Role of Estrogen Receptor Expression in Estrogen (E-2)-Induced Modulation of Th1 and Th2 Cytokine Responses

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Role of Estrogen Receptor Expression in Estrogen (E-2)-Induced Modulation of Th1 and Th2 Cytokine Responses

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Candidate for B.S. Degree and Renée Crown University Honors

May 2010

Honors Capstone Project in Biology

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Abstract

Previous studies have demonstrated that repeated administration of 17\(\beta\)-estradiol (E-2) at a dose of 1mg/kg to both (SWR x NZB)F\(_1\) (SNF\(_1\)) mice and (DBA x BALB/c)F\(_1\)(DBF\(_1\)) mice resulted in significantly decreased survival and increased appearance of lupus nephritis pathology. However, the ways in which estrogen modulates immune responses has yet to be fully understood, specifically in regard to modulations in T\(_{H1}\) and T\(_{H2}\) cytokine profile. Various studies have reported the presence of two estrogen receptors (ER), \(\alpha\) and \(\beta\), which have been found to be constitutively expressed in many immune cells. In the present project, ER chimeric alpha knockout (\(\alpha\)ERKO), beta knockout (\(\beta\)ERKO) and double knockout (\(\alpha\beta\)ERKO) as well as wild type(WT) mice were injected monthly with \(\beta\)-estradiol 17-valerate (E-2) at 1mg/kg. Control mice of each type received oil vehicle only. Results showed that T\(_{H1}\) cytokine production by cells from WT mice was suppressed by E-2 administration, although IFN-\(\gamma\) production was increased in cells from \(\beta\)ERKO mice treated with estrogen compared to those treated with oil vehicle. T\(_{H2}\) cytokine production was increased in cells from WT mice treated with estrogen compared to controls. Increases in the production of anti-double stranded DNA antibodies were also observed in cells from \(\alpha\)ERKO mice. These observations suggest a role for differences in ER-\(\alpha\) and ER-\(\beta\) expression in E-2 induced modulation of immune responses, including autoimmune diseases like Systemic Lupus Erythematosus (SLE).
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INTRODUCTION

According to one study, it is estimated that approximately 5 percent of populations in Western countries is affected by various autoimmune diseases (1). The immune system is essential in the recognition of and distinction between self and non-self entities. A typical immune system only generates a humoral and/or cellular response to non-self such as pathogens, bacteria, antigens etc. However, in some cases the immune system may recognize an individual’s own cells and proteins as foreign, causing an autoimmune response. Contrary to what might have been thought initially, autoimmunity is a normal process that is important in clearing the body of certain pathological self cells (such as the prevention of tumor metastasis) and cellular debris.

According to Davidson and Diamond (2001), autoimmune disease is defined as a clinical syndrome caused by the activation of T or B cells, or both, in the absence of an ongoing infection or other discernible cause. In other words, autoimmune disease is caused by the failure of the proper functioning of the body’s defense mechanisms, or a lack of tolerance of one’s own cells. There exist two categories of autoimmune diseases. Organ-specific autoimmune diseases, such as type 1 diabetes mellitus, are localized in specific tissues in the body. Systemic diseases, such as systemic lupus erythematosus (SLE), have a widespread effect on several tissue systems in the organism. In general, these diseases are caused by, either changes in selection and regulation of T cells or B cells, or aberrant reactions to particular self or foreign antibodies (1).
While there are many factors that can cause autoimmune disease, genetic susceptibility is suggested to play an important role in disease manifestation. This has been supported by evidence of familial clustering and higher rates of concordance for the diseases in monozygotic twins than in dizygotic twins. Certain autoimmune diseases are thought to be caused, in major part, by mutation in a single gene. Such is the case of autoimmune lymphoproliferative syndrome, a disease with an autosomal dominant heredity pattern resulting from defects in the Fas protein (1). Fas protein is essential in mediating apoptotic pathways in the body and serves to down-regulate immune responses (1). The defect in the gene for the Fas protein results in the organism’s inability to successfully perform apoptosis, and therefore delete, activated immune cells that are reactive with self antigens.

It is important to note, however, that not all individuals who possess this single gene mutation will develop the disorder. There are many other factors that interact to enhance one’s susceptibility to autoimmunity. In fact it has been revealed that susceptibility to most autoimmune diseases results from the stochastic or simultaneous affects of mutations in many genes. Thus there are many susceptibility genes that contribute to the onset of disease in patients. For example, there is evidence that the genes for the class I and class II HLA major histocompatibility complex molecules, which are responsible for antigen presentation by antigen presenting cells (APCs), in association with the tumor necrosis factor α gene (TNF-α), are involved in most autoimmune diseases.
Further, many autoimmune diseases share common susceptibility genes, predisposing individuals to a combination of diseases.

Although certain individuals possess genes that confer susceptibility to disease, in many the onset of autoimmunity may be triggered by environmental factors. Two categories of such factors can be distinguished: infectious and non-infectious agents. In the case of multiple sclerosis for instance, the manifestation of the disease is primarily due to both T cell auto-reactivity to a peptide from the auto-antigen myelin basic protein, and T cells with reactivity to peptides from the Epstein-Barr virus, influenza virus type A, and human papillomavirus. A potential explanation is that the onset of disease is initiated by a normal immune response to a viral or bacterial infection, which is then sustained, even after elimination of the threat, by T cell reactivity to autoantigens that are homologous or “mimic” the foreign antigen.

Various studies have noted a significant sex difference in immune responses to antigens. It appears that females in many species are seen to have heightened immune responses and are more susceptible to autoimmunity (2, 3). Particularly, in Systemic Lupus Erythematosus (SLE), we see a 9:1 ratio in predominance of the disease in women as compared to men (6-7). There is also evidence of the correlation between steroid hormone levels and the changes in clinical manifestation of SLE, which is additionally enhanced during pregnancy and the postpartum period (8-10). Male and female patients with SLE often have abnormally high levels of estrogen (hyperestrogenic) and/or abnormally low levels of androgen (testosterone) hypoandrogenic (11-13). This correlation is also
reinforced by the higher incidence of the disease in patients with Klinefelter’s syndrome (14, 15), which is 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 of estrogen in the gender-specific modulation of immune responses involved in autoimmunity.

Research done in the Gavalchin Lab using the (SWR x NZB) F1 (SNF₁) mouse model for the human autoimmune disease Systemic Lupus Erythematosus (SLE) has provided a more direct evidence of the correlation between estrogen levels and SLE. Specifically, repeated administration of 17β-estradiol (E-2) at a dose of 1mg/kg to male (SWR x NZB)F₁ (SNF₁) mice, which do not develop lupus unlike SNF₁ female 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. By the 3rd dose (about 20 weeks of age), there was a significant development of excess serum protein in the urine (proteinuria), with an additional increase in autoantibodies expressing a pathogenic biomarker for lupus, Id⁵⁺, in the serum of E-2 treated male SNF₁ mice. Id⁵⁺-reactive T cells with a memory phenotype (CD44⁺CD45⁻) had also increased significantly in E2-treated mice and were found in the kidneys. Splenocytes from E-2 treated mice produced significantly more Id⁵⁺⁺ (a subset of Id⁵⁺⁺) IgG&M in vitro than splenocytes from control mice when incubated with control T cells, which is evidence for the pathogenic role of the Id⁺⁺ reactive T cells. Lastly, at 25 weeks of age, 80% of the estrogen-treated mice had glomerular deposits of Id⁺⁺ immunoglobulins (2). These
results point to the interaction of estrogen with immune cells. However, the ways in which estrogen modulates immune responses are still not yet well understood. Key to this knowledge would be an understanding of how estrogen interacts with its receptors. Two forms of estrogen receptors (ER), ERα and ERβ, have been identified. Each of these receptor subtypes or isoforms have been found to be normally expressed in immune cells (16-21).

