Summer 8-14-2015

Impact of Estrogen Receptor Alpha on SLE1-Induced Loss of Tolerance

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IMPACT OF ESTROGEN RECEPTOR ALPHA ON *SLE1*-INDUCED LOSS OF TOLERANCE

by

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A DISSERTATION

Submitted to the graduate faculty at the University of Nebraska Medical Center
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Genetics, Cell Biology, and Anatomy

Under the supervision of Karen A. Gould, PhD

OMAHA, NEBRASKA

JUNE 2015

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The autoimmune disease lupus shows a significant female sex bias. This sex bias may be due to the ability of estrogens to promote loss of tolerance to chromatin, the initial loss of tolerance event in lupus. Previously, we demonstrated that the ability of estrogens to promote lupus in (NZBxNZW) F1 mice is dependent on signaling via estrogen receptor alpha (ERα). The *Sle1* lupus susceptibility allele controls loss of tolerance to chromatin, and C57BL/6 (B6) mice carrying the *Sle1* lose tolerance and develop anti-chromatin autoantibodies and spontaneously activated immune cells. Loss of tolerance occurs earlier and with a higher penetrance in B6.*Sle1* females than in males, illustrating a sex bias, although the specific impact of estrogens/ERα signaling in this phenotype has not been described. Furthermore, nothing is known about the impact of sex and estrogen signaling on *Sle1*-induced immune cell hyperactivation. Here, we show that a deficiency of either estrogens or ERα attenuates loss of tolerance and autoantibody development in B6.*Sle1* female mice. Additionally, we found *Sle1*-associated immune cell hyperactivation shows a female sex bias, and furthermore, that ERα deficiency diminishes *Sle1*-associated immune cell hyperactivation in B6.*Sle1* females. Furthermore, our preliminary data indicate that the actions of *Sle1* subloci *Sle1a* and *Sle1b* are modulated to ERα signaling. Finally, we demonstrate that ERα may influence the *Sle1* phenotype in females by modulating the expression of *Pbx1*, a gene that lies within the *Sle1* interval. Altogether, estrogen signaling via ERα controls the female sex bias in the *Sle1* phenotype.

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ACKNOWLEDGEMENTS

First and foremost, I need to thank my husband, Collin. Without his patience, encouragement, and selflessness, I would not have reached this point in my career. From volunteering to work weekend overnight shifts to allow my sometimes ridiculous work schedule, to doing more than his fair share of parenting and maintaining our household, he is the reason I've survived all that has been the last five years of graduate school. Thank you, Collin. I love you and appreciate you more than I could ever say.

To my son, Graham. You are the reason I continue to do my best. I am so blessed to be your mom, and I love you with all of my heart.

To my labmates – Jenny, Dana, Kim, Karla, and Richie. Thank you for your help with my project, and your critiques and advice along the way. I couldn't have asked for a better group of people to share all that is graduate school with, and I wish you all the very best in whatever lies ahead.

To my supervisory committee members – Dr. Gould, Dr. Joshi, Dr. Keim, Dr. McDonald, and Dr. Thiele. Thank you for your guidance and advice these past five years. I have learned more from each of you than you'll ever know. Thanks for sticking with me, for entrusting in me your ideas, and for challenging me to think differently.

To my teaching mentor, Ryan. Thank you for changing my mind. You are the reason I stuck with this program, and you are truly the reason I am the teacher that I am today. Thank you for your 'teaching points', for your patience, and for the ridiculous amount of coffee. I will always admire your ability to connect to students, to teach by inspiring critical thinking, and for seeing the potential in me to do the same. I could not have imagined a better teaching mentor, and I hope I make you proud in my career as a teacher. I owe you.

And to my research mentor, Karen, who may deserve more credit for my successes in graduate school than even I do. Thank you for your patience, your ability to embrace my career goals, and most importantly, for sharing with me your time and providing guidance to help me succeed. Not only did you allow me to pursue an academic career, you helped to create an Anatomy Teaching Track Program to ensure that I received the training I need to be both a researcher and an educator. I cannot thank you enough for your dedication to my success, and your attention, advice, and encouragement along the way. I am so very lucky to have had you as my mentor. Thank you, thank you, thank you.

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CHAPTER I:

INTRODUCTION

1.1 *Lupus in human patients*

Lupus is a chronic autoimmune disease characterized by a loss of tolerance to nuclear antigens, leading to the production of anti-nuclear autoantibodies. The most common form of lupus is systemic lupus erythematosus, or SLE, in which the inflammation and tissue damage occurs systemically. Inflammation due to this autoimmune disease can lead to vasculitis, synovitis, pericarditis, and pleuritis. Additionally, SLE may present as inflammation in the kidneys, or lupus-induced glomerulonephritis, which can result in severe kidney damage. This potentially fatal complication occurs in about 50% of lupus patients (Petri et al. 1991). While other forms of lupus exist (i.e. discoid lupus erythematosus, drug-induced lupus, etc.), SLE accounts for more than 70% of lupus diagnoses (LFA 2008).

SLE (referred to as 'lupus' hereafter) results from an initial breach in tolerance that leads to the production of autoreactive IgG antibodies. These autoantibodies form complexes with nuclear antigens, including double-stranded DNA (dsDNA) and chromatin, and then deposit into various tissues. The deposition of these anti-nuclear IgG immune complexes into tissues induces an inflammatory response, leading to the recruitment of pro-inflammatory cells which amplify and sustain the immune response. Locally produced cytokines then perpetuate the inflammatory response by hyperactivating pro-inflammatory cells, including neutrophils and lymphocytes. The inability of the immune system to combat chronic invasion and hyperactivation of immune cells leads to chronic inflammation and subsequent tissue damage.

Lupus development is multifactorial, with evidence supporting both genetic predisposition and environmental triggers contributing to the pathogenesis of disease. The concordance rate in human lupus is high in monozygotic twins (25-69%), and the high heritability

between first degree relatives indicates that lupus has a significant genetic basis (for a review, see Nguyen et al. 1995). In addition, environmental triggers such as ultraviolet light, hormones, and certain medications contribute to disease pathogenesis and progression, indicating that lupus is a complex disease (Kotzin, 1996). Patients are diagnosed with lupus based on cumulative presentation of both symptoms and laboratory abnormalities, according to clinical and immunological criteria used in Systemic Lupus International Collaborating Clinics classification system (SLICC, 2012). These symptoms include acute cutaneous lupus presentation (i.e. malar rash), mucosal ulcers, synovitis, pericarditis, neurologic symptoms, and lupus nephritis. Laboratory abnormalities include elevated anti-nuclear autoantibody values and anemia. The clinical presentation is cumulative, although presentation of biopsy-indicated lupus nephritis in the presence of anti-nuclear antibodies in the absence of additional symptoms is regarded as sufficient for lupus diagnosis (Isenberg et al. 2014).

The Lupus Foundation of America estimates the current prevalence of lupus may be nearly 1.5 million in the U.S., with a significant African American race bias demonstrated by an incidence of nearly 1 in 250 African American women. An estimated 90-95% of lupus patients are females, indicating a significant female sex bias. Furthermore, this female sex bias is specific to women of childbearing age; the female to male ratio in the juvenile and elderly populations is much less remarkable (Isenberg et al. 2014). The influence of sex and stage in life on disease occurrence is thought to reflect the impact of female sex hormones on the immune system. In fact, several decades of research have indicated that female sex hormones, specifically estrogen, significantly impact lupus development and progression (Steinberg and Steinberg, 1985; Barrett et al 1986; Ostensen, 1999). Although evidence consistently supports a link between estrogens and lupus risk, very little is understood on a cellular or molecular level about the basis of this effect.

Because the etiology of lupus has proven to be quite complex, the current treatment options for those affected with lupus are ineffective and often induce significant adverse effects. Compared to the 1950s, when the 4-year survival was just 50%, overall mortality rates have drastically decreased due to the introduction of key immunosuppressive drugs such as cyclophosphamide (Monneaux and Muller, 2009). Today, antimalarials, corticosteroids, and cytotoxic drugs are the classic medications used in lupus treatment. However, these treatment options induce significant adverse reactions that severely outweigh the clinical benefits, as general immunosuppression often leads to recurrent infections and in some cases, malignant diseases. For these reasons, intense research over the last decade in particular has led to the development of more targeted approaches to treat lupus patients without the complications of current treatments. The majority of these therapies focus on targeting B cells, as one hallmark of lupus is the presence of autoantibodies produced by activated B cells. Rituximab, a monoclonal anti-CD20 antibody first approved in 1997 for the treatment of non-Hodgkin lymphoma, showed initial promise in mouse studies as a B-cell targeted therapy for lupus, however, results from most clinical studies indicated no statistically significant difference in disease parameters between rituximab and placebo groups (Harvey and Gordon, 2013). It should be noted, however, that rituximab has shown some clinical effectiveness when used as a last-line effort in treatment of patients with severe lupus that was unresponsive to traditional therapies (Dall'Era and Chakravarty, 2011).

Another B cell-targeted lupus treatment, belimumab, has shown similar, disappointing clinical effectiveness in human trials. As the first drug in 50 years to be approved specifically for the treatment of lupus, belimumab initially showed promise in reducing autoantibody production and disease flares in patients (Dall'Era and Chakravarty, 2011). However, more recent studies have determined belimumab to be ineffective in treating African American lupus patients, the most affected racial background, and no studies to date have investigated the effectiveness of

belimumab on lupus patients exhibited severe complications of lupus, such as neurological manifestations or lupus nephritis. In summary, while recent efforts in identifying more targeted treatments for lupus have led to somewhat disappointing clinical outcomes, the more specific treatment options have allowed lupus patients to decrease usage of immunosuppressants and corticosteroids, at least minimizing the severe adverse effects associated with long-term use of these treatments. However, continued research in identifying the specific cell types or pathways altered in lupus will shed light on potential therapeutic targets, and should highlight new treatment options for those affect by lupus without the adverse effects of conventional treatments.

1.2 The study of lupus using murine models

Lupus susceptibility in human patients is inherited in a polygenic manner, although the specific genes, or genomic intervals, underlying the disease have yet to be fully characterized. In order to better characterize the genetic factors contributing to disease susceptibility, several inbred mouse strains that spontaneously develop a lupus-like disease have served as experimental models. Much of what is known about the impact of estrogen on lupus has been demonstrated in three autoimmune mouse strains: MRL.lpr, BXSB, and (NZBxNZW) F1 strains. Each of these strains spontaneously develops loss of tolerance leading to significant autoantibody production and immune complex-mediated glomerulonephritis (Andrews et al. 1978; Wakeland et al. 2001). While each lupus-prone model has strengths and weaknesses in their emulation of human disease, all three strains have greatly aided to the understanding of lupus development and progression albeit via different genetic mechanisms. The MRL.lpr mouse model develops systemic autoimmunity and glomerulonephritis, however, the strain is dependent on the lpr mutation, which affects FAS-mediated apoptosis. Because mutations in the FAS pathway are not associated with lupus in humans specifically, the MRL.lpr strain may not the most accurate model to use in understanding the genetic mechanisms underlying lupus (Wakeland et al. 2001). The BXSB mice carry the *Y chromosome autoimmune accelerator* (*yaa*) gene on the autoimmune-prone BXSB

background (Wakeland et al. 2001). Because this strain is dependent on a gene found on the Y chromosome, only males can be used for experimental purposes. While BXSB male mice also develop severe systemic autoimmunity, this strain cannot emulate the female sex bias associated with lupus in humans, and therefore cannot be used to study the impact of estrogens/ERα in depth.

Finally, the (NZBxNZW) F1 strain has been characterized genetically and phenotypically in great detail, and has served as a classic model of lupus for decades. This hybrid strain develops a disease very similar to human lupus, involving the production of autoantibodies against nuclear antigens culminating in kidney failure due to the immune complex-mediated glomerulonephritis (Howie and Helyer, 1968; Andrews et al. 1978; Morel et al. 1997). Interestingly, the (NZBxNZW) F1 strain also recapitulates the female sex bias seen in human lupus patients, in that the incidence of disease is higher and survival time is reduced in female (NZBxNZW) F1 mice compared to male counterparts (Andrews et al. 1978). Given the similarity in disease presentation to the lupus phenotype in humans, the (NZBxNZW) F1 strain is most often used to further delineate the mechanisms by which estrogens may promote lupus.

1.3 Hormones and the immune system

Given the female sex bias in lupus, the impact of sex hormones, specifically estrogens, has been a focus of research for several decades. Although studies have consistently demonstrated a role of estrogens in promoting a loss of tolerance, very little is known about the mechanisms through which this occurs. Estrogens have been clearly demonstrated to play a role in both normal immune responses and autoimmunity. In general, the immune system of females is more robust, with females producing more total immunoglobulins in response to infection or immunization, with this difference first appearing at the onset of puberty and persisting throughout female reproductive years (Beeson, 1994). In human patients with lupus, estrogens are thought to influence disease flares during both the menstrual cycle and pregnancy (Lahita, 1999;

Cunningham and Gilkeson, 2011). Furthermore, exogenous estrogens, such as those administered in the form of oral contraceptives, can also increase lupus risk (Petri et al. 2001). Lupus risk is also increased in men with Klinefelter's syndrome, a genetic condition in males associated with increased endogenous estrogen production (Lahita and Bradlow, 1987). Finally, both men and women with lupus have been shown to have abnormal estrogen metabolism, leading to increased serum estrogen metabolites with estrogenic activity (Lahita et al. 1979; Lahita et al. 1981).

Production of mature $CD4^+$ and $CD8^+$ T cells in the thymus has been shown to be dependent on estrogen (Pernis, 2007). Development of these cells in the thymus involves the migration of progenitor cells from the bone marrow, acquisition of CD4 and CD8 markers, and then eventual maturation into single positive $CD4^+$ or $CD8^+$ T cells. While in the thymus, T cells undergo both positive and negative selection processes that are essential for the prevention of autoreactivity. Estrogen has been shown to affect thymocyte development by decreasing the migration of precursors from the bone marrow to the thymus, and by depleting the early progenitor T cell population in the thymus (Zoller et al. 2006). Furthermore, estrogen modulates the expression of IL-7, which plays a role in T lymphopoiesis, and thereby influences thymocyte development (Ryan et al. 2005). While the effects of estrogen on mature T cells have proven to be complex and have yet to be fully understood, studies have nonetheless identified broad effects of estrogen on the action of T cells in immune responses. Estrogens have been shown to influence both $CD4^+$ Th1 and Th2 responses, seemingly at differing levels of estrogen, where low levels of estrogen are associated with enhanced $CD4^+$ Th1 responses and increased cell-mediated immunity, and high levels of estrogen instead result in increased $CD4^+$ Th2 responses. Additionally, estrogen is thought to play a role in the production of T regulatory cells, although relatively little is known about the role of estrogen on this cell population (Cunningham and Gilkeson, 2011). Tai et al. found that physiologic doses of estrogen increases the expression of FoxP3, and plays a role in the conversion of T cells from $CD4^+CD25^-$ to $CD4^+CD25^+$ T cells

(2007). Furthermore, this Treg conversion was inhibited by an estrogen receptor (ER) antagonist, indicating a vital role for estrogens in this process (Tai et al. 2007).

Estrogens also regulate B cell lymphopoiesis and maturation. B cell production is decreased in response to estrogen, while ovariectomy results in increased B lymphopoeisis (Medina et al. 1993; Masuzawa et al. 1994). Estrogens enhance production and secretion of Th2 associated cytokines, such as IL-4, IL-5, IL-6, and IL-10, which are potent stimulators of B cell maturation and differentiation into plasma cells, thereby influencing antibody production (Kassi and Moutsatsou, 2010). Furthermore, estrogen has been shown to upregulate expression of anti-apoptotic *bcl-2* in naïve B cells, which is thought to block tolerance induction and potentiate autoreactive T and B cells (Kassi and Moutsatsou, 2010). In summary, although much is still unknown about the direct and indirect influences of estrogen on lymphocytes, it is clear that estrogen has a dramatic impact on the immune system.

