally prone to develop disease.

4.1 Introduction

Sex hormones, especially the estrogens, can have potent effects on the immune system, and are thought to contribute greatly to the gender dimorphism in the immune responses. In general, females tend to have a stronger immune response to invading pathogens, resulting in higher immunoglobulin concentrations, and also a more rapid rejection of allografts [190, 201, 209]. But on the other hand, this increased immune response may also predispose females to an increased susceptibility of developing autoimmune diseases such as SLE [78, 79, 201], which occurs at a female to male ratio of 9:1 or 10:1 [1, 2, 35, 186]. Despite the well-established female predominance in SLE, the molecular mechanisms by which the estrogens and androgens modulate the immune system remain to be identified. Furthermore, the term “female predominance”, which refers to the sex differences of incidence, not severity, is also being questioned with the thought that the sex hormones may actually affect the severity of autoimmune disease rather than its incidence through a threshold or permissive mechanism [76, 210].

Previous work using an autoimmune murine model of lupus, the cross between NZB and SWR (SNF₁), showed that female mice developed and succumbed to a more severe disease much earlier than males, with severe immune complex glomerulonephritis [54, 211]. Studies in this model supported the pathological significance of an idiotype, Id^{LN}F₁, found on immunoglobulins deposited in diseased female kidneys [73, 201]. We hypothesized in the SNF₁ mouse model, the pathogenesis of nephritis might be caused primarily by the dysregulation of these Id^{LN}F₁ idiotypes, with the production of anti-DNA antibodies, a diagnostic marker for disease, having less significance [27]. Specifically, we found that the ratio of CD4⁺ to CD8⁺ Id^{LN}F₁-reactive T cells increased and peaked at 24 weeks, exactly mirroring a rise to maximum serum Id^{LN}F₁ IgG production and its deposition in the kidney

glomeruli [54]. Furthermore, we have identified a pathogenic member of the Id^{LN}F₁ family, referred to as 540, which was found to induce the proliferation of T cells derived from nephritic mice or pathogenic autoreactive T cell clones. Adoptive transfer of Id^{LN}F₁-reactive T cells was able to accelerate disease pathogenesis in murine models, while regulatory anti-Id^{LN}F₁⁺ antibodies treatment suppressed the disease development [29, 30]. The relevant idiotypic determinant at the peptide level (aa62-73) was also identified from the CDR2 region of the 540 antibody (D.K. Price, in preparation). Interestingly, we also found that repeated administration of pharmacological doses (1mg/kg) of 17 β -estradiol (E-2) to male SNF₁ mice caused accelerated disease with significantly decreased survival and increases in serum Id^{LN}F₁-540 IgG production as well as more severe renal damage, similar to what was found in female SNF₁ mice.

These results as well as other studies suggested that E-2 accelerates and exacerbates autoimmune disease in autoimmune mouse models, which are genetically predisposed to develop lupus. Here, we showed that E-2 treatment of non-autoimmune DBF₁ mice (the cross between DBA and BALB/C, H-2^{d/q}) also led to autoimmune disease, with Ig depositions in the kidney, nephritis development and significantly decreased survival. The proliferative response of splenocytes to the pathogenic peptide 62-73 was increased. Id^{LN}F₁⁺ immunoglobulin production was also increased, as well as production of both T_H1 and T_H2 cytokines.

4.2 Materials and methods

4.2.1 Mice

Six to eight weeks old female DBA and male BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). (DBA \times BALB/c) F₁ (DBF₁) mice were bred and maintained in the Cornell University College of Veterinary Medicine,

Center for Research Animal Resources and Education (CARE). Only male mice were used in this study.

4.2.2 Treatment with E-2

Male DBF₁ mice were injected with β -estradiol 17-valerate (1mg/kg and 5mg/kg, Sigma) in olive oil vehicle subcutaneously once every month starting at 7 weeks of age; control mice were injected with vehicle alone. Urine protein and body weight were measured monthly. Mice from each group were sacrificed at age of 25 weeks and 50 weeks. The thymus, spleen and kidney were harvested and single cell suspensions were prepared from individual spleens and thymuses. Kidneys were harvested and fixed, and sections were stained with hematoxylin and eosin (H&E) for glomerular histology analysis.

4.2.3 Estrogen levels in the serum

The E-2 level in the serum was detected using EIA kits (Diagnostic Systems Laboratories, Inc), according to the manufacturer's instruction with slightly modification. Briefly, the serum sample, diluted in PBS-0.1% Tween, and each standard were added in duplicates. The Estradiol-Biotin Conjugate was also added. The plates were incubated shaking at fast speed (500-700rpm) at room temperature for an hour. After washing, Streptavidin-Enzyme Conjugate was added to the plates. The plates were again incubated and shaken at room temperature for 30 min. Then, the plates were washed again and TMB Chromogen solution was added. After 30min, the color was quantitated using a microplate reader at 450nm and the concentration for each sample was calculated from the standard curve. The data were expressed as pg/ml.

4.2.4 Flow cytometric analysis of splenic lymphocytes

The splenocyte suspension was treated with tris-ammonium chloride (TAC) and then enhanced for T cells by affinity chromatography (T cell Recovery Column,

Biotex). 1×10^5 cells were stained with the appropriate dilution of biotin-, fitc-, and phycoerythrin-conjugated antibodies and streptavidin-red670 (PharMingen), including anti-CD44-PE, anti-CD4-PE, anti-B220-PE, anti-CD45RB-Bio, anti-CD8a-Bio, anti-CD3-Bio, anti-CD25-Bio, anti-CD4-Fitc, and Id^{LN}F₁-Fitc (PharMingen, with the exception of Id^{LN}F₁⁺ fitc-conjugated antibody which was derived from monoclonal 540 antibody-producing hybridoma cell culture supernatants and labeled in-house using a fitc-conjugation kit). The stained cells were incubated on ice for 20 min, and then fixed in 1% paraformaldehyde. Flow cytometry was carried out on a Becton-Dickison FACSCalibur[®] flow cytometer and analyzed with WinMDI program.

4.2.5 Cytokine production

Splenic lymphocytes were cultured in triplicate at a concentration of 1×10^6 per well with ConA or aa62-73 for three days at 37°C under 5% CO₂. Supernatants were harvested and analyzed by ELISA (eBioscience) for IL-2, IL-4, IL-5, IL-6 and IFN- γ according to the manufacturer's procedure. The data were expressed as pg/ml.

4.2.6 T cell Proliferative response

Five hundred thousand splenocytes/well were mixed with aa62-73 peptide at 100ug/ml or ConA at 2ug/ml. After incubation at 37°C with 5% CO₂ for three days, 1mCi/well of [³H]-thymidine (Amersham, Arlington Heights, IL) was added, and incubation continued overnight. The cells were then lysed, collected onto filter mats and incorporated radioactivity measured by a scintillation counter (Packard, Meriden, CT). The data were expressed as the proliferation index (cpm of sample versus ConA treated control).

4.2.7 ELISPOT determination of B cell frequencies.

For determination of the frequency of Id^{LN}F₁-producing B cells, 96-well microtiter plates with nitrocellulose bottoms (Millipore) were coated with rabbit anti-Id^{LN}F₁ Ab, aa62-73, control peptide Mal, anti-540 Ab, or normal rabbit Ig overnight

at 4°C. The plates were washed with PBS-0.1%Tween and blocked with PBS-3% BSA, then washed again. Splenic lymphocytes diluted in culture medium were added at a concentration of 5×10^6 mononuclear cells/well in triplicate. The plates were incubated overnight at 37°C with 5% CO₂. Specific Igs secreted by B cells bound to the plate-bound antibody were detected by the addition of biotin-conjugated anti-mouse IgG, followed by SA-HRP (streptavidin conjugated to horseradish peroxidase, Amersham Life Sciences) and 3-amino-9-diethylcarbazole and H₂O₂ as substrate. Brown spots corresponding to cells secreting specific Ig were counted, and the data were expressed as the frequency of specific B cells per 10⁵ cells.

4.2.8 Renal histology

One hundred glomeruli from hematoxylin and eosin (H&E)-stained sections of kidneys were blindly evaluated and graded as either normal, with no or only segmental lymphocyte infiltration or damaged, with globular infiltration, and/or sclerosis. The data were expressed as the percent damaged glomeruli from each treatment group.

4.2.9 Survival

At least 5 mice for each group were kept for the survival, and were euthanized when moribund, as determined by CARE veterinarians. The data were expressed as the percentage of surviving mice.

4.2.10 Statistics

Statistical significance of treatment on survival was determined by the Logrank test. All other data were analyzed by the Student's t-test or ANOVA. Statistical significance was ascribed when p values were less than 0.05.

4.3 Results

4.3.1 Estrogen levels in the serum are increased after the injection of E-2

To determine the serum level of estradiol after E-2 injection, the mice were bled at 1 day, 3 days, 6 days and 14 days after injection, and then the serum E-2 was measured by EIA. As shown in Fig 4.1, after E-2 injection, serum E-2 levels rose rapidly in both 1 mg/kg and 5 mg/kg E-2 treatment groups to 158 ± 32 pg/ml and 1240 ± 185 pg/ml respectively, while in control mice the E-2 levels were less than 10 pg/ml. The serum E-2 levels in the E-2 treated mice remained at a higher level up to 14 days after injection, after which levels were equal to control mice.

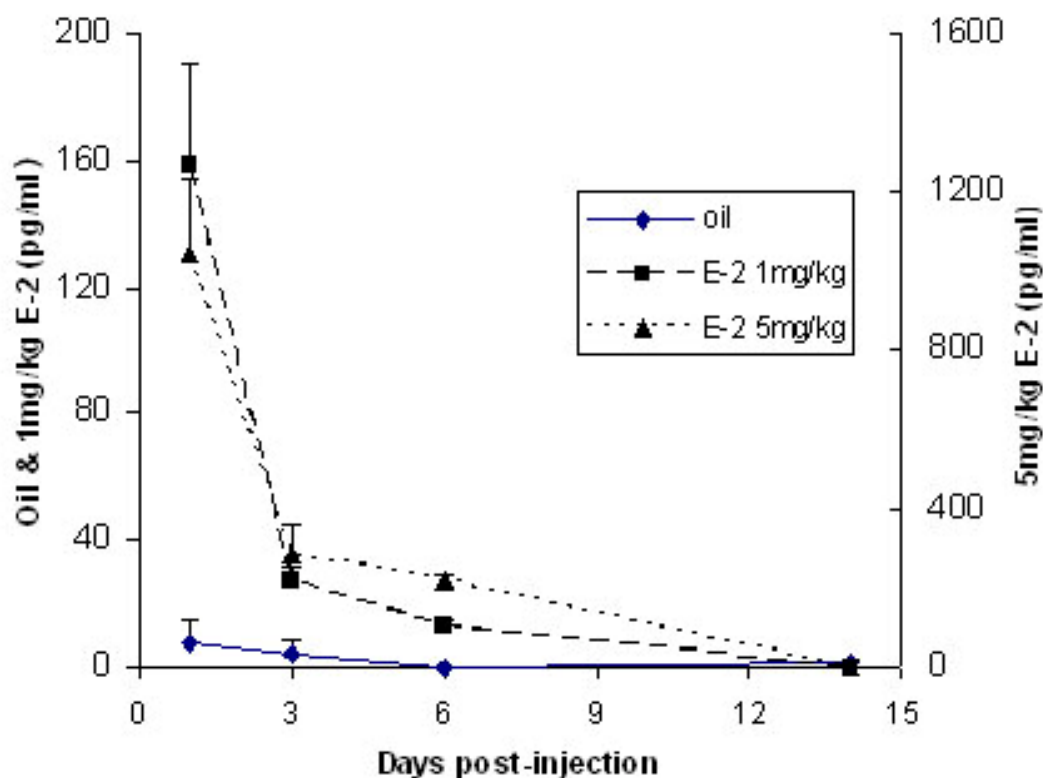


Figure 4.1 Serum E-2 levels in DBF₁ mice after injection with estradiol. DBF₁ mice were injected with 1mg/kg, 5mg/kg E-2 or oil monthly, and serum samples were

collected on different day after injection. Estradiol levels were determined by EIA. Data were expressed as Mean level \pm SE (n=6 for each group).

4.3.2 Estrogen treatment decreases the survival of male DBF₁ mice

Our previous work showed that repeated estradiol (E-2) treatment of male SNF₁ mice led to significantly reduced survival [212, 213]. To examine whether E-2 treatment of DBF₁ also led to decreased survival, we treated these mice with either 1mg/kg or 5mg/kg E-2 in olive oil monthly, while the control mice were treated with olive oil alone. We found that the mean survival for control mice was greater than 100 weeks, while the E-2 treated mice had a significantly reduced lifespan, with a mean survival of 55 weeks (E-2 5mg/kg treated group, $p < 0.01$) and 80.5 weeks (E-2 1mg/kg treated group, $p < 0.05$ compared to control).

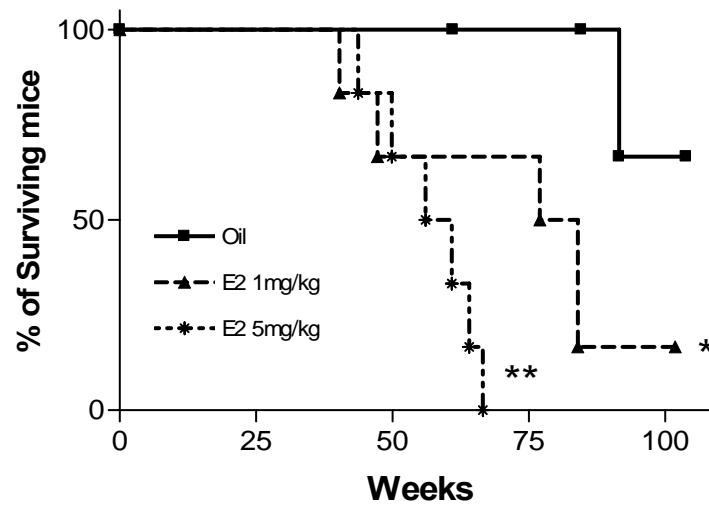


Figure 4.2 E-2 treatment of male DBF₁ decreases survival. DBF₁ mice were injected subcutaneously with E-2 (1mg/kg or 5mg/kg) in olive oil monthly, while control mice were treated with olive oil alone. Mice were followed until their natural deaths or were euthanized when moribund as determined by Cornell CRAR veterinarians. The y-axis denotes the percentage of mice alive in each treatment of group, and the X-axis indicates their age in weeks. (*, $p < 0.05$; **, $p < 0.01$) (n=6 for each group)

4.3.3 Estrogen treatment of DBF₁ mice leads to the development of glomerular nephritis

We found previously that E-2 treatment of male SNF₁ mice led to accelerated and increased renal damage [212, 213]. Hematoxylin & Eosin stained kidney sections from DBF₁ male mice obtained two weeks subsequent to the 4th dose were blindly evaluated and graded for severity of damage as being normal, with no lymphocytic infiltration, or damaged, having segmental and/or globular infiltration, or sclerosis. A significant increase ($p < 0.05$) in damaged glomeruli with a concomitant significant decrease in normal glomeruli (not shown) was seen in DBF₁ mice after 1mg/kg estradiol treatment (Fig 4.3). The increase in damaged glomeruli was also seen in 5mg/kg E-2 treated mice, although statistical significance was not achieved ($p = 0.06$).

4.3.4 Estrogen treatment induces phenotypic changes associated with lupus pathogenesis in splenocytes of DBF₁ mice

To examine the phenotypic changes in splenocytes induced by E-2 treatment, flow cytometric analysis of immune cells was performed at two time points, 25 weeks and 50 weeks of age. At 25 weeks of age, within the Id^{LN}F₁-reactive T cell population, the percentages of both CD44⁺ and CD25⁺ cells in E-2 treated mice were increased significantly ($p < 0.05$, Table 4.1A). Further, the expression of CD44⁺ and CD25⁺ on the Id^{LN}F₁-reactive T cells was increased ($p < 0.05$) after E-2 treatment, as determined by mean channel fluorescence. These data suggested that E-2 treatment of DBF₁ led to an increase in the number and level of activation of Id^{LN}F₁-reactive splenic T-lymphocytes compared to oil treatment.

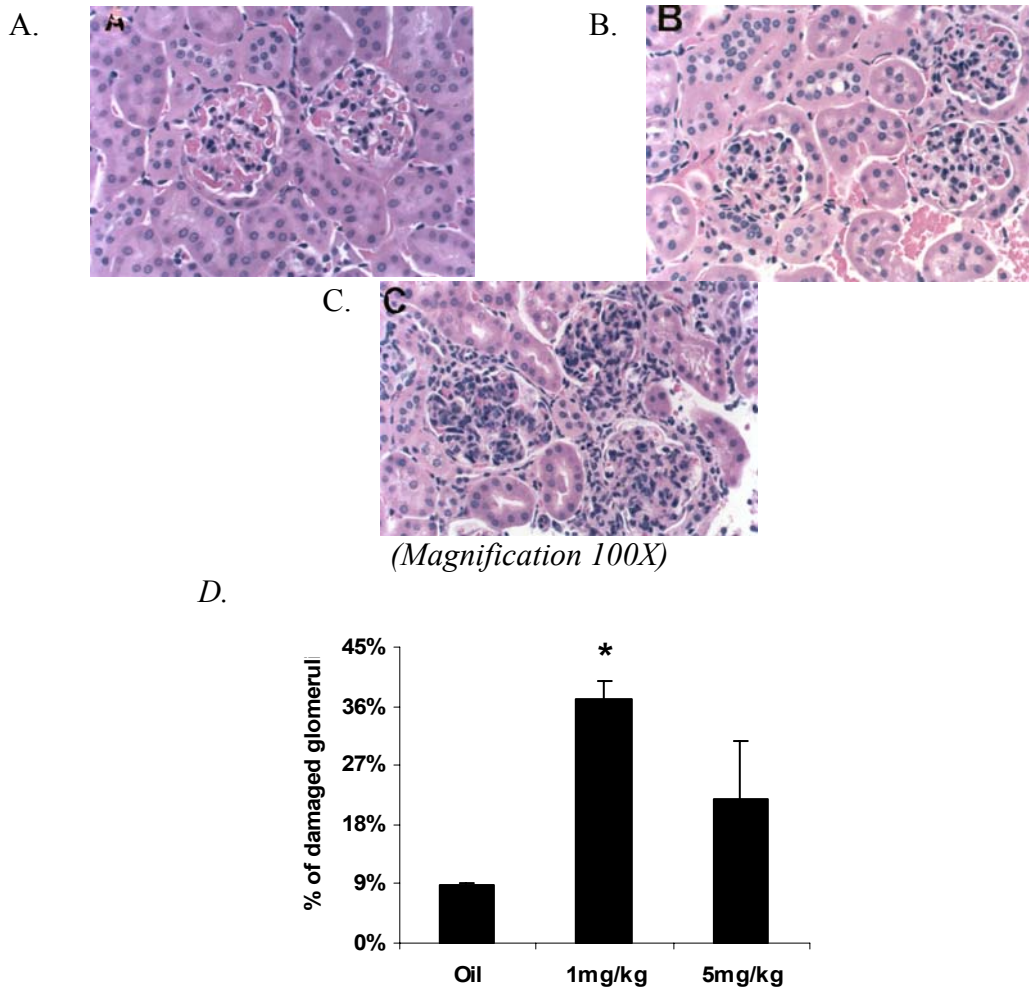


Figure 4.3 E-2 treatment leads to accelerated and increased kidney damage. Male DBF₁ mice were injected subcutaneously with E-2 (1mg/kg or 5mg/kg) in olive oil monthly, and control mice were treated with olive oil alone. Kidney sections from 25-week old mice from each treatment group were stained with H&E for pathological grading. A. Oil treated DBF₁. Capillary loops were blood filled and there were a small number of cells in the mesangium; B. 1mg/kg E-2 treated DBF₁. E-2 treatment led to an increase in mesangial cells and a reduction in blood capillaries; C. 5mg/kg E-2 treated DBF₁. In these glomeruli, the cellular number was increased and the cells that were present are enlarged. There was also an obliteration of much of the capillary structure in the glomeruli; D, Summary of glomerular changes caused by E-2 treatment. One hundred glomeruli from hematoxylin and eosin (H&E)-stained sections of kidneys were graded as normal (with no or only segmental lymphocyte infiltration), or damaged (with globular infiltration and/or sclerosis). The data were expressed as percent damaged glomeruli from each treatment group (*, p<0.05 compared with oil control group).

E-2 treated mice that were 50 weeks old, produced more Id^{LN}F₁ cells both in B220⁺ and CD3⁺ cell populations and also more Id^{LN}F₁ memory cells (Table 4.1B). Moreover, the ratio of Id^{LN}F₁-reactive CD4⁺ T cells to Id^{LN}F₁-reactive CD8⁺ T cells was also increased by E-2 treatment, which we have previously identified as a hallmark phenotypic change in SNF₁ female mice, and was due to both an increase of Id^{LN}F₁-reactive CD4⁺ T cells and a decrease of regulative (or suppressive) Id^{LN}F₁-reactive CD8⁺ T cells [54]. We also found that the Id^{LN}F₁⁺-T cells were now in a lower state of activation with low CD44 and CD25 molecule expression, which reflected that the long term of estrogen exposure led to the immunosuppression.

4.3.5 Estrogen treatment modulates in vitro proliferative responses of splenocytes

We wanted to determine whether E-2 administration would affect the proliferative response of lymphocytes. We found that the antigen-independent T-cell stimulation and proliferation, as measured by response to concanavalin A (ConA), were decreased by monthly E-2 treatment at both 25 and 50 weeks of age at both doses of E-2 (Fig 4.4A and Fig 4.4B), although statistical significance was achieved only in the 5mg/kg E-2 treated group at 25 weeks. Interestingly, we found that the proliferative response to pathogenic peptide aa62-73, calculated as the fraction of the aa 62-73 proliferative response to the ConA proliferative response (denoted PI), was increased after monthly E-2 treatment at 25 weeks of age (Fig 4.4B), but not at 50 weeks (Fig 4.4D).