Thus, the purpose of this research project was to further investigate the influence of estrogen on autoimmunity, specifically in SLE. It has been suggested that estrogen may act on helper T lymphocytes (T\textsubscript{H}), which are an essential part of the normal immune response to antigens. Two types of T\textsubscript{H} cells are known: T\textsubscript{H}1 and T\textsubscript{H}2 cells, which are distinguished by the specific cytokines they produce. T\textsubscript{H}1-type cytokines lead to pro-inflammatory responses in the host and while they participate in the initiation of autoimmune responses, they are protective later in disease. T\textsubscript{H}2-type cytokines are important in immunoglobulin production and predominate in later disease (22). Experiments on male estrogen receptor (ER) knockout mice that were administered estrogen (E-2) showed that the shifts from T\textsubscript{H}2 to T\textsubscript{H}1 responses were protective while a T\textsubscript{H}2 response exacerbated autoimmune disease (16-21). These results suggested that estrogen has a differential effect in promoting T\textsubscript{H}1 and T\textsubscript{H}2 responses, as well as raise the question of the importance of the ER in inducing these responses since CD4+ T cells have been show to express ER. Consequently, the hypothesis that was tested in this project was that exposure to estrogen will lead to modulation of immune cells via interaction with the specific ERs that they express. To do this, we
exposed male ERα, ERβ and ERαβ knockout chimeric mice to chronic doses of estrogen with the aims of identifying the consequences of estrogen (E-2) exposure in the numbers and functions of immune cells, in order to determine whether these modulations resulted from the interaction of E-2 and ER expression.

MATERIALS AND METHODS

Derivation of ER deficient chimeric mice

Thirty C57 Black 6 (C57BL/6) male mice were placed on an acid water regimen two days before irradiation to reduce nasal *Pseudomonas* infection. They were then irradiated with a total of 1100 rads, distributed in two doses, with five hours between the two irradiations. The day after irradiation, the mice were reconstituted with cells from donor bone marrow prepared from either of four types of male ER-deficient mice: wild type (WT), alpha knockout (αERKO), beta knockout (βERKO) or double knockout (αβERKO or KO/KO) for the E-2 receptor. To prepare the donor cells, 2-3 mice of the appropriate ER genotype were euthanized by CO₂ asphyxiation. Their femurs and tibias were removed and placed in a dish of sterile HBSS containing 5ml RPMI with 5% FBS. All muscle was removed from the bones. The ends were cut off and the bone marrow was flushed out using 5ml solution of RPMI with a syringe and a 25g needle. The marrow was flushed into another dish containing HBSS and was passed through a 22g needle in order to break up the clumps. The mixture was then spun down and
suspended in media. The cells were counted, and resuspended at a concentration of $1 \times 10^7$ cells/mL.

Groups of seven irradiated recipient mice were reconstituted with 0.2 ml ($5 \times 10^6$) of the appropriate cell type by intravenous injection in the tail vein. Two mice served as controls for irradiation and were not reconstituted. All mice were maintained on acid water, with antibiotics for one month. Within ten days of irradiation, the two control mice were moribund. This confirmed that the lethal irradiation was successful.

Seven weeks after reconstitution, four mice from each group were injected with E-2 (17$\beta$-estradiol valerate-1 mg/kg) in oil and three mice were injected with oil vehicle by bolus injection subcutaneously between the shoulder blades. These injections were performed subsequently every month (4 weeks) for 5 months. Body weights were measured before each injection. This dosing regimen elevated mouse serum E-2 level to the peak physiological serum E-2 level in female mice of 150–200pg/ml (23). The level of E-2 was maintained for about 7 days and decreased to normal levels found in male mice 2 weeks after treatment.

*Cell harvesting*

Two weeks after the 5th injection of estrogen, the treated and control mice were euthanized for experimental analysis. Body weight was recorded, and blood was collected. Tissue from thymus, spleen and kidneys were harvested individually from each mouse and cell suspensions were prepared in order to determine specific phenotypes. Red blood cells were lysed by resuspending the
cell suspension in 5mL TAC (tris-ammonium chloride), and the cells were counted.

*In vitro cytokine enzyme linked immunosorbent assay (ELISA)*

Lymphocytes from individual ER chimeric mice from each treatment group were cultured with Con A or idiopeptide aa62-73 (24-27). Cells and supernatant were collected on day 3 for cytokine production and day 7 for immunoglobulin production. Supernatants were frozen at -70°C until assays were conducted. ELISA was used to measure Interleukins 2, 4, 10, 12, IFNγ, to identify Th1/Th2-type cytokine shifts (R & D Diagnostics, e-Biosciences). Briefly, 96 well Nunc Maxisorp plates were coated with 50µl of the appropriate dilution of anti-cytokine capture antibodies in 0.1M carbonate coating buffer pH 9.5 (IL-2, IL-4, IFN-γ) or 0.2M phosphate coating buffer pH 6.5 (IL-10 and IL-12). Plates were incubated overnight at 4°C, washed two times with PBS/0.05% Tween (wash solution), and then blocked with 50 µl of the assay diluent (PBS/10%FBS pH7) for 1 hour at room temperature (RT). The plates were then washed two times, and cytokine standard and supernatant samples diluted 1:2 in assay diluents were added to the wells. After an overnight incubation at 4 °C, the plates were washed three times with wash solution, and then 50µl of appropriately diluted biotin-conjugated detection antibodies was added to the wells and incubated overnight at 4 °C. The plates were then washed four times, and 50µl strepavidin-conjugated horseradish peroxidase (HRP), diluted 1:250 in assay diluent, was added and incubated for 30 minutes at RT. Lastly, the plates were washed five
times, and 50µl TMB substrate was added to each well. Color change corresponding to bound antibody was monitored for 15-30 minutes at RT, and then the reaction stopped by adding 50µl of H₃PO₄ to each well. The absorbance was then read at 450nm. The concentration of each cytokine in the culture supernatant was determined from a standard curve.

Table 1. Cytokine assays capture and detection antibody dilutions and standard range.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Capture Antibody Dilution</th>
<th>Detection Antibody Dilution</th>
<th>Standard Range (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>1:250</td>
<td>1:250</td>
<td>0-200</td>
</tr>
<tr>
<td>IL-4</td>
<td>1:250</td>
<td>1:250</td>
<td>0-500</td>
</tr>
<tr>
<td>IL-10</td>
<td>1:1000</td>
<td>1:500</td>
<td>0-2000</td>
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<tr>
<td>IL-12</td>
<td>1:250</td>
<td>1:250</td>
<td>0-100</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1:1000</td>
<td>1:500</td>
<td>0-1000</td>
</tr>
</tbody>
</table>

In vitro immunoglobulin ELISA

For this assay, 96 well Immulon 1B plates were coated with 50µL per well of single-stranded DNA (ssDNA or double-stranded DNA (dsDNA) at 5µg/mL in 1X SSC. The plates were incubated overnight at 4º C and were subsequently washed 2 times with PBS/1%Tween and 2 times with PBS. The plates were blocked with 50µL of PBS/1%Tween per well, incubated for an hour at RT and washed as previously described. Then 50µL of each supernatant sample diluted 1:2 in PBS/0.1%Tween in duplicate, was added to the wells of the plate and incubated overnight at 4º C. The plates were washed twice with PBS/1%Tween and twice with PBS. Next 50µL of anti-goat IgM and IgG alkaline-phosphatase conjugate in PBS/0.1% Tween (1/2000 dilution) was added to all of the wells. The plates were incubated overnight at 4º C and washed as in previous steps.
Lastly, 50µL of substrate solution paranitrophenyl phosphate (PNPP) was added, and allowed to develop until color change was apparent (approximately 30 minutes). Color development was then read at 405nm.