Androgens, including testosterone, have also been shown to play a role in immune regulation, however, unlike estrogen, they are thought to act as natural immune suppressors by downregulating T and B cell interactions (Shames, 2002). Administration of testosterone in mice has been shown to decrease thymic mass and decrease the B cell population size, whereas castration of mice leads to an expanded B cell population and increased autoantibody production (Viselli et al. 1997). Although the protective effects of testosterone may play a role in the decreased occurrence of autoimmune diseases in males compared to females, the evidence suggests that it is more likely the removal of estrogenic action rather than a compensatory increase in serum testosterone that protects male and ovariectomized female mice from developing lupus (Bynote et al. 2008). It has been shown previously by Roubinian and colleagues (1978) that administration of exogenous testosterone to ovariectomized female (NZBxNZW) F1 mice beginning at an early age prolongs survival of these mice, in part by decreasing production of pathogenic IgG autoantibody production. However, intact (NZBxNZW) F1 mice with

disrupted estrogen signaling pathways were also found to be protected from IgG autoantibody production, even without exogenous testosterone administration, indicating that it is likely the lack of estrogen signaling, as opposed to increased serum testosterone, that is responsible for the prolonged survival of these mice (Bynote et al. 2008).

Because estrogen has been thought to promote autoimmunity in both human lupus patients as well as experimental mouse models of lupus, recent studies have investigated the impact of disrupting estrogen signaling on lupus development and progression. One study, by Wu and colleagues, used tamoxifen to act as an estrogen antagonist in the (NZBxNZW) F1 strain. It was determined by this study that (NZBxNZW) F1 mice who were treated with tamoxifen had a prolonged lifespan compared to controls, in part due to reduced renal complications (Wu et al. 2000). However, in human patient studies using tamoxifen to antagonize estrogen signaling in lupus, it was determined that tamoxifen was ineffective at alleviating disease, as no patients improved while undergoing treatment, and 2 patients actually suffered from increased disease symptoms. This is thought to be due to differences between mice and humans in their response to tamoxifen, as this drug is known to function as either an estrogen signaling agonist or antagonist depending on the tissue. While this study did not have a large sample size to evaluate the effectiveness of tamoxifen treatment in human patients, it must be noted that the typical lupus patient, a female of childbearing age, is unlikely to use a broad, systemic anti-estrogen treatment as a first line of defense against disease progression and therefore it is difficult to achieve a significant sample number in these conditions.

In addition to tamoxifen, two other anti-estrogenic medications have been developed in recent years, and clinically evaluated in female lupus patients – raloxifene and fulvestrant. In a study evaluating the usage of raloxifene on lupus in (NZBxNZW) F1 mice, raloxifene treatment significantly suppressed estrogen-mediated effects on the survival and maturation of activated B cells, thereby reducing autoantibody production (Zhang et al. 2010). In human clinical trials,

raloxifene was shown to have fewer adverse effects compared to tamoxifen, but was unable to significant improve disease parameters in female lupus patients. However, raloxifene was well-tolerated and did not induce disease flares, so it remains a treatment option in postmenopausal women to combat the osteoporosis frequently associated with both aging and longterm corticosteroid use in these patients (Mok et al. 2005). Finally, fulvestrant has shown much promise as a selective estrogen antagonist in the treatment of lupus. Unlike, tamoxifen, which acts as both an estrogen signaling agonist or antagonist depending on the target, fulvestrant has no agonistic function. In a study of 16 premenopausal lupus patients, treatment with fulvestrant was demonstrated to significantly improve SLE Disease Activity Index in these patients (Abdou et al. 2008). Therefore, unlike the previously described anti-estrogen treatment options for lupus patients, fulvestrant shows promise as a safe and effective therapeutic options for the management of lupus, although more studies with larger sample sizes are warranted.

1.4 Estrogen and estrogen receptor signaling

As the works cited previously illustrate, estrogen plays a vital role in the development and maintenance of a functional immune system. Estrogen modulates the production of T and B lymphocytes from progenitor cells, aids in the maturation of these cells, and regulates the cytokine production and cytokine receptor expression (Karpuzoglu and Zouali, 2009; Pernis, 2007; Cunningham and Gilkeson, 2011). Previous studies have demonstrated that estrogen modulates B cell tolerance and CD4+ T-helper cell function, and alterations in estrogen signaling through the use of estrogen receptor antagonists or mouse models with disrupted estrogen signaling have been shown to influence lupus development and progression (Wu et al. 2000; Li and McMurray, 2007; Svenson JL et al. 2008). In recent studies involving 3 different autoimmune-prone mouse strains ((NZBxNZW) F1, MRL.lpr, and NZM2410), lack of estrogen signaling attenuated disease and prolonged survival of the mice, in part by decreasing autoantibody production and attenuating glomerulonephritis (Bynote et al. 2008; Svenson et al.

2008; Cunningham and Gilkeson 2011). Altogether, results from studies using autoimmune-prone mouse strains strongly suggest that estrogens are responsible for the sexual dimorphism observed in autoimmune diseases such as lupus.

Although previous studies strongly suggest a major role of estrogen signaling in lupus development, relatively little is known about the mechanism by which estrogens promote autoimmunity. Importantly, the effect of estrogens is mediated via estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), both of which are expressed on most immune cells. These receptors are encoded by two different genes located on two different chromosomes, and despite having a considerable degree of sequence similarity, the amino termini of the two estrogen receptors is poorly conserved (Kuiper et al. 1997). ERα and ERβ are differentially expressed in many cells and tissues, and interact with an overlapping set of cofactors. Both estrogen receptors are nuclear hormone receptors that act as ligand-dependent transcription factors (Cunningham and Gilkeson, 2011). Classically, the binding of estrogen to its receptor causes a conformation change in the receptor, freeing it from its association in an inactive complex. ERs then form dimers, either homo- or hetero-, and subsequently translocate to the nucleus, where it may bind either directly to estrogen response elements in gene promoters or serve as cofactors with other transcription factors (Cunningham and Gilkeson, 2011). In addition, non-classical ERα signaling occurs via cell surface receptors to activate signal transduction pathways independent of genomic estrogen response elements. Together, both classical and non-classical estrogen receptor signaling are proposed to play a role in modulating lymphocyte cytokine production, activation of effector cells, and likely the loss of tolerance seen in lupus (Cunningham and Gilkeson, 2011).

It should be noted that both ERα and ERβ have splice variants due to alternative splicing of each transcript. Full-length, wildtype ERα is also referred to as ERα66, in reference to its size (66 kDa). In addition, alternative splicing produces two additional ERα isoforms, ERα36 and ERα46, which differ from ERα66 in the amino-terminal end. Interestingly, ERα46 is thought to

act at the cell membrane in immune cells, although very little is known about the function of ERα46 in these cells, specifically (Pierdominici et al. 2010). Furthermore, nothing is known to date about the function, if any, of ERα36 in immune cells. Because ERα36 and ERα46 differ from full-length ERα66 at the transcriptional regulation domain, it is likely that they differ significantly in binding partners (Taylor et al. 2010). However, further investigation is warranted in the specific functions of each ERα isoform in immune cell function and signaling.

Because estrogen signaling occurs via two unique estrogen receptors, ERα and ERβ, recent studies have aimed to identify the specific roles of each estrogen receptor in promoting autoimmune disease in the lupus- prone mice. By using estrogen receptor subtype-specific agonists to selectively activate ERα and ERβ individually, Li and McMurray (2007) demonstrated that the estrogen-mediated exacerbation of the lupus phenotype in (NZBxNZW) F1 mice is predominantly via ERα. Furthermore, previous work by our lab using a mouse strain carrying an *ERα* knock-out allele (referred to as ERαKO hereafter) crossed to (NZBxNZW) F1 autoimmune mice demonstrated that *ERα* deficiency attenuates lupus (Bynote et al. 2008). The ERαKO mouse strain, first developed by Lubahn et al. (1993), was made by insertional deletion of exon 2 of the *ESR1* gene using a neomycin cassette. This insertion disrupts the reading frame of the *ERα* transcript, preventing a functional receptor from being produced. Although the homozygous ERαKO mice produced are sterile, both the male and female ERαKO mice survive to adulthood and show no gross abnormalities (Lubahn et al. 1993). Importantly, it is noteworthy that the specific deletion in Lubahn et al's ERαKO strain involves an exon not coded by the shortened isoforms of ERα, ERα46 and ERα36. Because it is predicted that ERα46 may localize to the cell membrane, the studies using mice generated with the ERαKO strain created by Dr. Lubahn and colleagues cannot eliminate the possible actions of ERα46 in membrane-associated signaling cascades. However, because no studies to date have investigated the functional effects

of ERα46 in immune cells specifically, further investigation is warranted to understand the mechanism of ERα46 action.

Previous work in our lab investigated the impact of global ERα66-deficiency in lupus-prone (NZBxNZW) F1 mice, through which we demonstrated that *ERα* deficiency attenuates loss of tolerance and the female sex bias in these mice (Bynote et al. 2008). Our study was the first to demonstrate that ERα66 (hereafter referred to as ERα) deficiency inhibited lupus development in (NZBxNZW) F1 females, in part by reducing anti-nuclear antibody production (Bynote et al. 2008). Importantly, we found that *ERα* deficiency is associated with reduced development of anti-H2A/H2B/DNA antibodies in (NZBxNZW) F1 females, indicating that ERα promotes the initial loss of tolerance leading to lupus development. Altogether, we demonstrated that ERα signaling plays a crucial role in stimulating loss of tolerance to chromatin, and is responsible for the female sex bias in lupus development and progression in (NZBxNZW) F1 mice (Bynote et al. 2008).

Together, the use of ERα-specific agonists and elimination of ERα signaling clearly illustrate the crucial role of ERα in loss of tolerance. In contrast, the use of ERβ agonists illustrated that selective activation of ERβ has relatively little effect on lupus development and progression (Li and McMurray, 2007). These data suggest a crucial role for ERα in promoting the female-biased loss of tolerance displayed by the (NZBxNZW) F1 mice. Furthermore, it has been demonstrated that the impact of estrogens on the regulation of B cell immunoglobulin production is dependent upon ERα, and not ERβ, signaling (Erlandsson et al. 2003). In addition, while both ERα and ERβ play a role in B cell maturation, it has been demonstrated that can ERα but not ERβ can promote the survival of autoreactive B cells and therefore the production of autoantibodies (Hill et al. 2011). Altogether, work by our lab and others strongly suggests that estrogen signaling via ERα promotes loss of tolerance leading to lupus.

1.5 Loss of tolerance

An initial breach in tolerance is necessary for the development of lupus. Early work by Burlingame et al. (1994) identified that in both the MRL.lpr and BXSB strains of lupus-prone mice, the initial autoantibodies produced are reactive specifically to nuclear antigens. Furthermore, this work demonstrated that the loss of tolerance in these autoimmune strains resulted first in the development of anti-chromatin autoantibodies, specifically reactive to the H2A/H2B/dsDNA subnucleosome, the most exposed component of chromatin (Burlingame et al, 1994; Mohan et al, 1998). In most of these mice, epitope spreading occurred later in life leading to the production of anti-dsDNA autoantibodies (Burlingame et al. 1994). Additionally, it was determined that the patterns of autoantibody reactivity to chromatin in human SLE patients were remarkably similar to those of the MRL.lpr and BXSB mice (Burlingame et al. 1994). However, neither MRL.lpr nor BXSB strains recapitulate the female sex bias seen in both human lupus and (NZBxNZW) F1 mice, so the effect of sex and specifically, estrogens, was not determined in the development or progression of loss of tolerance in the lupus-prone mice described by Burlingame et al (1994).

Although much is still unknown about the cellular and molecular mechanisms by which loss of tolerance occurs, and furthermore, why loss of tolerance is a female-biased occurrence, work by Bynoe et al. (2000) has shed light on the mechanisms by which estrogen may alter the tolerance threshold in immune cells. The generation of transgenic mice that harbored the γ2b heavy chain of the R4A anti-DNA antibody, in which the heavy chain can associate with various light chains to generate antibodies with varying reactivity to DNA allowed Bynoe and colleagues to investigate the role of estrogen in modulating tolerance induction of B cells, and at which stage of development this occurs (Bynoe et al. 2000). It was determined that sustained, high physiologic doses of exogenous estradiol can block B cell tolerance induction and induce an autoimmune phenotype in this transgenic strain, demonstrating that estrogen signaling plays a

crucial role in loss of tolerance. Additionally, estrogen has been shown to impair negative selection checkpoints in autoreactive B cells (Grimaldi et al. 2006). Importantly, the effect of estrogen signaling on BCR signaling and increased survival of autoreactive B cells is mediated by ERα, not ERβ, in part by an upregulation of anti-apoptotic *Bcl-2* and BCR signaling-associated *cd22* (Hill et al. 2011). However, because these studies involved continuous administration of high doses of estrogen in transgenic mice of a non-autoimmune background, more research is necessary to determine the impact of endogenous estrogen on loss of tolerance in lupus-prone mice.

1.6 Lupus susceptibility locus Sle1

As a well-characterized mouse model of the lupus phenotype, the (NZBxNZW) F1 strain has been further dissected to identify the specific NZB- and NZW-derived loci that contribute to disease in this strain. To do so, Rudofsky and colleagues (1993) produced a collection of strains derived from an intercross of NZB and NZW, to generate a strain called NZM2410, which develops acute, highly penetrant lupus. NZM2410 male and female mice demonstrate splenomegaly, a significant expansion of hyperactive immune cells, high-titered anti-nuclear antibodies, and early-onset fatal glomerulonephritis (Mohan et al. 1999). Linkage analysis using the NZM2410 strain identified several loci that are strongly associated with lupus susceptibility, termed *Sle1*, *Sle2*, and *Sle3*, located on chromosomes 1, 4, and 7, respectively. (Morel et al. 1994). Each of these loci was identified as genomic intervals contributing to the lupus phenotype in the NZM2410, as determined by association with anti-dsDNA IgG production and GN susceptibility (Morel et al. 1994). However, the unique phenotype of each locus could not be determined by linkage analysis alone.

In order to understand the contribution of each of these three loci to the overall NZM2410 lupus phenotype, congenic strains were produced by transferring each of the loci onto a resistant, non-autoimmune C57BL/6 background to allow phenotypic analysis of *Sle1*, *Sle2*, and

Sle3 individually (Wakeland et al. 2001). *Sle2* was shown to lower the activation threshold of B cells causing general polyreactivity, and is associated with elevated IgM, but not IgG, autoantibodies (Morel et al. 1997). In contrast, *Sle3* causes T cell dysregulation, the production of both IgM and IgG autoantibodies against both nuclear and non-nuclear antigens, and is associated with the development of lupus nephritis (Morel et al. 1997). However, phenotypic analysis of B6.*Sle2* and B6.*Sle3* demonstrated that neither *Sle2* nor *Sle3* alone are able to induce the initial loss of tolerance necessary for lupus development, indicating that they may play more of a role in lupus progression by synergizing with other lupus susceptibility (i.e. *Sle1*) rather than initiating an autoimmune phenotype (Morel et al. 1997).

In contrast, phenotypic analysis of the B6.*Sle1* congenic strain revealed that *Sle1* alone is sufficient to cause the breach in tolerance and lead to the production of IgG autoantibodies with high specificity to chromatin (Wakeland et al. 2001). Importantly, *Sle1* has shown strong linkage in all genomic scans conducted in both NZM2410 and (NZBxNZW) F1 strains, as well as human linkage analysis, indicating that this genomic interval is consistently associated with autoimmunity (Morel et al. 2001). B6.*Sle1* mice develop autoantibodies specific to chromatin, and this phenotype is thought to be due to altered tolerance checkpoints rather than secondary to an increase in apoptosis and nonspecific generation of uncleared cellular debris (Mohan et al. 1998). Finally, B6.*Sle1* mice exhibit significantly greater total serum IgG levels, but not IgM levels, compared to controls, indicating that the immune response associated with *Sle1* is specific in the generation of anti-chromatin IgG autoantibodies (Mohan et al. 1999).