4.3.6 Estrogen treatment modulates cytokine production in DBF₁ mice

Abnormal cytokine production plays an important role in the pathogenesis of certain autoimmune diseases including SLE [39, 97]. It has been shown that the

Table 4.1 E-2 treatment leads to increased numbers and activation of Id^{LN}F₁-reactive T cells. DBF₁ mice were injected subcutaneously with E-2 (1mg/kg or 5mg/kg) in olive oil monthly, and control mice were treated with olive oil alone. At 25 and 50 weeks, mice from each group were sacrificed, and splenocyte (1 × 10⁵) cells were stained with the appropriate antibodies. A, Phenotypic changes at 25 weeks; B, Phenotypic changes at 50 weeks. Data were expressed as Mean±SD, (*, p<0.05; **, p<0.01 compared with oil control group).

A. 25 weeks.			
	Oil	E-2 1mg/kg	E-2 5mg/kg
CD44+/Id^{LN}F₁+			
%	90.61±0.53%	92.07±0.46%*	95.32±0.61%**
Mean Fluorescence of CD44	178.85±28.45	211.67±22.48*	301.54±34.42**
CD25+/ Id^{LN}F₁+			
%	47.21±5.04%	59.49±6.39%*	74.00±4.42%**
Mean Fluorescence of CD25	53.43±3.19	56.98±4.37	62.43±2.00**
B. 50 weeks			
	Oil	E-2 1mg/kg	E-2 5mg/kg
B220⁺			
%	75.60±3.66	71.13±0.84	50.87±4.45**
CD3⁺			
%	28.22±1.51	23.63±1.96	12.51±0.19**
Id^{LN}F₁+/B220+			
%	33.07±6.70	45.93±2.35**	62.38±3.45**
Id^{LN}F₁+/CD3+			
%	4.83±1.28	6.83±1.61*	11.93±3.53**
Id^{LN}F₁⁺CD44+CD45-			
%	0.04±0.01	0.54±0.27**	0.63±0.29**
CD44+/ Id^{LN}F₁+			
%	96.32±1.41	96.59±1.04	97.66±0.75
Mean Fluorescence CD44	430.7±48.1	332.2±34.1**	310.6±26.8**
CD25+/ Id^{LN}F₁+			
%	31.29±7.18	17.4±1.51**	11.10±3.05**
Mean Fluorescence of CD25	87.5±21.5	68.1±17.5	58.4±12.4*
(Id^{LN}F₁+CD4+CD3+)÷ (Id^{LN}F₁+CD8+CD3+)			
	0.94±0.15	1.19±0.43	1.58±0.58*

production of specific cytokines by both mature immune cells and cells in the bone marrow and thymus can be modulated by sex hormones [171, 174, 193, 194, 214, 215]. In order to investigate the effect of E-2 administration on the cytokine production in male DBF₁ mice, the splenic lymphocytes from each treatment group were co-cultured with ConA or aa62-73 in vitro, and ELISA was used to detect the specific cytokines produced in the supernatant. As shown in Fig 4.5A and Fig 4.5B, both T_H1 and T_H2 cytokine production after ConA stimulation were increased at 25 weeks in mice treated with E-2 compared to oil control mice (P<0.05). Furthermore, when the splenic lymphocytes were co-cultured with pathogenic aa62-73, we found that IFN- γ production by lymphocytes isolated from E-2 treated mice at 25 weeks of age was decreased significantly (p<0.05), while the levels of other cytokines were unchanged (data not shown). At 50 weeks, cytokine production by cells from mice in both E-2 treatment groups was similar to that of the control group.

4.3.7 E-2 treatment leads to an increased frequency of B cells producing pathogenic Id^{LN}F₁ antibody and protective anti-Id^{LN}F₁ antibody

We next investigated whether the E-2 treatment led to changes in the numbers of pathogenic Id^{LN}F₁ antibody-producing B cells, as well as the numbers of the anti-Id^{LN}F₁ antibody-producing B cells by ELISPOT assay. We found that at both 25 and 50 weeks of age (in Fig 4.6), both the numbers of the anti-pathogenic antibody producing B cells and the numbers of pathogenic antibody-producing B cells were increased significantly (p<0.05) in the E-2 treated mice groups compared with oil treated mice. Further, the numbers of antibody-producing B cells decreased at 50 weeks.

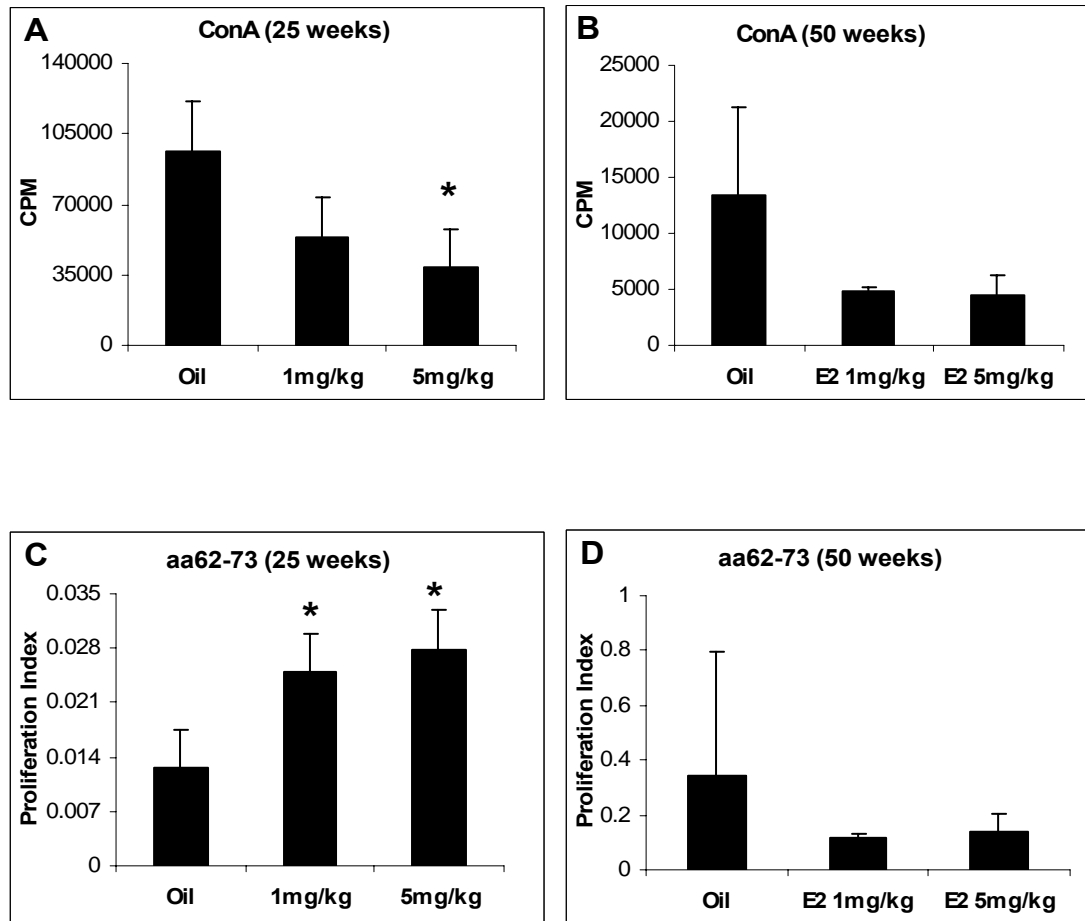
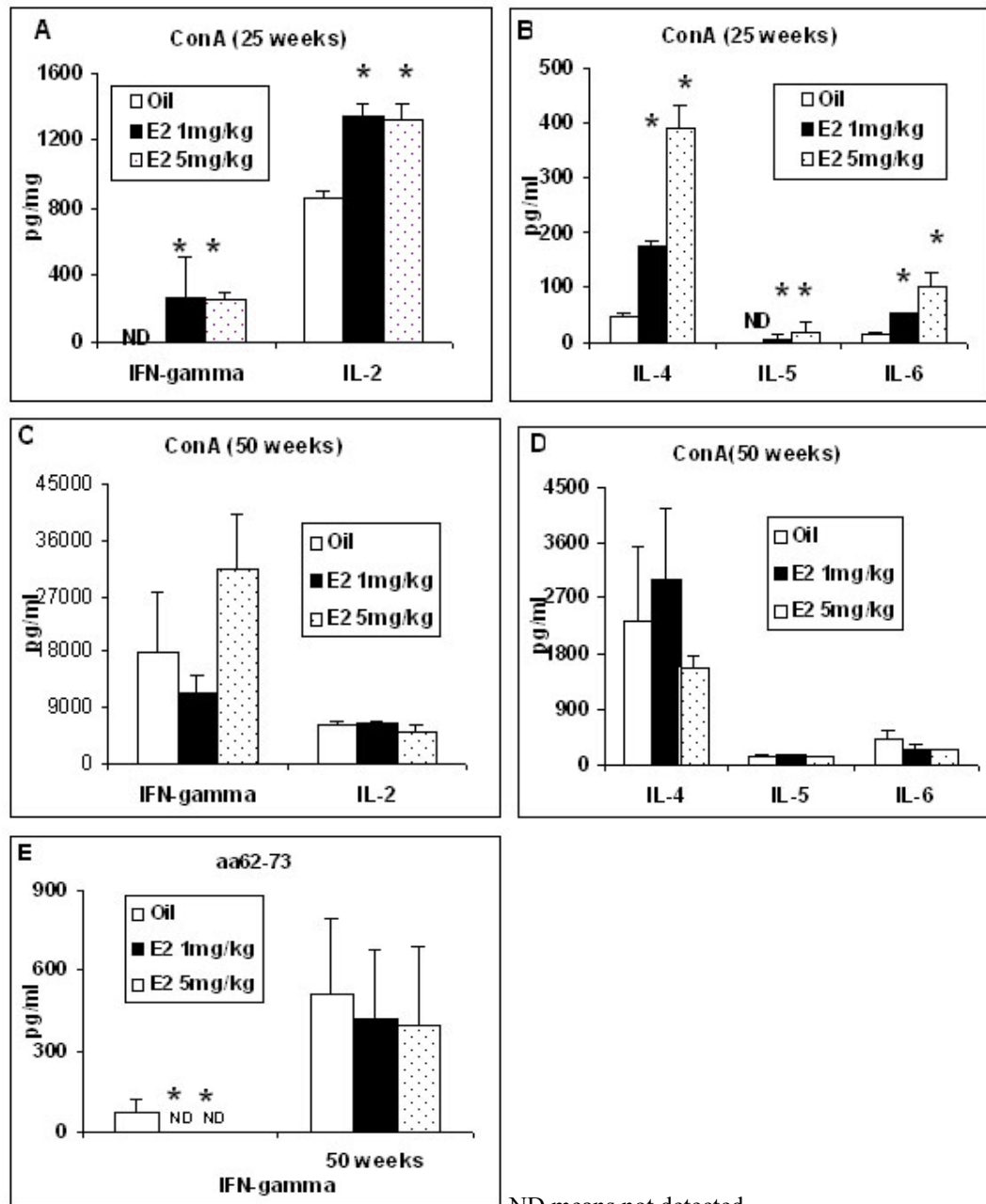


Figure 4.4 E-2 treatment leads to modulation of lymphocyte proliferation. Splenic lymphocytes were isolated from mice treated with 1mg/kg or 5mg/kg E-2 or controls at 25 and 50 weeks of age. The cells were cultured in the presence of ConA or aa62-73 for 72 hours, pulsed with [³H]-Thymidine overnight, and then harvested. Incorporated radioactivity was determined. A and B. Proliferative response to ConA expressed as CPM at 25 weeks and 50 weeks, respectively; C and D. Proliferative response to aa62-73 at 25 and 50 weeks. Data were expressed as a proliferation index for aa62-73, which was calculated as the ratio of the proliferative response to aa 62-73 to the proliferative response to ConA. (*, p<0.05 compared to oil group)



ND means not detected.

Figure 4.5 E-2 treatment leads to modulation of *in vitro* cytokine production. Splenic lymphocytes were isolated from oil treated control mice, or mice treated with E-2 at 1mg/kg or 5mg/kg when the mice were 25 or 50 weeks of age. The cells were cultured in the presence of ConA or aa62-73 for 72 hours, and the cytokine levels in the supernatant were determined by ELISA. A and C. Th-1 cytokine production in the presence of ConA at 25 and 50 weeks of age; B and D. Th-2 cytokine production after ConA stimulation at 25 and 50 weeks; E, Production of IFN- γ in response to aa62-73 at 25 and 50 weeks of age. (*, $p < 0.05$, compared to oil group; **, $p < 0.01$, compared to oil group).

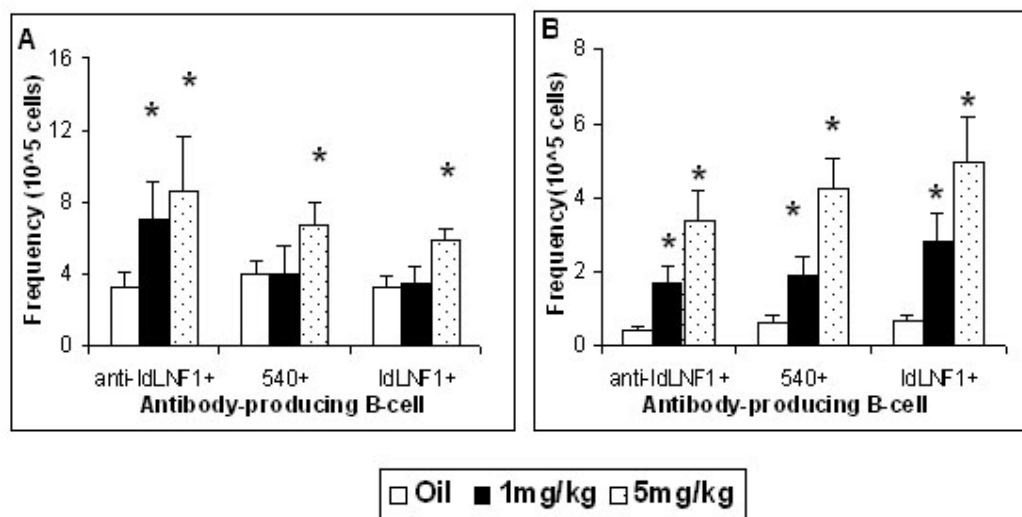


Figure 4.6 E-2 treatment leads to an increased frequency of B cells producing pathogenic Id^{LN}F₁⁺ antibodies. Splenic lymphocytes were isolated from oil treated control mice, or mice treated with 1mg/kg or 5mg/kg, and then antibody-producing cells were detected by ELISPOT at 25 (A) and 50 weeks (B). (*, p<0.05 compared to oil group).

4.4 Discussion

Many factors can influence the development of SLE, including hormones, genetic factors and environmental and dietary influences [38]. Although genetic factors are of extreme importance in disease development, recently it has been shown that sex hormones such as estrogen and androgen have marked impact on the immune responses [59, 216, 217]. Estradiol (E-2) administration results in decreased mitogen-induced T cell responses [90], a suppressed delayed type hypersensitivity [218], a decreased NK cell activity [92], generation of autoreactive T cells [93], and a shift towards a T_H2 type cytokine profile [217]. B cell functions can also be affected, with a polyclonal B cell activation, an increase in the number of Ig-secreting B cells, and the autoantibody formation [159, 219]. Increased macrophage [220] and lymphocyte proliferation [202, 208], increased phagocytosis and augmented expression of surface

antigens [220] have also been reported. In addition, other studies have shown specific effects of estrogen on autoimmune disease, and in general, estrogen exposure accelerates the course of B-cell-mediated autoimmune diseases [81, 221]. For example, treatment of female B/W mice with estrogen accelerated the onset of disease and nephritis with increased and earlier mortality [76]. Similar effects were also seen in other autoimmune mice model including MRL lpr/lpr and BALB/c mice injected with anti-DNA (16/6 id) antibodies [3, 202, 217]. However, to date most studies have focused on the strains that are genetically predisposed to develop autoimmune disorders. Little is known about the effects of estrogen on the induction or regulation of autoimmunity in normal or non-autoimmune individuals. In one study, it was found that exposure of C57BL/6 and BALB/c mice to estrogen resulted in increased numbers of plasma cells producing antibodies to both self- and non-self-antigens, including autoantibodies to dsDNA and anti-cardiolipin antibodies [159, 160, 222]. But these studies did not go further to investigate whether these effects persisted or led to autoimmune disease.

In the SNF₁ model for lupus nephritis, the female mice spontaneously develop a disease resembling human lupus, which is characterized by the production of anti-DNA immunoglobulins and a fatal nephritis before one year of age. Male mice of this strain develop much lower incidence of disease, and its onset is much later, at approximately 15 months. Our laboratory has shown that a family of pathogenic cross-reactive idiotypes, known as Id^{LN}F₁ [72, 73], can be eluted from nephritic kidneys, and that a defect(s) in the regulation of Id^{LN}F₁ Ig production is a primary defect leading to the nephritis in SNF₁ mice, with anti-DNA antibodies as an important diagnostic marker but playing a less important role in the pathogenesis of SLE. T cells reactive with Id^{LN}F₁-derived peptides lead to the production of Id^{LN}F₁ IgG, which is deposited in the kidneys, resulting in nephritis [28]. The production of Id^{LN}F₁ IgG is

increased at 20-24 weeks of age due to an increase in CD4⁺ Id^{LN}F₁-reactive T cells and a concomitant decrease in CD8⁺ Id^{LN}F₁-reactive T cells. This event is also coincident with a sudden, significant increase in serum levels of Id^{LN}F₁⁺ IgG and first detectable Id^{LN}F₁⁺ Ig deposition in the kidneys [54]. In support of this mechanism, Id^{LN}F₁-reactive CD4⁺ T cells induced Id^{LN}F₁⁺ Ig production *in vitro* and accelerated disease when adoptively transferred into young pre-nephritic SNF₁ mice [28]. Furthermore, treatment with the anti- Id^{LN}F₁ antibody can inhibit the production of Id^{LN}F₁⁺ Ig production and delay the onset of nephritis. We have also shown that the pathogenic Id^{LN}F₁ idiotope(s) is located on the CDR2 region of 540 from aa62 to aa73 (D.K. Price, in preparation).

In a recent study, we found that repeated administration of 1mg/kg E-2 to male SNF₁ mice led to accelerated nephritis and resulted in almost the same pathological changes as that we identified in the females [212, 213]. In this paper, we showed that administration of E-2 to MHC-matched non-autoimmune mice DBF₁, (DBAxBALB/c)F1 (H-2^{d/q}), could induce autoimmunity and autoimmune disease with pathological changes similar to those associated with disease in SNF₁ mice. Male DBF₁ were injected subcutaneously with either 1mg/kg or 5 mg/kg E-2 in oil monthly starting from 7 weeks old, and control mice were injected with oil only. E-2 treated mice died significantly earlier (p<0.05 for E-2 1mg/kg and p<0.01 for E-2 5mg/kg) than the control mice. The effect of E-2 on survival was striking, so that all of the DBF₁ mice treated with 5mg/kg E-2 did not survive beyond 12-15 months, exactly like that of female SNF₁ mice, while the survival of mice injected with 1mg/kg E-2 was similar to unmanipulated male SNF₁ mice. This result clearly showed that estrogen exposure led to disease development in the non-autoimmune mice. Furthermore, nephritis as determined by renal histology correlated with our findings of decreased survival. At 25 weeks, E-2 treated mice had decreased numbers of normal

glomeruli, with a concomitant increase in the percentage of damaged glomeruli compared to oil-treated control mice; however, the kidney damage induced by E-2 in these nonautoimmune mice was not as severe as that found in female SNF₁ mice or male SNF₁ mice after E-2 treatment. These data support that at least one outcome of E-2 exposure is the induction of autoimmune nephritis regardless of genetic susceptibility to develop autoimmune disease.

Other results also supported the above conclusion. Flow cytometric analysis showed the same phenotypic changes in DBF₁ mice after E-2 treatment as those detected in female SNF₁ mice as disease develops. For example, by 25 weeks, Id^{LN}F₁-reactive T cells were expanded and activated, as is seen in SNF₁ mice [28, 54]. E-2 treatment also led to more Id^{LN}F₁⁺ B cells, as well as more Id^{LN}F₁-reactive memory T cells. There was also an increase in the ratio of Id^{LN}F₁-reactive CD4⁺ cells to Id^{LN}F₁-reactive CD8⁺ cells in E-2 treated mice. This suggested a decrease in the regulatory T cells, and /or an increase in the autoreactive T cells which provided help to the autoreactive B cells. Interestingly, although E-2 treatment led to the suppression of the mitogen-stimulated T cell proliferation, at 25 weeks the proliferation to pathogenic aa62-73 peptide was increased. Mitogen-induced cytokine production was increased at 25 weeks but not at 50 weeks. On the other hand, IFN- γ production in response to pathogenic aa62-73 *in vitro* was decreased significantly. More evidence towards the induction of autoimmune disease came from ELISPOT assay, which showed an increased number of Id^{LN}F₁⁺ antibody-producing B cells as well as regulatory anti-Id^{LN}F₁⁺ antibody-producing B cells in E-2 treated DBF₁ mice. Furthermore, the increased numbers of pathogenic Id^{LN}F₁ antibody-producing B cells was maintained at the same higher level over time in E-2 treated mice but with a concomitant decrease in the number of regulatory anti- Id^{LN}F₁ antibody-producing B

cells. All of those results supported a role for E-2 in the induction of autoimmunity in non-autoimmune DBF₁ mice.