*Statistical analyses*

Data was analyzed by one-way ANOVA with post hoc comparisons by the Student-Newman-Keuls multiple comparisons test. Differences in significance will be considered at p <0.05.
RESULTS

Effect of estrogen treatment on survival of WT and estrogen receptor-deficient chimeric mice

All WT mice survived throughout the duration of the experiment. Estrogen treated mice αERKO mice showed a lower survival rate than those that were treated with oil. One out of four mice died during the third month of injections. Mice from the βERKO estrogen treatment group also showed a lower survival rate than those that were treated with oil. There was one death out of four during the third month of injections. Additionally, estrogen-treated αβERKO mice also had a lower survival rate, with half of the mice in this group moribund within three months and four months of the experiment. This is in contrast with the control αβERKO mice which all survived the duration of the experiment.

Table 2. Deaths of estrogen receptor-deficient chimeric mice. There were no deaths among any of the oil treated ER chimeric mice, while 1-2 mice from each E-2 treated ER KO chimera became moribund during the course of the injections.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Ratio of Death of Total Group Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT + Oil</td>
<td>0</td>
</tr>
<tr>
<td>WT + E-2</td>
<td>¼</td>
</tr>
<tr>
<td>αERKO + Oil</td>
<td>0</td>
</tr>
<tr>
<td>αERKO + E-2</td>
<td>¼</td>
</tr>
<tr>
<td>βERKO + Oil</td>
<td>0</td>
</tr>
<tr>
<td>βERKO + E-2</td>
<td>¼</td>
</tr>
<tr>
<td>αβERKO + Oil</td>
<td>0</td>
</tr>
<tr>
<td>αβERKO + E-2</td>
<td>½</td>
</tr>
</tbody>
</table>
Effect of estrogen treatment on body weights of WT and estrogen receptor-deficient chimeric mice

In general, oil treated (control) WT mice had higher body weights than the αβERKO, αERKO and βERKO control mice. But estrogen treatment resulted in lowered body weights in the WT and the ER-deficient chimeras, compared to controls (Figure 1).

E2 treatment effect on body weight
The weights of the wild-type (WT) mice treated with estrogen were significantly lower than the weights of the oil vehicle treatment group (p-value=0.014). Data showed that both groups averaged approximately the same weight at the first injections (25g), but diverged from then on. At the final injection, the WT oil-treated mice had an average weight of 32.3g (+/- 1.9g) while the estrogen-treated group weighed an average of 29.6g (+/-2.1g), a difference of 2.7g.
b. αβERKO mice

Data of body weights for the αβERKO mice showed that although the oil-treated mice maintained slightly higher weights than estrogen-treated mice throughout the experiment, with the exception of the 5th injection, the weight differences were not significant. At the 5th injection, the average body weight of the αβERKO oil-treated mice was 5.7g higher than the estrogen-treated αβERKO mice, but this was not significant.

c. αERKO mice

The weights of the oil-treated αERKO chimeric mice were significantly higher that weights of estrogen-treated mice at later injections. On average, the oil-treated mice weighed 2.9g (+/- 2.4) more than the estrogen-treated groups, with the greatest significant difference of 5.6g (+/- 2.4) at the 5th time point (p-value=0.01).

d. βERKO mice

The body weights of βERKO mice in both the oil and estrogen treatment groups were similar weight until the 4th injection, when the estrogen-treated mice showed a sharp decrease in body weight with a difference of 4.5g (+/- 2.5) between two groups at the 5th injection. This difference in weight, although not significant, persisted until the end of the study.
Effects of E2 receptor expression in the modulation of Th1 Cytokine production

Cells from each estrogen receptor chimeric mice were harvested and cultured with Con A or idiopeptide aa62-73, to determine the effects of ER receptor expression on cytokine production. An enzyme-linked immunosorbent assay (ELISA) was used to determine the relative amount of Th1 cytokines, IL-2, IFN-λ, IL-12, produced in media alone, Con-A and aa62-73.

a. IL-2

Cytokine production in cells cultured in media alone

When cells were cultured in media alone, we found that there was no significant difference in IL-2 production by cells from the oil and estrogen-treated mice (Figure 2a). Interestingly, for the αERKO chimeric mice, the production of IL-2 by cells from estrogen-treated mice was significantly higher than production by cells from oil-treated mice (p=0.0038). There was no statistically significant difference in IL-2 production by cells from either βERKO or αβERKO chimeric mice, with or without estrogen treatment.
Figure 2a. Production of interleukin 2 (IL-2) cytokines by cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone (blank), media containing Con-A mitogen. IL-2 cytokine production was measured by ELISA and expressed in terms of optical density (OD) at 450nm. *Indicates results of statistical significance (e.g. p-value<0.05) were found.

Con A–induced cytokine production

Data showed a significantly higher production of Con-A induced IL-2 in the WT control group, compared to the estrogen-treated group (p =0.008) (Figure 2a). Thus, it appeared that E-2 suppressed the production of IL-2 by the WT cells. Comparison of the different knockout groups showed that estrogen treatment did not result in significant differences in IL-2 production compared to oil control mice. Further, IL-2 production by cells from all of the knockout groups was significantly higher than that produced by cells from WT mice.
Peptide 62-73 induced cytokine production

In general, IL-2 production was significantly higher in all estrogen-treated and oil control chimeric receptor mice regardless of ER expression when cells were stimulated with peptide 62-73. Figure 2b shows a significantly higher production of IL-2 in the WT control group, compared to the estrogen group (p=0.04). Thus, similar to what we saw with Con-A, it appears that E-2 suppressed the production of αα62-73-induced IL-2 production in the WT cells. Although IL-2 production was slightly higher for αβERKO cells that for the other ERKO chimeric mice, the differences between the knockout groups were not statistically significant.

Figure 2b. Production of interleukin 2 (IL-2) cytokines by cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone (blank) and aa62-73 peptide (100µg/mL). IL-2 cytokine production was measured by ELISA and expressed in terms of optical density (OD) at 450nm. * Indicates results of statistical significance (e.g. p-value<0.05) were found.
b. IFN-gamma

Cytokine production in cells cultured in media alone

Unstimulated cells from WT mice treated with E-2 produced relatively higher levels of IFN-γ compared to cells from WT mice treated with oil (p-value=0.018) (Figure 3a). Similar results were also seen for αERKO and αβERKO mice; however this increase was not significant. In contrast, IFN-γ production by cells from βERKO mice treated with oil was greater than that produced by cells from mice that had been treated with E-2.

Figure 3a. Production of interferon-γ (IFN-γ) cytokines by cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone (blank) and media containing Con-A mitogen. IFN-γ cytokine production was measured by ELISA and expressed in terms of optical density (OD) at 450nm. * Indicates results of statistical significance (e.g. p-value<0.05) were found.
Con A-induced cytokine production

Overall, Con-A stimulated cells showed higher productions of IFN-γ compared to cells that were cultured in media alone or with peptide 62-73 (Figure 3a). Production by cells from WT mice treated with oil was slightly higher than that produced by WT mice treated with E-2; however this increase was not statistically significant. There was no difference in IFN-gamma production by cells from the oil or estrogen treated αERKO mice. Data showed a higher production of the cytokine by cells from the βERKO E-2 treated group as opposed to cells from the βERKO control group (p-value=0.018). IFN-gamma levels produced by cells from either the oil or the E-2 treated βERKO group were also higher than those observed for any of the other WT or receptor knockout groups. Finally, while the amount of IFN-γ produced by cells from the αβERKO control group was higher than that produced by cells in the E-2 treated group, this difference was not statistically significant.