Additionally, B6.*Sle1* mice display spontaneous hyperactivity of both B and T cells (Mohan et al. 1998). Mixed bone marrow chimeras demonstrated that *Sle1* is functionally expressed in both T and B cells, suggesting that both cell types may contribute to immune cell dysregulation, and therefore autoantibody production, in this strain (Sobel et al. 2002). Importantly, the *Sle1*-induced T and B cell hyperactivation has been shown to be intrinsic defects

of each cell type through the use of uMT and Tcrα$^{-/-}$ mutations bred onto B6.*Sle1* to deplete circulating *Sle1* B and T cells, respectively (Sobel et al. 2002). Furthermore, using polycongenic mouse strains carrying various combinations of lupus susceptibility loci on a B6 background, Morel et al. (2000) determined that *Sle1* is essential for the loss of tolerance to occur in a polycongenic background. These data illustrate the indispensable role of *Sle1* in lupus pathogenesis, and illustrate the importance of further identification and characterization of *Sle1*.

Importantly, the *Sle1*-induced loss of tolerance occurs earlier and with a higher penetrance in females compared to males (Mohan et al. 1998; Morel et al. 2001). The sex bias associated with *Sle1* strongly suggests that hormones, specifically estrogen, play a role in disease development and progression and is responsible for the female sex bias in *Sle1*-associated loss of tolerance. However, the impact of estrogen signaling on *Sle1*-induced loss of tolerance has yet to be described. Although *Sle1* is associated with spontaneous hyperactivation in B and T cells, it has not yet been determined if there is a sex bias in the penetrance or magnitude of this phenotype (Sobel et al. 1999; Sobel et al. 2002). Altogether, further studies are warranted to determine the role of hormones, and specifically estrogens, in the *Sle1*-induced loss of tolerance and immune cell hyperactivation.

1.7 Sle1 subloci

Given the essential role of *Sle1* in lupus pathogenesis, recent studies have focused on further characterizing how *Sle1* induces loss of tolerance and identifying the gene(s) that underlie this locus. Fine mapping analysis of the *Sle1* interval by Morel et al. (2001) demonstrated three distinct subloci within *Sle1* that contribute to loss of tolerance. These subloci, referred to as *Sle1a*, *Sle1b*, and *Sle1*c, independently cause a loss of tolerance with varying penetrance, demonstrated by anti-chromatin IgG production, yet each displays a unique immune cell phenotype contributing to the overall *Sle1* presentation (Morel et al. 2001). Each of the three subloci span a non-overlapping interval of approximate 1-2 cM within the larger *Sle1* interval,

and each sublocus contained several known genes that may play a role in the autoimmune phenotype. To better characterize the contributions of each sublocus to the overall *Sle1* phenotype, subcongenic strains were produced each carrying an individual *Sle1* sublocus on a B6 background (Morel et al. 2001). Phenotypic analysis of B6.*Sle1a,* B6.*Sle1b,* and B6.*Sle1c* mice identified unique components of each subcongenic strain that contribute to the overall *Sle1* phenotype. Further identification and characterization of the genes underlying the *Sle1*, through evaluation of both the entire *Sle1* locus as well as each *Sle1* subloci, will aid in our understanding of the molecular mechanisms leading to *Sle1*-induced loss of tolerance.

The *Sle1a* sublocus is associated with anti-chromatin IgG production, with a higher penetrance in female B6.*Sle1a* mice compared to male B6.*Sle1a* mice (Morel et al. 2001). Furthermore, the autoantibodies produced in B6.*Sle1a* mice are selectively reactive to the H2A/H2B/dsDNA subnucleosome, which is characteristic of the entire *Sle1* interval and represents the initial loss of tolerance described by Burlingame et al. (1994). Additionally, B6.*Sle1a* mice display greater proportions of activated CD4+ T cells, higher levels of CD4+ T cell activation markers, increased cytokine production, and generate histone-reactive T cells (Chen et al. 2005). Furthermore, B6.*Sle1a* mice have reduced levels of $CD4^{+}CD25^{+}$ $FoxP3^{+}$ T regulatory cells preceding the loss of tolerance, suggesting the *Sle1a*-induced diminished T regulatory cell numbers contribute to the generation of autoreactive T cells and therefore production of autoantibodies (Chen et al. 2005). Importantly, the alteration of the *Sle1a* T cell compartment is similar to that in B6.*Sle1* mice, indicating a major contribution of *Sle1a* to the immune cell phenotype displayed in B6.*Sle1* mice. However, B6.*Sle1a* mice had a lower penetrance of autoantibody production than that seen in B6.*Sle1* mice. Furthermore, B6.*Sle1a* mice did not display the remarkable alteration in the B cell compartment that is characteristic ofB6.*Sle1* mice, indicating that *Sle1a* likely synergizes with other subloci within the *Sle1* interval to cause the *Sle1* phenotype in its entirety.

Sle1a consists of several known genes, including *Pbx1* and *EAT-2*, which are thought to play a role in immune cell regulation and function (Cuda et al. 2010). *Pbx1*-containing protein complexes interact with other transcription factors, including nuclear steroid hormone receptors, and modulate the ability of these transcription factors to bind to DNA binding sites (Laurent et al. 2008; Chang et al. 1996). Differential expression of *Pbx1* isoforms has been demonstrated to correlate with lupus susceptibility, however, the basis for this is unclear. Importantly, because these *Pbx1* isoforms differ in crucial activation and binding domains, differential expression of *Pbx1* isoforms may have a dramatic impact in their ability to interact with other transcription factors and modulate transcription. Furthermore, because *Pbx1* has been shown to play a critical role in self-renewal and lineage commitment of lymphoid lineage cells, alterations in relative expression of *Pbx1* isoforms could have profound effects on autoimmunity. Additionally, another gene within *Sle1a*, *EAT-2*, encodes an adaptor protein which associates with immune cell surface receptors and activates signaling cascades thought to regulate B cell tolerance (Eissmann and Watzl, 2006. Wang et al. 2010). Because ERα functions as a ligand-dependent transcription factor, it is possible that ERα may promote *Sle1a*-associated phenotypes by regulating the expression of genes such as *Pbx1* and *EAT-2*.

In contrast, B6.*Sle1b* mice display the most potent phenotype, with anti-chromatin autoantibody production penetrance reaching nearly 80% in females (Morel et al. 2001). *Sle1b* is associated with hyperactivation of both T and B cells, but predominantly affects the autoreactive B cell population to promote autoantibody production. Furthermore, B6.*Sle1b* mice have been shown to exhibit altered B cell tolerance, leading to an impairment of negative selection mainly at peripheral tolerance checkpoints (Wong et al. 2012). *Sle1b* is also associated with the formation of spontaneous germinal centers and increased follicular T helper cells, which correlate strongly with high titers of anti-chromatin IgG autoantibodies by autoreactive B cells (Wong et al. 2012). Despite the high penetrance of autoantibody production and immune cell phenotypes induced by

Sle1b, B6.*Sle1b* mice do not fully recapitulate the phenotype of *Sle1*, especially in T cell phenotype, indicating that, similar to *Sle1a*, synergy likely occurs between genes within the *Sle1b* interval and those underlying additional loci.

The *Sle1b* interval contains the SLAM/CD2 cluster genes, which encode cell surface receptors with extracellular binding domains and intracellular signaling domains (Wang et al. 2010). Polymorphisms within the SLAM/CD2 gene family cause structural and transcriptional variations shown to alter BCR signaling strength and, therefore, B cell tolerance (Wandstrat et al. 2004; Kumar et al. 2006). In particular, *Sle1b* causes an upregulation of the autoimmune variant of *Ly108*, *Ly108-1*, which is associated with crippled B cell tolerance through reduced BCR signaling strength, allowing autoreactive B cells to escape deletion (Kumar et al. 2006; Menard et al. 2013). Because ERα is known to modulate transcription and gene expression, it is of great interest to determine the impact of *ERα* genotype on expression of SLAM/CD2 genes, as well as other genes within *Sle1b*.

Finally, *Sle1*c has been demonstrated to induce a relatively modest loss of tolerance (~30%) demonstrated by autoantibody production. However, and of importance, the autoantibodies produced by B6.*Sle1*c mice are not specific for the H2A/H2B/DNA subnucleosome, in contrast to B6.*Sle1a* and B6.*Sle1b* mice (Morel et al. 2001). Additionally, *Sle1*c has a more modest immune cell phenotype, in which B6.*Sle1*c T cells provide help to only *Sle1*-expressing but not B6-derived chromatin-specific B cells (Chen et al. 2005). Therefore, it is likely that *Sle1*c requires synergistic interactions with other loci to influence *Sle1*-associated autoantibody production and immune cell activation.

Sle1c contains several genes including *Estrogen related receptor γ (Esrrg)* and *Complement receptor 1/2 (Cr2).* Decreased expression of *Esrrg* associated with *Sle1c* has been shown to alter CD4+ T cell function, in part by altering the mitochondrial metabolism of these T cells (Perry et al. 2012). Additionally, *Sle1c* is associated with a polymorphism within a crucial

binding domain of *Cr2*, leading to reduced ligand binding of CR1/CR2 that interferes with receptor signaling. This alteration is thought to affect negative selection of autoreactive B cells by impairing receptor ligand binding (Boackle et al. 2001). Together, alterations in the expression of *Esrrg* and *Cr2* due to *Sle1c* play a key role in the impaired T and B cell function associated with *Sle1*.

Despite evidence of higher penetrance of the lupus phenotype in female mice of each *Sle1* subcongenic strain, very little research has focused on identifying the mechanisms through which this occurs. Morel et al. (2001) identified a female sex bias in the penetrance of anti-chromatin autoantibodies in each *Sle1* subcongenic strain, significantly in the B6.*Sle1b* strain, however, the role of estrogens or ERα in this sex bias was not identified. Both *Sle1b* and estrogen signaling are thought to impair B cell tolerance induction, therefore illustrating a possible synergism between ERα signaling and the female-biased *Sle1b* phenotype, however this has not yet been explored (Wandstrat et al 2004; Grimaldi et al 2002). Wong and colleagues (2012) have demonstrated a crucial alteration in the GC checkpoint leading to loss of tolerance in *Sle1b* mice, and this perturbation was sex-biased where only female B6.*Sle1b* mice exhibited the phenotype, however the mechanisms underlying this have yet to be identified. Furthermore, although it has been shown that a greater proportion of B cells from female B6.*Sle1b* mice are spontaneously activated compared to males, the effect of estrogens or *ERα* genotype on this phenotype was not investigated (Wong et al 2012). Nothing is known about the impact of estrogens or ERα signaling on the *Sle1a*-induced loss of tolerance and immune cell hyperactivation. Identifying the mechanisms though which estrogen signaling via ERα synergizes with *Sle1* and its subloci will aid in our understanding of how estrogens regulate genetically controlled pathways to promote loss of tolerance, and will shed light on potential novel therapies for more effective lupus treatment.

CHAPTER II.

PURPOSE

2.1 Hypothesis and rationale

In summary, previous work by others and in our own lab has illustrated a vital role of estrogen in the regulation of genetically controlled pathways affecting immune tolerance, however, the mechanism through which this occurs remains unknown. Given the previous studies, we hypothesize that estrogens, acting via ERα, promote the *Sle1*-induced loss of tolerance and contributes to the sex bias demonstrated by B6.*Sle1* congenic mice. To better understand the role of estrogen signaling on *Sle1*-induced loss of tolerance, we tested our hypothesis that ERα signaling synergizes with the *Sle1* lupus susceptibility loci to promote the *Sle1*-associated loss of tolerance and immune cell activation by intercrossing the B6.*ERα*KO strain with B6.*Sle1* congenic mice to determine the impact of *ERα* deficiency on the actions of *Sle1*. Furthermore, we will begin to determine the impact of ERα signaling on *Sle1a*- and *Sle1b*-associated loss of tolerance and immune cell hyperactivation to help us better understand the specific cellular and molecular mechanisms through which ERα and *Sle1* subloci synergy occurs. In whole, these data will shed light on the mechanisms by which estrogen signaling enhances the female sex bias in lupus, and aid in the identification of key genetic factors that are modulated by ERα to promote the loss of tolerance associated with lupus development.

2.2 Specific Aims

(1) Determine the impact of *ERα* deficiency on *Sle1*-induced loss of tolerance.

(2) Investigate the impact of sex and *ERα* deficiency on *Sle1*-associated immune cell hyperactivation.

(3) Determine the impact of *ERα* deficiency on gene expression of *Sle1* candidate genes.

CHAPTER III.

MATERIALS AND METHODS

3.1 Care and Treatment of Mice:

The Institutional Animal Care and Use Committee of the University of Nebraska Medical Center approved all procedures involving live animals. The ERα knockout strain (B6.129-Esr1tm1Ksk or B6.ERα) was originally obtained from Dennis Lubahn (Lubahn et al. 1993). The B6.*Sle1* congenic strain was provided by Laurence Morel (Morel et al. 2001). The B6.*Sle1a* and B6.*Sle1b* subcongenic strains were also provided by Laurence Morel (Morel et al.1996; Morel et al. 1997; Mohan et al. 1999). Animals were housed under controlled temperature, humidity, and 12h light/12h dark lighting conditions in a facility accredited by the American Association for Accreditation of Laboratory Animal Care and operated in accordance with the standards outlined in Guide for the Care and Use of Laboratory Animals (The National Academies Press, 1996). Mice were provided Harlan irradiated rodent diet 7904 (Harlan Teklad, Madison, WI), which contains soy, milk, and meat-based protein sources, and allowed to feed ad libitum.

B6 females heterozygous for targeted disruption of the *ERα* gene ($ER\alpha^{+/-}$) were crossed to B6.*Sle1* congenic males. The resulting $ER\alpha^{+/-}$ males were backcrossed to B6.*Sle1* females. Resulting $ER\alpha^{+/-}$ offspring were genotyped at markers (D1Mit47, D1Mit159, D1Mit111, D1Mit206, D1Mit426, and D1Mit17) that are polymorphic between the NZW and B6 strains and span the *Sle1* congenic interval to identify mice that were homozygous for NZW alleles throughout the interval. These mice were interbred to generate the experimental mice. PCR-based genotyping was performed as described previously (Bynote et al. 2008). For the ovariectomy studies, mice were randomized to the sham and ovariectomy groups at 5-6 weeks of age and subjected to either a sham procedure in which the ovaries are externalized and then returned to the abdominal cavity or removed, respectively. Success of these surgical procedures

was confirmed by measuring serum estradiol levels using a quantitative ELISA assay (Alpha Diagnostics International, San Antonio, TX) from serum collected prior to sacrifice. Testosterone levels were also measured by using a quantitative ELISA assay (Alpha Diagnostics International, San Antonio, TX).

To produce *Sle1a* and *Sle1b* subcongenic strains, B6 females heterozygous for targeted disruption of the *ERα* gene ($ER\alpha^{+/-}$) were crossed to B6.*Sle1a* or B6.*Sle1b* congenic males. The resulting $ER\alpha^{+/-}$ males were backcrossed to B6.*Sle1a* or B6.*Sle1b* females, respectively. Resulting $ER\alpha^{+/-}$ offspring were genotyped at markers that are polymorphic between the NZW and B6 strains and flank the *Sle1a* subcongenic interval or *Sle1b* subcongenic interval to identify mice that were homozygous for NZW alleles at each interval (Morel et al. 2001). The markers used for *Sle1a* were D1mit15 and D1mit353 (Morel et al. 2001). The markers used for *Sle1b* were D1mit113 and D1mit206 (Morel et al. 2001). These mice were interbred to generate the experimental mice. PCR-based genotyping was performed as described previously (Bynote et al. 2008).

3.2 Serological Analysis

Autoantibody levels were assessed by ELISA using serum isolated from blood collected monthly via the saphenous vein and stored at -80C. Samples were assayed in duplicate for each ELISA. The anti-chromatin IgG and anti-dsDNA IgG concentrations were determined using plates prepared as described previously (Bynote et al 2008). Autoantibody levels in these samples were quantitated in arbitrary ELISA units (U/µl) based upon a standard curve generated by serial dilution of a positive control sample that was made by pooling serum from a group of (NZB x NZW) F1 females with heavy albuminuria. The threshold for a positive autoantibody titer in the experimental mice was set at 2 standard deviations above the mean of a group of age-matched control B6 mice (Morel et al. 2001). Total serum concentrations of antibodies of each isotype were determined using the clonotyping kit (Southern Biotech, Birmingham, AL) according to the

manufacturer's instructions. All optimal density measurements were made using a BioRad 680 Microplate reader and Microplate Manager software, version 5.2.1 (Hercules, CA). Sera were diluted serially from 1:100 to 1:2000 for measurement of autoantibody concentrations and to 1:50,000 for measurement of total serum immunoglobulins.