The mechanism underlying the role of E-2 in the autoimmunity still has not been well characterized, but based on our laboratory's work and that of several others, multiple mechanisms are possible. First, estrogen may directly act on the T cells to induce their hyperactivity. Estrogen receptors have been found on several subsets of T cells [39]. Both estrogen receptors (ER), α and β [129-132] were found in the thymus, and ER α level is increased after administration of E-2. Further, gender differences in ER expression have been noted [223]. Another study found that administration of low doses of E-2 resulted in a striking increase in antigen-specific CD4⁺ T cell responses, that was ER- α -dependent [153], and was due to the direct activation of T cells by estrogen via cell membrane ER [154]. This suggests the possibility that differences in the numbers and types of ER expressed on the immune cells of some individuals and between the genders could explain the differences in their T cell responses to antigen. This hypothesis was further supported by the observation that calcineurin, CD40L expression and PP2B phosphatase activity were increased when SLE T cells were cultured with E-2 [149, 155]. Moreover, estrogen binding to a cell surface ER was shown to directly activate T cells, as measured by an increase in [Ca²⁺] within seconds after binding [154]. Therefore, in the SNF₁ model estrogen could act directly on the Id^{LN}F₁-reactive T cells leading to the up-regulation of the pathogenic T cell and/or down-regulation of the Id^{LN}F₁ suppressor T cells, as shown in this study. Others have shown that estrogen acts on B cells to induce hyper-reactivity. In support of this, estrogen receptors have been found on pro-B cells and stromal cells of bone marrow [224, 225]. B cell development in the bone marrow may also be modified by exposure to estrogen [157]. It was found that the ER-dependent up-regulation of several genes involved in B cell activation and survival [44, 102, 158], which resulted in the

modulation of B cell populations including autoreactive B cells, as suggested by autoantibody production by non-autoimmune mice treated with estrogen [159, 160]. Furthermore, estrogen can break the B cell tolerance and induce the lupus-like phenotype [102]. Both our work (unpublished data) and other's work have indicated that E-2 may also affect presentation of antigen by B cells [226]. Third, estrogen treatment may lead to an altered milieu for T cells or/and B cells, and indirectly results in autoimmunity. NK cells may also be modulated [161, 162] after estrogen exposure presumably through ER [163]. Estrogen treatment promoted differentiation of a CD11c+, CD11b (int) DC population that displayed high levels of MHC Class II and CD86 (B7-2). In another study, anti-estrogen treatment resulted in mature dendritic cells (DC) that were less effective than immature DC in presenting antigen to allogeneic T cells [164], possibly by altering cytokine production in these cells [227], suggesting that E-2 could augment the numbers of potent APCs [166]. Estrogen induces a T_H2-type cytokine response and gender differences in cytokine secretion after E-2 exposure [167-173], have been postulated as an important mechanism in E-2-induced modulation of T cells. Not unexpectedly, estrogen has been shown to modulate autoimmune diseases such as multiple sclerosis (MS), possibly by altering cytokines as suggested in a study showing that CD4+ myelin-basic protein specific T cells clones derived from patients with MS [174], showed changes in the levels of IL-10, IFN- γ and TNF α production after culture with estrogen. Estrogen exposure also leads to increased expression of adhesion molecules including E-selectin and VCAM-I [176].

Based on current study as well as previous studies in our laboratory, we can hypothesize that E-2 treatment induces the lupus nephritis by acting on both T and B cells, with expansion and activation of pathogenic Id^{LN}F₁-reactive T cells and Id^{LN}F₁+ B cells. These pathogenic B cells with the help from pathogenic T cells produce higher

level of pathogenic $\text{Id}^{\text{LN}}\text{F}_1 + \text{IgG}$, which in turn leads to pathological damage associated with lupus nephritis. This pathogenesis of lupus nephritis induced by E-2 in DBF_1 is similar to that found in lupus-prone SNF_1 mice.

In summary, this study showed that exposure to pharmacological estrogen alone could induce autoimmunity and autoimmune disease in nonautoimmune mice by activating the pathogenic auto-reactive T cells and B cells. This finding is of great significance to those patients exposed in the long term to estrogenic compounds, such as for hormone replacement therapy, contraceptive drugs, and certain ovarian tumors, because change in estrogen level is a potential trigger of perturbed immune function and autoimmune disease. Another source of concern is the recent identification of hormone disrupting effects of a large number of pesticides and insecticides currently on the market as well as phytoestrogens.

CHAPTER 5

Thymectomy prolongs the survival of lupus-prone (SWR x NZB)_{F1} mice and decreases the Id^{LN}F₁-reactive T cells

Abstract

Females of the (SWR x NZB)_{F1} (SNF₁) (H-2^{d/q}) mouse model for lupus nephritis develop spontaneously accelerated nephritis, while male mice develop later disease. Previous studies have shown that T cells reactive with Id^{LN}F₁, a family of idiotypic determinants found on pathogenic Igs in the kidneys, are of critical importance in the pathogenesis of the disease in this model. However, where and how this pathogenic T cell population originates and is regulated is not well known. Here we thymectomized the male and female SNF₁ mice at the age of 30 days in order to investigate the role of thymus in the development and regulation of the pathogenic T cells and their effect on the lupus progression. We found that both the female and male thymectomized mice had significantly prolonged survival. The mice also showed less IL-5, IL-6 and TNF- α production and more IL-12 and IL-2 production *in vitro* after stimulation with the pathogenic idiotypic peptide, aa62-73. The proliferative responses to aa62-73 peptide were also decreased compared to those of intact control mice. Furthermore, thymectomized mice also had a lower frequency of Id^{LN}F₁⁺ antibody producing B cells, as well as decreased production of Id^{LN}F₁⁺ IgG, *in vitro*. Phenotypic analysis indicated that thymectomy led to a significantly lower percentage of Id^{LN}F₁-reactive T cells. Taken together, these data suggest that thymus is critical to maintain the high levels of pathogenic Id^{LN}F₁-reactive T cells, which in turn lead to the progression of lupus in this model.

5.1 Introduction

Systemic Lupus Erythematosus (SLE) is a multi-factorial autoimmune disease characterized by an excessive immune and inflammatory response targeting multiple tissues, organs, and systems of the body. The pathogenesis of SLE arises from defective central or peripheral tolerance mechanisms, an unusually high activation of T and B lymphocytes including an alteration in cytokine levels and antibody production, and/or insufficient suppression of autoreactive lymphocytes [48]. T cells have definitively been shown to be critical to the development of disease, with both CD4⁺CD8⁻ and CD4⁻CD8⁺ population involved [64]. In the (SWR x NZB)F₁ (SNF₁) (H-2^{d/q}) mouse model for autoimmune lupus nephritis, female mice spontaneously develop a disease resembling human lupus, that is characterized by the production of anti-DNA immunoglobulins and a fatal nephritis before one year of age. Male mice of this cross develop a much lower incidence of disease, and the onset of lupus is mainly after one year [54, 211]. Previous studies in our laboratory using these mice identified the pathological significance of a family of cross-reactive idiotype, Id^{LN}F₁ (idiotypes-lupus nephritis-SNF₁), found on immunoglobulins deposited in diseased female kidneys [72, 73]. Data also suggest that the pathogenesis of lupus nephritis in SNF₁ mouse model might be caused primarily by the dysregulation of this pathogenic idiotype, with the production of anti-DNA antibodies having less significance [27, 54, 72, 73]. Moreover, we found that the ratio of CD4⁺ to CD8⁺ Id^{LN}F₁-reactive T-cells increased and peaked at 24 weeks, exactly mirroring a rise to maximum of serum Id^{LN}F₁ IgG production, followed by its deposition in the kidney glomeruli [54]. We also have identified the relevant pathogenic Id^{LN}F₁ peptide (aa62-73) derived from the CDR2 region of the 540 antibody (Price K.D. *et al*, in preparation); This peptide induces the proliferation of syngeneic T cells derived from nephritic mice as well as

pathogenic T cell clones that accelerated disease in the nephritic SNF₁ mice (Price K.D. *et al*, in preparation).

In the thymus in mammals, potentially auto-reactive $\alpha\beta$ -T cells are removed by negative selection, while the process of positive selection selects $\alpha\beta$ -T cells that are capable of recognizing foreign antigen in the context of self-MHC on thymic epithelial cells [107]. When the thymus is removed in newborn mice, defects in development and selection are noted [119], such as a decrease in the total number of peripheral T cells and a concomitant increase in the number of potentially auto-reactive T cells [107, 228-230]. The thymus also generates an important population of regulatory T cells, CD4⁺CD25⁺ T cells, which are capable of regulating autoreactive cells that may escape thymic selection [231]. In non-autoimmune mice, neonatal thymectomy can result in various organ specific autoimmune diseases such as prostatitis, orchitis and oophoritis, supposedly through elimination of this protective population of CD25⁺CD4⁺ cells [119, 121-123]. In contrast, neonatal thymectomy protects SNF₁ mice from genetically prone lupus-like glomerulonephritis [121]; the underlying mechanism is unknown but appears to be unrelated to CD25⁺CD4⁺ regulatory T cells [122]. These results illustrated the importance of the thymus in the development and regulation of the SLE in SNF₁ model. Hence the current study was undertaken to investigate the effect of pre-pubertal thymectomy on disease progression and the appearance and function of pathogenic Id^{LN}F₁-reactive T cell population in order to identify the mechanism(s) involved in thymectomy-induced modulation of SNF₁ nephritis.

5.2 Materials and methods

5.2.1 Mice

Six to eight week old female SWR and male NZB mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The (SWR x NZB) F_1 progeny (SNF $_1$) were bred and maintained in the Cornell University College of Veterinary Medicine, Center for Animal Resources and Education (CARE). Mice were weighed monthly.

5.2.2 Thymectomy and E-2 treatment

Thymectomies were performed by suction thymectomy [232] at 4 weeks of age. Briefly, the mice were anesthetized with isoflurane administered by face mask. Both lobes of the thymus were aspirated through a mid-sternal incision using a glass pipette. The wound was closed with surgical glue and microclamps. At the time of sacrifice, tissue from the superior mediastinum was visually examined for the presence of residual thymus. Mice with thymic remains were excluded from the study. E-2 at 1mg/kg or oil treatment began at 7 weeks, and continued monthly thereafter. Mice from each group were sacrificed two weeks after the 4th dose of E-2 or oil (at age of 24 weeks). The thymus, spleen and kidney were harvested and single cell suspensions were prepared from individual spleens and thymuses. Kidneys were harvested and fixed, and sections were stained with hematoxylin and eosin (H&E) for glomerular histology analysis.

5.2.3 Cytokine production

Splenic lymphocytes were cultured in triplicate at a concentration of 1×10^6 per well with ConA (2 μ g/ml) or aa62-73 (300 μ g/ml) for three days at 37°C under 5% CO $_2$. Supernatants were harvested and analyzed by ELISA (eBioscience) for IL-2, IL-4, IL-5, IL-6 and IFN- γ according to the manufacturer's procedure. The data were expressed as pg/ml.

5.2.4 T cell proliferative response

Five hundred thousand splenocytes/well were cultured with aa62-73 peptide at 100ug/ml or ConA at 2ug/ml. After incubation at 37°C with 5% CO₂ for three days, 1mCi/well of [³H]-thymidine (Amersham, Arlington Heights, IL) was added, and incubation continued overnight. The cells were then lysed, collected onto filter mats and incorporated radioactivity measured by a scintillation counter (Packard, Meriden, CT). The data were expressed as the proliferation index (cpm of sample versus ConA treated control).

5.2.5 ELISPOT determination of B cell frequencies

For determination of the frequency of Id^{LN}F₁-producing B cells, 96-well microtiter plates with nitrocellulose bottoms (Millipore) were coated with rabbit anti-Id^{LN}F₁ Ab, aa62-73, control peptide Mal, anti-540 Ab, or normal rabbit Ig overnight at 4°C. The plates were washed with PBS-0.1%Tween and blocked with PBS-3% BSA, then washed again. Splenic lymphocytes diluted in culture medium were added at a concentration of 5 x 10⁶ mononuclear cells/well in triplicate. The plates were incubated overnight at 37°C with 5% CO₂. Specific Ig secreted by B cells bound to the plate-bound antibody was detected by biotin-conjugated anti-mouse IgG, followed by SA-HRP (streptavidin conjugated to horseradish peroxidase, Amersham Life Sciences) and 3-amino-9-diethylcarbazole and H₂O₂ as substrate. Brown spots corresponding to cells secreting specific Ig were counted, and the data were expressed as the frequency of B cells per 10⁵ cells.

5.2.6 Antibody production

In triplicate, 5x10⁵ Ig⁺ splenocytes purified by Ig panning were cultured with 1x10⁵ affinity-purified T cells in the combinations with 50μl aa62-73. The cultures were incubated for seven days at 37°C with 5% CO₂. The supernatants were then harvested and frozen at -20°C until analyzed by ELISA for total anti-Id^{LN}F₁ IgG, as

previously described [29]. Briefly, Immulon 1B 96-well plates (Krackeler Scientific, Albany, NY, USA) were coated with prepared rabbit anti-Id^{LN}F₁ Ig. Then the diluted sample in PBS/0.1% Tween was added in triplicate. A standard curve was generated using duplicates of eight, two-fold serial dilutions of affinity-purified specific IgG starting at 1 µg/ml in PBS/0.1% Tween. Incubation was continued overnight, and then the plates were washed by PBS/2% Tween. Goat anti-mouse IgG or IgM-horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) was added per well and the incubation was continued overnight. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was added to each well. Color development proceeded for five minutes to one hour at room temperature (RT) until significant blue color developed, and the reaction was stopped by addition of 1M phosphoric acid. The O.D. was read at 450nm. Ig concentrations were determined from the corresponding linear standard curves. The data were calculated as µg/ml.

5.2.7 Flow cytometric analysis of splenic lymphocytes

The splenocyte suspension was treated with tris-ammonium chloride (TAC) and then enhanced for T cells by affinity chromatography (T cell Recovery Column, Biotex). 1×10^5 cells were stained with the appropriate dilution of biotin-, fitc-, and phycoerythrin-conjugated antibodies and streptavidin-red670 (PharMingen). Following antibodies were used, anti-CD44-PE, anti-CD4-PE, anti-B220-PE, anti-CD45RB-Bio, anti-CD8a-Bio, anti-CD3-Bio, anti-CD25-Bio, anti-CD4-Fitc, and Id^{LN}F₁-Fitc (PharMingen, with the exception of Id^{LN}F₁⁺ Fitc-conjugated antibody which was derived from monoclonal 540 antibody-producing hybridoma cell culture supernatants and labeled in-house using a fitc-conjugation kit). The stained cells were fixed in 1% paraformaldehyde and flow cytometry was carried out on a Becton-Dickison FACSCalibur[®] flow cytometer and analyzed with WinMDI program.

5.2.8 Renal histology

Individual glomeruli from each kidney were blindly evaluated and graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis.

5.2.9 Survival

At least 5 mice for each group were kept for the survival, and were euthanized when moribund, as determined by CARE veterinarians. The data were expressed as the percentage of surviving mice.

5.2.10 Statistics

Statistical significance of treatment on survival was determined by the Logrank test. Glomerulus damage grades were analyzed by nonparametric Mann-Whitney test. All other data were analyzed by the Student's t-test or ANOVA. Statistical significance was ascribed when p values were less than 0.05.

5.3 Results

5.3.1 Thymectomy prolongs survival of *SNF₁* mice.

The thymus in mammals is the primary lymphoid organ for T cell development. T cells undergo “education”, a process whereby potentially auto-reactive $\alpha\beta$ -T cells are deleted and $\alpha\beta$ -T cells that are capable of recognizing foreign antigens in the context of self-MHC are selected. Many studies have shown that removal of the thymus leads to modulation of autoimmunity, with an increase in the number of potentially auto-reactive T cell clones and induced/accelerated organ-specific autoimmune diseases [107, 121, 122, 228-230]. Here we thymectomized the mice at 30 days of age, and compared the survival of thymectomized female and male mice with that of intact female and male, respectively. As indicated in Figure 5.1,

thymectomized mice of both genders had prolonged lifespans. Thymectomy in male mice led to significantly increased survivals ($p < 0.01$), with a median survival of 98.9 weeks, in contrast to the intact male mice, which only had a median survival of 73.8 weeks. In female mice, the median survivals were 50.1 and 44.4 weeks for thymectomized and intact mice respectively, which was also significantly different ($p < 0.01$). As depicted in Figure 5.2, there was no difference in the body weights of mice that were thymectomized compared to control mice for either gender.

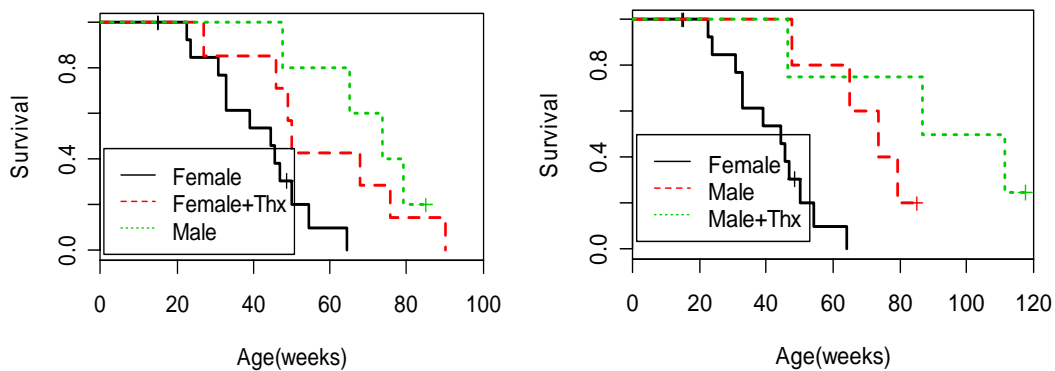


Figure 5.1 Thymectomy prolongs survival of SNF₁ mice. Female and Male SNF₁ mice were thymectomized by suction at 30 days of age ($n=6$). The y-axis denotes the percentage of mice in each treatment of group that were alive at a specific age, and the X-axis indicates the age in weeks. Both female and male thymectomized mice showed a significantly increased survival compared to control female and male intact mice ($p < 0.01$ for female; $p < 0.005$ for male).

5.3.2 Effect of thymectomy on kidney histology

Hematoxylin & Eosin stained kidney sections from male and female thymectomized mice obtained at 24 weeks of age were blindly evaluated and graded for order of severity of damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. A decrease in glomerular damage was seen in both male ($p=0.08$) and female ($p=0.6$) mice after

thymectomy (Table 5.1) compared to intact controls. The mean grade of damaged glomeruli was 1.0 ± 0.1 for male thymectomized mice and 3.0 ± 0.1 for intact controls, while the grades were 3.3 ± 0.33 and 3.7 ± 0.34 for female thymectomized and intact mice, respectively.

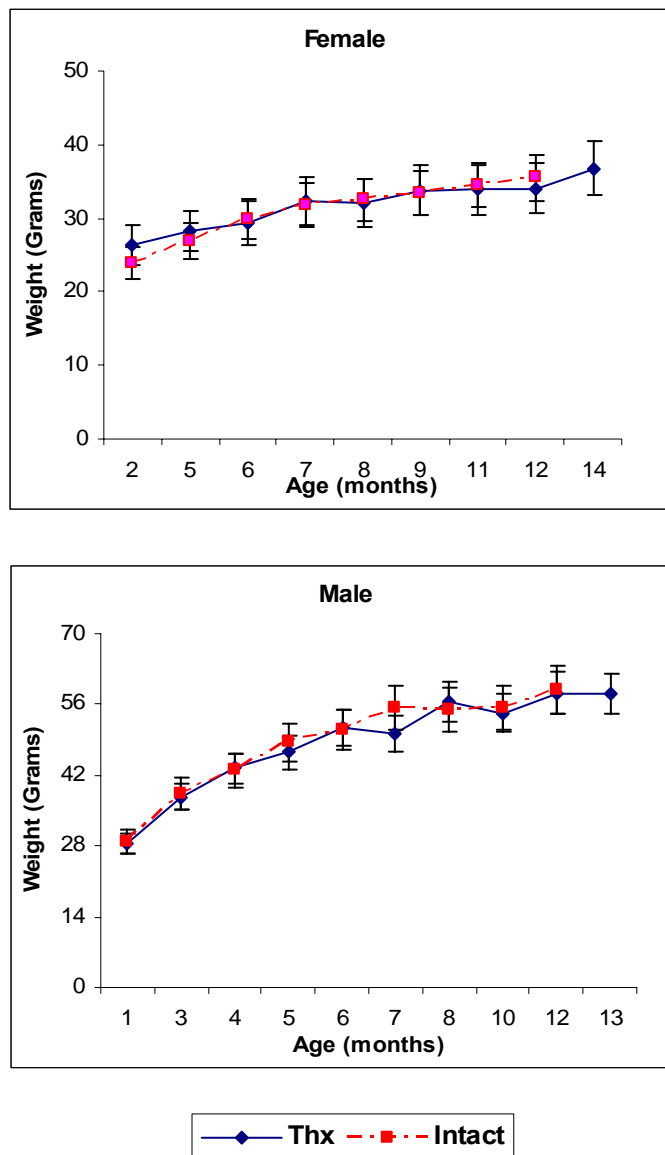


Figure 5.2 Body weights of SNF₁ were not affected by thymectomy. The mice were weighed monthly starting from 2 months of age. Results are expressed as the mean body weights \pm SE (n=6).

Table 5.1 Thymectomy leads to decreased kidney damage. Female and Male SNF₁ mice were thymectomized by suction at 30 days of age. Kidney sections from 24-week old mice in each treatment group were stained with hematoxylin and eosin (H&E) for pathological examination. Individual glomeruli from each kidney were graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. The data were expressed as mean grade \pm SE for each treatment group.