Peptide 62-73 induced cytokine production

Production of IFN-γ by cells from WT mice treated with E-2 stimulated with peptide 62-73 was greater than that produced by cells from WT mice treated with oil (p-value=0.018) (Figure 3b). In general, however, IFN-gamma production by cells from WT mice treated with oil was higher than levels produced by cells from αERKO and βERKO chimeric mice, but similar to that produced by cells from αβERKO mice. Further, cells from αβERKO chimeric mice treated with E-2 produced slightly higher levels of IFN-γ compared to cells
from αβERKO chimeric oil-treated control mice, while no effect was seen in the αERKO and βERKO mice.

![IFN-gamma Production in Vitro](image)

**Figure 3b. Production of interferon-γ (IFN-γ) cytokines by cells from ER chimeric mice treated with estrogen and vehicle.** Cells isolated from each treatment group were cultured in media alone (alone) and media containing aa62-73 peptide (100µg/mL). IFN-γ cytokine production was measured by ELISA and expressed in terms of optical density (OD) at 450nm.

* Indicates results of statistical significance (e.g. p-value<0.05) were found.

**c. IL-12**

**Cytokine production in cells cultured in media alone**

Unstimulated cells from WT mice treated with E-2 displayed greater production of IL-12 than cells from WT mice treated with oil, although this difference was not statistically significant (Figure 4a). Cells from αERKO mice showed no significant differences in IL-12 production; however, significant differences were seen for cells from the βERKO treatment groups. Cells from E-2
treated βERKO chimeric mice produced more IL-12 than cells from the oil-treated βERKO control mice (p-value=0.01).

**Figure 4a. Production of interleukin 12 (IL-12) cytokine by cells from ER chimeric mice treated with estrogen and vehicle.** Cells isolated from each treatment group were cultured in media alone (blank) and media containing Con-A mitogen. IL-12 cytokine production was measured by ELISA and expressed in terms of optical density (OD) at 450nm. *Indicates results of statistical significance (e.g. p-value<0.05) were found.

**Con A-induced cytokine production**

IL-12 production in response to Con was increased in cells from WT mice treated with E-2 compared to that produced by cells from WT mice treated with oil. On the other hand, cells from αERKO mice treated with E-2 produced lower levels of IL-12 than cells from the αERKO oil control mice, but these results were not statistically significant. Cells from αβERKO mice treated with estrogen
produced significantly less IL-12 than the controls (p-value=0.03). No significant differences were observed for βERKO mice.

![IL-12 Production in Vitro](image)

*Figure 4b. Production of interleukin 12 (IL-12) cytokine by cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone (blank) and media containing aa62-73 peptide (100µg/mL). IL-12 cytokine production was measured by ELISA and expressed in terms of optical density (OD) at 450nm. * Indicates results of statistical significance (e.g. p-value<0.05) were found.

**Peptide 62-73 induced cytokine production**

IL-12 production in WT cells stimulated with peptide 62-73 was slightly higher than for the other receptor chimera groups, with cells from βERKO E-2-treated group producing the lowest levels (Figure 4b). Cells from WT mice treated with E-2 and stimulated with peptide 62 produced significantly higher levels of IL-12 compared to cells from mice treated with oil (p-value=0.02). No significant differences in IL-12 production by cells from oil or E-2 treated αERKO and βERKO mice were observed. However, we did note significant
differences for αβERKO mice. Cells from E-2 treated αβERKO chimeric mice produced lower levels of IL-12 compared to cells from αβERKO mice treated with oil (p-value=0.007).

Effects of E2 receptor expression on the modulation of Th2 cytokine production

Cytokine ELISA was used to determine the relative amount of Th2 cytokines, IL-4 and IL-10, produced by cells from estrogen or oil treated WT or ER chimeric mice cultured in media alone, or with Con-A or aa62-73.

a. IL-4

Cytokine production in cells cultured in media alone

There was no significant difference in IL-4 production by unstimulated cells from the WT oil and estrogen-treated mice, although IL-4 levels in the E-2 treated mice were slightly higher (Figure 5a). There was also no significant difference in IL-4 production by cells from αERKO and αβERKO chimeric mice. However, cells from βERKO chimeric mice treated with estrogen produced significantly less IL-4 than cells from oil-treated mice (p-value=0.006).
Con A-induced cytokine production

Significantly higher levels of Con A-induced IL-4 were produced by cells from WT mice treated with estrogen compared to that produced by cells from the oil treated control group (p-value=0.025) (Figure 5a). In the βERKO chimeric mice however, the cells from mice that had been treated with estrogen produced less IL-4 than cells from oil-treated mice (p-value=0.002). Comparison of the αERKO and αβERKO groups showed no significant difference in IL-4 production by cells from control and E-2 treated mice.
Peptide 62-73 induced cytokine production

There were no significant differences in peptide 62-73 induced IL-4 production by cells from oil or estrogen-treated mice for WT or any of the ER receptor knockout chimeras (Figure 5b).

* Indicates results of statistical significance (e.g. p-value<0.05) were found.
b. **IL-10**

**Cytokine production in cells cultured in media alone**

IL-10 production by cells from WT mice treated with E-2, and culture in media alone, was greater than that by cells from WT oil treated control mice, but this difference was not statistically significantly different (Figure 6a). Estrogen treatment of βERKO mice resulted in the production of significantly lower levels of IL-10 than their oil control counterparts (p-value=0.001). This was also seen in the αβERKO mice (p-value=0.002). In general, cells from βERKO mice produced lower levels of IL-10 compared to the WT, αERKO and αβERKO groups.

*Figure 6a. Production of interleukin 10 (IL-10) cytokine by cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone (blank) and media containing Con-A mitogen. IL-10 cytokine production was measured by ELISA and expressed in terms of optical density (OD) at 450nm. *Indicates results of statistical significance (e.g. p-value<0.05) were found.*
Con A-induced cytokine production

Cells from WT mice treated with E-2 and cultured in Con-A produced higher levels of IL-10 compared to WT control mice; however this difference was not statistically significantly different (Figure 6a). In general, cytokine levels produced by cells from the oil or E-2 treated WT were higher than levels produced by cells from the other ER chimeric mice. This difference was particularly significant when compared to IL-10 levels produced by cells from βERKO chimeric mice (p-value=3.78x10^{-11}). Cells from αERKO mice treated with E-2 produced significantly lower levels of IL-10 compared to cells from control αERKO (p-value=0.001). The same trend was seen in cells from βERKO mice (p-value=7.0x10^{-4}) and αβERKO mice (p-value=0.005). In general, cells from βERKO mice in both estrogen and oil treatment groups produced lower levels of IL-10 than cells from the WT, αERKO and αβERKO groups. Cells from αβERKO mice treated estrogen or oil also produced greater amounts of cytokine than αERKO and more significantly βERKO cells (p-value=4.42x10^{-9}).
Peptide 62-73 induced cytokine production

Production of IL-10 by cells from WT mice treated with E-2, and stimulated with peptide 62-73, was greater, although not significantly, than levels of IL-10 produced by the WT control (Figure 6b). In general, peptide 62-73 induced IL-10 production by cells from WT mice, whether control or E-2 treated, were greater than the levels produced by cells from αERKO and βERKO chimeras, but similar to that produced by cells from αβERKO chimeras. Further, E-2 treatment of αERKO chimeric mice resulted in the production of significantly less IL-10 than cells from oil-treated αERKO mice (p-value=0.02). Similar results were observed for cells from βERKO chimeras (p-value=1.0x10^-4). On the other hand,
hand, cells from αβERKO mice treated with E-2 produced slightly higher levels of IL-10 compared to αβERKO controls treated with oil.