3.3 Flow Cytometry

The antibodies (BD Biosciences, San Jose, CA) used for flow cytometry were: CD4-PE (RM4-5), CD4-v450 (RM4-5), CD8-APC (53-6.7), CD69-FITC (H1.2F3), CD134-Biotin (OX-86), CD62L-APC (MEL-14), B220-APC (RA3-6B2), CD86-PE (GL1), CD22-PE (Cy34.1), CD25-APC.Cy7 (PC61), CD95-PE.Cy7 (Jo2), CXCR5- PE.Cy7 (2G8), PD-1-APC (J43), PNA-FITC (L7281), and FoxP3-PE (150D). Biotinylated antibodies were detected using FITC-conjugated streptavidin (BD Biosciences). Flow cytometric analysis was performed using various combinations of these antibodies on single cell suspensions of splenocytes. For intracellular FoxP3 staining, surface-stained cells were treated with fixation/permeabilization buffer and stained with FoxP3-PE (150D) using the BioLegend FoxP3 flow kit, following the manufacturer's protocol (BioLegend). Stained cells were analyzed in the UNMC Flow Cytometry Research Facility using the BD LSR II flow cytometer. Data were analyzed using FACSDiva software, version 6.1.2 (BD Biosciences). For analysis of T regulatory cells, splenocytes were isolated from mice that were 3-4 months of age. For all other flow cytometry analyses, splenocytes were collected from mice that were 6–12 months of age.

3.4 Cell Isolation

Splenic B cells were isolated from mice at 5-9 months of age using the B Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) and collected using MACS columns and the VarioMACS separator (Miltenyi Biotec). Splenic $CD4^+$ T cells were isolated from mice at 5-9 months of age using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec) and collected using MACS columns and

either the VarioMACS or AutoMACS separator (Miltenyi Biotec). Flow cytometry was used to confirm the purity of the isolated cell population. RNA was prepared from isolated cells was extracted using the Absolutely RNA Miniprep Kit (Stratagene Corporation, La Jolla, CA). cDNA was generated using either the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Carlsbad, CA) or SuperScript VILO Master Mix (Invitrogen, Carlsbad, CA) according the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed using Power SYBR Green PCR Master Mix, the ABI 7500 Real-Time PCR System, and Sequence Detection Software, version 1.4 (Applied Biosystems). Quantitative data regarding gene expression was extracted from the PCR data and the abundance of each gene transcript was normalized to that of *Gapdh* for each sample.

3.5 Immunoprecipitation and SDS-PAGE Analysis

CR1/CR2 expression and glycosylation were quantified using a modification of the method described previously by Boackle and colleagues (2001). Briefly, single cell suspensions of splenocytes from mice at 5-9 months of age were depleted of RBC with ACK Lysing Buffer (Life Technologies, Carlsbad, CA) and surface biotinylated with the EZ-LINK Sulfo-NHS-LC-Biotin (Thermo Scientific, Waltham, MA). Cells were lysis in RIPA Buffer (1XPBS, 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS.) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO) diluted to final concentrations of 1 mM AEBSF, 0.8 μM Aprotinin, 40 μM Bestatin, 14 μM E-64, 20 μM Leupeptin, and 15 μM Pepstatin A. Lysates were incubated with anti-mouse CR1/CR2 antibody (7E9) for 30 min on ice. After the addition of Protein G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA), samples were incubated on a rotating mixer at 4° C. Samples were electrophoresed through an 8% non-reducing gel and transferred to a nitrocellulose membrane. Membranes were incubated with streptavidin-Alexa Fluor® 700 conjugate (Life Technologies) and imaged and quantified using the Li-Cor Odyssey Imaging system (Li-Cor, Lincoln, NE).

3.6 Statistical Methods

Comparisons were performed using Fishers exact test, independent or paired samples t-test, or one-way ANOVA with Tukey's post hoc test where appropriate. Statistical analyses were performed using SPSS software (version 19.0). A two-sided $P \leq 0.05$ was considered significant. Two-sided p-values are provided. Mean ± standard error of the mean is presented.

CHAPTER IV. ESTROGEN RECEPTOR ALPHA PROMOTES LOSS OF TOLERANCE AND FEMALE SEX BIAS IN B6.*Sle1* CONGENIC MICE

INTRODUCTION

Approximately 90% of lupus patients are women. This dramatic sex bias is thought to be due, in large part, to endogenous estrogens. Although abundant evidence links exposure to estrogens with enhanced lupus risk and increased lupus manifestations (Lahita et al. 1987; Lahita et al. 1979; Lahita et al. 1981; Lui et al. 2002; Lee et al. 2004), relatively little is understood in regard to cellular and molecular mechanisms. Much of our understanding of how estrogens may promote lupus has come from studies in lupus prone mice, such as (NZB x NZW) F1 mice (Howie et al. 1968; Andrews et al. 1978). Lupus development in these mice is influenced by sex; lupus incidence is higher and survival time is reduced in female (NZB X NZW) F1 mice relative to males (Howie et al. 1968; Andrews et al. 1978). We demonstrated previously that disruption of estrogen receptor α (ERα) in (NZB X NZW) F1 mice eliminated the sex bias in the development of lupus in these mice (Bynote et al. 2008). The decreased incidence of lupus and increased survival in ERα deficient (NZB X NZW) F1 females was associated with a dramatic attenuation of loss of tolerance to nuclear antigens and development of pathogenic autoantibodies (Bynote et al. 2008). These data indicate that the sex bias in lupus pathogenesis in this model is due, at least in part, to estrogens, acting via ERα. These data also suggest that estrogens, acting through ERα, may augment the effects of at least some lupus susceptibility loci, leading to an enhanced lupus phenotype in females.

Loss of tolerance to chromatin is thought to represent an initial step in the development of lupus (Burlingame et al. 1993; Burlingame et al. 1994). In (NZB x NZW)F1 mice, the loss of tolerance to chromatin is controlled by NZB- and NZW- derived lupus susceptibility alleles (Mohan et al. 1999; Mohan et al. 1998; Gubbels et al. 2005). Among these loci, the NZW-derived *Sle1* locus is the best characterized (Morel et al. 1994; Morel et al. 1996; Morel et al.

1999). B6.*Sle1* congenic mice, in which the NZW-derived *Sle1* allele is carried on the non-autoimmune C57BL/6 (B6) genetic background, spontaneously lose tolerance to chromatin and develop anti-chromatin IgG (Mohan et al. 1998). The incidence of loss of tolerance in B6.*Sle1* females is significantly greater than in males, suggesting that sex hormones may impact the actions of *Sle1* (Mohan et al. 1999; Morel et al. 2001). *Sle1* also leads to increased activation in B and T cells (Sobel et al. 2002; Sobel et al. 2012), although it is not known if these aspects of the *Sle1* phenotype also display a sex bias. Virtually nothing is known about how hormones, including estrogens, influence *Sle1* action.

The *Sle1* interval represents at least three distinct subloci, *Sle1a*, *Sle1b* and *Sle1c*, each of which acts via distinct pathways to independently contribute to loss of tolerance and the development of anti-chromatin IgG (Morel et al. 2001). Analysis of congenic strains carrying these individual subloci has shown that *Sle1b*-induced loss of tolerance to chromatin displays a robust and significant female sex bias (Morel et al. 2001). Consistent with the idea that *Sle1b* enhances loss of tolerance preferentially in females, a recent study indicates that B cell activation and proliferation is more robust in B6.*Sle1b* congenic females than males (Wong et al. 2012). Both *Sle1a*- and *Sle1c*- induced loss of tolerance to chromatin also appear to be more pronounced in female mice than in male mice, although these differences fall short of statistical significance (Morel et al. 2001).

We postulate that estrogens, acting via ERα-dependent pathways, synergize with the pathways controlled by certain lupus susceptibility loci to preferentially enhance loss of tolerance and the development of lupus in females. Given the fact that development of anti-chromatin IgG in B6.*Sle1* congenic mice shows a clear sex bias, we hypothesize that the effects of *Sle1* are likely to be influenced by estrogens via ERα signaling. To test this hypothesis, we examined the impact of a targeted mutation in *ERα* on the phenotype in B6.*Sle1* congenic mice. We observed that *ERα* deficiency attenuated loss of tolerance and the development of anti-chromatin IgG in B6.*Sle1*

congenic females but not males. *ERα* deficiency significantly decreased *Sle1*-induced immune cell hyperactivation in females, and to a lesser extent, in males. These effects of *ERα* deficiency were associated with a decrease in the relative expression of *Pbx1a*, an isoform of one of the genes that underlies *Sle1*. The impact of removal of the ovaries, the primary source of estrogens, on B6.*Sle1* congenic females was similar to that associated with *ERα* deficiency. Furthermore, the phenotype of *ERα* deficient B6.*Sle1* congenic females was similar to that in *ERα* wildtype B6.*Sle1* congenic males, suggesting that the sex bias in the phenotype of B6.*Sle1* congenic mice is both estrogen- and ERα-dependent.

RESULTS

4.1 ERα deficiency attenuates Sle1-induced loss of tolerance and epitope spreading in females

To explicitly test the hypothesis that ERα signaling promotes the *Sle1*-induced loss of tolerance, we intercrossed B6.*Sle1* congenic mice with the B6.*ERα* knockout strain, which has been maintained in our lab since 1999. In doing so, we generated B6.*Sle1* mice that were estrogen receptor α wildtype ($ERα^{+/+}$), heterozygous ($ERα^{+/-}$), or null ($ERα^{-/-}$). Consistent with previous studies, B6.*Sle1*.$ER^{+/+}$ exhibited a significant loss of tolerance demonstrated by a high penetrance (77%) of anti-chromatin IgG production (Figure 1A-B). Whereas *ERα* heterozygosity did not significantly impact the penetrance of autoantibody production, *ERα* deficiency decreased the population of B6.*Sle1* mice that produced significant levels of anti-chromatin IgG autoantibodies (33%; $p< 0.005$). These results suggest that ERα signaling promotes loss of tolerance in B6.*Sle1* females.

In a parallel study, we performed ovariectomies on B6.*Sle1* females to explore the impact of removing the primary source of estrogens on loss of tolerance. Similar to B6.*Sle1*.$ERα^{+/+}$ females, a significant proportion of sham-operated B6.*Sle1* females developed anti-chromatin IgG autoantibodies (82%; Figure 1C-D). In contrast, only 33% of ovariectomized B6.*Sle1* females developed significant levels of anti-chromatin IgG autoantibodies, a proportion identical to that observed in B6.*Sle1*.$ERα^{-/-}$ females ($p<0.05$) (Figure 1C-D). These results suggest that the removal of the ovaries, the primary source of endogenous estrogen in females, has a similar impact on loss of tolerance in B6.*Sle1* females as the disruption of ERα signaling. These data indicate the necessity of ERα signaling in *Sle1*-induced loss of tolerance in B6.*Sle1* females.

Some studies have identified a subset of B6.*Sle1* mice that develop anti-dsDNA IgG as a result of epitope spreading (Morel et al. 1997). These autoantibodies are produced secondary to the initial anti-chromatin-specific IgG autoantibodies, and are not consistently found in B6.*Sle1*

mice. Furthermore, the level of anti-dsDNA autoantibodies produced by B6.*Sle1* is low, and only 10% of the mice show evidence of lupus nephritis (Morel et al 1997). Nevertheless, we wanted to determine the impact of *ERα* genotype on epitope spreading in B6.*Sle1* females (Figure 1E-F). We found that 46% of our B6.*Sle1*.$ER^{+/+}$ females developed measurable levels of anti-dsDNA IgG autoantibodies. Whereas heterozygosity for *ERα* did not significantly impact the proportion of B6.*Sle1* females that developed anti-dsDNA IgG autoantibodies, we found that only 9% of B6.*Sle1*.$ER^{-/-}$ females developed anti-dsDNA autoantibodies ($p<0.05$; Figure 1E-F). These results strongly suggest a role of ERα signaling in epitope spreading demonstrated by some female B6.*Sle1* mice.

Previous studies have shown that B6.*Sle1* congenic mice produce greater total serum IgG levels compared to controls (Mohan et al. 1999). Consistent with the findings by Mohan and colleagues, we found that total serum IgG levels were significantly elevated in B6.*Sle1*.$ERα^{+/+}$ females compared to B6.$ERα^{+/+}$ ($p<0.05$; Figure 1G). However, we did not observe a significant difference in total serum IgG between B6.*Sle1*.$ERα^{+/+}$ and B6.*Sle1*.$ERα^{-/-}$, indicating that *ERα* genotype does not significantly impact the total serum IgG produced, and furthermore, confirming previous studies that showed no sex bias in the elevated total serum IgG of B6.*Sle1* mice (Mohan et al. 1999) (Figure 1G). These data strongly suggest that the attenuated development of anti-chromatin IgG we observed in B6.*Sle1*.$ERα^{-/-}$ females compared to B6.*Sle1*.$ER^{+/+}$ is not due to a generalized defect in IgG production or maturation of the immune response. Finally, no significant differences were observed in total serum IgM levels, which have been inconsistently observed as increased in B6.*Sle1* mice (Figure 1H).

FIGURE 1

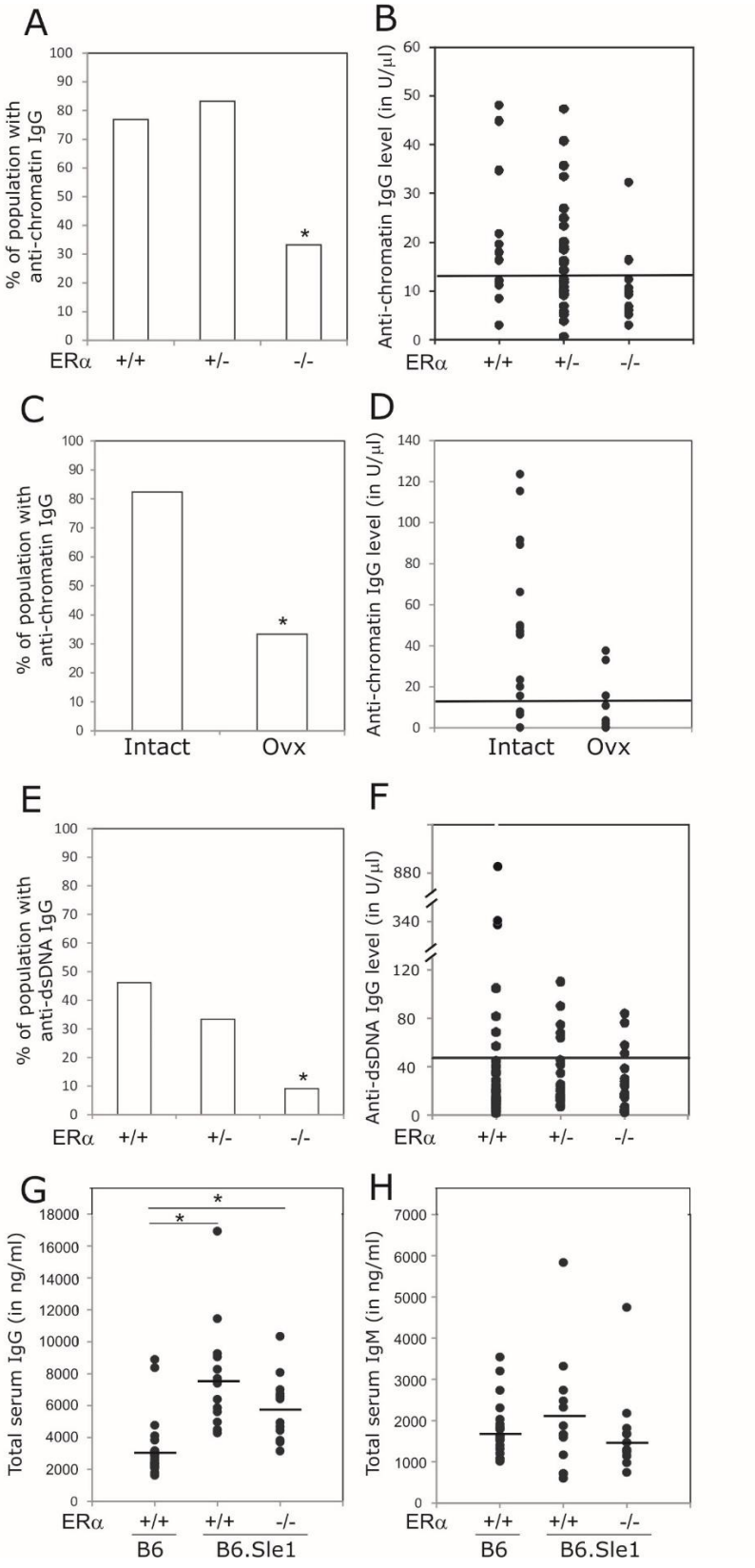
A
% of population with anti-chromatin IgG
ERα +/+ +/- -/-
B
Anti-chromatin IgG level (in U/μl)
ERα +/+ +/- -/-
C
% of population with anti-chromatin IgG
Intact Ovx
D
Anti-chromatin IgG level (in U/μl)
Intact Ovx
E
% of population with anti-dsDNA IgG
ERα +/+ +/- -/-
F
Anti-dsDNA IgG level (in U/μl)
ERα +/+ +/- -/-
G
Total serum IgG (in ng/ml)
ERα +/+ +/+ -/-
B6 B6.Sle1
H
Total serum IgM (in ng/ml)
ERα +/+ +/+ -/-
B6 B6.Sle1

Figure 1. Attenuation of *Sle1*-induced loss of tolerance by ERα deficiency and ovariectomy.