	Glomerular damage
Female Thx	3.3 \pm 0.33
Female Intact	3.8 \pm 0.34
Male Thx	1.0 \pm 0.0
Male Intact	3.0 \pm 0.0

Note: n=3 for each treatment group

5.3.3 Thymectomy modulates cytokine production in SNF₁ mice.

Abnormal cytokine production plays an important role in the pathogenesis of certain autoimmune diseases including SLE [39, 97, 233]. In order to investigate the effect thymectomy on cytokine production in the SNF₁ model for lupus nephritis, the splenic lymphocytes from each treatment group were co-cultured with ConA or aa62-73 *in vitro*, and then ELISAs were performed to detect the concentrations of specific cytokines in the supernatant. The results were expressed as the fold over a non-relevant peptide control (Mal peptide). As shown in Figure 5.3, IFN- γ , IL-12, IL-4, IL5, IL-6, and TNF- α cytokine productions stimulated by Con A were decreased in thymectomized SNF₁ female mice compared to intact female mice, with statistical significance ($p < 0.05$) for IFN- γ , IL-4, IL-5 and IL-6. In thymectomized male mice, we found that ConA stimulation led to an increase in IL-2 and IL-12 production (*in vitro*)

compared to control, while other cytokine levels were similar to control. Furthermore, when the splenic lymphocytes from female thymectomized mice were co-cultured with pathogenic peptide aa62-73, IL-5 and IL-6 cytokine production was suppressed ($p < 0.05$), and there also was a decrease in levels of the pro-inflammatory cytokine TNF- α ($p < 0.05$). Cells from thymectomized female mice also produced increased IL-2 and IL-12, although statistical significance wasn't achieved. In male mice, decreased IL-5 and increased IL-12 were observed ($p < 0.01$) after thymectomy. These changes in cytokine production in the presence of pathogenic aa62-73 peptide in thymectomized mice most likely are part of the protective mechanism of thymectomy, since SLE generally was a T_H2 response and the switch from a T_H2 to T_H1 response is associated with protection [39, 49]. Pro-inflammatory cytokine TNF- α was also associated with the lupus pathogenesis and disease development [39, 97].

5.3.4 Thymectomy results in suppression of proliferative responses to pathogenic idiotypic peptide (aa62-73).

Splenocytes from each treatment group were cultured with Concanavalin A (ConA, mitogen), anti-CD3mAb, Mal peptide (non-relevant peptide as control) or pathogenic aa62-73 peptide to measure antigen-independent and dependent T-cell stimulation and proliferation. We found that the proliferative responses to ConA (38337 ± 17216 cpm vs. 78065 ± 30930 cpm in females; 16000 ± 9400 cpm vs. 85101 ± 6000 cpm in males), anti-CD3mAb (11724 ± 5290 cpm vs. 23850 ± 5507 cpm in females; 14023 ± 3801 cpm vs. 21001 ± 9299 cpm in males) and aa62-73 peptide (772 ± 187 cpm vs. 1123 ± 204 cpm in females; 640 ± 480 cpm vs. 1754 ± 295 cpm in males) were all decreased in thymectomized mice for both genders (Fig 5.4), although a statistical significant difference in proliferation was only achieved in thymectomized

Figure 5.3 *In vitro* cytokine production in SNF₁ mice is modulated by thymectomy. Splenic lymphocytes were isolated from thymectomized mice and control mice at 24 weeks of age, and then cultured in the presence of ConA or aa62-73 idiotypic peptide for 72 hours. The cytokine levels in the supernatant were detected by ELISA. Results are expressed as (mean fold over peptide control)±SE. (*, p<0.05, compared with intact mice).

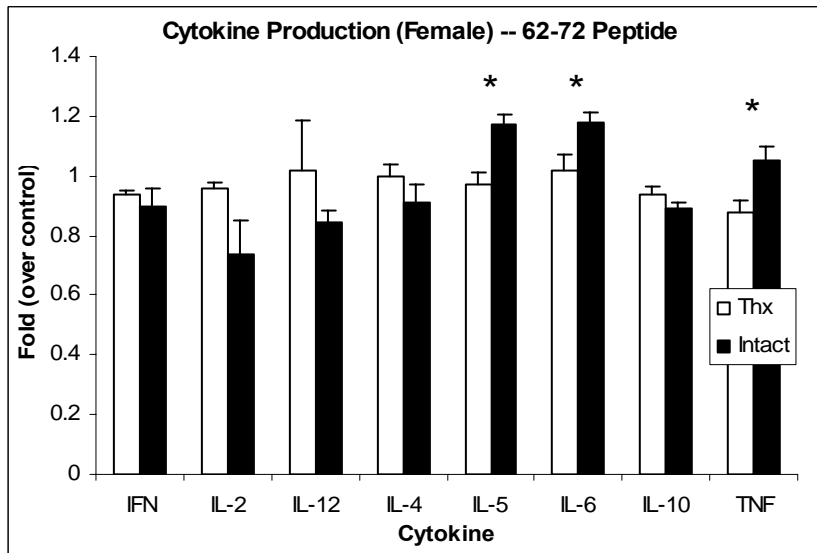
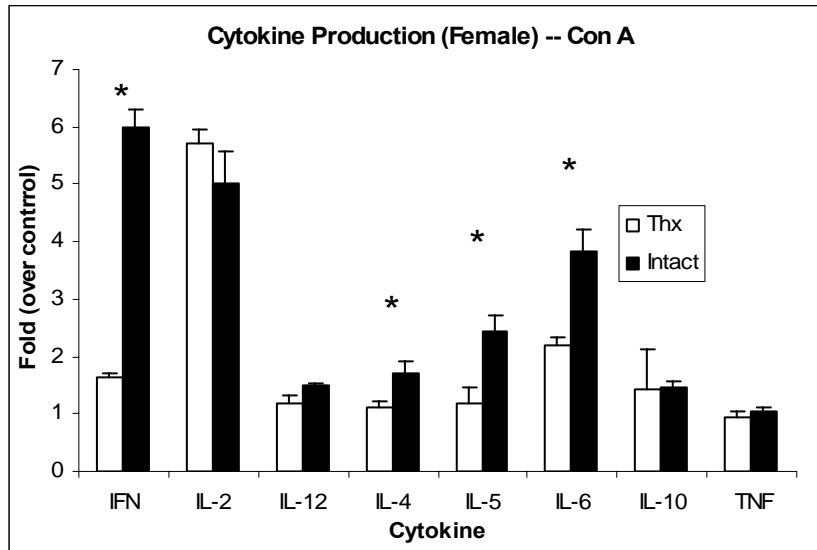
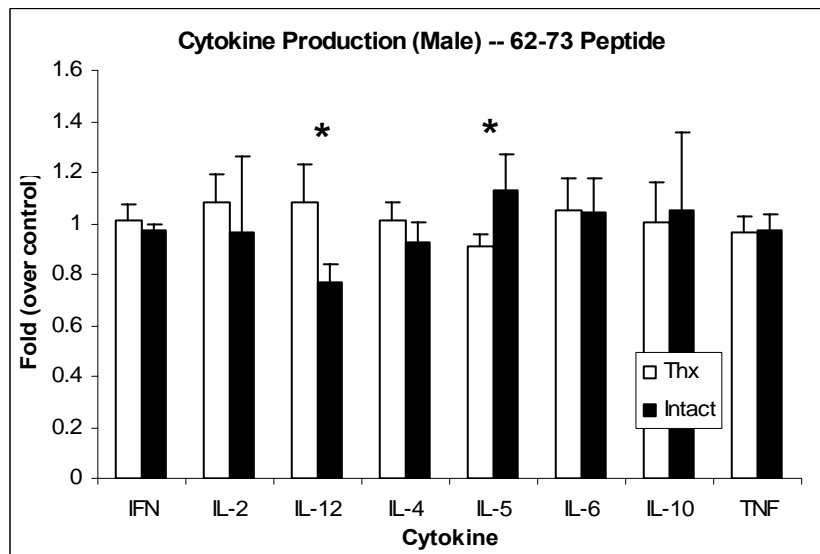
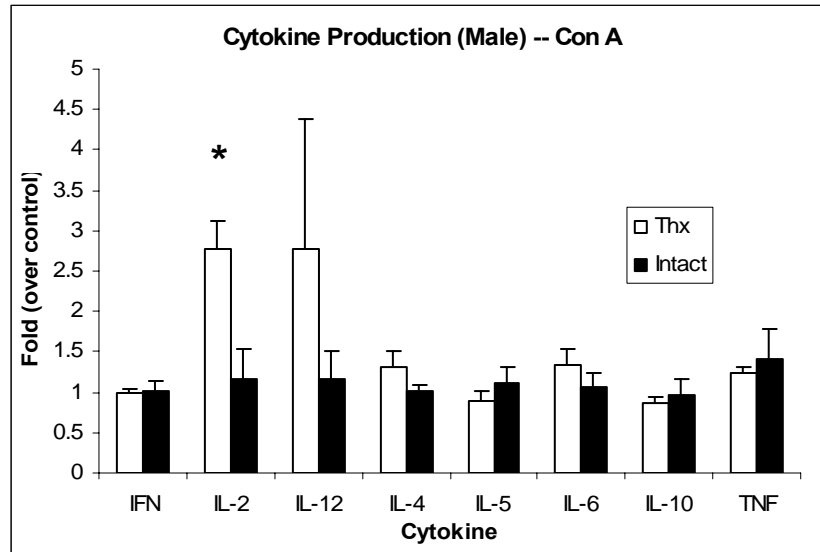


Figure 5.3 (Continued)



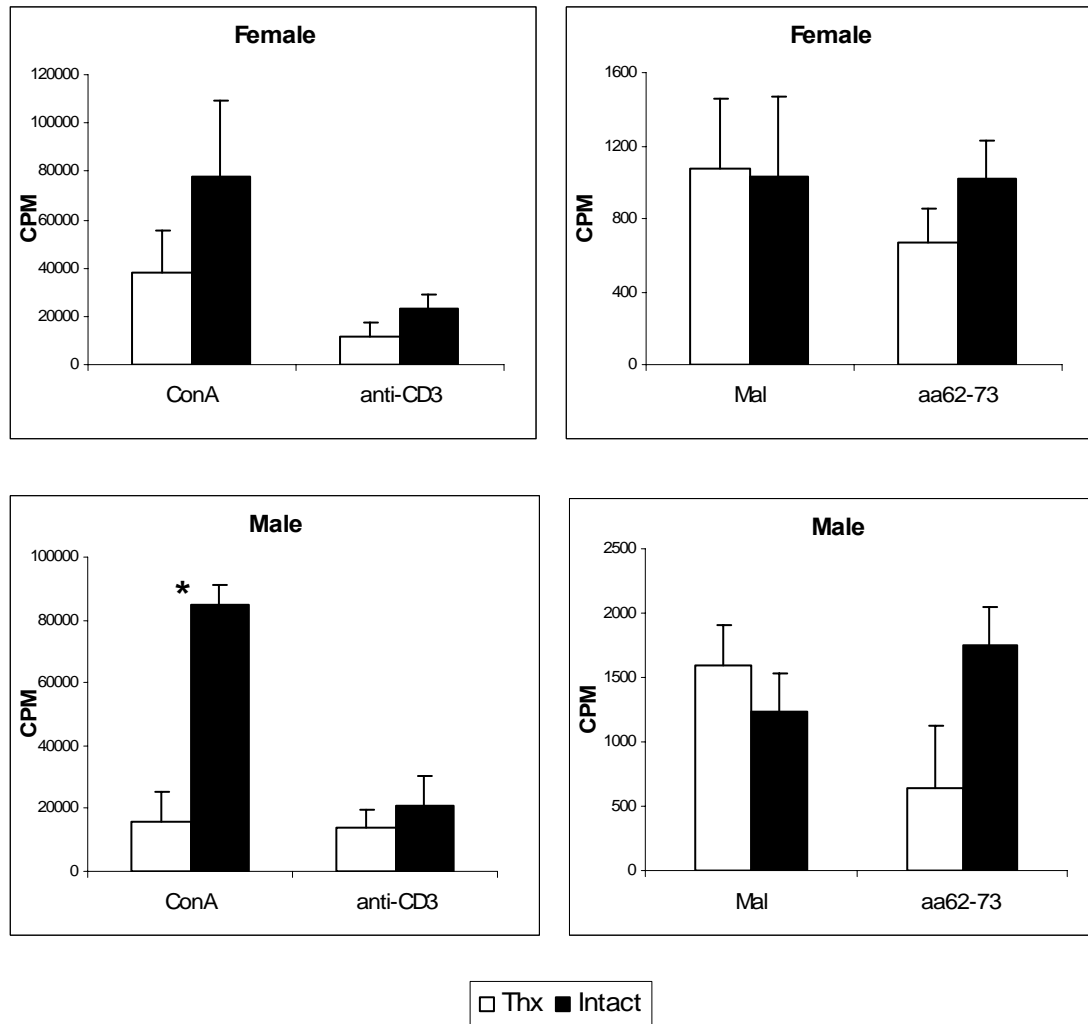


Figure 5.4 Proliferative responses are suppressed in thymectomized mice. Splenic lymphocytes were isolated from thymectomized and control intact mice at 24 weeks of age, and then cells were cultured with ConA, anti-CD3 mAb, Mal peptide (non-relevant peptide as control) or aa62-73 for 72 hours followed by pulsing with [3 H]-Thymidine overnight. Then the cells were harvested and incorporated radioactivity was measured by a scintillation counter. Data are expressed as mean cpm \pm SE (*, $p < 0.05$ compared with control intact mice). We also determined the frequencies of B cells producing pathogenic antibodies by ELISPOT. As indicated in Figure 5.7, thymectomized female SNF $_1$ mice had a higher frequency of anti-Id $^{LN}F_1^+$ antibody compared to controls. Similarly thymectomized male mice had a lower frequency of anti-Id $^{LN}F_1^+$ antibody producing B cells, but in addition fewer Id $^{LN}F_1^+$ antibody-producing B cells, although the statistical significance was not achieved.

males with ConA stimulation. Responses to Mal control peptide were not different for thymectomized and intact mice.

5.3.5 Antibody production by splenocytes (in vitro) is reduced in thymectomized mice

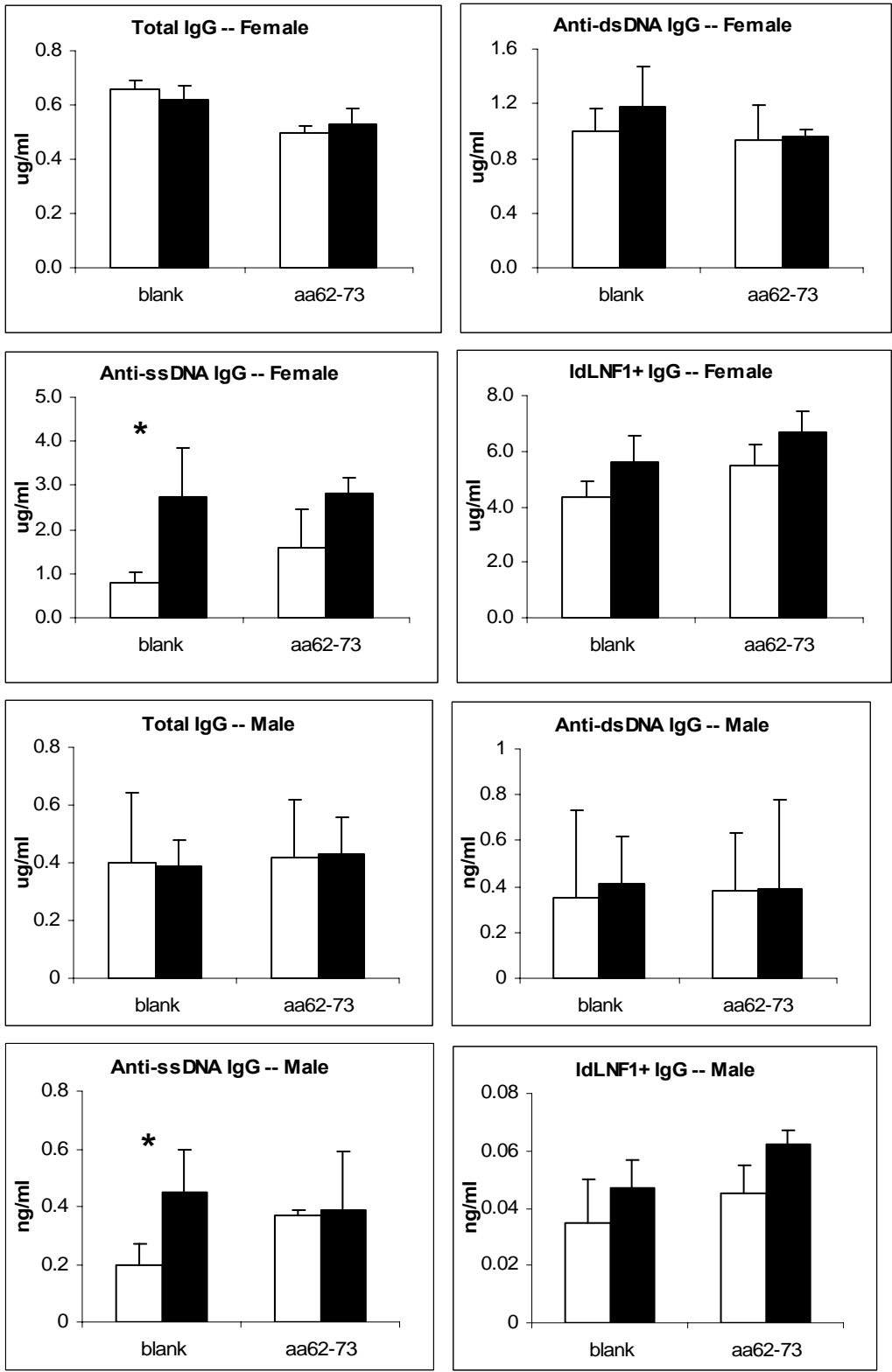
SLE is an autoimmune disease characterized by the production of high titers of auto-antibodies. Previous studies in our laboratory have shown that dysregulation of the production of pathogenic $\text{Id}^{\text{LN}}\text{F}_1$ antibodies is part of the pathogenetic mechanism, with anti-DNA antibodies having less significance [28, 54]. We next examined the *in vitro* antibody production by splenocytes from thymectomized and intact mice.

Antibody production was measured in the presence of aa62-73 peptide or medium alone. We found that splenocytes from thymectomized mice produced significantly lower levels of anti-ssDNA antibody ($p < 0.05$) spontaneously or in the presence of aa62-73 peptide ($p > 0.05$) (Fig 5.5). Further, production of $\text{Id}^{\text{LN}}\text{F}_1^+$ IgG antibody was also decreased both in thymectomized mice. However, there was no difference in total IgG and anti-dsDNA IgG production between the control and thymectomized group of either gender.

5.3.6 Thymectomy leads to decreased pathogenic $\text{Id}^{\text{LN}}\text{F}_1$ -reactive CD4^+ T cells

Phenotypic modulation of splenocytes and splenic T cells after thymectomy was measured by flow cytometry. The results are shown in Table 5.2. We found that the thymectomized mice of both genders produced significantly fewer $\text{Id}^{\text{LN}}\text{F}_1$ -reactive T cells ($\text{Id}^{\text{LN}}\text{F}_1^+\text{CD4}^+\text{CD3}^+$) than intact control mice of either gender ($p < 0.05$). Further, There were also fewer cells with a memory phenotype ($\text{Id}^{\text{LN}}\text{F}_1^+\text{CD44}^+\text{CD45}^-$) in female thymectomized mice ($p < 0.05$), but the $\text{Id}^{\text{LN}}\text{F}_1$ -reactive T cells expressed higher levels of maturation markers ($\text{Id}^{\text{LN}}\text{F}_1^+\text{CD44}^+$).

Figure 5.5 Modulation of antibody production by thymectomy in SNF₁. Ig⁺ splenocytes and the affinity-purified T cells were isolated from thymectomized mice and intact control mice at 24 weeks of age, and then cells were cultured with or without aa62-73 for 7 days. The supernatants were then harvested and frozen at -20°C until analyzed by ELISA for total IgG, anti-Id^{LN}F₁ IgG, anti-ssDNA IgG, and anti-dsDNA IgG. (*, p<0.05, compared with intact control mice).



□ Thx ■ Intact

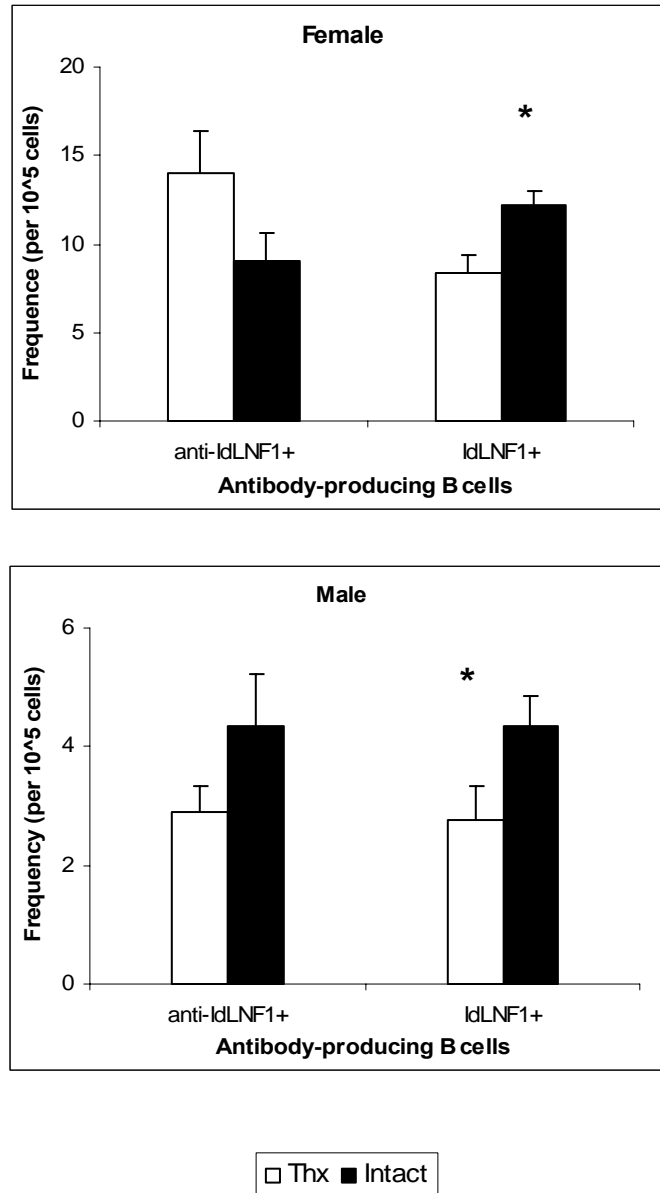


Figure 5.6 Thymectomy leads to a reduced frequency of B cells producing pathogenic antibody. Splenic lymphocytes were isolated from thymectomized and control mice at 24-week of age, and then antibody-producing cells were detected by ELISPOT. Results are expressed as mean±SE (*, $p < 0.05$ compared to oil).