**Effects of ER expression on anti-single stranded DNA total immunoglobulin (IgG and IgM) production**

Cell culture supernatant from each group of estrogen receptor chimeric mice was tested in an ELISA to determine the relative amount of anti-single stranded DNA total IgG and IgM produced in the presence of media alone, LPS or aa62-73.

a. **Anti-ssDNA immunoglobulin production in cells cultured in media alone**

Figure 7 shows the levels of anti-single stranded DNA antibodies (IgG and IgM) produced by unstimulated cells in each ER knockout chimera group of mice. In general, antibody production was highest for cells from αERKO mice and lowest for cells from αβERKO mice. Differences in total immunoglobulin production in WT estrogen-treated and control mice were recorded, however, they were not of statistical significance. In contrast, there was a significant difference in the production of total immunoglobulin by cells from both αERKO and βERKO mice. Cells from αERKO mice treated with oil produced increased anti-ssDNA antibodies compared to cells from αERKO mice treated with E-2 (p-value=0.006). A similar trend was observed between control cells from βERKO mice and cells from βERKO mice treated with E-2 (p-value=0.02). We also noted a significant increase in antibody production by cells from αERKO mice treated
with E-2 compared to cells from WT mice treated with E-2 (p-value=3.1x10⁻³).

Cells from βERKO mice treated with oil only also produced higher levels of immunoglobulin compared to WT cells treated with oil (p-value=3.4x10⁻⁴).

**Figure 7.** Effects of ER expression on anti-single stranded DNA IgG and IgM production by unstimulated cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone (blank), media containing lipopolysaccharide (LPS), and aa62-73 peptide. Immunoglobulin production was measured by ELISA and expressed in terms of optical density (OD) at 405nm.

* Indicates results of statistical significance (e.g. p-value<0.05) were found.

a. **LPS-induced anti-ssDNA immunoglobulin production**

Figure 8 compares levels of the anti-single stranded DNA immunoglobulin produced by cells from the different ER knockout chimeric mice groups stimulated with lipopolysaccharide (LPS). LPS is found in Gram negative bacteria and is an endotoxin that elicits strong immune responses in animals,
including polyclonal production of immunoglobulin. We found an increase in LPS-induced immunoglobulin production for both oil and estrogen-treated cells from αERKO mice compared to cells from WT mice treated with oil and estrogen (p-values=2.3x10^{-5} and 2.28x10^{-5} respectively). A similar trend was found in each treatment group (oil or estrogen) for cells from βERKO and αβERKO mice compared to the cells from WT mice, however results were lower for αβERKO mice than for WT mice (p-values=0.0004, 0.003, 0.0003 and 0.0003, respectively).

Figure 8. Effects of ER expression on anti-single stranded DNA IgG and IgM production by LPS-stimulated cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone (blank), media containing lipopolysaccharide (LPS), and aa62-73 peptide. Immunoglobulin production was measured by ELISA and expressed in terms of optical density (OD) at 405nm.

* Indicates results of statistical significance (e.g. p-value<0.05) were found.
b. Peptide 62-73 induced anti-ssDNA immunoglobulin production

Figure 9 shows the levels of anti-single stranded DNA immunoglobulin production by cells stimulated with the pathogenic peptide aa62-73 for each ER knockout chimera treatment group. As expected, given that this is an antigen-specific response, there was a general trend towards less antibody production in aa62-73-stimulated cells compared to unstimulated and LPS-stimulated cells. However, there was a significant increase in antibody production by cells from αERKO mice treated with estrogen compared to E-2 cells from WT mice (p-value=0.024). No differences were found between cells from oil treated control mice, either WT or any of the ER knockout chimera. Decreased immunoglobulin production by cells from αβERKO mice was seen whether treated with oil or estrogen compared to the WT groups (p-values=0.001 and 0.004, respectively).

We did not find any differences in anti-ssDNA production by cells from either oil or estrogen treated βERKO chimeric mice.
**Figure 9. Effects of ER expression on anti-single stranded DNA IgG and IgM production by aa62-73-stimulated cells from ER chimeric mice treated with estrogen and vehicle.** Cells isolated from each treatment group were cultured in media alone (blank), media containing lipopolysaccharides (LPS), and aa62-73 peptide. Immunoglobulin production was measured by ELISA and expressed in terms of optical density (OD) at 405nm.

* Indicates results of statistical significance (e.g. p-value<0.05) were found

**Effects of ER expression in the modulation of anti-double stranded DNA total immunoglobulin (IgG and IgM) production**

Cells from each estrogen receptor chimeric mouse were harvested and an ELISA was used to determine the relative amount of total anti-double stranded DNA IgG and IgM produced in media alone, LPS and aa62-73.

a. **Anti-dsDNA immunoglobulin production in cells cultured in media alone**

Figure 10 shows the levels of anti-double stranded DNA antibodies (IgG and IgM) produced by unstimulated cells for each ER knockout chimera group. Generally, the production of anti-dsDNA antibodies was similar for all treatment groups except for a statistically significant increase in the production of antibodies by cells from αERKO chimeric mice treated with oil as compared to cells from the WT mice (p-value=0.01).
b. LPS-induced anti-dsDNA immunoglobulin production

Figure 10. Effects of ER expression on anti-double stranded DNA IgG and IgM production by unstimulated cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone, media containing lipopolysaccharides (LPS), and aa62-73 peptide. Immunoglobulin production was measured by ELISA and expressed in terms of optical density (OD) at 405nm. * Indicates results of statistical significance (e.g. \( p-value < 0.05 \)) were found.

Figure 11 compares anti-double stranded DNA immunoglobulin production by cells stimulated with LPS in the different ER knockout chimera mice. The only significant difference found was an increase in anti-dsDNA immunoglobulin production in estrogen treated cells from \( \beta \)ERKO mice compared to cells from \( \beta \)ERKO mice treated with oil vehicle (\( p-value = 0.035 \)).
Figure 11. Effects of ER expression on anti-double-stranded DNA IgG and IgM production by aa62-73-stimulated cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone, media containing lipopolysaccharides (LPS), and aa62-73 peptide. Immunoglobulin production was measured by ELISA and expressed in terms of optical density (OD) at 405nm.

* Indicates results of statistical significance (e.g. p-value<0.05) were found.

c. Peptide 62-73 induced anti-dsDNA immunoglobulin production

Figure 12 shows the levels of total anti-double stranded DNA immunoglobulin produced by cells stimulated with the pathogenic peptide aa62-73 for each ER knockout chimeric group. We observed an increase in antibody production by cells from αβERKO estrogen treated chimeric mice compared to cells from αβERKO chimeric mice treated with oil (p-value=0.01). This trend was also seen for cells from βERKO and αERKO chimeric mice treated with estrogen compared to oil treated cells, however, these results were not significant.

Compared to the estrogen-treated cells from WT mice, cells from estrogen-treated αβERKO chimeric mice had a higher production of the antibody (p-value=0.02).
Increased immunoglobulin production by cells from βERKO chimeras treated with estrogen compared to cells from βERKO mice treated with oil was also seen (p-values=0.01). There were no other significant differences seen.

Figure 12. Effects of ER expression on anti-double-stranded DNA production by aa62-73-stimulated cells from ER chimeric mice treated with estrogen and vehicle. Cells isolated from each treatment group were cultured in media alone, media containing lipopolysaccharides (LPS), and aa62-73 peptide. Immunoglobulin production was measured by ELISA and expressed in terms of optical density (OD) at 405nm.