ELISAs were used to assess the abundance of anti-chromatin IgG (A –D), anti-dsDNA IgG (E-F), total serum IgG (G) and IgM (H) in 5-9 month old female mice. The proportion of B6.*Sle1*.*ERα*$^{+/+}$ (N=13), B6.*Sle1*.*ERα*$^{+/-}$ (N=12), and B6.*Sle1*.*ERα*$^{-/-}$ (N=12) females that developed anti-chromatin IgG (A) and anti-dsDNA IgG (E) is shown. The proportion of sham-treated, ovary-intact B6.*Sle1* (N=10) and ovariectomized B6.*Sle1* (N=16) females that develop anti-chromatin IgG (C) is shown. The absolute levels of anti-chromatin IgG (B, D) and anti-dsDNA IgG (F) are also shown. For panels G, H, the horizontal bar denotes the mean. In panels B, D and F, the horizontal line represents the threshold for a positive autoantibody titer in the experimental mice. This threshold was set at 2 standard deviations above the mean of a group of age-matched control B6 mice as has been described previously (Morel et al. 2001). The mean (± standard error of the mean) abundance of total serum IgG (G) and IgM (H) in B6 (N=20), B6.*Sle1*.*ERα*$^{+/+}$ (N=13), and B6.*Sle1*.*ERα*$^{-/-}$ (N=12) females is shown. The * indicates a $P<0.05$ compared to B6.*Sle1*.*ERα*$^{+/+}$ females (A and E), intact females (C), and B6 females (G).

4.2 ERα deficiency attenuates Sle1-induced B and T cell activation in females

Several studies have identified a *Sle1*-induced T and B cell hyperactivation, intrinsic defects in each cell type as autoreactivity still occurs in B6.*Sle1* mice depleted of either peripheral T or B cells (Sobel et al. 2002). However, the impact of sex has not yet been investigated on *Sle1*-induced immune cell activation. Additionally, because estrogen has been shown to impact both T and B cell activation, identifying the impact estrogens, via ERα signaling, have on *Sle1*-associated immune cell activation is warranted. To investigate the impact of *ERα* deficiency on immune cell activation in B6.*Sle1* female mice, flow cytometry was used to assess the expression of activation markers on both T and B cells. As expected from previous studies, we found a greater proportion of B cells from B6.*Sle1*.*ER*$^{+/+}$ female mice expressed CD86 compared to B6.*ER*$^{+/+}$ females ($p<0.05$; Table 1). This confirms the reported *Sle1*-induced B cell hyperactivation by Morel and colleagues (2001). Importantly, we found that the *Sle1*-induced B cell hyperactivity is completely attenuated with *ERα* deficiency, as B6.*Sle1*.*ERα*$^{-/-}$ female mice had a significantly smaller population of B220^{+}CD86^{+} cells than their B6.*Sle1*.*ER*$^{+/+}$ female counterparts ($p<0.01$; Table 1). The proportion of B220^{+}CD86^{+} cells in B6.*Sle1*.*ER*$^{-/-}$ female mice was not statistically different than B6.*ER*$^{+/+}$ or B6.*ER*$^{-/-}$ female mice, indicating that *Sle1*-induced B cell hyperactivation is dependent on ERα signaling.

To determine the impact of *ERα* deficiency on *Sle1*-associated T cell hyperactivation, we evaluated the expression of CD4^{+} T cell activation markers CD69 and CD134. Compared to B6.*ER*$^{+/+}$ female mice, the percentage of CD4^{+}CD69^{+} T cells was significantly greater in B6.*Sle1*.*ER*$^{+/+}$ female mice ($p< 0.05$; Table 1). We also observed a smaller proportion of CD4^{+} T cells that expressed CD62L^{+}, a marker of naïve T cells, in B6.*Sle1*.*ERα*$^{+/+}$ female mice compared to B6.*ERα*$^{+/+}$ females ($p<0.05$; Table 1). Together, these data confirm the previously reported *Sle1*-induced T cell hyperactivation. Furthermore, we found this T cell hyperactivation phenotype to be nearly abolished due to *ERα* deficiency, as a significantly smaller proportion of CD4^{+} T

cells from B6.*Sle1*.*ER*$^{-/-}$ female mice expressed CD69 or CD134 ($p<0.05$; Table 1). We also identified a significantly larger population of $CD4^+CD62L^+$ T cells in B6.*Sle1*.*ER*$^{-/-}$ female mice compared to B6.*Sle1*.*ER*$^{+/+}$ ($p<0.05$; Table 1). Therefore, *ERα* deficiency significantly impacts the T cell phenotype associated with *Sle1*. However, although we observed a significant difference in T cell phenotype between B6.*Sle1*.*ER*$^{+/+}$ and B6.*Sle1*.*ER*$^{-/-}$ females, the proportion of $CD4^+CD62L^+$ T cells in B6.*Sle1*.*ER*$^{-/-}$ female mice was still significantly less than that of B6.*ER*$^{+/+}$ or B6.*ER*$^{-/-}$ control mice ($p<0.01$; Table 1), indicating that *ERα* deficiency did not completely abolish the *Sle1*-induced T cell hyperactivation. Interestingly, the percentage of $CD4^+CD62L^+$ T cells in B6.*ERα*$^{-/-}$ female mice was significantly less than that of B6.*ERα*$^{+/+}$ female mice, indicating that ERα signaling may promote certain aspects of T cell hyperactivation independently of *Sle1* ($p<0.05$; Table 1). This was not the case for activation markers $CD69^+$ or $CD134^+$, as we observed no significant differences between these subsets of $CD4^+$ T cells between B6.*ERα*$^{+/+}$ and B6.*ERα*$^{-/-}$ (Table 1). Altogether, these data strongly suggest that *ERα* deficiency attenuates *Sle1*-induced T cell hyperactivation.

We also found a significant decrease in the proportion of $CD4^+CD25^+$ T cells in B6.*Sle1*.*ERα*$^{+/+}$ female mice compared to B6.*ERα*$^{+/+}$ female mice ($p<0.05$; Table 1). Because previous studies have found a significant decrease in $CD4^+CD25^+$ T regulatory cells, we next investigated the impact of *ERα* deficiency on *Sle1*-induced T regulatory cell population size. Consistent with previous reports, we noted a significant decrease in population size of $CD4^+CD25^+$ T cells that express $CD62L^{hi}$ ($CD4^+CD25^+CD62L^{hi}$) in B6.*Sle1*.*ERα*$^{+/+}$ female mice compared to B6.*ERα*$^{+/+}$ ($p<0.01$; Figure 2A). However, we found no significant difference in $CD4^+CD25^+CD62L^{hi}$ T cell population size in B6.*Sle1*.*ER*$^{-/-}$ compared to B6.*Sle1*.*ER*$^{+/+}$ female mice, indicating that *ERα* deficiency does not impact the *Sle1*-associated decrease in Treg population size (Figure 2A). Consistent with this observation, *ERα* deficiency did not impact the relative population size of the $CD4^+CD25^+FoxP3^+$ Treg population or FoxP3 expression

Table1. Impact of *ERα* genotype on *Sle1*-induced immune cell activation in females

		B6		B6.Sle1	
Cell Type	ERα	+/+	-/-	+/+	-/-
$B220^+$		54.7±2.0	55.2±3.1	49.1±2.4*	49.3±2.7
$B220^+CD86^+$		8.6±1.3	9.3±2.5	12.9±2.5*	$6.2±1.4^{††}$
$CD4^+$		17.2±1.0	18.2 ±1.3	17.8±0.8	17.8±1.2
$CD4^+CD69^+$		13.3±2.3	12.8±4.3	22.6±3.2**	$8.8±1.4^{††}$
$CD4^+CD134^+$		13.5±3.6	9.4±3.8	24.8±4.1*	$11.9±3.2^{††}$
$CD4^+CD62L^+$		40.6±4.7	$59.2±4.7^{††}$	15.0±1.4**	$25.2±5.2^{**,††}$
$CD4^+CD25^+$		2.4±0.2	2.7±0.2	1.9±0.2*	1.9±0.3*

*,**Significant difference compared to B6 mice of the same ERα genotype are denoted by * (P=0.05) and **(P=0.01)

†,†† Significant difference compared to $ERα^{+/+}$mice of the same Sle1 genotype are denoted by †(P=0.05) and ††(P=0.01)

Table 1. Impact of *ERα* genotype on lymphocyte activation in female B6.*Sle1* mice.

Splenocytes from each mouse (6-12 months of age) were analyzed individually by flow cytometry (N=12-24 per group). The values presented represent the mean ± SEM for the samples in each group.

level (Figure 2B-C). Altogether, these studies indicate that *ERα* deficiency largely abolishes *Sle1*-induced T cell hyperactivation, but does not attenuate the *Sle1*-associated reduction in Treg population size.

4.3 Impact of ERα deficiency on expression of Sle1-associated genes in female mice

Sle1 is associated with several polymorphisms that have been shown to impact gene expression and/or alternative splicing patterns. Because many of these genes have been suggested to play a role in immune cell tolerance, it is likely that alternations in the expression or relative expression of isoforms of these genes could be responsible for the *Sle1*-induced loss of tolerance. Additionally, ERα functions as a ligand-dependent transcription factor and is known to influence gene expression. We therefore predicted that ERα signaling may promote *Sle1*-induced immune cell activation by impacting the gene expression or relative isoform expression of genes within the *Sle1* locus. Because the *Sle1* interval is composed of 3 subloci, each containing genes predicted to play a role in *Sle1*-induced loss of tolerance and immune cell hyperactivation, we next identified candidate genes within *Sle1a*, *Sle1b,* and *Sle1*c subloci to examine the impact of *ERα* deficiency on gene expression and/or splicing on genes within *Sle1*.

The *Sle1a* sublocus is comprised of several known and unknown genes, including the pre-B cell leukemia homeobox 1 (*Pbx1*) gene (Cuda et al. 2010). The polymorphisms associated with *Sle1a* have been shown to induce a differential splicing of the *Pbx1* transcript, leading to an increase in the relative abundance of the lupus-associated *Pbx1d* isoform relative to the non-autoimmune *Pbx1b* isoform (Cuda et al. 2012). This alteration is thought to impact $CD4^+$ T cell activation and tolerance associated with *Sle1* (Cuda et al. 2012). Because ERα is known to impact gene expression, we sought to determine if *ERα* deficiency could impact relative expression of either or both *Pbx1b* and *Pbx1d* isoforms. To evaluate the expression of each *Pbx1* genes, we

FIGURE 2

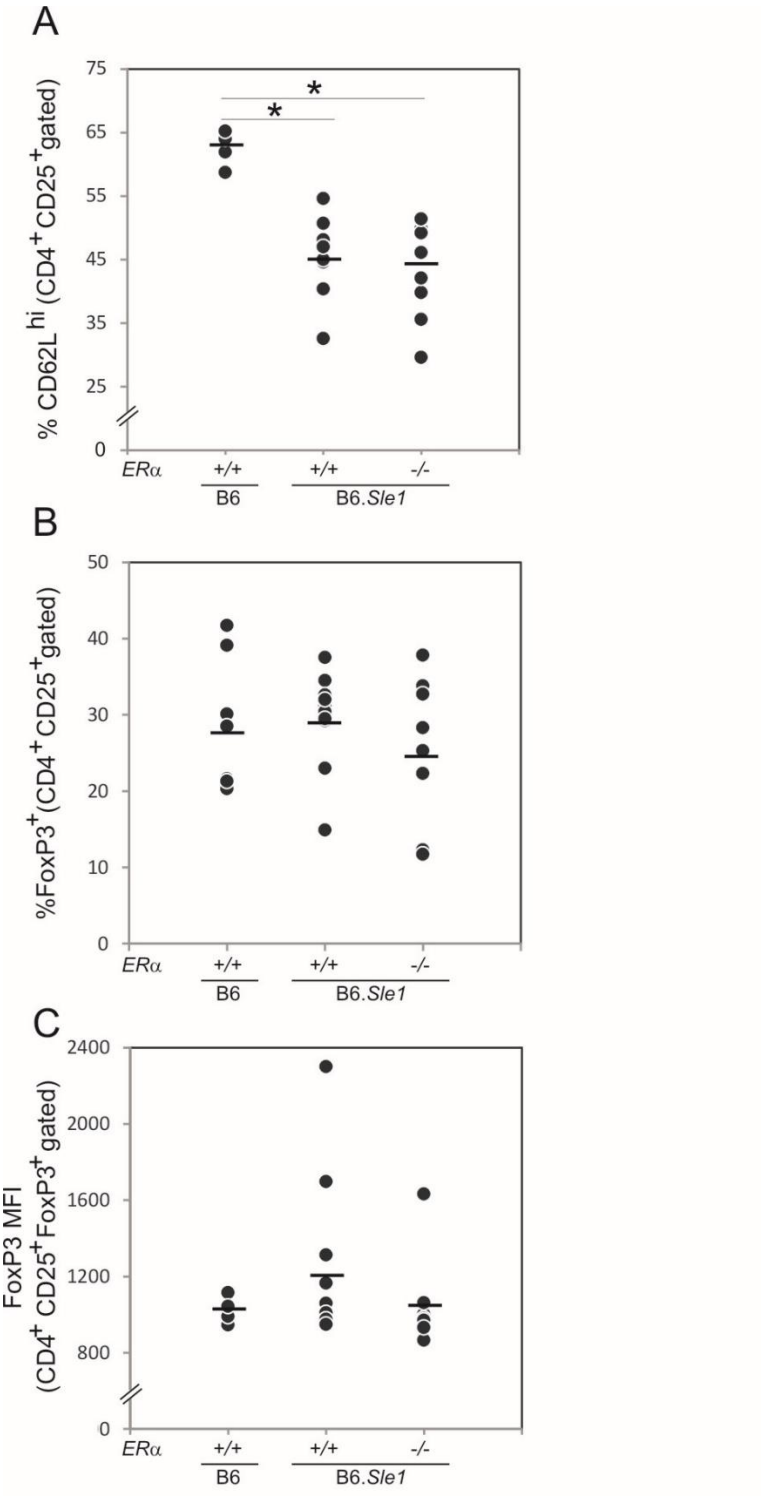
A
% CD62L^{hi} (CD4$^+$CD25$^+$gated)
75
65
55
45
35
25
0
*
*
ERα
+/+
+/+
-/-
B6
B6.Sle1
B
%FoxP3$^+$ (CD4$^+$CD25$^+$gated)
50
40
30
20
10
0
ERα
+/+
+/+
-/-
B6
B6.Sle1
C
FoxP3 MFI
(CD4$^+$ CD25$^+$FoxP3$^+$ gated)
2400
2000
1600
1200
800
0
ERα
+/+
+/+
-/-
B6
B6.Sle1

Figure 2. Impact of *ERα* genotype on the T regulatory population in B6.*Sle1* congenic mice. Flow cytometry was used to assess the T regulatory cell pool in female mice at 3-4 months of age. Splenocytes from each mouse were analyzed individually by flow cytometry (N=4-12 per group). The percentage of the $CD4^+CD25^+$ cells that were $CD62L^{hi}$ T regulatory cells (A) and $FoxP3^+$ T regulatory cells (B) is shown. The level of expression (mean fluorescence intensity or MFI) of FoxP3 in $CD4^+CD25^+FoxP3^+$ T regulatory cells was also assessed (C). The horizontal bar in each panel denotes the mean. The * indicates a $P<0.05$ compared to B6 females.

developed isoform-specific qRTPCR primers and performed real-time PCR (Figure 3).