Table 5.2 Phenotypic analysis. Thymectomized SNF₁ mice and control intact mice were sacrificed at 24 weeks of age. Splenocytes (A) or affinity-purified splenic T cells (B) were prepared, and 1×10^5 cells were stained with the appropriate antibodies. Phenotypes were analyzed by flow cytometry. Data were expressed as mean percentage \pm SE (*, $p < 0.05$; **, $p < 0.01$ compared to intact control mice).

A. Splenocytes

	Female		Male	
	Intact	Thx	Intact	Thx
B220+	66.76 \pm 4.37	71.86 \pm 4.30	64.17 \pm 8.38	62.37 \pm 10.66
CD3+	28.64 \pm 4.40	21.20 \pm 3.50	24.08 \pm 9.32	18.23 \pm 3.80
CD4+CD3+	22.01 \pm 2.84	15.42 \pm 2.51	16.62 \pm 5.39	12.32 \pm 1.96
Id^{LN}F₁+CD3+	6.53 \pm 0.63	2.19 \pm 0.13**	1.07 \pm 0.31	0.38 \pm 0.15*
Id^{LN}F₁+CD4+CD3+	4.24 \pm 0.09	1.32 \pm 0.02**	1.07 \pm 0.15	0.29 \pm 0.03**

B. Splenic T cells

	Female		Male	
	Intact	Thx	Intact	Thx
CD4+	53.10 \pm 3.00	60.02 \pm 5.39	55.80 \pm 0.95	45.91 \pm 8.04
CD8+	23.30 \pm 5.30	25.48 \pm 2.07	21.62 \pm 6.50	23.53 \pm 7.14
Id^{LN}F₁+	14.83 \pm 9.86	6.44 \pm 1.33	6.36 \pm 1.70	2.65 \pm 0.86*
Id^{LN}F₁+CD4+	5.16 \pm 1.16	3.03 \pm 0.66*	2.03 \pm 0.70	1.01 \pm 0.09*
Id^{LN}F₁+CD8+	2.21 \pm 0.53	3.41 \pm 1.99	2.74 \pm 0.61	1.40 \pm 0.51
CD44+	42.69 \pm 3.64	50.57 \pm 4.09	91.67 \pm 1.29	90.16 \pm 4.31
CD45+	64.55 \pm 1.99	59.19 \pm 3.44	60.94 \pm 7.58	51.75 \pm 2.09
CD45-	35.52 \pm 2.049	40.92 \pm 3.51	39.15 \pm 7.58	48.35 \pm 2.15
CD44+CD45-	27.02 \pm 0.67	33.14 \pm 1.63 *	38.30 \pm 7.42	45.38 \pm 2.09
Id^{LN}F₁+CD44+	14.27 \pm 2.50	6.82 \pm 1.87*	5.03 \pm 2.65	2.53 \pm 0.57
Id^{LN}F₁+CD44+CD45-	5.08 \pm 0.33	2.43 \pm 0.97*	0.76 \pm 0.45	1.04 \pm 0.19
(Id^{LN}F₁+CD44)/(Id^{LN}F₁+CD8+)	1.70 \pm 0.65	1.38 \pm 0.13	0.79 \pm 0.42	0.76 \pm 0.21

5.4 Discussion

Neonatal thymectomy (before day 3 after birth) in non-autoimmune mice leads to the development of organ-specific autoimmune disease [119-123]. The organs affected are dependent on the strain of mouse [124], including prostate, testis, ovaries, thyroid and stomach. The underlying mechanism is thought to be related to the loss of regulatory T cells (naturally arising CD25⁺CD4⁺ T cells) in the thymectomized mice and the ensuing activation of the neonatal T cell repertoire. The results of thymectomy on systemic autoimmune diseases, such as SLE, are different depending on the strain of mice and the time of thymectomy [121, 122, 125, 126]. No effect, protection and aggravation by thymectomy have all been reported in different mouse strains [125, 126]. Steinberg *et al* reported that neonatal thymectomy of MRL/lpr mice led to a marked reduction in their characteristic massive lymphadenopathy, as well as significantly reduced antibodies to native DNA and prolonged survival [126]; in contrast, neonatal thymectomy of NZB x NZW F₁ mice led to accelerated disease [126]. Further, the protective effect by thymectomy in MRL/lpr mice diminished when thymectomy was delayed beyond 3 weeks post-natally [126]. Similar results were obtained in another study using additional mouse strains [125]. In addition, Bagavant *et al.* reported a differential effect of neonatal thymectomy on systemic and organ-specific autoimmune disease, in which thymectomy paradoxically protected SNF₁ mice from genetically prone lupus-like glomerulonephritis, yet promoted *de novo* organ-specific autoimmunity [121]. However, in this case the mechanism of the protection has not been identified and recently was proved to not be related to the suppressive effect of regulatory CD25⁺CD4⁺ T cells [122]. Taken together, the variability in the responses of the mice by thymectomy suggests that the pathogenic and protective mechanisms might vary among different lupus mouse models. In the current study, we investigated the effect of pre-pubertal thymectomy on SLE

pathogenesis in the murine lupus model, SNF₁ mice, with an emphasis on the modulation of the pathogenic Id^{LN}F₁-reactive CD4⁺ T cells, as well as other immunological changes that we have found to be associated with pathogenesis in SNF₁ mice [28-30, 54, 72, 73].

SLE has been considered to be an immune complex-mediated disease. The anti-dsDNA and anti-nuclear antibodies produced in this disease have long been thought to be of great importance in the pathogenesis and diagnosis of the lupus nephritis [234, 235]. However, recently Mannik et al have reported that anti-dsDNA, anti-snRNP, anti-Ro/La, and anti-histone autoantibodies only account for less than 25% of Ig eluted from kidneys of SLE patients with severe glomerulonephritis [26]. Waters et al. have demonstrated that anti-dsDNA and related antibody production and chronic glomerulonephritis are under independent genetic control, and furthermore breaking tolerance to dsDNA, nucleosome, and other nuclear antigens is not required for the pathogenesis of lupus nephritis [27]. Previous studies in our as well as other laboratories have shown the pathogenic importance of cross-reactive idiotypes, found on immunoglobulins deposited in diseased kidneys and auto-antibodies [72, 73, 236]. In support of this, induction of an experimental disease resembling murine SLE has been achieved by immunization of normal mice with idiotypic determinants from anti-DNA antibodies [236]. Anti-Id^{LN}F₁ antibody has been shown to modulate the production of anti-DNA antibody both *in vitro* and *in vivo* [237], and both Id^{LN}F₁-reactive CD4⁺ helper [238] and CD8⁺ suppressor cells [195] appear to be involved. Auto-reactive T-cells that induced (NZB X NZW) F₁ (B/W) B cells to produce anti-DNA antibodies were recently shown to proliferate in response to several peptides derived from the amino acid sequence of an anti-DNA antibody [74]. Immunization with one of these peptides accelerated disease [239]. In contrast, we have found that administration of an idiotypic peptide derived from an Id^{LN}F₁⁺ antibody, aa62-73

(KFKGKATLTSDK), to pre-nephritic SNF₁ mice prolonged their survival. We have identified the pathogenic cells involved, and further, our laboratory has shown that T cell clones that react to this idiotypic family induce SNF₁ B cells to produce only Id^{LN}F₁⁺ IgG but not anti-DNA antibodies, and accelerate disease [28]. Thus, our data supports expansion of Id^{LN}F₁ as part of the pathogenic mechanism in SNF₁.

As previously reported, neonatal thymectomy (before day 3) could protect SNF₁ mice from lupus-like glomerular nephritis [121, 122]. In the current study, pre-pubertal thymectomy also prolonged survival of both male and female SNF₁ mice significantly, and led to decreased kidney damage with lower glomerulus grades by the histological examination. In addition, the phenotype analysis of thymectomized mice showed a significant decrease in the pathogenic Id^{LN}F₁-reactive T cell population in both male and female mice. And in female mice, the Id^{LN}F₁-reactive memory T cells (CD44^{hi}CD45RA^{low}) were found to be decreased after thymectomy compared to intact mice. As expected, we also found a decrease in the ratios of (Id^{LN}F₁⁺CD4⁺)/(Id^{LN}F₁⁺CD8⁺) T cells in thymectomized mice, which we have previously found to be a hallmark for the onset and progression of lupus nephritis in SNF₁ mice [54]. Furthermore, there was no apparent increase in regulatory CD25⁺CD4⁺ T cell population in thymectomized mice (data not shown). This finding is consistent with other studies, that found the suppressive effect to be associated with a CD8⁺ T cell population [195]; in another study the CD25⁺CD4⁺ T cell failed to suppress the lupus nephritis in NZM2328 mice [122]. Taken together, these findings suggested that the thymus might be required for generating the pathogenic T cells, rather than regulatory cells in SNF₁ nephritis.

In addition to a reduction in the pathogenic Id^{LN}F₁-reactive T cell populations, the function of these cells was also found to be affected following the thymectomy, as demonstrated by suppressed proliferative responses to pathogenic peptide (aa62-73)

and modulation of cytokine production in thymectomized mice. We found that cytokine production by splenocytes in thymectomized mice stimulated with pathogenic peptide aa62-73 was biased, evident as significantly decreased production of IL-4 and IL-5 and increased production of IL-12 in males and significantly decreased production of IL-5, IL-6 and TNF- α and increased production IL-12 and IL-2 in females. This is consistent with earlier reports proposing a T_H2 response as the relevant pathogenic response in lupus-like glomerular nephritis, and that a change to a T_H1 response was associated with protection [96, 240, 241]. We also found significantly decreased levels of IFN- γ produced after Con A stimulation in female mice following thymectomy compared with control mice, which also could contribute to the protective effect mediated by the thymectomy, since deficiency of IFN- γ or IFN- γ receptor has been proved to beneficial in this disease [242, 243]. Moreover, in the present study, there were no apparent changes in the levels of IL-10 or TGF- β between thymectomized and intact control mice, which suggested that the protection from disease after the thymectomy in SNF₁ mice was not mediated by these 2 suppressive cytokines.

As expected, Id^{LN}F₁⁺IgG production by splenocytes stimulated with pathogenic peptide aa62-73 was decreased in thymectomized mice, although statistical significance was not achieved ($p > 0.05$). This reduction in Ig production appeared to be restricted to Id^{LN}F₁⁺ antibody, because there was no change in the total IgG, anti-dsDNA or anti-ssDNA levels following thymectomy in both male and female mice. Using ELISPOT, we found that following thymectomy male SNF₁ mice tended to have lower frequency of B cells producing Id^{LN}F₁⁺ immunoglobulins. On the other hand, although thymectomized female SNF₁ mice had comparable number of B cells producing Id^{LN}F₁⁺ immunoglobulins to intact mice, they produced a relatively higher frequency of B cells producing regulatory anti-Id^{LN}F₁⁺ immunoglobulins [29],

although statistical significance was not reached ($p=0.06$). This discrepancy in the frequency of B cells producing $\text{Id}^{\text{LN}}\text{F}_1^+$ / anti- $\text{Id}^{\text{LN}}\text{F}_1^+$ antibodies between male and female mice following thymectomy could possibly be explained by the effects of sex hormone, especially the effect of estrogen. It has been reported that estrogen exposure induces the expansion of anti-DNA autoantibodies and increases the number of plasma cells producing antibodies to both self and non-self antigen [160, 222]. In another study, we also found that estrogen could preferentially increase the number of B cells producing $\text{Id}^{\text{LN}}\text{F}_1^+$ Ig (unpublished data). The elevated frequency of B cells producing anti- $\text{Id}^{\text{LN}}\text{F}_1^+$ in female mice also suggests the importance of the balance between $\text{Id}^{\text{LN}}\text{F}_1^+$ /anti- $\text{Id}^{\text{LN}}\text{F}_1^+$ antibodies in the pathogenesis of lupus in SNF_1 mice [29]. Previously, Uner, et al, have found that treatment with these anti- $\text{Id}^{\text{LN}}\text{F}_1^+$ leads to prolonged survival of SNF_1 mice [29].

SLE is a complex systemic autoimmune disease, and the pathogenesis of disease development is not well understood. Recently, data is accumulating in support of a lesser role for the production of the anti-DNA and anti-nuclear auto-antibodies in disease development. Furthermore, as our and other laboratory's data suggests, immune responses involved in the production of cross-reactive $\text{Id}^{\text{LN}}\text{F}_1^+$ autoantibodies and $\text{Id}^{\text{LN}}\text{F}_1$ -reactive T helper cells appear to be an important mechanism leading to increased production of nephritogenic antibody, just prior to its deposition in lupus nephritis. In the current study, we found that the prolonged survival and ameliorated glomerulonephritis in SNF_1 male and female mice following thymectomy was associated with a significant decrease in the percentage and function of pathogenic $\text{Id}^{\text{LN}}\text{F}_1$ -reactive CD4 T cells and a shifted cytokine production in favor of lupus development. This was accompanied by a reduction of function in the B cell compartment that included decreased $\text{Id}^{\text{LN}}\text{F}_1^+$ auto-antibody production perhaps due to a decreased frequency of B cells producing this auto-antibody in thymectomized mice.

Taken together, these findings suggest that the thymus is important for the generation and expansion of the pathogenic populations in lupus nephritis in SNF₁ mice.

CHAPTER 6

Development of lupus nephritis after 17 β -estradiol treatment is via estrogen receptor- α dependent pathway

Abstract

Previous studies in our laboratory have demonstrated that repeated administration of 17 β -estradiol (E-2) at a dose of 1mg/kg to both (SWR x NZB)F₁ (SNF₁) mice and (DBA x BALB/c)F₁(DBF₁) mice results in significantly decreased survivals and increased kidney damages by glomerulonephritis. But how estrogen modulates immune responses remains a fundamental unanswered question. Both nuclear and membrane forms of the two estrogen receptors (ER), α and β , have been cloned, and found expressed in many immune cells normally. Therefore, in the present study, ER α -deficient (ER α -/-) and wild type mice (WT) were injected subcutaneously with β -estradiol 17-valerate (E-2) at 1mg/kg beginning at 8 weeks and then monthly. The control ER α -/- and WT were treated with oil vehicle only. In WT mice, repeated E-2 treatment appeared to induce the lupus phenotype similar to the observations in previous studies, with accelerated death and increased kidney damage, as well as elevated serum T_H2 and pro-inflammatory cytokines and pathogenic Id^{LN}F₁⁺ IgG/IgM. In contrast, in ERKO mice, only minimal changes were observed in the E-2 treated group compared to control groups, which strongly suggested a role of ER α in E-2 induced development of the lupus phenotype in these mice. When ER expression on immune cells was analyzed by flow cytometer in both DBF₁ and SNF₁ with or without E-2 treatment, we observed that both ER α and ER β were differentially expressed. Specifically, in SNF₁ mice we noted that there were more CD4⁺ and CD8⁺ T cells constitutively expressing ER α , while in DBF₁ mice expression of ER α in those populations was upregulated only after E-2 treatment. We also found that the

percentage of ER α positive dendritic cells and macrophages were increased after E-2 exposure in SNF₁ mice, but not in E-2 treated DBF₁ mice. These observations suggest a role for ER α expression in E-2 induced modulation of lupus nephritis in SNF₁ mice.

6.1 Introduction

Sex differences in immune responses are well documented. Females in many species produce more robust immune responses to variety of antigens, reject allografts more readily and show a higher incidence of many autoimmune disease [80, 190].

Systemic Lupus Erythematosus (SLE) is one of the autoimmune diseases that show a high female predominance with a female to male ratio of 9:1 [60, 61, 158, 200].

Indeed, modulation of the clinical manifestations of SLE correlates with alterations in steroid hormone levels, and may be exacerbated during pregnancy, postpartum and, in some cases, with oral contraceptive use [82, 83, 244]. Male and female patients with SLE are often hyperestrogenic and/or hypoandrogenic [84-86], and SLE is found at a higher incidence in patients with Klinefelter's syndrome [87, 88], a genetic disease of males that is characterized by a variety of sex hormone abnormalities, including increased production of estrogen. These data, therefore, point to a potential significant role for estrogen in the gender-specific modulation of immune responses involved in autoimmunity.

More direct evidence for the role of sex hormones in the pathogenesis of SLE has been provided by studies of murine models of lupus. It has been reported that estrogen administration accelerated the disease, resulting in increased mortality, in (NZB x NZW)F₁ mice [199]. Work in our laboratory towards understanding the role of sex hormones in autoimmunity has also found that repeated administration of 17 β -estradiol (E-2) at a dose of 1mg/kg to (SWR x NZB)F₁(SNF₁) mice, beginning at 7 weeks and then monthly thereafter for another 4 months, resulted in significantly decreased survival,

similar to that of female SNF₁ mice, compared to vehicle-treated male mice (manuscript submitted). By the 3rd dose (about 20 weeks of age), significant proteinuria had developed, with Id^{LN}F₁⁺ immunoglobulins increased to significant levels in the serum of E-2 treated male SNF₁ mice. Id^{LN}F₁-reactive T cells with a memory phenotype (CD44⁺CD45⁻) also were significantly increased in E2-treated mice and found in the kidneys. A pathogenetic role for these T-cells was suggested by the finding that splenocytes from E-2 treated mice produced significantly more Id540⁺ IgG&M *in vitro* than splenocytes from control mice when incubated with control T cells. Finally, at 25 weeks of age (2 weeks after the 5th dose), 80% of the treated animals had glomerular deposits of Id^{LN}F₁⁺ immunoglobulins, and significant histopathological manifestations. Furthermore, the E-2 treatment in non-autoimmune (DBA x BALB/c)F₁ (DBF₁) mice led to similar lupus phenotype, although with milder manifestations (unpublished data). In direct contrast, estrogen can suppress the development of the autoimmune exocrinopathy, Sjögren's syndrome [245] and encephalomyelitis [175], the pathology of which is mainly mediated by T_H1 responses, as opposed to T_H2 responses in SLE.

How estrogen modulates immune responses remains a fundamental unanswered question. Two estrogen receptors (ER), α and β [129-134] have been cloned, and are expressed in many immune cells normally and at increased levels after administration of E-2. A recent study that investigated ER α and ER β expression among resting and activated PBMC subsets found the ER α was expressed at higher levels in CD4⁺ T cells, while levels were lower and similar in CD8⁺ T cells and monocytes. B cells, on the other hand, expressed higher ER β . Further, gender differences in ER expression were noted [223]. Administration of low doses of E-2 resulted in a striking increase in antigen-specific CD4⁺ T cell responses, that was ER- α -dependent [153], and in another study, it was shown that estrogen exposure directly activated T cells, via cell membrane ER [154], suggesting the possibility that

differences in the numbers and types of ER expressed on the immune cells of some individuals could explain the differences in their T cell responses to antigen. This hypothesis was further supported by the observation that calcineurin, CD40L expression and PP2B phosphatase activity were increased when SLE T cells, but not T cells from normal women, were cultured with E-2 [149, 155]. Since these responses could be specifically inhibited by the ER antagonist, ICI 182 780, it appeared that estrogen activation of these T cells was ER-dependent [156]. B cell development in the bone marrow may also be modified by exposure to estrogen [157], and estrogen exposure also may result in the ER-dependent upregulation of several genes involved in B cell activation and survival [44, 102, 158]. NK cells may also be modulated by estrogen and were found to either increase [161] or decrease [162] after estrogen exposure, presumably through ER β [163]. In one study, estrogen treatment has also been shown to promote the differentiation of a CD11c⁺, CD11b (int) DC population that displayed high levels of MHC Class II and CD86 (B7-2), while in another study, anti-estrogen treatment resulted in mature dendritic cells (DC) that were less effective than immature DC in presenting antigen to allogeneic T cells [164], possibly by altering cytokine production in these cells [165]. These observations suggest that E-2 could augment the numbers of potent APCs [166], as well as modulate cytokine and chemokine expression.

In the present study, we investigated the role of ER α in the development of lupus nephritis treating the ER α -deficient mice (ER α ^{-/-}) with E-2. ER expression on immune cells was also determined by flow cytometric analysis in both autoimmune and non-autoimmune mice, with or without estrogen treatment.

6.2 Materials and methods

6.2.1 Mice and E-2 Treatment

All mice were bred and maintained in Cornell University College of Veterinary Medicine, Center for Research Animal Resources. Male heterozygous mice ($ER\alpha^{+/-}$, a kind gift of Dennis Lubahn, University of Missouri) were mated with female heterozygous mice ($ER\alpha^{+/-}$) on a mixed C57BL/6 background resulting in WT ($ER\alpha^{+/+}$) and $ER\alpha^{-/-}$ offspring [246]. Genotyping of tail DNA was performed using polymerase chain reaction (PCR). The $ER\alpha$ gene was analyzed with primer pair fmerex2/c 5'-ctacggccagtcgggcat-3' and rmerex2/c 5'-agacctgtagaaggcgggag-3', leading to a 200bp fragment for the normal $ER\alpha$ gene. The mutant $ER\alpha$ gene was detected with the primer pair fneo/c 5'-tgaatgaactgcaggacgag-3' and rneo/c 5'-aatatcacgggtagccaacg-3', resulting in a 500bp fragment. Only the male homozygous mice (WT or $ER\alpha^{-/-}$) were used in the study. Mice received β -estradiol 17-valerate (E-2, Sigma) at 1mg/kg in olive oil vehicle subcutaneously once every month starting at 8 weeks of age; control mice were injected monthly with oil vehicle alone. Body weights were recorded monthly. Mice from each group were sacrificed two weeks after the 4th dose of E-2 or oil (at age of 24 weeks). The thymus, spleen and kidney were harvested and single cell suspensions were prepared from individual spleens and thymuses. Kidneys were harvested and fixed, and sections were stained with hematoxylin and eosin (H&E) for glomerular histology analysis.