* Indicates results of statistical significance (e.g. p-value<0.05) were found.
DISCUSSION

The higher occurrence of SLE in females versus males, as well as the significant increase in the disease incidence in females after puberty, and the fluctuation in disease severity throughout the menstrual cycle and pregnancy suggests that sex hormones, specifically the female sex hormone estrogen, play an important role in SLE and in autoimmunity (6, 7). This project sought to gain further knowledge about the mechanisms by which estrogen mediates immune responses that could explain the predilection of SLE in females. Particularly, we investigated the significance of estrogen’s interactions with its various receptors in modulating $\text{T}_1$ and $\text{T}_2$ immune response, since $\text{T}_2$-type cytokines predominate in SLE. To address this question we examined cytokine and autoantibody production in estrogen and oil vehicle-treated estrogen-receptor knockout chimeric mice. In these mice, ER receptor expression was modulated on immune cells only, so that these cells expressed only ER-$\alpha$, ER-$\beta$, or no ER, while non-immune cells expressed normal levels and types of all estrogen receptor isoforms. The results in these mice were compared to results in WT mice, which would express both ER. In addition, only male mice were used, in order to obviate the effects of physiological levels of estrogen.

First, we looked at the effect of E-2 treatment on body weight. No body weight differences were seen between the mice prior to estrogen treatment period. E-2 administration of 1mg/kg monthly to male WT C57/black 6 mice and the
three ER receptor knockout chimeric groups led to a decrease in survival rate, as well as weight loss, compared to the control mice treated with oil vehicle only. There was a lower survival and a significant decrease in body in estrogen treated WT mice compare to the control oil-treated WT mice. The same trend was seen in αERKO chimeric mice. Estrogen-treated mice in the βERKO and αβERKO chimera groups also showed a large decrease in body weight, but only after the 4th injection of E-2. The difference was particularly significant for the WT and αERKO chimeric mice, suggesting a differential effect of α and β receptors in mediating estrogen-induced cytokine production, with ER-α possibly playing a greater role. Our observations for estrogen and oil treated WT mice are in agreement with results reported by Feng et al. (28), which looked at the development of lupus nephritis in αERKO mice and reported a significant decrease in weight in WT mice treated with E-2. Other studies in both human and animal models (29-32) have shown that estrogens serve to mobilize body fat and induce the apoptosis of adipose tissue, thereby leading to a decrease in body weight. This effect seems to be quite rapid; in fact, in the current study, body weights of E-2 treated WT mice decreased significantly following the first and second injections and remained lower than the control mice throughout the duration of the experiment.

However, in contrast to our data, Feng et al. (28) did not observe a significant difference in body weights in E-2-treated αERKO mice compared to those treated with oil. However, in the previous work, the mice studied were ER-α knockout mice and not ER-α knockout chimeric mice; the former would not
express ER receptors on any cells or tissue while, the latter would be deficient in ER expression on immune cells only, but other cells and tissues in the recipient mice would express ER-α. This suggests that in our studies, estrogen’s effect in the modulation of body weight was mediated by expression of ER-α on non-immune cells.

Other research has shown varying effects of the sex hormone estrogen on autoimmune disease manifestation. Some studies have shown that estrogen has immunosuppressive effects in some organ-specific autoimmune diseases. One study revealed that administration of E-2 to αERKO C57BL/6 mice resulted in the suppression of the clinical signs of Experimental Autoimmune Encephalomyelitis (EAE), an animal model of the autoimmune disease Multiple Sclerosis (MS) (33). It has also been observed that the severity of multiple sclerosis in human decreases as sex hormone levels increase during pregnancy, but this effect is reversed postpartum due to alterations in sex hormones (34-36). Another study of MS reported an enhanced stimulation of IL-10 secretion at pregnancy levels of E-2, as well as an increase in IFN-γ in patients with the disease (37). On the other hand, estrogen exposure has been shown to accelerate systemic autoimmune diseases. Together, these findings have supported the important role of sex hormones on immune responses and specifically autoimmune diseases.

One explanation for the differences in the response to estrogen in organ-specific and systemic autoimmune diseases could be its role in cytokine production. Estrogen stimulates the production of Th2 cytokines and promotes interactions between T and B cells. It has also been reported that estrogen leads to
increased secretion of IL-4, IL-5, IL-6 and IL-10 by Th2 lymphocytes (4, 38-40). These cytokines stimulate the proliferation antibody-producing B cells. SLE patients have increased levels of IL-6 and IL-10, which can be directly correlated with clinical disease severity (39, 40). Increased levels of IL-4 in NZB/W and MRL/lpr mice are also evident.

In our project, both Th1 and Th2 cytokine production was determined using ELISA. Our findings show that when cells were cultured in media alone, in the absence of any antigen or mitogen, IL-4 and IL-10 production by cells from estrogen-treated WT mice was generally higher compared to cells from control WT mice. Although these results were not statistically significant, they point to a role for estrogen in Th2 cytokine production. Consistent with the findings of Feng et al (28), which reported that serum levels of Th2 cytokines were not increased in oil and estrogen-treated αERKO mice, we did not find differences in cytokine production by cells from oil or estrogen-treated αERKO knockout chimeric mice. This suggested that ERβ, which would be expressed in the ERα KO chimeric mice, is likely not involved in estrogen-induced Th2 cytokine production. Interestingly, our results showed a decreased in both IL-4 and IL-10 production by cells from estrogen-treated βERKO mice compared to oil-treated βERKO mice, suggesting that estrogen-induced modulation of Th2 cytokines production may be mediated by ER-α expression. Furthermore, this trend was similar across all cytokines examined, both Th1 and Th2, as consistently lower levels of cytokines were produced by βERKO cells treated with E-2 compared to those treated with oil vehicle. Production of IL-4 by cells from both αERKO and
αβERKO mice did not prove to be statistically significant. However, IL-10 production followed a similar trend for αβERKO as for βERKO cells, discussed previously, with decreased production of the cytokine in αβERKO estrogen-treated mice versus oil-treated mice. Taken together, these results point to the importance of ER-α in estrogen-mediated T_{H}2 cytokine production and a potential role for ER-β in down-regulating this response, particularly with respect to IL-10 levels.

In response to Concanavalin A (Con A) WT mice treated with estrogen produced higher levels of IL-4 and IL-10, although the increase in IL-10 levels was not significant. We also observed a decrease in IL-4 and IL-10 production in βERKO mice treated with estrogen compared to βERKO mice treated with oil. This further supports the conclusion that estrogen-induced T_{H}2 cytokine modulation is not mediated by the ER-β isoform. Interestingly, a similar trend was observed for IL-10 production by cells from αERKO mice. This suggests that expression of both receptors is important in estrogen-induced T_{H}2 cytokine production after mitogen stimulation, with one or the other playing a more predominant modulatory role depending on the specific cytokine.

We also looked at specific cytokine production in response to the pathogenic idiopeptide, aa62-73. As expected from previous data (28), stimulation of cells with the pathogenic Id^{LN}_1 antigen resulted in an increase in T_{H}2 cytokine (IL-10) in cells from WT mice treated with estrogen compared to those treated with oil only, although this difference was not statistically significant. This result suggests that the pathogenic response in lupus nephritis may be enhanced directly
by estrogen treatment. Interestingly, production was significantly decreased in cells from αERKO mice treated with estrogen when compared to oil treated αERKO mice. We found a similar trend for βERKO mice. These results suggest that both ER receptors are important in estrogen-induced Th2 cytokine production in response to pathogenic autoimmune peptides.