Consistent with previous reports, we found a significant increase in the ratio of *Pbx1d* to *Pbx1b* isoform expression in $CD4^+$ T cells isolated from B6.*Sle1*.*ERα*$^{+/+}$ female mice compared to B6.*ERα*$^{+/+}$ female control mice ($p<0.05$; Figure 4A). However, we did not see a significant difference in relative *Pbx1d*/*Pbx1b* isoform expression between $CD4^+$ T cells of B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ER*$^{-/-}$ female mice, indicating that the *ERα* genotype did not impact the relative abundance of these *Pbx1* transcripts (Figure 4A). A third *Pbx1* isoform, *Pbx1a*, has been reported to be expressed by human T cells, but not T cells in B6.*Sle1a*.1 subcongenic mice (Cuda et al. 2012). However, by developing isoform specific PCR primers based on sequencing information, we were able to detect the *Pbx1a* isoform in splenic $CD4^+$ T cells isolated from B6.*Sle1* congenic mice. Therefore, we sought to evaluate the impact of *ERα* deficiency on the relative expression of the *Pbx1a* isoform in $CD4^+$ T cells of the B6.*Sle1* female mice. The *Pbx1a*/*Pbx1b* ratio in $CD4^+$ T cells isolated from B6.*Sle1*.*ERα*$^{+/+}$ female mice was significantly higher than that of $CD4^+$ T cells isolated from B6.*ERα*$^{+/+}$ female mice, indicating that the expression of the *Pbx1a* isoform is also modulated by the *Sle1* locus ($p<0.05$; Figure 4A). Interestingly, the *Pbx1a*/*Pbx1b* ratio in $CD4^+$ T cells from B6.*Sle1*.*ERα*$^{-/-}$ female mice was significantly less than that of B6.*Sle1*.*ERα*$^{+/+}$ females, suggesting that the relative isoform of *Pbx1a* is regulated by ERα ($p<0.05$; Figure 4A).

The *Sle1b* sublocus contains the SLAM/CD2 cluster genes, which encode cell surface receptors with extracellular binding domains and intracellular signaling domains known to activate signaling cascades within $CD4^+$ T and B cells (Veillette et al. 2009). Polymorphisms within the SLAM/CD2 genes due to *Sle1b* cause transcriptional and structural changes that have been shown to significantly impact B cell tolerance (Wandstrat et al. 2004). Previous studies have shown that *Sle1b* causes a significant decrease in expression of *CD48*, as well as alternative splicing of *Ly108* leading to an increase in the ratio of the autoimmune *Ly108-1* isoform relative to the 'normal' *Ly108-2* isoform (Wang et al. 2010). Consistent with previous studies, we found

FIGURE 3

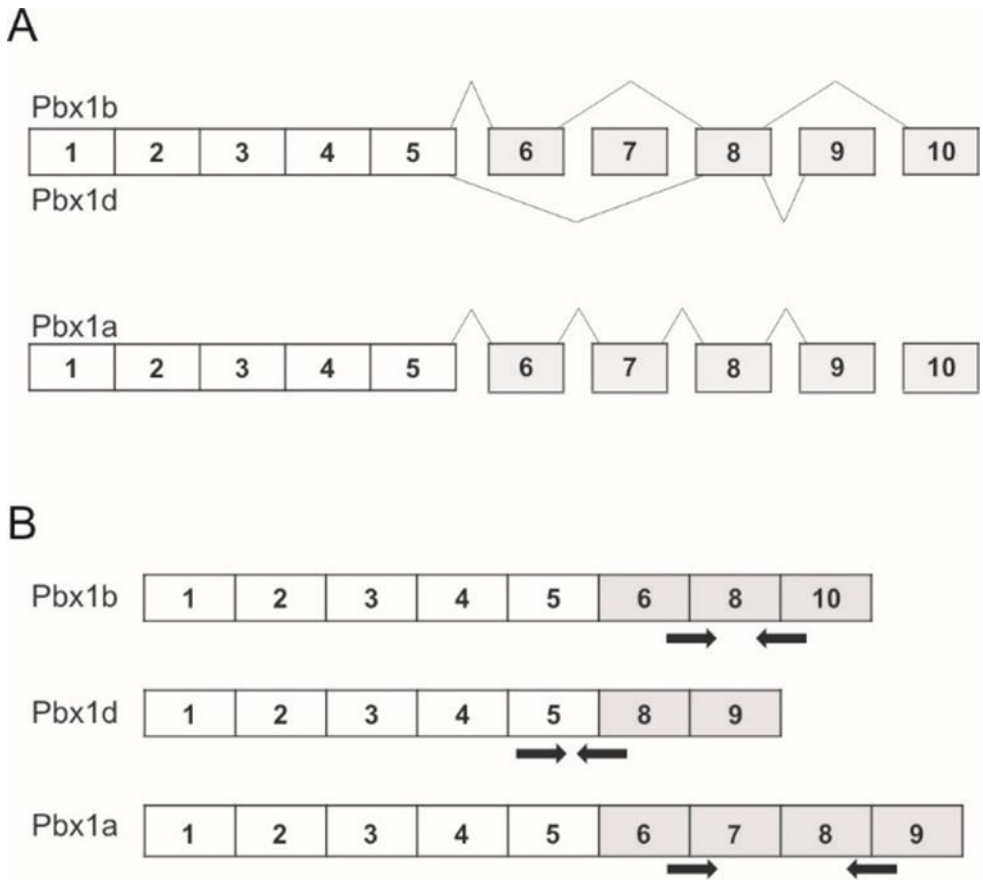

Figure 3. Alternative splicing of *Pbx1* yields multiple isoforms at the mRNA and protein level.

Alternative splicing of exons 6-10 of the *Pbx1* gene yields 3 unique isoforms, *Pbx1b*, *Pbx1d* and *Pbx1a* (A). PCR primers (indicated by arrows) were designed to independently quantify each transcript (B). These primers, with the exception of the *Pbx1d* forward primer, span exon junctions that are unique to each transcript.

that B cells derived from B6.*Sle1*.*ERα*$^{+/+}$ female mice expressed significantly greater levels of *Ly108-1* and significantly lower levels of both *Ly108-2* and *CD48* compared to B6.*ERα*$^{+/+}$ female mice, confirming the impact of *Sle1* on the expression of these genes ($p<0.05$; Figure 4B). However, we found no difference in the expression levels of *Ly108-1*or *Ly108-2* due to ERα deficiency in B cells isolated from B6.*Sle1* or B6 mice, indicating that ERα signaling does not significantly impact gene expression or splicing of *Ly108* (Figure 4B). We also saw no difference in expression of *CD48* due to *ERα* deficiency in B6 control mice, however, we did note a small, but significant increase in expression of *CD48* in B cells of B6.*Sle1*.*ERα*$^{-/-}$ females compared to B6.*Sle1*.*ERα*$^{+/+}$ ($p<0.01$; Figure 4B). Despite the increase in *CD48* expression in B6.*Sle1*.*ERα*$^{-/-}$ females, *ERα* deficiency was not sufficient to restore the levels of *CD48* to that of B6 controls.

A decrease in expression of *Ly108-2* and *CD48* has also been reported in CD4^{+} T cells from B6.*Sle1* mice. In our study, we found a similar decrease in expression of these genes due to *Sle1* (data not shown). However, there was no significant difference in *Ly108-2* or *CD48* expression between T cells isolated from B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ERα*$^{-/-}$ female mice, indicating that *ERα* deficiency does not have a major impact on expression of these genes. Furthermore, although previous studies have shown that *Sle1* is associated with an alternation of *CD84* expression, we did not find that *Sle1* or *ERα* genotype impacted the expression of *CD84* (data not shown).

Finally, *Sle1*c contains several genes including Estrogen Related Receptor γ (*Esrrg*) and Complement Receptor ½ (*Cr2*). CD4^{+} T cells from B6.*Sle1*c mice express lower levels of *Esrrg* compared to T cells from B6 control mice (Perry et al. 2012). Consistent with the findings from Perry and colleagues, we found a decrease in expression of *Esrrg* in CD4^{+} T cells isolated from B6.*Sle1* female mice, however, this difference did not reach statistical significance (Figure 4C). Furthermore, *ERα* deficiency did not significantly alter the expression of *Esrrg* in T cells from B6.*Sle1* mice (Figure 4C). *Cr2* expression has also been reported to be altered in B6.*Sle1*c

subcongenic mice, where *Sle1*c induces an alternative splicing pattern and altered glycosylation of the protein products CR1/CR2 (Boackle et al 2001). Consistent with previous findings, we found that *Sle1* impacts the relative abundance of CR1 and CR2 proteins, and furthermore, that B6.*Sle1*.*ERα*$^{+/+}$ female mice had a higher CR1/CR2 ratio compared to B6.*ER*$^{+/+}$ mice, indicating that *Sle1* affects the alternative splicing of the *Cr2* gene ($p<0.05$; Figure 4E). However, B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ER*$^{-/-}$ mice had similar CR1/CR2 ratios, indicating that *ERα* genotype does not significantly impact *Sle1*-induced alternative splicing of *Cr2*. To investigate the potential impact of *ERα* deficiency on glycosylation of CR1/CR2 proteins, we looked at the migration of bands and found that the CR1 and CR2 expressed on splenocytes isolated from B6.*Sle1* mice migrated at a higher molecular weight compared to that of B6 control mice (Figure 4D). However, we did not find a significant difference in migration between B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ER*$^{-/-}$ mice, suggesting *ERα* genotype does not have a major impact on glycosylation of *Cr2*.

4.4 ERα deficiency does not impact loss of tolerance to chromatin in B6.Sle1 congenic male mice

Our previous work investigating the impact of *ERα* deficiency in (NZBxNZW) F1 mice demonstrated that even low levels of endogenous estrogens, signaling via ERα, induce loss of tolerance in male mice (Bynote et al. 2008). Furthermore, work by Morel and colleagues illustrated a loss of tolerance as demonstrated by anti-chromatin autoantibody production in B6.*Sle1* male mice, although the loss of tolerance was much less dramatic than in B6.*Sle1* females. (Morel et al. 1999). Consistent with the findings by Morel et al., we found that B6.*Sle1* male mice developed significant levels of anti-chromatin IgG autoantibodies, and this level was significantly lower than that of B6.*Sle1* female mice, confirming the proposed sex bias in this *Sle1* phenotype (Figure 5A-D. However, among those mice who did loss tolerance to chromatin, sex did not impact the absolute levels of anti-chromatin IgG (Figure 5B). In sharp contrast to

FIGURE 4

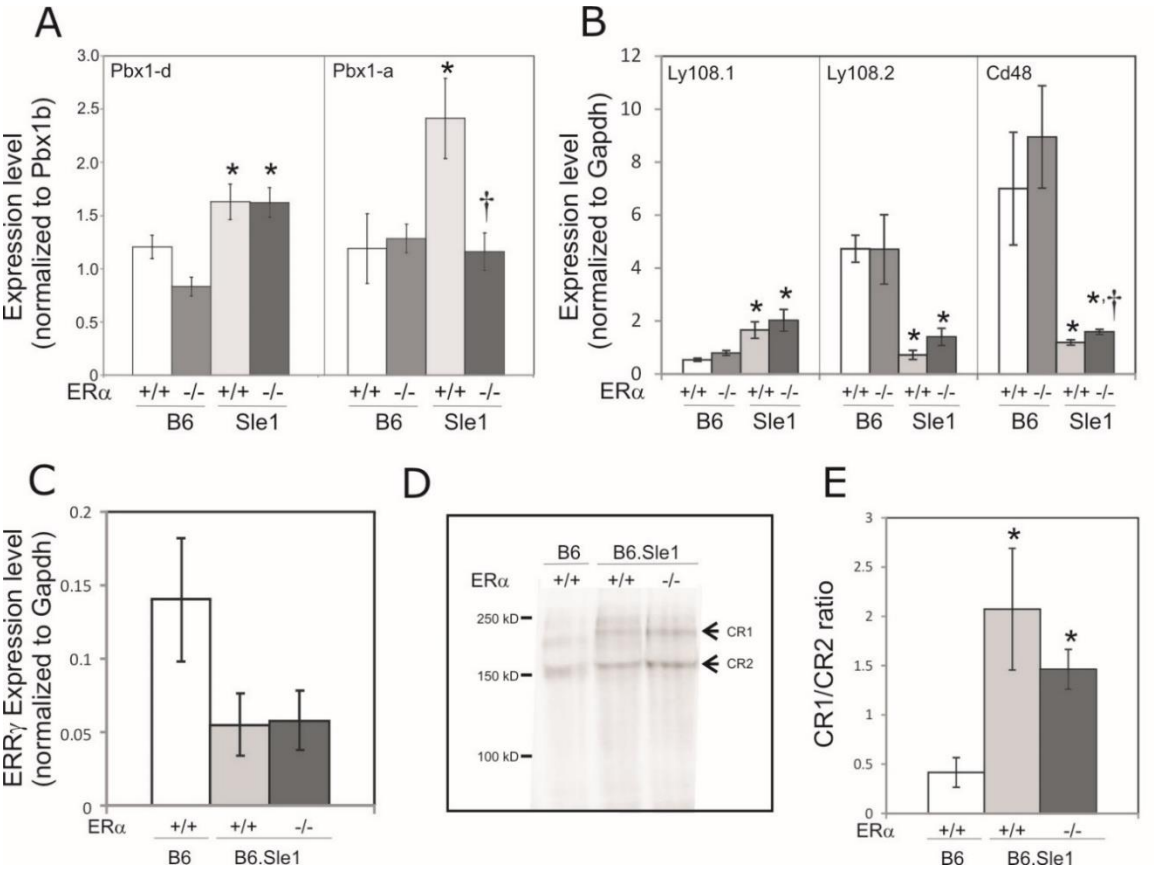

Figure 4. Impact of *ERα* genotype on the expression of *Sle1*-associated genes in female mice.

The relative abundance of transcripts produced by genes that underlie *Sle1* was assessed using quantitative RT-PCR. Data for panels A, B, C and E presented as mean ± standard error of the mean. The expression of *Pbx1d* and *Pbx1a* relative to *Pbx1b* in T cells (A) and *Ly108-1*, *Ly108-2* and *CD48* relative to *Gapdh* in B cells (B) isolated from 5-9 month old B6.*ERα*$^{+/+}$, B6.*ERα*$^{-/-}$, B6.*Sle1*;*ERα*$^{+/+}$ and B6.*Sle1*;*ERα*$^{-/-}$ females (N=5-9 per genotype) is shown. Expression of *Esrrg* in T cells isolated from B6.*ERα*$^{+/+}$, B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ERα*$^{-/-}$ females (N=5-7 per genotype) is shown (C). The relative molecular weight of CR1 and CR2 (indicated by arrows)

expressed in splenocytes from B6.*ERα*$^{+/+}$, B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ERα*$^{-/-}$ females (N=5-7 per genotype) was assessed by western blotting following immunoprecipitation and native gel electrophoresis. A representative image of each genotype is shown (D). CR1 and CR2 bands were quantified and the mean ratio (± standard error of the mean) for each genotype (N=5-7) is shown (E). The * indicates a $P<0.05$ compared to B6 females of the same *ERα* genotype (A and B) or B6.*ERα*$^{+/+}$ females (E). The † indicates a $P<0.05$ compared to B6.*Sle1*.*ERα*$^{+/+}$ females (A and B).