For ER expression study, six to eight week old female SWR, male NZB, male DBA and female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), to derive (SWR x NZB) F_1 progeny (SNF $_1$) and (DBA x BALB/c) F_1 progeny (DBF $_1$), both H-2^{d/q}. Male SNF $_1$ and DBF $_1$ mice at about 12 weeks of age were injected subcutaneously with E-2 at 1mg/kg, and control mice were injected with oil vehicle. Two weeks later, spleens, thymuses and lymph nodes from

these mice were harvested, and lymphocytes were analyzed for ER expression by flow cytometry.

6.2.2 Serum collection

Retro-orbital eye bleeds were performed monthly under isoflurane anesthesia in order to determine the kinetics of disease progression, beginning two weeks after the first E-2 treatment. For terminal bleeds, mice were euthanized via CO₂ asphyxiation, and cardiac puncture was used to obtain whole blood samples. Sera was isolated and then frozen at -20°C until further use.

6.2.3 Determination of serum Ig levels

A direct binding ELISA was used to determine the levels of total serum IgG, anti-dsDNA IgG, anti-ssDNA IgG, and Id^{LN}F₁+ IgG as previously described [29]. Immulon 1B 96-well plates (Krackeler Scientific, Albany, NY, USA) were coated with goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN, USA), calf-thymus dsDNA (Sigma), or rabbit anti-Id^{LN}F₁ Ig respectively. Then the diluted serum was added in triplicate. A standard curve was generated using duplicates of eight, two-fold serial dilutions of affinity-purified specific IgG starting at 1 µg/ml in PBS/0.1% Tween. Incubation continued 12 hours at 4°C. Then the plates were washed with PBS/2% Tween and goat anti-mouse IgG or IgM-horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) was added per well. Incubation was continued overnight. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was added to each well. Color development proceeded for five minutes to one hour at room temperature (RT) until significant blue color developed, then the stop solution (1M phosphoric acid) was added and the O.D. was read at 450nm. Serum Ig concentrations were determined from the corresponding linear standard curves.

6.2.4 Determination of serum cytokine levels

Mouse serum was diluted 1:3 or 1:4 in PBS/0.1%Tween and analyzed by ELISA (eBioscience) for IL-2, IL-4, IL-5, IL-6 and IFN-gamma as per the manufacturer's instructions. The data were expressed as pg/ml.

6.2.5 Renal histology

Individual glomeruli from each kidney were blindly evaluated and graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis.

6.2.6 Survival

At least 6 mice for each treatment group were remained in order to determine the effect of treatment on survival. Mice were euthanized when moribund. The data were expressed as the percentage of surviving mice.

6.2.7 Detection of ER on immune cells by flow cytometry

Male DBF₁ and SNF₁ mice were injected with E-2 1mg/kg or oil subcutaneously. Two weeks later, the mice were euthanized and thymuses, spleens and lymph nodes were harvested. The organs were homogenized and single cell suspensions were made. Purified CD3⁺ T cells, B220⁺ B cells, CD11b⁺ macrophages and CD11c⁺ dendritic cells (DC) were isolated using the Magnetic Cell Sorting and Separation (MACS) system (Miltenyi Biotec, Auburn, CA, USA).

The isolated cells ($1-2 \times 10^5$ cells) were stained with the appropriate dilution of phycoerythrin-conjugated goat-anti-ER α IgG or rabbit-anti-ER β IgG (PharMingen) and streptavidin-red670 (PharMingen) to detect surface ER. Another set of samples were permeabilized by addition of 70% ethanol in order to detect intracellular staining of ERs. Flow cytometry was carried out on a Becton-Dickison FACSCalibur[®] flow cytometer and the data were analyzed with WinMDI program

6.2.8 Statistics

Statistical significance of treatment on survival was determined by the Logrank test. Glomerulus damage grades were analyzed by nonparametric Mann-Whitney test. Other data were analyzed by the Student's t-test or one-way ANOVA test to compare means from two treatment groups. One-way ANOVA followed by Fisher LSD test was used for multiple comparisons of means. Statistical significance was ascribed when p values were less than 0.05.

6.3 Results

6.3.1 *ER α expression mediates E-2 induced effects on survival and body weight*

Previous studies in our and other laboratories have shown that E-2 treatment of male non-autoimmune DBF₁ mice and autoimmune prone SNF₁ mice (Feng F. *et al*, in preparation) leads to the development of lupus disease phenotype, with an upregulation of pathogenic Id^{LN}F₁-reactive T cell populations, decreased survival, and accelerated glomerulonephritis. In order to further identify the role of ER receptors in the pathogenesis of lupus, we examined the effect of E-2 exposure on the survival of male ER α ^{-/-} and WT mice that had been treated with either E-2 or oil monthly starting from 8 weeks of age (Figure 6.1A). The results showed that male WT mice treated with E-2 had a significantly ($p < 0.01$) decreased survival (median survival of 75 weeks) compared to oil-treated WT male mice (median survival greater than 95 weeks). This result was consistent with what we found previously in another non-autoimmune DBF₁ mouse; the E-2 treated male DBF₁ mice had a median survival of about 80 weeks, while the median survival of oil treated controls was greater than 100 weeks. However, E-2 treatment did not lead to significantly decreased survival of ER α ^{-/-} mice compared to oil treated ER α ^{-/-}, and both groups of mice had median survival of greater

than 95 weeks; survival of these mice was similar to that of oil-treated WT mice. These results suggested that the E-2 mediated effect on survival was ER α dependent.

Both human and animal studies have shown that estrogens are important modulators of body weight, by mobilizing body fat and inducing the apoptosis of adipose tissue [247-250], leading to weight loss. In the present study, body weights of WT mice treated with E-2 were shown a significantly decrease after 2 injections of E-2 compared to those of the other 3 groups, and were kept at the lower level until the end of the study (in Fig 6.1B). ER α ^{-/-} mice treated with E-2 or oil had normal body weights, similar to those of oil-treated WT mice, throughout the study.

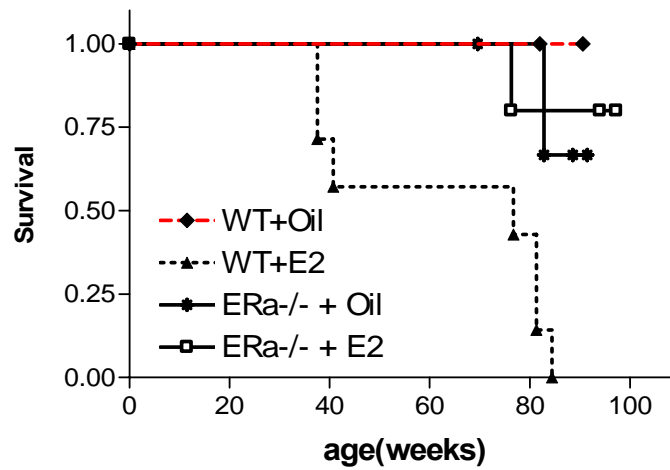
6.3.2 Effect of E-2 exposure on kidney pathology.

Hematoxylin & Eosin stained kidney sections from E-2 treated WT and ER α ^{-/-} male mice obtained two weeks subsequent to the 4th dose were blindly evaluated and graded for order of severity of damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. An increase ($p=0.08$) in damaged glomeruli was only seen in WT mice after 1mg/kg E-2 treatment (Table 6.1). The mean grade of damaged glomeruli for E-2 treated WT mice was 2.7 ± 0.33 , while the grade in other three groups (WT treated with oil, ER α ^{-/-} treated with E-2 and ER α ^{-/-} treated with Oil) were 2.0 ± 0.0 .

6.3.3 Effect of E-2 treatment on cytokine production.

Sera were collected monthly from E-2 or oil treated ER α ^{-/-} and WT mice, and cytokine levels were determined by ELISA. The data were expressed as the difference between E-2 and oil treated mice in WT or ERKO mice in order to highlight the effect of E-2 treatment on the serum cytokine levels in the presence or absence of ER α . The

A.



B.

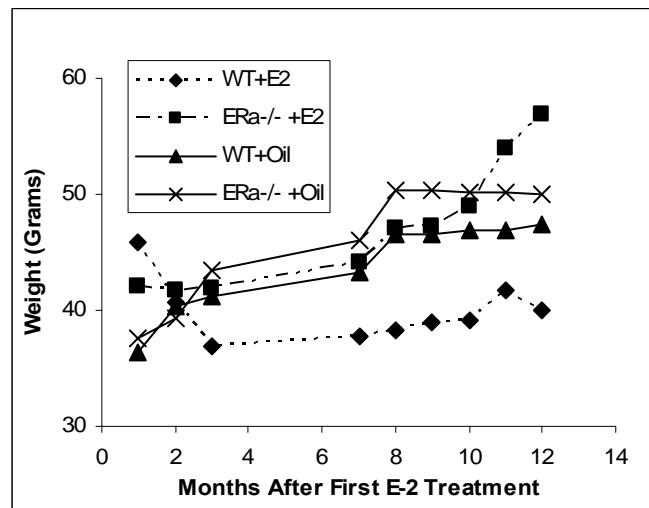


Figure 6.1 ER α mediates E-2 induced modulation of survival and body weight. Male ER α -deficient mice (ER α ^{-/-} C57BL/6) and WT mice (ER α ^{+/+} C57BL/6) were treated with 1mg/kg E-2 or oil monthly starting at 7 weeks of age. Treatment groups included WT mice with E-2 (WT+E2), WT mice with oil (WT+Oil), and ER α ^{-/-} mice with E-2 (ER α ^{-/-}+E-2) and ER α ^{-/-} mice with oil (ER α ^{-/-} +Oil) (A), and the differences in survival were analyzed with the Logrank Test. WT mice exposed to E-2 had a significantly reduced survival ($p < 0.01$), compared to the other three groups. WT mice treated with E-2 also had significantly mean body weights ($p < 0.05$), beginning as early as 2 month after the first E-2 treatments (B), in contrast to the 2 groups of KO mice and WT mice with oil treatment. (n=6 for all groups)

Table 6.1 E-2 treatment leads to increased kidney damage in WT mice. Male WT and ER α ^{-/-} mice were injected subcutaneously with E-2 (1mg/kg) in olive oil monthly, and the control WT and ER α ^{-/-} mice were treated with oil vehicle alone. Kidney sections from 24-week old mice in each treatment group were stained with hematoxylin and eosin (H&E) for pathological examination. Individual glomeruli from each kidney were graded for damage using a scale from 0 to 5, with (0) defined as normal, having no lymphocyte infiltration and/or minimal segmental infiltration and (5), defined as damaged, with lymphocytic infiltration, and/or sclerosis. The data were expressed as mean grade \pm SE for each treatment group.

	Glomerular damage
WT + Oil	2.0 \pm 0.0
WT + E-2	2.7 \pm 0.33
ER α ^{-/-} + Oil	2.0 \pm 0.0
ER α ^{-/-} + E-2	2.0 \pm 0.0

Note: n=3 for each treatment group

results (Fig 6.2) show that WT mice treated with E-2 had increased T_H2 serum cytokine levels (IL-5, IL-6 and IL-10), which is consistent with the cytokine profile associated with SLE [3, 39, 49, 100]. In contrast, the levels of serum T_H2 cytokines in oil and E-2 exposed ER α ^{-/-} mice were not increased. E-2 treated WT mice also developed a higher level of IFN- γ relative to the oil control WT mice, which was not seen in E-2-treated ER α ^{-/-} mice. IFN- γ has also been reported to be important in the initiation of lupus nephritis [96, 99, 194]. On the other hand, level of IL-2 (a T_H1 cytokine) was decreased in WT mice treated with E-2, but not in ER α ^{-/-} mice with E-2 treatment compared to oil treated ER α ^{-/-} mice. Levels of the pro-inflammatory cytokine TNF- α , which is important in mediating the pathology of lupus nephritis[3, 39, 99, 194], were also found to be increased in the serum of E-2 treated WT mice compared to oil control mice, but not in E-2 treated ER α ^{-/-} mice. Finally, serum IL-12 cytokine levels were decreased after E-2 exposure in both ERKO and WT mice.

Figure 6.2 Effect of E-2 on the serum cytokine levels in ERKO and WT mice. $ER\alpha^{-/-}$ and WT mice were treated with E-2 or oil monthly starting at 8 weeks of age. Serum was collected monthly to measure serum cytokine levels. The data were expressed as the difference between E-2 treated mice and their matched oil controls. A value close to zero means that serum cytokine levels were equivalent in E-2 and oil treated mice; a value greater than zero corresponds to higher level of serum cytokine production with E-2 treatment, while a value less than zero corresponds to decreased cytokine production in E-2 treated mice. The results suggested that E-2 exposure led to increased production of IL-5, IL-6, IL-10, IFN- γ , TNF- α cytokines in E-2 treated WT mice at multiple time points but not in E-2 treated $ER\alpha^{-/-}$ mice. (*, $p < 0.05$, compared WT mice with $ER\alpha^{-/-}$ mice).

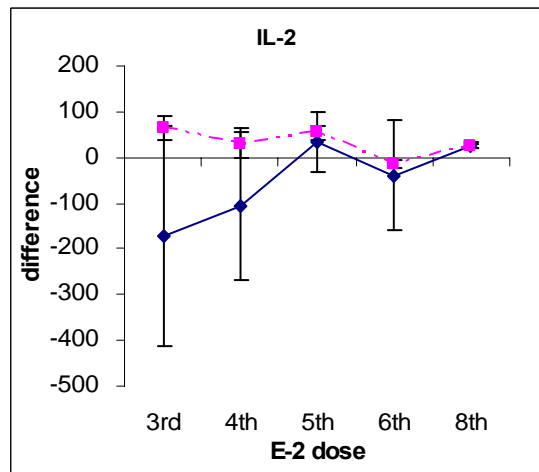
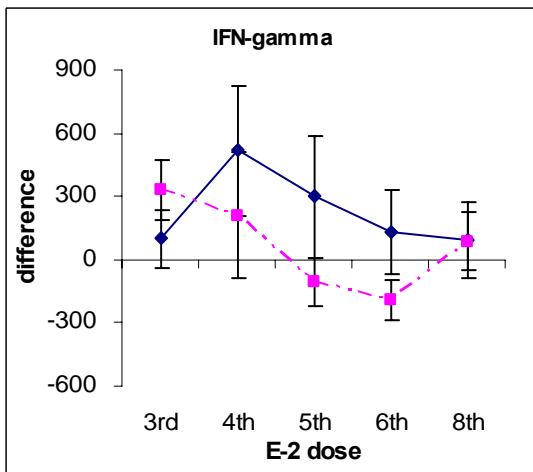
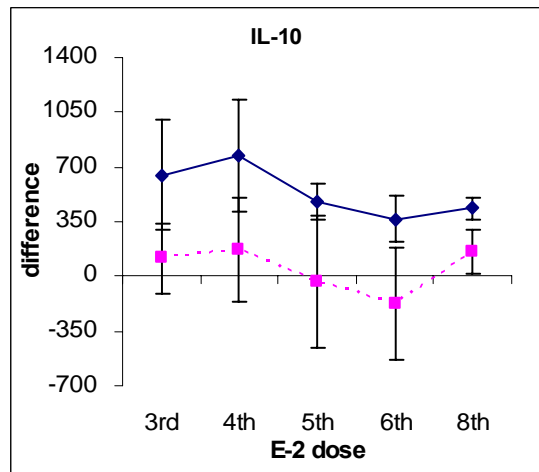
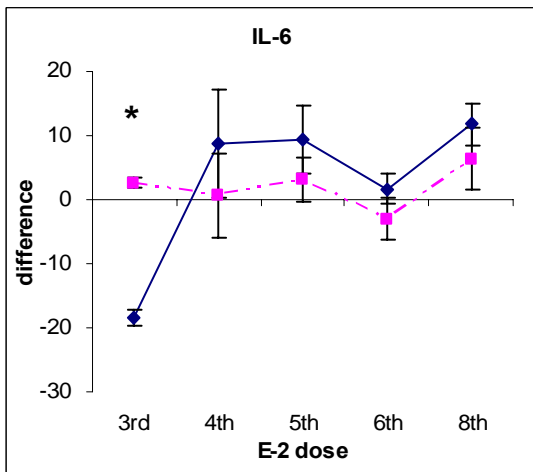
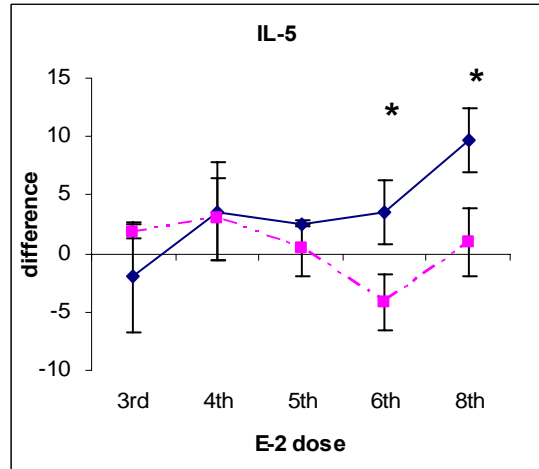
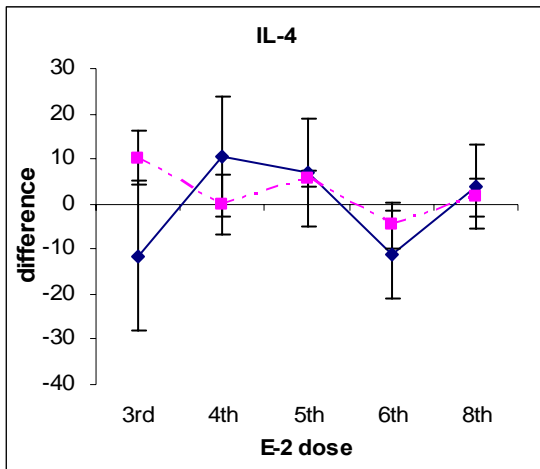
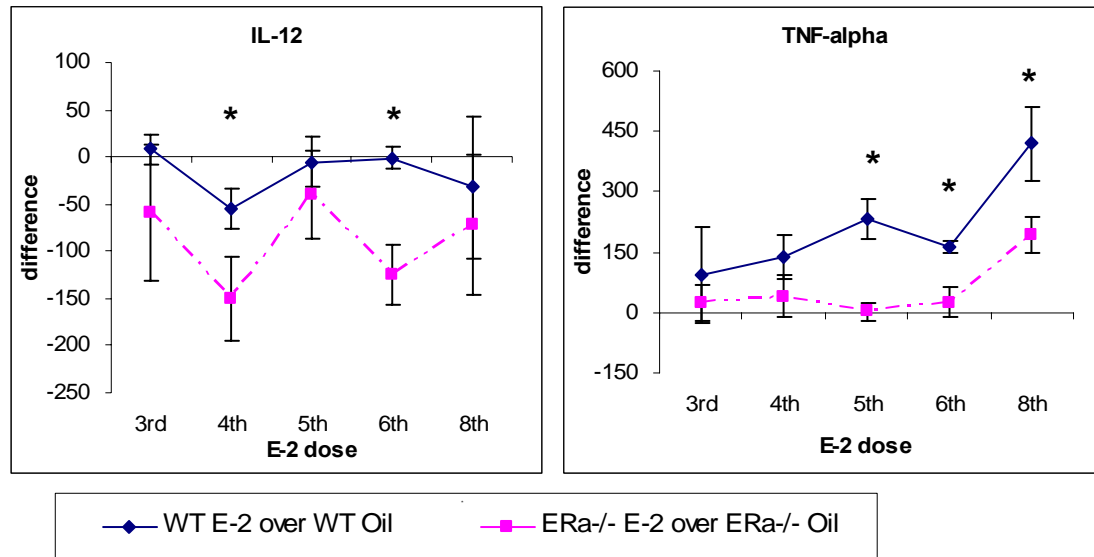


Figure 6.2 (Continued)



6.3.4 E-2 administration significantly up-regulates the production of pathogenic immunoglobulin (Ig) levels in WT mice but not ERα^{-/-} mice.

The effect of E-2 treatment on immunoglobulin production is presented in Figure 6.3. The data were expressed as the fold over Ig production for E-2 treated mice compared to controls. We found that E-2 treatment led to increased levels of total IgG after the 3rd injection and increased levels of total IgM after 6th injection in WT mice only, while no differences were seen in ERα^{-/-} mice. Similarly, both serum anti-ssDNA IgG/M and anti-dsDNA Ig/M were increased in E-2 treated WT mice, but not in E-2 treated ERα^{-/-} mice. Id^{LN}F₁+ IgG and M, which have been shown to be pathogenic and more closely correlated to the disease progress in both autoimmune SNF₁ and non-autoimmune DBF₁ mice [28, 54], were also found to be increased significantly (p<0.05) after E-2 treatment of WT mice but not ERα^{-/-} mice. Taken together, these results suggest an essential role for ERα in E-2 induced autoantibody production, since only E-2 treated WT mice produced significantly increased levels of

these antibodies, while in ER α ^{-/-} mice, the levels were similar between E-2 or oil treated controls.