While in most autoimmune diseases, Th2 cytokines (IL-4, IL-10, and IL-6) are important in later stages of disease, pro-inflammatory Th1 cytokines (IL-2, IFN-γ, IL-12) are essential in initiation of pathology. Cytokine production in male estrogen receptor (ER) knockout mice that were administered estrogen (E-2) showed that the shifts from Th2 to Th1 responses were protective while a Th2 response exacerbated autoimmune disease (28). To further define the role of ER in this process, we measured Th1 cytokine production in estrogen-treated ERKO chimeric mice. Cells from αERKO chimeric mice treated with E-2 and cultured in media alone produced higher levels of IL-2 and IFN-γ (but not IL-12) than those mice treated with oil. Interestingly, this result was different from that observed by Feng et al, who found no differences in Th1 cytokine production by cells from αERKO mice. However, differences in ER expression between the two models, as discussed previously, may have contributed to this difference. Conversely, as expected, IL-12 and IFN-γ production in WT mice was greater in the estrogen treatment group compared to the oil treatment group. These data are in agreement with previous studies that supported a role for IFN-γ in the onset, but not later stages, of autoimmune disease (27).
Mitogen-induced IL-2 and IFN-γ production by cells from WT estrogen-treated mice was decreased compared to cells from WT oil-treated mice. From this observation, it appears that T_H1 cytokine production was suppressed by the administration of estrogen in wild type mice, and this suppression may be mediated by ER. This was further supported by the observation that there were no differences in IL-2 production in any of the estrogen-treated ER knockout chimeric mice. Interestingly, however, we found that IFN-γ production by cells from estrogen-treated βERKO mice was greater compared to that produced by cells from control βERKO mice. This, combined with the fact that no differences were found for αERKO mice, suggests a role of ER-α in IFN-γ production. In the case of IL-12 production, we noted that levels produced by cells from αERKO and αβERKO estrogen-treated mice were decreased compared to the oil controls of these groups, while the opposite results were seen for βERKO mice. Thus it appears that ER-α expression may play a role in IFN-γ and IL-2 production but not in IL-12 production. Additionally, because none of the cells from ER knockout chimeric mice treated with estrogen showed any significant changes in IL-2 production compared to the oil treatment groups, it is possible that both ER-α and ER-β expression on immune cells play significant roles in promoting the estrogen-induced T_H1 cytokine production.

Finally, we found that in general, there was greater production of T_H1 cytokines in the presence of the idiopeptide 62-73. While there was an increase in IL-2 levels produced by cells from WT control mice compared to cells from estrogen-treated mice, the opposite was observed for both IL-12 and IFN-γ, with
cells from estrogen-treated mice producing higher levels of these cytokines. Our results for IFN-γ were consistent with previous research (41), which reported an increase in IFN-γ–producing cells in castrated female C57BL/6 mice, which was ER-α and not ER-β dependent. However, with the exception of IL-12, we did not find differences in T_H1 cytokine production in αERKO, βERKO or αβERKO mice. Thus our data suggests the importance of both ER-α and ER-β in estrogen-induced modulation of the pathogenic autoantibody response, with ER-α expression being possibly more significant. Differences in the levels of expression of each ER isoform, as observed by Feng et al (28) may also play a role; in that study, ER-α was found to be expressed in a higher density than ER-β on immune cells, which could explain why it may play a more significant role than ER-β in modulating the immune response. Further, levels of ER were higher in mice that were genetically susceptible to develop autoimmune disease relative to MHC haploype-matched nonautoimmune mice. Finally, another study showed that estrogen exposure directly activated T cells through the cell membrane ER-α (42). Thus, differences in the numbers of each ER isoform expressed on the immune cells of certain individuals could explain differences in T cell responses to antigen, which could contribute to the development of autoimmune diseases. If the levels of receptor expression between males and females also differ, this could play a role in the predominance of autoimmune diseases in females as opposed to their male counterparts.
To further explore the role of ER expression in modulating the immune response, particularly in terms of the development of autoimmunity, the effect of ER-α and ER-β expression on estrogen-induced modulation of anti-single stranded DNA and anti-double stranded DNA immunoglobulin levels was also determined. Interestingly, we found that B cells from both αERKO and βERKO mice treated with estrogen produced less anti-single stranded DNA than oil-treated mice. This same trend was seen in WT mice, although the difference was not significant. Compared to cells from oil-treated WT mice, cells from oil-treated βERKO mice produced significantly less immunoglobulin. Conversely, compared to WT estrogen treated mice, cells from αERKO chimeras treated with estrogen produced significantly greater levels of immunoglobulin. These data would suggest a potential role for E-2 in modulating immunoglobulin production that may involve estrogen interaction with both ER isoforms. The latter conclusion is further supported by the observations that αERKO chimeric mice treated with E-2 produced greater amounts of anti-single stranded DNA immunoglobulin when compared to cells from WT mice treated with E-2, as well as the fact that cells from βERKO mice treated with oil produced higher levels of immunoglobulin compared to WT cells treated with oil. These results suggest that ER-β expression might have an up-regulating effect on E-2 mediated anti-single stranded DNA immunoglobulin production, while the presence of ER-α expression would have a down-regulating effect.

LPS-induced anti-single stranded DNA antibody production was increased in both control and E-2 treated mice in all of the ERKO chimeric mice when
compared to WT mice. This supports the idea that both ER-α and ER-β are involved in anti-single stranded DNA immunoglobulin modulation by estrogen. In the presence of Id\textsuperscript{LN}F\textsubscript{1} antigen (peptide 62-73), we found lower anti-single stranded antibody production by cells from WT mice treated with estrogen when compared to cells from WT mice treated with oil, suggesting that E-2 may suppress the pathogenic B cell response to the antigen. We found that in the presence of Id\textsuperscript{LN}F\textsubscript{1} antigen, as in the absence of it, there was a significant increase in antibody production in cells from αERKO mice treated with estrogen compared to E-2 cells from WT mice. However, no significant differences were found when βERKO chimeric mice were compared to WT, suggesting that ER-β expression was important in this response.

Previous studies have reported that the production of anti-double-stranded DNA antibody is a specific biomarker for SLE in some patients (26). When cells were cultured in media alone, we found a significant increase in production of anti-DNA specific immunoglobulin by cells from αERKO mice treated with oil when compared to cells from WT mice treated with oil, suggestive of the role of ER-β expression in production of this antibody. No differences were found with regard to E-2 treatment. However, LPS-induced anti-DNA specific immunoglobulin production was increased when cells from βERKO E-2 mice were compared to βERKO control mice, suggesting that in mitogen-induced immunoglobulin production, ER-α may be involved.

In the presence of Id\textsuperscript{LN}F\textsubscript{1} antigen, as expected from previous studies (28), we found an increase in immunoglobulin production by cells from Wild Type
mice after E-2 administration compared to control. This result points to a role of E-2 in modulating pathogenic autoantibody production. We also found that anti-double stranded DNA antibody production by cells from \( \alpha \)ERKO estrogen treated mice was decreased compared to \( \alpha \)ERKO oil-treated cells. The same trend was seen in \( \beta \)ERKO mice, suggesting that both ER-\( \alpha \) and ER-\( \beta \) may be important in this response. Significantly greater levels of total immunoglobulin was produced by cells from estrogen treated \( \alpha \)ERKO and \( \beta \)ERKO mice compared to WT mice treated with estrogen. These data taken together suggest that ER-\( \alpha \) and ER-\( \beta \) may modulate pathogenic autoantibody-producing B cell responses. Our data also showed that compared to the cells form oil-treated WT mice, cells from oil-treated \( \alpha \beta \)ERKO produced less ani-DNA antibody while the opposite was seen when estrogen treated cells from these two groups were compared. This further supports a role for estrogen in exacerbating the pathogenic B cell response.