B6.*Sle1* females, *ERα* deficiency did not significantly impact the penetrance of loss of tolerance, or epitope spreading, in B6.*Sle1* males (Figure 5E-H). Therefore, these results suggest that the loss of tolerance in B6.*Sle1*.*ERα*$^{+/+}$ males may represent a basal loss of tolerance due primarily to the *Sle1* locus, and that the sex bias in penetrance of loss of tolerance we found in B6.*Sle1* females is due to estrogen signaling. Furthermore, because *ERα* deficiency attenuated this loss of tolerance, it is likely that ERα is synergizing with *Sle1* to induce the highly penetrant loss of tolerance demonstrated by B6.*Sle1*.*ERα*$^{+/+}$ female mice.

4.5 Estrogens and ERα control sex bias in Sle1-induced loss of tolerance

The proportion of B6.*Sle1*.*ERα*$^{-/-}$ females that developed a loss of tolerance was not different from either ovariectomized B6.*Sle1* females or B6.*Sle1* males, regardless of *ERα* genotype. This strongly suggests that disruption of estrogen signaling via ERα is responsible for the female sex bias in *Sle1*-induced loss of tolerance. Nevertheless, because *ERα* deficiency leads to elevated serum testosterone levels (Couse et al. 2003; Eddy et al. 1996), we considered the possibility that increased androgens in ovariectomized B6.*Sle1* and B6.*Sle1*.*ERα*$^{-/-}$ mice contributed to the attenuation of loss of tolerance. Consistent with previous reports, we found that B6.*Sle1*.*ERα*$^{-/-}$ female mice have serum testosterone levels that are significantly higher than B6.*Sle1*.*ERα*$^{+/+}$ female mice, but similar to that seen in B6.*Sle1* male mice (Figure 6A). From this information, it seemed possible that the attenuation in loss of tolerance in B6.*Sle1*.*ERα*$^{-/-}$ females could be a result of elevated testosterone levels. However, the incidence of loss of tolerance in B6.*Sle1*.*ERα*$^{-/-}$ females is identical to that of ovariectomized B6.*Sle1* female mice, which have very low levels of serum testosterone (Figure 6A). Furthermore, there was no inverse correlation between anti-chromatin IgG autoantibody levels and serum testosterone levels in these mice (Figure 6B). We therefore concluded that estrogens, signaling via ERα, are the primary drivers of the female sex bias and loss of tolerance in B6.*Sle1* congenic mice.

FIGURE 5

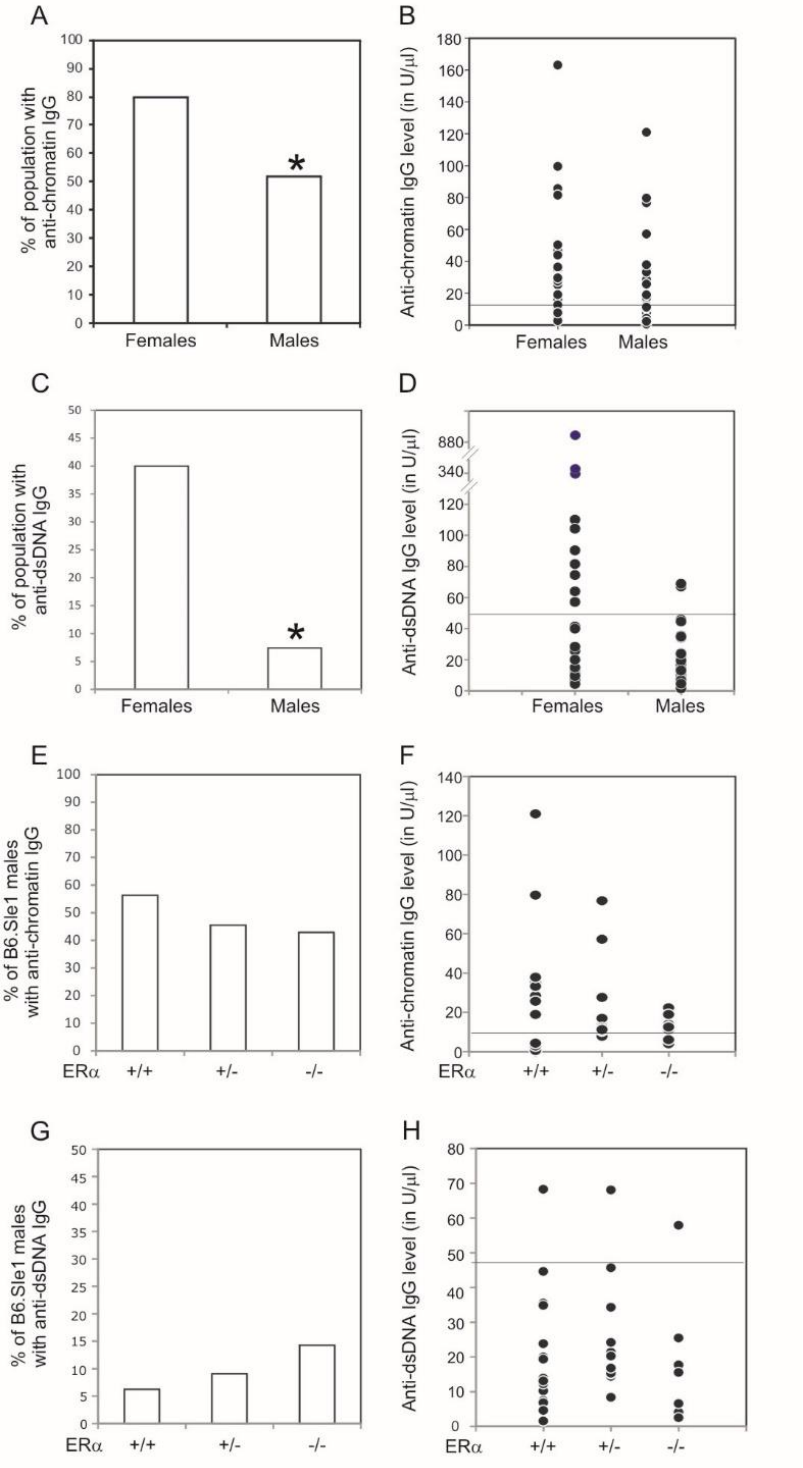
A
% of population with anti-chromatin IgG
Females
Males
B
Anti-chromatin IgG level (in U/μl)
Females
Males
C
% of population with anti-dsDNA IgG
Females
Males
D
Anti-dsDNA IgG level (in U/μl)
Females
Males
E
% of B6.Sle1 males with anti-chromatin IgG
ERα +/+ +/- -/-
F
Anti-chromatin IgG level (in U/μl)
ERα +/+ +/- -/-
G
% of B6.Sle1 males with anti-dsDNA IgG
ERα +/+ +/- -/-
H
Anti-dsDNA IgG level (in U/μl)
ERα +/+ +/- -/-

Figure 5. *ERα* deficiency does not impact loss tolerance in B6.*Sle1* males.

ELISAs were used to assess the abundance of anti-chromatin IgG (A, B, E, and F), anti-dsDNA IgG (C, D, G, and H) in 5-9 month old mice. The proportion of B6.*Sle1*.*ERα*$^{+/+}$ females (N=25) and B6.*Sle1*.*ERα*$^{+/+}$ males (N=27) that develop anti-chromatin IgG (A, B) and anti-dsDNA IgG (C, D) is shown. The proportion of B6.*Sle1*.*ERα*$^{+/+}$ (N=16), B6.*Sle1*.*ERα*$^{+/-}$ (N=11) and B6.*Sle1*.*ERα*$^{-/-}$ (N=10) males that develop anti-chromatin IgG (E, F) and anti-dsDNA IgG (G, H) is shown. For panels B, D, F and H, the horizontal bar denotes the mean serum testosterone levels in mice of the indicated genotypes is shown (N=9-11 of each sex per genotype). The * indicates a $P<0.05$ compared to B6.*Sle1*.*ERα*$^{+/+}$ females (A, C).

FIGURE 6

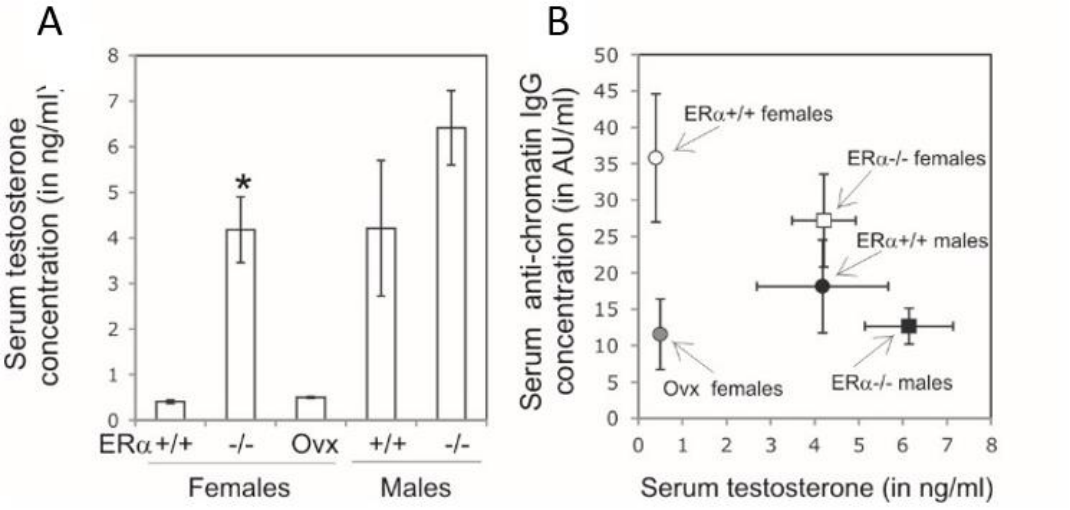

Figure 6. Impact of *ERα* deficiency on serum testosterone levels and serum anti-chromatin IgG concentration.

Mean serum testosterone levels (A) in mice of the indicated genotypes are shown (N= 9-11 per sex per genotype). For each group in panel A, the mean concentration of anti-chromatin IgG (in arbitrary units/mL) was plotted in relation to the mean serum testosterone concentration (B) to determine if these two parameters were correlated. For panels A and B, the error bars represent the standard error of the mean. The * indicates a $P < 0.05$ compared to B6.*Sle1*.*ERα*$^{+/+}$ females (A).

4.6 ERα deficiency has a modest impact on Sle1-induced T cell activation in males

Although *ERα* deficiency had little impact on *Sle1*-induced loss of tolerance in B6.*Sle1* males, we considered the possibility that ERα signaling may still play a role in *Sle1*-induced immune cell hyperactivation, as no previous studies have investigated the impact of sex or ERα signaling on *Sle1*-induced T and B cell hyperactivity. Surprisingly, in contrast to B6.*Sle1* females, B6.*Sle1* males showed no evidence of B cell hyperactivation as there was no difference in expression of B cell activation marker CD86 between B6.*Sle1.ERα*$^{+/+}$ and B6.*ERα*$^{+/+}$ male mice (Table 2). This observation was confirmed with CD25 analysis (~12.9% B220^{+}CD25^{+} in B6.*ERα*$^{+/+}$ males vs ~13.5% B220^{+}CD25^{+} in B6.*Sle1.ERα*$^{+/+}$ males). B6.*Sle1* males did demonstrate CD4^{+} T cell hyperactivity, as indicated by an increase in CD4^{+}CD69^{+} and CD4^{+}CD134^{+} population sizes ($p<0.05$; Table 2). However, it should be noted that the T cell hyperactivation in B6.*Sle1* males is less remarkable than in B6.*Sle1* females, indicating a sex-biased phenotype in this parameter. Interestingly, *ERα* deficiency had relatively little impact on T and B cell hyperactivation in B6.*Sle1* males (Table 3). Altogether, these data illustrate a female sex bias in *Sle1*-induced immune cell hyperactivation, and demonstrate that this phenotype is due to estrogen signaling via ERα.

4.7 ERα deficiency does not alter the expression of Sle1-associated genes in male B6.Sle1 mice

Similar to our observations in female B6.*Sle1* mice, *ERα* deficiency did not have a significant impact on the expression of *Ly108-1*, *Ly108-2*, or *CD48* in male B6.*Sle1* mice (Figure 7A). Furthermore, we did not see a significant impact of sex on the expression of these genes in B6.*Sle1* mice (Figure 7B). Likewise, the magnitude of effect of *Sle1* on the expression of these genes was not different between B6.*Sle1* female and male mice. *ERα* deficiency did not have a significant effect on relative expression of either *Pbx1d* or *Pbx1a* in CD4^{+} T cells from B6.*Sle1* male mice ($p<0.05$; Figure 7C). Interestingly, the relative expression of both *Pbx1d* and *Pbx1a* in

Table 2. Sex differences in *Sle1*-induced immune cell activation

	Females		Males	
Cell Type	B6	B6.Sle1	B6	B6.Sle1
$B220^+$	60.9±2.4	44.7±3.5*	65.2±6.6	$57.0±2.2^{††}$
$B220^+CD86^+$	4.7±1.3	13.8±2.5*	3.4±0.3	$4.0±0.5^{††}$
$CD4^+$	15.0±1.0	17.4±1.0	15.1±2.3	14.3±0.7
$CD4^+CD69^+$	6.7±1.1	16.3±2.5**	9.6±2.9	18.3±2.9*
$CD4^+CD134^+$	5.5±0.8	17.6±2.8*	9.3±5.6	14.6±2.5*
$CD4^+CD62L^+$	40.5 ±7.1	13.9 ±1.7**	$62.3 ±9.8^{†}$	$35.6±3.4^{**,††}$
$CD4^+CD25^+$	2.4±0.2	1.9±0.1*	$2.7±0.1^{†}$	2.0±0.1*

*,**Significant difference compared to B6 mice of same sex are denoted by * (P=0.05) and **(P=0.01)

†,†† Significant difference compared to female mice of the same Sle1 genotype are denoted by †(P=0.05) and ††(P=0.01)

Table 2. Impact of sex on lymphocyte activation in B6.*Sle1* mice.

Splenocytes from each mouse (6-12 months of age) were analyzed individually by flow cytometry (N=12-25 per group). The values presented represent the mean ± SEM for the samples in each group.

Table 3. Impact of *ERα* genotype on *Sle1*-induced immune cell activation in males

		B6		B6.Sle1	
Cell Type	ERα	+/+	-/-	+/+	-/-
$B220^+$		57.3±2.2	52.9±3.7	58.6±2.0	59.4±2.2
$B220^+CD86^+$		6.8±1.1	6.6±1.8	7.0±2.2	7.9±2.2
$CD4^+$		15.6±1.9	15.9±1.7	16.2±0.7	15.2±0.9
$CD4^+CD69^+$		17.6±2.7	14.7±3.4	25.5±2.5	15.0±3.9†
$CD4^+CD134^+$		17.8±3.4	21.3±6.4	25.7±3.4	15.3±4.6
$CD4^+CD62L^+$		52.9±6.9	55.9±6.3	31.4±3.0**	33.7±4.6**
CD^+CD25^+		2.3±0.2	2.5±0.3	2.2±0.1	2.3±0.2

*,**Significant difference compared to B6 mice of the same ERα genotype are denoted by * (P=0.05) and **(P=0.01)

†,†† Significant difference compared to $ER\alpha^{+/+}$mice of the same Sle1 genotype are denoted by †(P=0.05) and ††(P=0.01)

Table 3. Impact of *ERα* genotype on lymphocyte activation in male B6.*Sle1* mice.

Splenocytes from each mouse (6-12 months of age) were analyzed individually by flow cytometry (N=14-32 per group). The values presented represent the mean ± SEM for the samples in each group.