6.3.5 Expression of ER on immune cells.

Results of our study examining the effect of ER α expression on E-2 induced lupus nephritis clearly suggested that ER α was required for E-2 induction of the lupus phenotype and the development of lupus nephritis even in a non-autoimmune mouse. In order to further investigate the role of ER expression on immune cells in E-2 induced modulation of immune responses, we examined ER expression in both DBF₁ and SNF₁ mice with or without E-2 treatment. Male mice of each cross were treated with E-2 or oil, and the immune cells were isolated two weeks later for phenotype analysis (Table 6.2). Both ER α and ER β were found to be expressed on T cells, B cells, dendritic cells (DC) and macrophages as previously reported [149, 196, 208]. ER α was expressed in DCs and macrophages at a higher density, while B cells had a relatively lower density of ER α expression. Most B cells and dendritic cells expressed ER α constitutively (about 90% cells were ER α positive), and its expression did not change after E-2 treatment in either mouse strain. In contrast, CD4⁺ and CD8⁺ T cells from DBF₁ and SNF₁ mice showed different patterns of expression. SNF₁ mice had a higher percentage of T cells that constitutively expressed ER α (~99%) compared to DBF₁ T cells (60~70%), but the ER α expression in DBF₁ T cells was increased to ~99% after E-2 treatment. ER α expression in macrophages was approximately same in oil treated DBF₁ and SNF₁ mice, but E-2 exposure up-regulated ER α expression by macrophages only in SNF₁ mice. ER β in both DBF₁ and SNF₁ showed similar patterns of expression. Most of the T cells, B cells and dendritic cells expressed ER β at low density (about 90% cells and MCF of 100); however, while 60~70% macrophages were ER β positive, its levels of expression were higher (MCF= 200) than for other

Figure 6.3 Effect of E-2 treatment on serum immunoglobulin (Ig) levels in ER α ^{-/-} and WT mice. ERKO and WT mice were treated with 1mg/kg E-2 or oil monthly beginning at 8 weeks of age. The mouse serum was collected every month two weeks after E-2 administration, and the serum Ig levels were measured by ELISA. The data are expressed as fold of Ig level in E-2 treated mice over oil control mouse in order to show the effect of E-2 exposure on the Ig levels in ER α ^{-/-} and WT mice. The results suggest that E-2 treatment of WT mice led to increased production of serum Igs, including atuo-antibodies, compared to E-2 treated ER α ^{-/-} mice. (*, p<0.05, compared WT mice with ER α ^{-/-} mice).

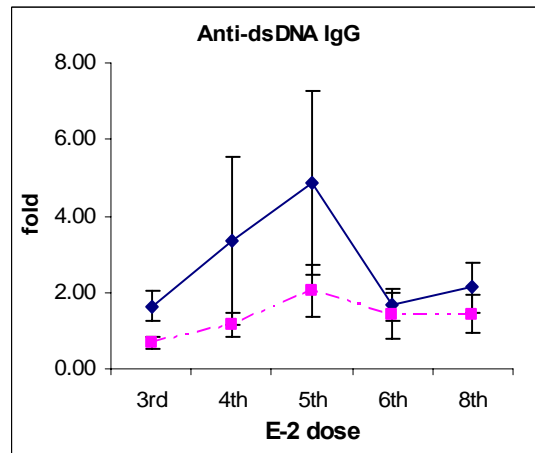
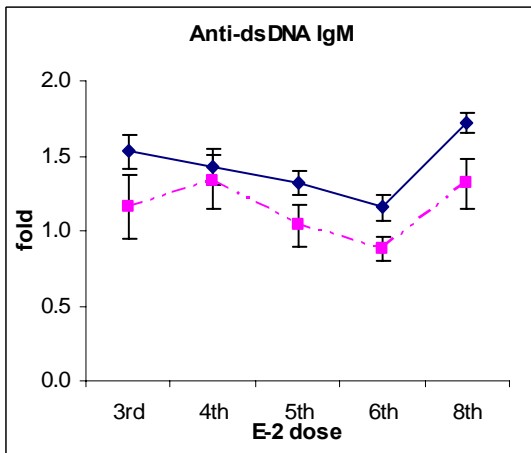
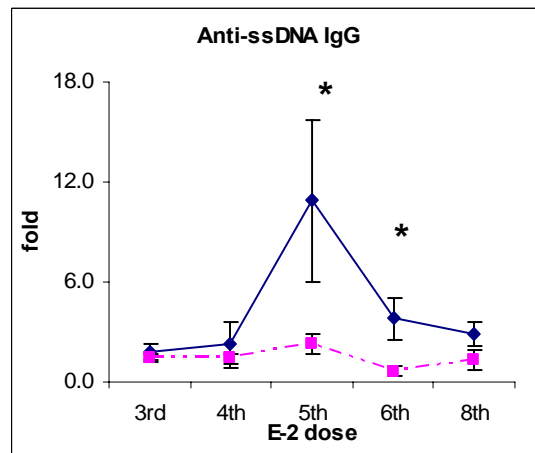
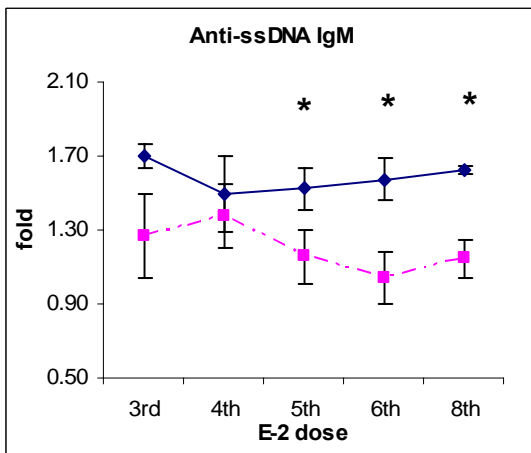
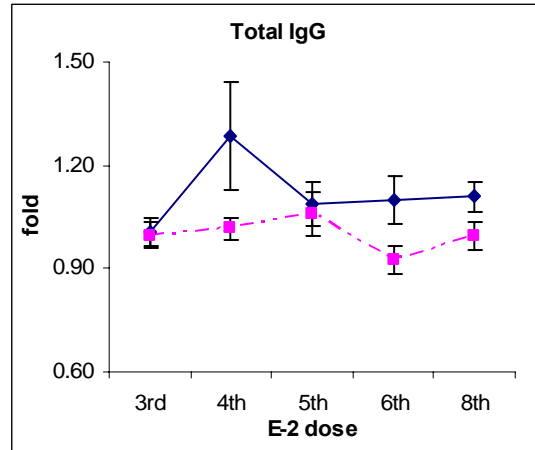
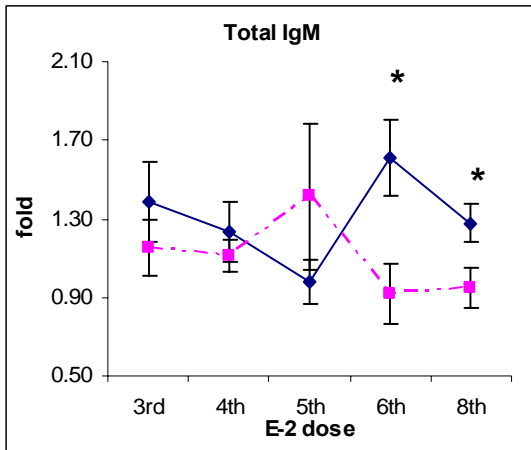
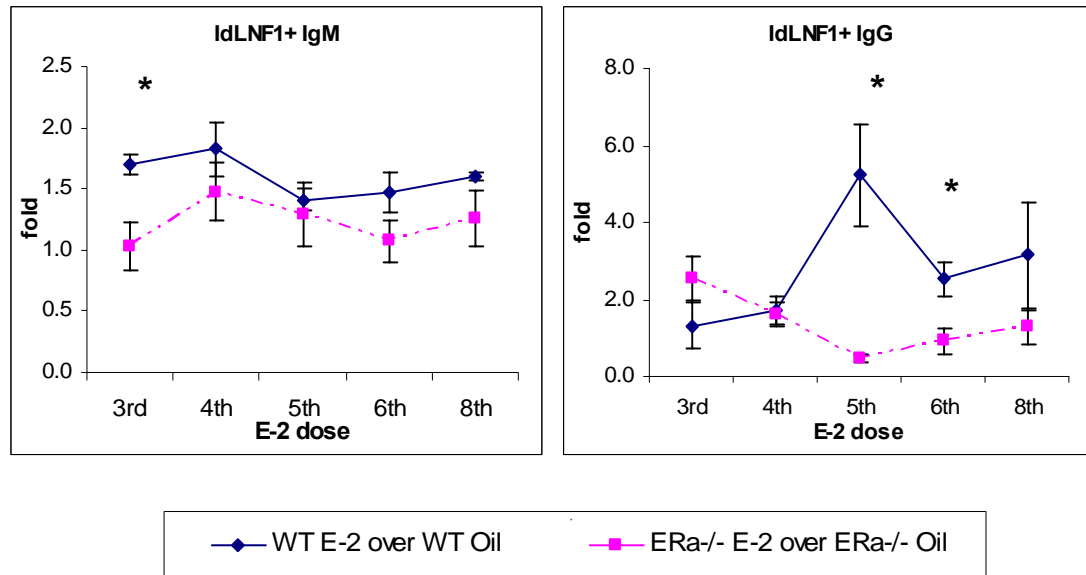


Figure 6.3 (Continued)



cells. Interestingly, in contrast to ER α expression, E-2 treatment did not alter the expression of ER β by cells from either DBF₁ or SNF₁ mice.

6.4 Discussion

As our previous studies have shown, repeated E-2 administration to both autoimmune (SNF₁) mice and non-autoimmune (DBF₁) mice induced the lupus phenotype with increased pathogenic Id^{LN}F₁⁺ Ig production and deposition in kidney, glomerulonephritis and decreased survival (Feng F. *et al*, in preparation). In the present study, we reproduced the E-2 effects in another non-autoimmune mouse, C57BL/6. Repeated E-2 treatment of C57BL/6 mice at 1mg/kg resulted in significantly decreased survival, with a median survival of approximately 75 weeks, similar to that found for E-2 treated DBF₁ mice (median survival of approximately 80 weeks). WT mice were also found to develop lupus glomerulonephritis after the 4th injection of E-2 as determined by histological examination, which was also found for

Table 6.2 Effects of E-2 exposure on ER expression determined on immune cells by flow cytometric analysis. Autoimmune male SNF₁ mice and non-autoimmune DBF₁ mice (n=3 for each group) were treated with E-2 (1mg/kg) or oil. Two weeks later, the mice were sacrificed, and T cells, B cells, macrophages, and dendritic cells were isolated and pooled. The cells were stained with the appropriate ER α and ER β antibodies, and analyzed by flow cytometry. (% , percentage of ER positive cells; MCF, mean channel fluorescence of ER positive cells)

		DBF ₁		SNF ₁	
		Oil	E2	Oil	E2
ERα	CD4+ T cell (%)	62.48	99.56	99.38	99.58
	MCF	2265.58	1158.84	1218.24	1238.88
	CD8+ T cell (%)	79.83	96.05	97.9	84.35
	MCF	2275.58	1390.38	1652.23	1227.41
	B Cell (%)	99.21	97.87	96.67	94.21
	MCF	1119.88	1536	1498.87	1445.41
	DC (%)	87.7	84.7	83.7	94.6
	MCF	3531.21	3871.45	5916.46	6136.28
	Macrophage (%)	62.3	64.9	43.1	78.7
	MCF	4529.92	3941.79	5327.98	5215.4
ERβ	CD4+ T cell (%)	98.3	99.48	98.43	98.92
	MCF	86.42	68.27	49.16	50.36
	CD8+ T cell (%)	99.39	99.16	97.41	98.04
	MCF	77.46	57.23	48.89	44.87
	B Cell (%)	99.52	99.3	98.54	99.36
	MCF	85.72	66.74	45.14	56.96
	DC (%)	90.9	93.8	84.7	85.6
	MCF	187.34	187.12	280.8	195.81
	Macrophage (%)	68.1	74.8	66.6	72.5
	MCF	213.58	195.7	231.2	220.1

E-2 treated DBF₁ mice. In contrast to E-2 treated WT mice, the median survival of E-2 treated ER α ^{-/-} mice was greater than 100 weeks, similar to oil control WT and ER α ^{-/-} mice, with no obvious kidney damage found by histological examination. These data strongly suggest the ER α was required at least in part for E-2 to induce the development of lupus nephritis in this murine model. This finding is consistent with studies in other models, including the SJL model for autoimmune encephalomyelitis, in which E-2 treatment suppresses experimental autoimmune encephalomyelitis (EAE) via ER α -dependent mechanism [175].

More evidence for the essential role of ER α in lupus phenotype induction by E-2 treatment was obtained from examination of serum cytokine and Ig levels. In WT mice, E-2 treatment induced more IL-5, IL-6 and IL-10 (T_H2 cytokines) as well as significantly higher levels of the pro-inflammatory cytokine TNF- α . Induction of these cytokines has been postulated as an important mechanism in E-2-induced modulation of T cell function and their role in lupus pathogenesis [3, 39, 49, 96, 99, 100, 194]. The serum IFN- γ level was also increased in WT mice after the 3rd injection of E-2 compared to oil controls; this cytokine is postulated to be important in the initiation of autoimmune disease but not in later stage of the disease [96]. More importantly, the E-2 induced changes on cytokine production in WT mice were not detectable in E-2 treated ER α ^{-/-} mice. Except for IL-12, all of the cytokine levels in E-2 treated ER α ^{-/-} mice were similar to those in oil-treated control ER α ^{-/-}, which confirmed the requirement of ER α for E-2 induced lupus-associated cytokine modulation in these mice. One mechanism whereby estrogen could act in the regulation of the cytokine profile may be through modulating the expression of T-bet in target cells [251], the transcription factor that directs the naïve T helper cell to differentiate along the T_H1 pathways. As shown in a recent study, estrogen suppressed T-bet expression in target cells by limiting the binding of transcription factors Stat1 and Stat5 to the T-bet

regulatory region; this in turn led to reduced secretion of T_H1 cytokines, especially IFN- γ [251]. However, how ER α may be specifically involved in this process is unknown.

The effect of ER α expression on E-2 induced modulation of serum Ig levels was also determined in the study. There was greater total IgG and total IgM in WT mice after E-2 administration. Similarly, anti-ssDNA IgG/M and anti-dsDNA IgG/M were all also increased in WT mice after E-2 treatment compared to controls. More importantly to the pathogenesis of lupus nephritis, Id^{LN}F₁⁺ IgG serum levels were elevated after E-2 administration to WT mice, after 3~4 injections. Our studies over many years have shown that dysregulation of the production of pathogenic Id^{LN}F₁⁺ antibodies is part of the pathogenic mechanism of SNF₁ nephritis [28, 54], and that anti-DNA antibodies are of less significance [27]. Therefore, based on these data, the increased Id^{LN}F₁⁺ IgG/M in E-2 treated WT mice could be responsible for the lupus nephritis development in these mice. Our data suggest that ER α is important to the production of these antibodies, since E-2 administration to ER α ^{-/-} did not increase Id^{LN}F₁⁺ Ig levels.

E-2 could act on many types of immune cells. Besides T cells, B cell development in the bone marrow may also be modified by E-2 exposure. Estrogen exposure has been shown to result in the ER-dependent up-regulation of several genes involved in B cell activation and survival, including Bcl-2, which could lead to the modulation of B cell populations, due to decreased apoptosis. Expanded autoreactive B cells then produce more autoantibodies [44, 102, 158]. APC's may be affected by E-2 as well [166].

Estrogen receptor expression on immune cells has been shown to be modulated after E-2 exposure. In one study, both ER α and ER β were found to be constitutively expressed in the thymus normally, and at increased levels after E-2 administration

[131]. Further, gender differences in ER expression were noted [152]. Administration of low doses of E-2 resulted in a striking increase in antigen-specific CD4⁺ T cell responses and the selective development of IFN- γ ⁺-producing cells in castrated female C57BL/6 mice, that was ER α -dependent instead of ER β -dependent [153]. In another study it was shown that estrogen exposure directly activated T cells, via cell membrane ER [154], suggesting the possibility that differences in the numbers and types of ER expressed on the immune cells of some individuals could explain the differences in their T cell responses to antigen. This hypothesis was further supported by the observation that calcineurin, CD40L expression and PP2B phosphatase activity were increased when SLE T cells, but not T cells from normal women, were cultured with E-2 [149, 155]. These findings led us to examine ER expression on different immune cells from both lupus-prone SNF₁ mice and non-autoimmune DBF₁ mice with or without E-2 treatment. We found that both ER α and ER β were expressed in CD4⁺ T cells, CD8⁺ T cells, B cells, DCs and macrophages. ER α was expressed in a higher density than ER β on immune cells (highest in macrophages and lowest in B cells). Moreover, there were more T cells (both CD4⁺ and CD8⁺) expressing ER α in SNF₁ mice compared to DBF₁, and the percentage of ER α ⁺ T cells in DBF₁ increased to a similar level as in SNF₁ after E-2 exposure. The expression of ER α on DCs and macrophages were similar in both DBF₁ and SNF₁ controls, but E-2 treatment led to the increased ER α expression in these two cell populations only in SNF₁ mice. These data combined with the observations that SNF₁ mice develop lupus nephritis spontaneously, but it is accelerated after E-2 treatment and that DBF₁ do not develop disease unless E-2 is administered suggest the possibility that differences in the expressions of ER, specifically ER α , on the immune cells between DBF₁ and SNF₁ may be responsible for the differences in susceptibility to develop lupus nephritis and to E-2 treatment. Further, since there was no difference for ER β expression on

immune cells between DBF₁ and SNF₁ either with or without E-2 treatment, it appears that ER β is not critical to the development of E-2 induced lupus nephritis.

This present study using ER α -deficient mice has identified the role of ER α in E-2 induced lupus nephritis. The absence of ER α in those mice sufficiently neutralized the negative effects of E-2 on the survivals and kidney damages seen in E-2 treated wild type mice. However, considering the expression of ER β on many immune cells a future study with ER β -deficient mice would be very advisable and necessary.

CHAPTER 7

Discussion

Autoimmune diseases, in general, are diseases in which the adaptive immune system initiates an injurious attack on normal tissues of self. More than 70 chronic disorders, which afflict ~5% of the US population, are classified as autoimmune diseases, and are either systemic (involving many body organs) or organ-specific. Sex differences were noted in the earliest descriptions of many autoimmune diseases, including systemic lupus erythematosus (SLE). SLE is a prototypic immune complex-mediated autoimmune disease with a striking sex difference and a female to male ratio of 9:1 [1, 2, 50, 51, 185-187]. The high female predilection of SLE, along with significantly increased disease incidence in females after puberty, the reversal of this phenomenon after menopause, and the fluctuation in disease severity throughout the menstrual cycle and pregnancy suggest the important role of sex hormones, especially female sex hormone, estrogen, in SLE pathogenesis [59-61]. However, the actual role of estrogen in the gender bias of autoimmune diseases is still controversial, and non-hormonal mechanisms, such as skewed X chromosome inactivation, exposure, behavior, etc, have also been suggested to contribute to loss of immune tolerance and therefore, increased incidence of autoimmune diseases in females (reviewed in [205]). A better understanding of the immunomodulatory effects of sex hormones will be required to reconcile some of the contradictions observed. In the current study, the impact of sex hormones (estradiol and testosterone) on the disease course of lupus was investigated by castration and in bone marrow chimeras, as well as after exogenous E-2 administration to both lupus-prone SNF₁ and non-autoimmune DBF₁ mice. Furthermore, the role of the thymus and estrogen receptor- α were also investigated in

order to understand the mechanisms involved in estrogen-induced pathogenesis of SLE.

Exogenous E-2 administration at 1mg/kg monthly to male SNF₁ mice led to an increase in serum E-2 level to 150~200pg/ml, which is the peak physiological level of E-2 in female mice [206]. This relatively high level persisted for about 7-10 days and returned to the low levels that are normal for male SNF₁ mice (<10pg/ml) by two weeks after treatment. We further found that this dose of E-2, administered monthly, led to significantly decreased survival of SNF₁ male mice compared to control mice treated with oil vehicle only. In fact, the survival curve for E-2 treated male SNF₁ mice was nearly identical to that of female mice that develop spontaneous disease. E-2 treated male SNF₁ mice also developed more severe lupus nephritis by the 4th injection of E-2 at 24 weeks of age compared to controls. Further, E-2 treated male mice showed significant changes in both T and B lymphocytes, which, as was expected, mirrored those found in SNF₁ females with disease. T lymphocytes from E-2 treated mice proliferated more than controls in response to pathogenic aa62-73 peptide *in vitro*, although the response to Con A was suppressed. B cell functions were also affected by E-2 treatment, and included increased numbers of auto-reactive Id^{LN}F₁⁺ antibody producing B cells. Further, Ig⁺ cells were also more effective as APC presenting aa62-73. Phenotypic analysis showed an up-regulation of the pathogenic Id^{LN}F₁-reactive T cell population after E-2 exposure, with increased percentages of Id^{LN}F₁+CD3⁺ splenocytes, Id^{LN}F₁+CD4⁺ T cells, Id^{LN}F₁-reactive memory T cells and an increased Id^{LN}F₁+CD4⁺ to Id^{LN}F₁+CD8⁺ ratio, as seen in female SNF₁ mice with nephritis [28, 54]. Serum levels of immunoglobulins and cytokines also confirmed the immunoregulatory effects of E-2, with increased pathogenic antibody levels and a cytokine profile skewed to favor the development of lupus in male mice after E-2 exposure, with an increase in IL-5, IL6, TNF- α and IFN- γ

and a decrease in IL-12. These observations suggested that E-2 could play a role in accelerated lupus in male SNF₁ mice.

As expected, ovariectomy of female SNF₁ mice effectively reduced the serum E-2 concentration to a level nearly identical to that of the male mouse. Body weight was increased in ovariectomized mice compared to intact control mice, and the median survival of ovariectomized mice was also increased, from 44.1 weeks to 62.1 weeks. This was accompanied with a notable reduction in glomerular damage in kidneys compared to sham-operated control mice. Also as expected, castration of male SNF₁ mice led to decreased serum T-1 levels. However, in contrast to ovariectomy, castration had no effect on survival compared to intact mice, and histological examination showed comparable immunopathology between castrated and intact control mice. However, the administration of E-2 to male SNF₁ mice, either castrated or intact, led to significantly reduced survival (55 weeks vs. 70 weeks) and more severe glomerulonephritis. In general, a reduction in E-2 levels in female SNF₁ mice appeared to be protective, and administration of exogenous E-2 to male mice induced lupus, while the reduction in T-1 levels in male mice had little effect on SNF₁ lupus disease.