In conclusion, our results provide further evidence supporting a critical role for E-2 in triggering and/or modulating the development of autoimmune lupus nephritis through its effect on cytokine production, since in general we saw an increase in the levels of T\(_{H2}\) cytokines with estrogen treatment, as well as an increase in levels of the T\(_{H1}\) cytokine IFN-\( \gamma \). Results from our study also provided evidence supporting a significant role for both ER-\( \alpha \) and ER-\( \beta \) in the development of the autoimmune disease cytokine and Ig profile, with ER-\( \alpha \) having a more significant effect. These findings support a role for estrogen and ER, and may, in part, explain the increased incidence of autoimmune diseases in females. Given our findings, the use of estrogen in contraceptives, hormone replacement therapy and dietary supplements should continue to be evaluated.
REFERENCES


Approximately 5 percent of the population in Western countries is affected by various autoimmune diseases (1). The immune system is essential in the recognition of and distinction between self and non-self entities. A typical immune system only generates a humoral (antibody-specific) and/or cellular response to non-self such as pathogens, bacteria, antigens etc. However, in some cases the immune system may recognize an individual’s own cells and proteins as foreign, causing an autoimmune response. Contrary to what might have been thought initially, autoimmunity is a normal process that is important in clearing the body of certain pathological self cells (such as the prevention of tumor metastasis) and cellular debris.

According to Davidson and Diamond (2001), autoimmune disease is defined as a clinical syndrome caused by the activation of T or B cells, or both, in the absence of an ongoing infection or other discernible cause. In other words, autoimmune disease is caused by the failure of the proper functioning of the body’s defense mechanisms, or a lack of tolerance of one’s own cells. There exist two categories of autoimmune diseases. Organ-specific autoimmune diseases, such as type 1 diabetes mellitus, are localized in specific tissues in the body. Systemic diseases, such as systemic lupus erythematosus (SLE), have a widespread effect on several tissue systems in the organism. In general, these diseases are caused by, either changes in selection and regulation of T cells or B cells, or aberrant reactions to particular self or foreign antibodies (1).
Various studies have noted a significant sex difference in immune responses to antigens. It appears that females in many species are seen to have heightened immune responses and are more susceptible to autoimmunity (2, 3). Particularly, in Systemic Lupus Erythematosus (SLE), we see a 9:1 ratio in predominance of the disease in women as compared to men (6, 7). There is also evidence of the correlation between steroid hormone levels and the changes in clinical manifestation of SLE, which is additionally enhanced during pregnancy and the postpartum period (8-10). Male and female patients with SLE often have abnormally high levels of estrogen (hyperestrogenic) and/or abnormally low levels of androgen (testosterone) hypoandrogenic (11-13). This correlation is also reinforced by the higher incidence of the disease in patients with Klinefelter’s syndrome (14, 15), which is 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 of estrogen in the gender-specific modulation of immune responses involved in autoimmunity. However, the ways in which estrogen modulates immune responses are still not yet well understood. Key to this knowledge would be an understanding of how estrogen interacts with its receptors. Two forms of estrogen receptors (ER) have been identified, ERα and ERβ. Each of these receptor subtypes or isoforms have been found to be normally expressed in immune cells (16-21). The purpose of this research project was to further investigate the influence of estrogen on autoimmunity, specifically in SLE. It has been suggested that estrogen may act on helper T lymphocytes (T_H), which are an essential part of the normal immune
response to antigens. Two types of $T_H$ cells are known: $T_{H1}$ and $T_{H2}$ cells, which are distinguished by the specific cytokines they produce. $T_{H1}$-type cytokines lead to pro-inflammatory responses and while they participate in the initiation of autoimmune responses, they are protective later in disease. $T_{H2}$-type cytokines are important in immunoglobulin production and predominate in later disease (22).

Experiments on male estrogen receptor knockout mice that were administered estrogen showed that the shifts from $T_{H2}$ to $T_{H1}$ responses were protective while a $T_{H2}$ response exacerbated autoimmune disease (16-21). These results suggested that estrogen has a differential effect in promoting $T_{H1}$ and $T_{H2}$ responses, as well as raise the question of the importance of the ER in inducing these responses. Consequently, the hypothesis that was tested in this project was that exposure to estrogen will lead to modulation of immune cells though an interaction with the specific ERs that they express. This hypothesis was tested by exposing male ER$\alpha$, ER$\beta$ and ER$\alpha\beta$ knockout chimeric mice to chronic doses of estrogen with the aims of identifying the consequences of estrogen exposure in the numbers and functions of immune cells, to determine whether these modulations resulted from the interaction of estrogen and its receptors.

**Methods**

Thirty C57 Black 6 (C57BL/6) male mice were irradiated with a total of 1100 rads. The day after irradiation, the mice were reconstituted with cells from donor bone marrow prepared from either of four types of male estrogen receptor (ER)-deficient mice: wild type (WT), alpha knockout (aERKO), beta knockout
(βERKO) or double knockout (αβERKO or KO/KO) for the ER. Donor cells were prepared from 2-3 mice of the appropriate ER genotype, which were euthanized by CO₂ asphyxiation. Their femurs and tibias were removed and all muscle was removed from the bones. The ends were cut off and the bone marrow was flushed out. The marrow was flushed into another dish containing and the clumps were broken. The mixture was then spun down and suspended in media.

Groups of seven irradiated recipient mice were reconstituted the appropriate donor cell type. Two mice served as controls for irradiation and were not reconstituted and within ten days of irradiation, these mice were moribund. This confirmed that the lethal irradiation was successful.

Seven weeks after reconstitution, four mice from each group were injected estrogen in oil and three mice were injected with oil vehicle by subcutaneous injection between the shoulder blades. These injections were performed subsequently every month (4 weeks) for 5 months. Body weights were measured before each injection. In order to study the effect of this estrogen treatment on immunity and specifically the production of cytokines and immunoglobulin, the mice were euthanized and their cells were harvested. We performed an enzyme-linked immunosorbent assay (ELISA) on these cells suspensions.

**Project Significance**

As aforementioned, much has yet to be understood about the mechanisms through with the sex hormone modulates autoimmune disease. Various autoimmune diseases, including lupus and rheumatoid arthritis have been shown to modulate in their clinical manifestation according to levels of estrogen. In fact, in addition to the gender
bias, lupus is also seen to manifest itself predominantly in women in child-bearing age. In light of this, the current project provides further evidence that estrogen plays a critical role in triggering the development of autoimmune lupus nephritis through its effect on cytokine production, since in general we saw an increase in T\textsubscript{H}2 cytokines, leading to activation of B cells and the production of auto-antibodies, with estrogen treatment. We also observed an increase in T\textsubscript{H}1 cytokine IFN-\(\gamma\) which is thought to play an important role in initiating autoimmune disease. Results from estrogen receptor knockout mice also provided evidence that both estrogen receptor subtypes, ER-\(\alpha\) and ER-\(\beta\), play significant roles in the autoimmunity disease cytokine and immunoglobulin profile. Our results also suggested that ER-\(\alpha\) might have a more significant effect.

These findings support a role for estrogen and ER in the increased incidence of autoimmune diseases in females. This study is significant in light of the growing use of estrogen in contraceptive, hormone replacement therapy and various dietary supplements nowadays. It is important for individuals who have been diagnosed with autoimmune disease to learn more about their condition and the mechanisms involved in order to better manage and cope with it. Furthermore, the findings from this project and others have been conducted in the field serve to open new avenues for the devising therapeutic agent that will specifically target the different mechanisms involved in autoimmune disease manifestation and progression.