The effects of sex hormones on survival and renal histopathology were accompanied by changes in the immune responses of castrated mice. In the female mouse, the reduction in serum E-2 level by ovariectomy led to decreased proliferative responses to pathogenic peptide aa62-73 and a shift in the *in vitro* cytokine profile, with more IL-12 and IL-2 produced and less IL-5, IL-6, and TNF- α . This was in agreement with the observations that, in general, SLE shows a T_H2 bias, with skewing to T_H1 beneficial or protective [39, 49]. We also found that ovariectomized mice produced more anti-Id^{LN}F₁⁺ antibody (anti-aa62-73) producing cells and fewer Id^{LN}F₁⁺ antibody-producing cells. Further, the splenocytes from intact female mice produced

more total IgG, anti-dsDNA and anti-ssDNA IgG, and Id^{LN}F₁⁺ IgG. Of a critical importance to disease pathogenesis, pathogenic T cell populations, CD3⁺Id^{LN}F₁⁺ and CD4⁺CD3⁺Id^{LN}F₁⁺ [28, 54], were decreased significantly by ovariectomy. Conversely, in male mice, the administration of E-2 had the opposite effect and exacerbated disease. E-2 treatment of both castrated and sham-castrated male mice led to increased proliferation to pathogenic peptide aa62-73. We also noted shifted cytokine production to T_H2 cytokines and increased numbers of Id^{LN}F₁⁺ antibody-producing cells in E-2 treated mice compared to oil-treated controls. However, castration alone did not lead to modulation of lupus-related changes in either the T or B cell compartments, which is consistent with its lack of effect on survival and renal histology. The changes we observed in the serum levels of autoantibodies and cytokines after E-2 treatment of male SNF₁ mice also confirmed that E-2 administration accelerated and exacerbated SNF₁ disease, with natural levels of androgens having little or no effect on disease pathogenesis.

Additional data supporting a role for E-2 in the pathogenesis of SNF₁ lupus nephritis were obtained from bone marrow transplantation experiments. In the first group of experiments, autoimmune SNF₁ mice of both genders were reconstituted with bone marrow from mice of either the same or the opposite gender. We found that regardless of the gender of the donor marrow, recipient mice tended to have survivals and a degree of glomerulonephritis similar to that of intact mice of the host gender. This suggested that the immune system of either male or female mice had the capacity to develop SNF₁ lupus nephritis. In contrast, host mice of different genders showed a striking gender bias in the development of lupus when reconstituted with either male or female bone marrow. Female mice reconstituted with either male or female marrow developed accelerated nephritis and died earlier than male mice reconstituted with marrow of either gender, which suggested that the female hosts provided a critical

factor or factors influencing the development of disease. We hypothesized that a possible non-genetic factor might be the sex hormone estrogen. Data in support of its importance in disease was obtained in bone marrow transplantation experiments using male recipients reconstituted with female or male donor marrow with or without E-2 treatment. E-2 treatment was required to reproduce the similar disease course in male mice reconstituted with female bone marrow. Survival of these mice was similar (with mean survival of about 30 weeks) to that of female mice reconstituted with marrow of either gender, as was the degree of kidney damage. In summary, these studies suggested that E-2, in addition to intrinsic genetic factors, was an important factor in the development and pathogenesis of lupus nephritis.

Furthermore, we found that non-autoimmune female DBF₁ mice reconstituted with bone marrow from female SNF₁ mice developed severe lupus glomerulonephritis and had a life span equal to that of the autoimmune SNF₁ female mice (mean survival of 30 weeks), while the control female DBF₁ mice reconstituted with female DBF₁ bone marrow had a much longer life span (mean survival >60 weeks) and minimum kidney damage. This result strongly suggested that the transplanted immune system from SNF₁ mouse was sufficient to induce the lupus disease even in a non-autoimmune host. In comparison, male DBF₁ mice had a normal lifespan (mean survival >70 weeks) when reconstituted with female SNF₁ bone marrow; however, E-2 treatment of these mice led to disease development with similarly shorten survivals (mean survival of 27 weeks) and kidney damage as we had seen previously in E-2 treated male SNF₁ chimera reconstituted with female SNF₁ bone marrow. Again, these results emphasized the important role of E-2 in lupus development and pathogenesis.

Interestingly, administration of exogenous E-2 to MHC-matched non-autoimmune mice DBF₁, (DBAxBALB/c)F1 (H-2^{d/q}), could induce autoimmunity and autoimmune disease with pathological changes similar to those associated with disease

in SNF₁ mice. Male DBF₁ were injected subcutaneously with either 1mg/kg or 5 mg/kg E-2 in oil monthly starting from 7 weeks old. E-2 treated mice died significantly earlier than the control mice. The effect of E-2 on survival was striking, so that all of the DBF₁ mice treated with 5mg/kg E-2 did not survive beyond 12-15 months, exactly like that of female SNF₁ mice, while the survival of mice injected with 1mg/kg E-2 was similar to that of unmanipulated male SNF₁ mice. This result clearly showed that estrogen exposure led to disease development in the non-autoimmune mice. Furthermore, nephritis as determined by renal histology correlated with our findings of decreased survival. At 25 weeks, E-2 treated mice had decreased numbers of normal glomeruli, with a concomitant increase in the percentage of damaged glomeruli compared to oil-treated control mice; however, the kidney damage induced by E-2 in these nonautoimmune mice was not as severe as that found in female SNF₁ mice or in male SNF₁ mice after E-2 treatment. These data support that at least one outcome of E-2 exposure is the induction of autoimmune nephritis regardless of genetic susceptibility to develop autoimmune disease.

Other results also supported the above conclusion. Flow cytometric analysis showed the same phenotypic changes in DBF₁ mice after E-2 treatment as those detected in female SNF₁ mice as disease developed. For example, by 25 weeks, Id^{LN}F₁-reactive T cells were expanded and activated, as was seen in female SNF₁ and E-2 treated male SNF₁ mice [28, 54]. E-2 treatment also led to more Id^{LN}F₁⁺ B cells, as well as more Id^{LN}F₁-reactive memory T cells. There was also an increase in the ratio of Id^{LN}F₁-reactive CD4⁺ cells to Id^{LN}F₁-reactive CD8⁺ cells in E-2 treated mice. This suggested a decrease in regulatory T cells, and/or an increase in the autoreactive T cells which provided help to the autoreactive B cells. More evidence in support of the induction of autoimmune disease by E-2 came from ELISPOT assays, which showed an increased number of Id^{LN}F₁⁺ antibody-producing B cells as well as

regulatory anti-Id^{LNF₁⁺} antibody-producing B cells in E-2 treated DBF₁ mice. All of these results supported a role for E-2 in the induction of autoimmunity in non-autoimmune DBF₁ mice.

Taken together, the results of our studies suggest that E-2 triggers as well as modulates lupus nephritis irregardless of genetic factors. These data strongly support a critical role for estrogen in mediating the sex difference in lupus disease incidence, as opposed to other postulated mechanisms such as X inactivation, imprinting, differential exposure, etc [205]. Exposure to pharmacological estrogen alone could induce autoimmunity and autoimmune disease in both lupus-prone and non-lupus-prone mice by activating pathogenic auto-reactive T cells and B cells, although in non-lupus prone mice the E-2-induced disease was of milder severity. Moreover, our studies in DBF₁ chimeras emphasized that the difference in development of nephritis was not due to differences in the target organs of lupus nephritis; disease was equivalent in both host strains. These findings are of great significance to persons exposed to estrogenic compounds for extended periods of time, such as for hormone replacement therapy, contraceptive drugs, and certain ovarian tumors, because our data shows that elevations in estrogen levels can be a potential trigger of perturbed immune function and autoimmune disease. Another source of concern is the recent identification of hormone-disrupting effects of a large number of pesticides and insecticides currently on the market, as well as phytoestrogens.

One consequence of E-2 treatment is thymic atrophy, which led us to consider the hypothesis that the effects of E-2 on the pathogenesis of SLE was through its effect on T lymphocyte development in thymus. In the current study, we found that pre-pubertal thymectomy prolonged the survival of both male and female SNF₁ mice significantly, and led to decreased kidney damage with less severe glomerular damage by histological examination. In addition, the phenotypic analysis of thymectomized

mice showed a significant decrease in the pathogenic Id^{LN}F₁-reactive T cell populations in both male and female mice. And in female mice, Id^{LN}F₁-reactive memory T cells (CD44^{hi}CD45RA^{low}) were found to be decreased after thymectomy compared to intact mice. As expected, we also found a decrease in the ratio of (CD4⁺Id^{LN}F₁⁺)/(CD8⁺Id^{LN}F₁⁺) T cells in thymectomized mice, which we have previously found to be a hallmark for the onset and progression of lupus nephritis in SNF₁ mice [54]. Furthermore, there was no apparent increase in the regulatory CD4⁺CD25⁺ T cell population in thymectomized mice. This finding is consistent with studies from other laboratories, that found the suppressive effect to be associated with a CD8⁺ T cell population [195]; in another study the CD4⁺CD25⁺ T cells failed to suppress the development of lupus nephritis in NZM2328 mice [122]. Taken together, these findings suggested that the thymus was very important for generating the pathogenic T cells, rather than regulatory cells, in SNF₁ nephritis. We also found that E-2 treatment could still induce/accelerate glomerulonephritis in thymectomized male SNF₁ mice, with significantly shortened survival and increased glomerular damage as well as the expansion of pathogenic Id^{LN}F₁-reactive T cell population, which clearly suggested that the E-2 induced effect on the pathogenesis of SLE was not thymus-dependent.

We reproduced the E-2 effects in another non-autoimmune mouse, C57BL/6 (ER^{+/+}, WT). Monthly treatment of C57BL/6 mice with 1mg/kg E-2 resulted in significantly decreased survival, with a median survival of approximately 75 weeks, similar to that found for E-2-treated DBF₁ mice (median survival of approximately 80 weeks). WT mice were also found to develop lupus glomerulonephritis after the 4th injection of E-2 as determined by histological examination, which was also found for E-2-treated DBF₁ mice. In contrast to E-2-treated WT mice, the median survival of E-2-treated ER α ^{-/-} mice was greater than 100 weeks, similar to oil control WT and ER α ^{-/-}

mice, with no obvious kidney damage found by histological examination. These data strongly suggested that ER α was required at least in part for E-2 to induce the development of lupus nephritis in this murine model. This finding was consistent with studies in other models, including the SJL model for autoimmune encephalomyelitis, in which E-2 treatment suppressed experimental autoimmune encephalomyelitis (EAE) via an ER α -dependent mechanism [175].

More evidence for the essential role of ER α in lupus phenotype induction by E-2 treatment was obtained from examination of serum cytokine and Ig levels. In WT mice, E-2 treatment induced more IL-5, IL-6 and IL-10 (T_H2 cytokines) as well as significantly higher levels of the pro-inflammatory cytokine TNF- α . Induction of these cytokines has been postulated to be an important mechanism in E-2-induced modulation of T cell function in lupus pathogenesis [3, 39, 49, 96, 99, 100, 194]. The serum IFN- γ level was also increased in WT mice after the 3rd injection of E-2 compared to oil controls; this cytokine is postulated to be important in the initiation of autoimmune disease but not in later stage of the disease [96]. More importantly, the E-2-induced changes in cytokine production in WT mice were not detectable in E-2-treated ER α ^{-/-} mice. Except for IL-12, all of the cytokine levels in E-2-treated ER α ^{-/-} mice were similar to those in oil-treated control ER α ^{-/-}, which confirmed the requirement of ER α for E-2 induced lupus-associated cytokine modulation in these mice. The effect of ER α expression on E-2-induced modulation of serum Ig levels was also determined. There were greater levels total IgG and total IgM in WT mice after E-2 administration. Similarly, anti-ssDNA IgG/M and anti-dsDNA IgG/M were all also increased in WT mice after E-2 treatment compared to controls. More relevant to the pathogenesis of lupus nephritis, Id^{LN}F₁⁺ IgG serum levels were elevated after E-2 administration to WT mice, after 3~4 injections. Our data suggest that ER α is

important to the production of these antibodies, since E-2 administration to ER α ^{-/-} mice did not lead to increased Id^{LNF1+} Ig levels.

E-2 could act on many types of immune cells. Besides T cells, B cell development in the bone marrow may also be modified by E-2 exposure. Estrogen exposure has been shown to result in the ER-dependent up-regulation of several genes involved in B cell activation and survival, including Bcl-2, which could lead to the modulation of B cell populations, due to decreased apoptosis. Expanded autoreactive B cells, would lead to increased production of more autoantibodies [44, 102, 158]. APC's may be affected by E-2 as well, with increased antigen presentation [166, 226]. Our studies indicate that E-2 treatment led to increased presentation of aa62-73 peptide by B cells (Ig+ cell) to pathogenic T cells and increased proliferation.

We further examined ER expression on different immune cells from both lupus-prone SNF₁ mice and non-autoimmune DBF₁ mice with or without E-2 treatment. Both ER α and ER β were expressed in CD4⁺ T cells, CD8⁺ T cells, B cells, DCs and macrophages. ER α was expressed in a higher density than ER β on immune cells (highest in macrophages and lowest in B cells). Moreover, there were more T cells (both CD4⁺ and CD8⁺) expressing ER α in SNF₁ mice compared to DBF₁, and the percentage of ER α ⁺ T cells in DBF₁ increased to a similar level as in SNF₁ after E-2 exposure. The expression of ER α on DCs and macrophages were similar in both DBF₁ and SNF₁ controls, but E-2 treatment led to the increased ER α expression in these two cell populations only in SNF₁ mice. These data combined with the observations that female but not male SNF₁ mice develop early lupus nephritis spontaneously, but that disease is accelerated in male SNF₁ mice after E-2 treatment and moreover, that DBF₁ do not develop disease unless E-2 is administered, suggest the possibility that differences in the expression of ER, specifically ER α , on the immune cells of DBF₁ and SNF₁ may be responsible in part for the differences in their susceptibility to

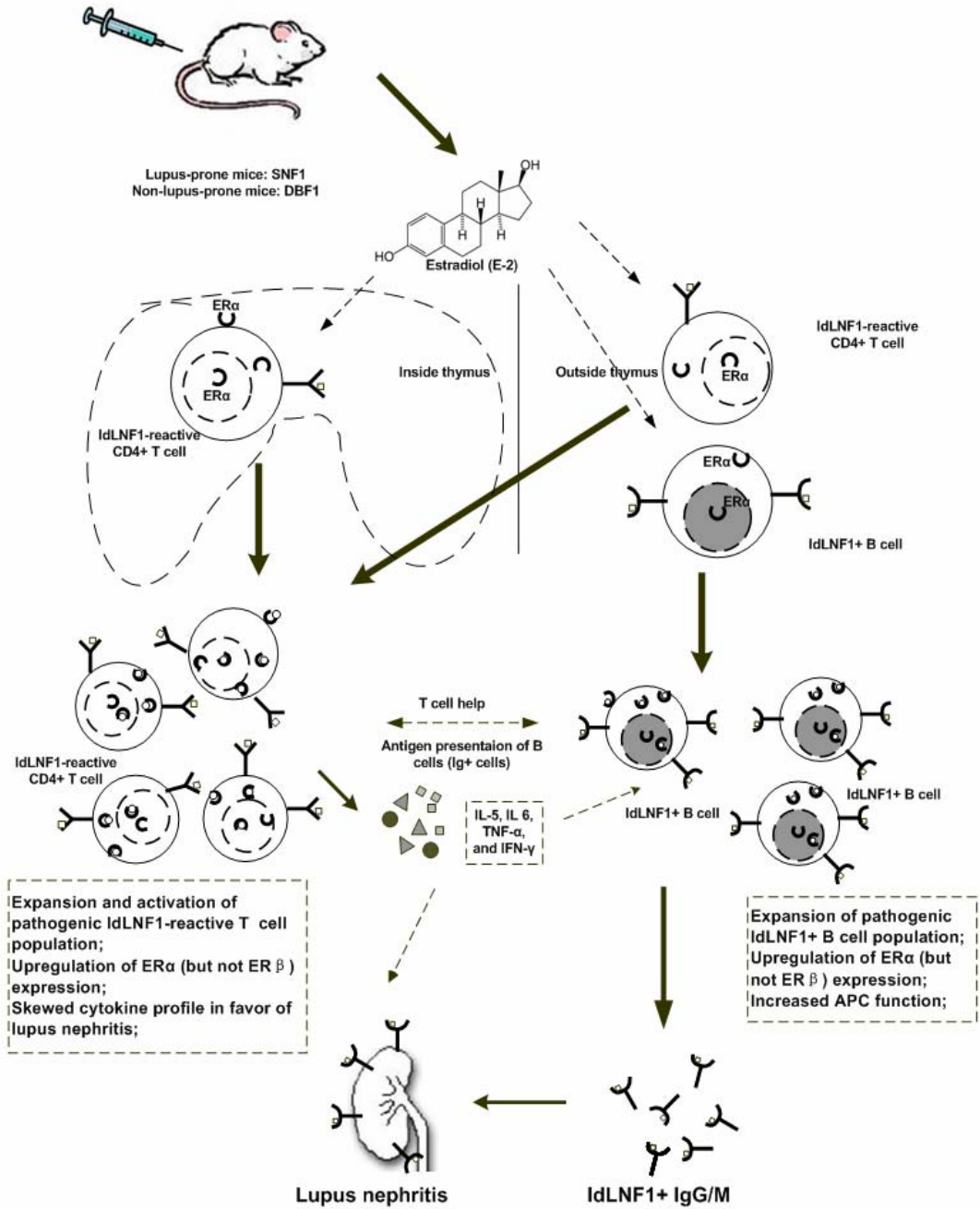
develop lupus nephritis. Further, since there was no difference in ER β expression on immune cells of DBF₁ and SNF₁ either with or without E-2 treatment, it appears that ER β does not play a direct role in the development of E-2 induced lupus nephritis.

The results reported in this study lead us to propose a model for E-2 induced pathogenesis of lupus nephritis in the SNF₁ and DBF₁ mice (in Figure 7.1). In this model, exogenous E-2 treatment of both lupus-prone and non-lupus-prone mice leads to expansion and activation of pathogenic Id^{LN}F₁-reactive CD4⁺ T cell populations. E-2 also acts on B cells, which results in expansion of Id^{LN}F₁+ B cell population and increased antigen presentation of pathogenic peptide by B cells (Ig⁺ cells). E-2 treatment also leads to a skewed cytokine profile in favor of development of lupus, including increased IL-5, IL-6, TNF- α and IFN- γ and decreased IL-12 and IL-2. The expansion of pathogenic B cells, with help from pathogenic Id^{LN}F₁-reactive T cells as well as skewed cytokines production, leads to increased levels of Id^{LN}F₁+ autoantibodies, which are contained in immune-complexes deposited in the kidney. Based on our results, we believe that a mechanism for the effect of E-2 in this process is via direct interaction with ER α on immune cells, resulting in direct expansion/activation of pathogenic T cell populations. We found that ER α is expressed on both CD4⁺ and CD8⁺ T cells, and further, that modulation of the expression of ER α on T cells by E-2 exposure was different in lupus-prone and non-lupus-prone mice. This suggests that differences in ER α expression may be responsible for the different responses to E-2 in those mice. B cells, which also express ER α , likely were also modulated directly by E-2, leading to the activation and expansion of Id^{LN}F₁ antibody-producing B cells. Another consequence of this expansion is increased antigen presentation of idiotypic peptide by B cells. Furthermore, the effect of E-2 on T cells occurs both in the thymus and in the periphery, which suggests that E-2 regulates autoreactive T cells at multiple stages,

including immature T cells during the development in the thymus and mature T cells in the periphery.

In future studies to further determine whether estrogen-induced modulation of the Id^{LN}F₁ response is ER-dependent, we would test whether the ER-specific antagonist, ICI 182 780 and the ER specific agonists, propyl pyrazole triol (PPT) (ER α), or diarylpropionitrile (DPN) (ER β), modulate Id^{LN}F₁ responses. Since ICI 182 780 is a pure antagonist, we expect that the E-2 induced immune responses to Id^{LN}F₁ should be significantly modulated by ICI 172 780 treatment, most likely decreased if E-2 induction of disease is ER-dependent. The use of the ER agonists should permit us to define whether one or both ER isoforms are involved in regulating E-2 induced Id^{LN}F₁ responses. Based on our work and that of others, we conclude that ER α plays a critical role in the pathogenesis of lupus nephritis. Therefore, we would expect to see an induction of the immune response to Id^{LN}F₁ in mice treated with ER α agonist, PPT. However, since ER β is also found on T and B cells, therefore studies will be required to identify any role for ER β in the pathogenesis of lupus. This could be accomplished in studies examining the effects of estrogen treatment in ER β -deficient mice, or whether treatment with the ER β agonist, DPN, modulates production of Id^{LN}F₁+ IgG.

Figure 7.1 Model for E-2 induced pathogenesis of SLE. Exogenous E-2 treatment leads to lupus nephritis in both lupus-prone (SNF₁ mice) and non-lupus-prone mice (DBF₁ mice). After binding to ER α on T and B cells, E-2 leads to upregulation of ER α expression on T cells, increased antigen-presentation of idiotypic peptide by B cells, and expansion/activation of pathogenic Id^{LN}F₁-reactive T cells and Id^{LN}F₁+ B cells. With increased cognate help as well as skewed cytokine production (IL-5, IL-6, TNF- α and IFN- γ) by expanded Id^{LN}F₁-reactive T cells, Id^{LN}F₁+ B cells produce higher levels of pathogenic Id^{LN}F₁+ autoantibodies. These autoantibodies are contained in pathogenic immune complexes which are deposited in the kidney and initiate the immunopathology of lupus nephritis. The effect of E-2 on T cells can occur both during T cell development as well on mature cells in the periphery.



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