$CD4^+$ T cells was greater in B6.*Sle1* congenic females compared to their male counterparts, but this difference only reached statistical significance for *Pbx1d* (Figure 7D). Consistent with our results in B6.*Sle1* females, *ERα* genotype had no impact on *Esrrg* expression or CR1/CR2 ratios in B6.*Sle1* males.

FIGURE 7

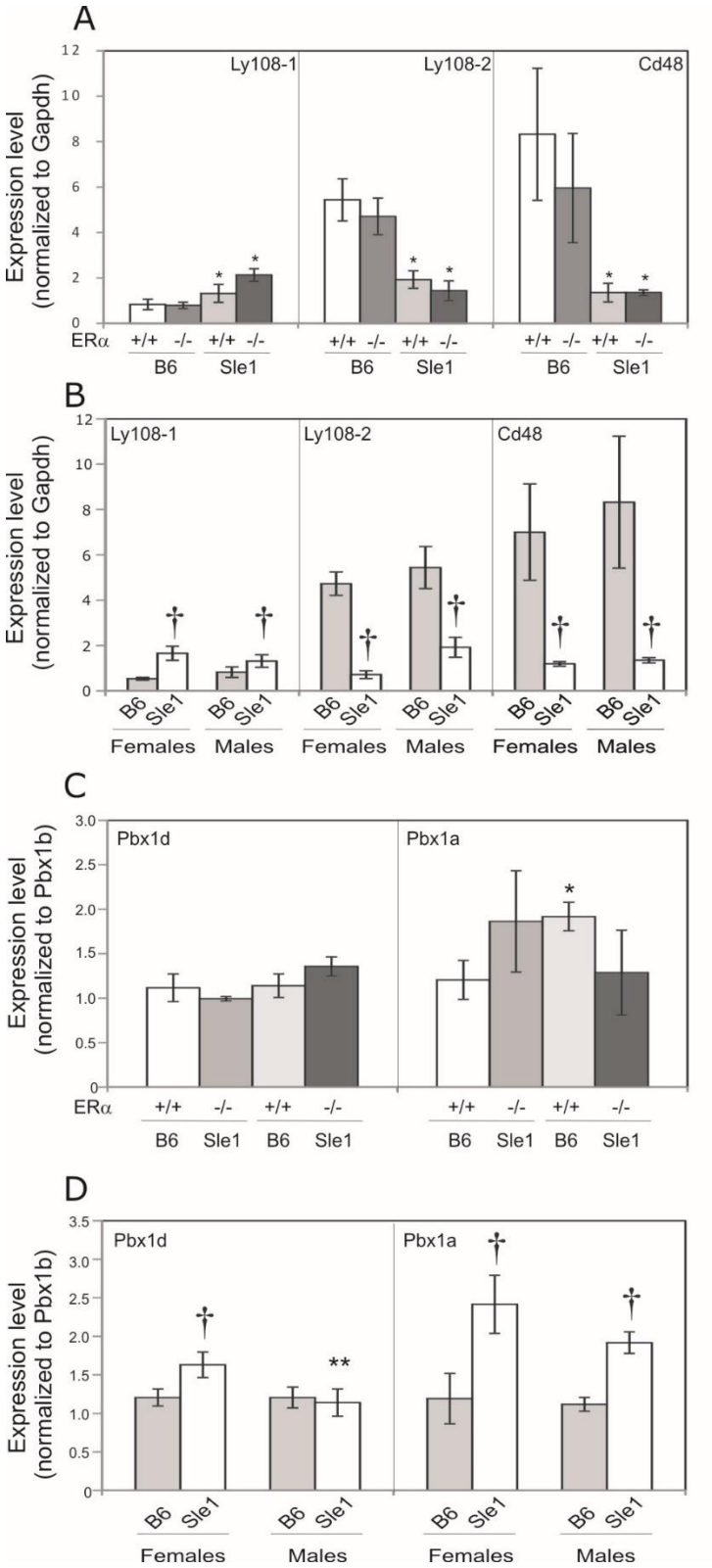
A
Ly108-1
Ly108-2
Cd48
Expression level (normalized to Gapdh)
ERα
+/+ -/- +/+ -/-
B6 Sle1
B
Females Males
C
Pbx1d
Pbx1a
Expression level (normalized to Pbx1b)
D

Figure 7. Impact of *ERα* genotype on the expression of *Sle1*-associated genes in male mice. The relative abundance of transcripts produced by genes that underlie *Sle1* was assessed using quantitative RT-PCR. Data is presented as mean ± standard error of the mean. The expression of *Ly108-1*, *Ly108-2* and *CD48* relative to *Gapdh* in B cells (A and B) and *Pbx1d* and *Pbx1a* relative to *Pbx1b* in T cells (C and D) is shown. The impact of *Sle1* and *ERα* genotype on the expression of *Ly108-1*, *Ly108-2* and *CD48* is shown by comparing relative expression in B6.*ERα*$^{+/+}$, B6.*ERα*$^{-/-}$, B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ERα*$^{-/-}$ males (N=4-5 per genotype) (A). The impact of sex on the expression of *Ly108-1*, *Ly108-2* and *CD48* is shown by comparing expression in B6 and B6.*Sle1* females and males (B). The impact of *Sle1* and *ERα* genotype on the expression of *Pbx1d* and *Pbx1a* is shown by comparing relative expression in B6.*ERα*$^{+/+}$, B6.ERα$^{-/-}$, B6.*Sle1*.*ERα*$^{+/+}$ and B6.*Sle1*.*ERα*$^{-/-}$ males (N=4-5 per genotype) (C). The impact of sex on the expression of *Pbx1d* and *Pbx1a* is shown by comparing expression in B6 and B6.*Sle1* females and males (D). The * indicates a $P<0.05$ compared to B6 male mice of the same *ERα* genotype (A and C). The † indicates a $P<0.05$ compared to sex-matched B6 mice (B and D). The ** indicates a $P<0.05$ compared to female mice of the same *Sle1* genotype (D).

DISCUSSION

Previous studies have indicated that the impact of lupus susceptibility locus *Sle1* is more robust in female mice than in males, however, the basis for this sex bias has been unknown. Given our previous work investigating the role of ERα signaling in (NZBxNZW) F1 lupus-prone mice, we hypothesized that estrogens, acting via ERα, may be responsible for the female sex bias in *Sle1*-induced loss of tolerance. Therefore, we examined the impact of removing the ovaries, the primary source of estrogens in females, on the *Sle1* phenotype. Furthermore, we generated B6.*Sle1* mice that were estrogen receptor wild-type ($ER\alpha^{+/+}$), heterozygous ($ER\alpha^{+/-}$), or null ($ER\alpha^{-/-}$) to determine if estrogen signaling via ERα could be synergizing with the *Sle1* locus to induce a female-biased loss of tolerance. Our results indicate that the female sex bias in the *Sle1* phenotype is in fact due to estrogen-dependent, ERα mediated processes. We found no evidence of attenuation of disease due to androgens, further illustrating the impact of estrogen signaling in the female sex bias associated with *Sle1*.

Based upon our results, we conclude that the *Sle1* lupus susceptibility locus induces a basal level of loss of tolerance that is independent of both sex and ERα signaling. *Sle1,* via these basal mechanisms, results in loss of tolerance in ~50% of mice, the proportion of B6.*Sle1* males the develop anti-chromatin IgG. Our observations also suggest that estrogen-dependent, ERα-mediated processes synergize with *Sle1*, increasing the proportion of B6.*Sle1* females that develop anti-chromatin IgG to more than 80%. Thus ERα signaling results in a significant increase in the penetrance of *Sle1* in females.

Sle1 also has been associated with hyperactivation of both B cells and T cells. We report here for the first time that *Sle1*-induced immune cell activation also shows a significant sex bias. *Sle1*-induced B cell hyperactivation was observed only in B6.*Sle1* females and was completely abrogated by *ERα* deficiency, suggesting that this phenotype in is fully dependent upon ERα

signaling. This observation is consistent with a previous report indicating that increased B cell activation is a feature of B6.*Sle1b* subcongenic female, but not male, mice (Wong et al. 2012).

By contrast, we found that *Sle1*-induced T cell activation was detectable in both B6.*Sle1* females and males. However, we noted that this aspect of the *Sle1* phenotype was more robust in B6.*Sle1* females than males. We also observed that *ERα* deficiency attenuated but did not completely eliminate *Sle1*-induced T cell activation. Altogether, these results indicate that *Sle1* induces some degree of T cell hyperactivation independent of sex and ERα. ERα-dependent signaling synergizes with *Sle1* to induce a more profound T cell hyperactivation phenotype in females. Here again, our results regarding the impact of sex on *Sle1*-induced T cell activation are consistent with the findings of Wong et al (2012), which indicate that *Sle1b*-induced T cell activation is more pronounced in female mice than male mice. We also noted that *ERα* deficiency alone slightly increased the proportion of naïve T cells, suggesting the ERα signaling can promote some degree of T cell activation. This effect was observed in females only and was independent of *Sle1* genotype. Finally, we also observed that *ERα* deficiency had no impact on the decrease in the number of regulatory T cells that is observed in B6.*Sle1* congenic mice.

The NZW-derived allelic variants that underlie the *Sle1* lupus susceptibility locus lead to differential expression and/or splicing of genes within the *Sle1* interval, and the altered expression of these genes is responsible for the immune dysfunction and loss of tolerance in B6.*Sle1* congenic mice. The impact of sex on expression and splicing of these genes has not been described. We postulated that ERα may impact the *Sle1* phenotype by directly regulating the expression of these same genes. Using qRT-PCR, we found that the relative expression of the *Pbx1d* isoform of *Pbx1* was increased in B6.*Sle1* congenic females but not males. However, *Pbx1d* expression was not attenuated in B6.*Sle1*.*ERα*$^{-/-}$ females, indicating that the expression of this isoform is not ERα dependent. We note that the present study, the relative proportion of *Pbx1d* to *Pbx1b* in non-autoimmune B6 control mice was somewhat higher than that reported

previously (Cuda et al, 2012). However, the *Pbx1d*:*Pbx1b* ratio in our other set of non-autoimmune control mice, the B6.*ERα*$^{-/-}$ mice, was not significantly different from that in our B6.*ERα*$^{+/+}$ controls and was also very similar to that reported by Cuda et al (2012). It is likely that any differences in the relative expression of *Pbx1* isoforms among control mice in these two studies reflects the differences in the methods used to evaluate and quantify gene expression. Nevertheless, despite differences in methods and relative expression in control samples, we observe that *Sle1* is indeed associated with an increase in the relative abundance of *Pbx1d*; this result is fully consistent with the findings of Cuda et al (2012).

We also detected *Pbx1a*, another isoform of *Pbx1*, in T cells from both B6 and B6.*Sle1* congenic mice. Although *Pbx1a* was reported to the undetectable by conventional RT-PCR in both the B6 strain and the B6.*Sle1a.1* subcongenic strain (Cuda et al. 2012), we were able to detect this isoform using an isoform specific qRT-PCR assay. The relative expression of the *Pbx1a* isoform was also increased in B6.*Sle1* congenic mice compared to B6 mice, but sex did not appear to impact *Pbx1a* expression. Surprisingly, *Pbx1a* expression was significantly reduced in B6.*Sle1*.*ERα*$^{-/-}$ congenic females. These observations suggest that ERα signaling may impact the *Sle1* phenotype, in part, by modulating the expression the *Pbx1a* isoform. It remains to be determined if the effects of ERα on *Pbx1* isoform expression are due to direct effects on *Pbx1* transcription and/or splicing or due to indirect effects. Neither sex nor *ERα* deficiency had an impact on the expression of the other genes that underlie *Sle1*. In this context, it is particularly noteworthy that neither sex nor *ERα* deficiency had an impact on the expression of *Ly108-1*, *Ly108-2* or *CD48*, which are associated with *Sle1b*, the sublocus that displays the most dramatic sex bias (Mohan et al. 1998; Mohan et al. 1999).

Pbx1 is a member of the three amino acid loop extension family of transcription factors (Kamps et al. 1990; Nourse et al. 1990). *Pbx1* proteins form homodimers and heterodimers with other homeodomain containing proteins. These *Pbx1*-containing dimers form ternary complexes

with other transcription factors, such as Hox proteins and nuclear steroid hormone receptors, and modulate the ability of these other transcription factors to bind to specific binding sites in the genome (Chang et al. 1996; Chang et al. 1995; Laurent et al. 2008). Evidence suggests that *Pbx1*-containing complexes impact transcription factor binding by recruiting co-repressors and co-activators and thus modulating the accessibility of specific binding sites (Laurent et al. 2008; Saleh et al. 2000). In this context, it is of particular interest that *Pbx1* has been shown to modulate the binding of ERα to specific estrogen response elements in ER^+ breast cancers (Magnani et al. 2011). However, the impact of *Pbx1* on either the accessibility of estrogen response elements or ERα function in other cell types, including those relevant to lupus susceptibility, has not been examined.

Our results suggest that sex and ERα impact the relative expression of *Pbx1* isoforms. Differential expression of these isoforms has been shown previously to correlate with lupus susceptibility (Cuda et al. 2012), but the basis for this effect is not fully clear. The *Pbx1a* isoform encodes a protein that contains the central domains required for DNA and homeodomain-containing protein binding as well as the c-terminal activation domain and co-repressor binding domain (Asahara et al. 1999; Peltenberg et al. 1997; DiRocco et al. 1997; Chang et al. 1997). The *Pbx1b* protein product has a c-terminal truncation that eliminates the c-terminal activation domain and co-repressor binding domains (Asahara et al. 1999; DiRocco et al. 1997). The *Pbx1d* peptide contains an internal deletion that eliminates the amino acids required for optimal Hox protein and DNA binding and is thus postulated to function as a dominant negative (Cuda et al. 2012). *Pbx1d* also lacks a portion of the c-terminal activation domain and co-repressor binding domain (Cuda et al. 2012). It is likely that the *Pbx1a*, *Pbx1b* and *Pbx1d* isoforms differ dramatically in their ability to interact with co-repressors, co-activator and transcription factors, and thus, in their ability to modulate transcription in different cellular contexts. Given the fact that *Pbx1* is a critical regulator of self-renewal and lineage commitment in the hematopoietic and

lymphoid lineage (Riddell et al. 2014; Ficara et al. 2013; Ficara et al. 2008), changes in the relative expression of the *Pbx1* isoforms could have a dramatic impact on autoimmunity. Further studies are required to determine the mechanism through which different *Pbx1* isoforms modulate lupus susceptibility and to determine the impact of ERα signaling of these processes.

CHAPTER V. IMPACT OF ERα DEFICIENCY ON *Sle1a*- AND *Sle1b*-INDUCED LOSS OF TOLERANCE AND IMMUNE CELL HYPERACTIVATION

INTRODUCTION

Our initial work investigating the interaction of *Sle1* with estrogen signaling has illustrated that ERα signaling strongly enhances *Sle1*-induced loss of tolerance to chromatin in females and contributes to T and B cell hyperactivation in B6.*Sle1* female mice, and to a lesser degree, B6.*Sle1* male mice. However, because the *Sle1* interval has been further characterized into distinct subloci, each contributing to the loss of tolerance and immune cell hyperactivation, it is necessary to tease out the interaction of ERα signaling with *Sle1* subloci to better understand the synergy between the genetic and hormonal components of lupus susceptibility. In order to do so, we have chosen to focus our future studies on *Sle1a* and *Sle1b*, two *Sle1* subloci that display a female sex bias in loss of tolerance penetrance. By better understanding the interplay between *Sle1* subloci and ERα signaling, we will shed light on the cellular and molecular pathways that are impacted by both potent lupus susceptibility loci and estrogens, and take the first step in developing novel therapeutics to target molecular pathways regulated by both *Sle1* and estrogen signaling.

Previous work by Morel and colleagues (2001) demonstrated that subcongenic B6.*Sle1a* mice display a ~30% penetrance of loss of tolerance. This phenotype is reported to be female sex-biased, although this difference has not been reported to reach statistical significance. Although sex has been suggested to influence anti-chromatin IgG production in B6.*Sle1a* mice, no studies to date have investigated the impact of hormones on lupus susceptibility or progression in these mice. Because our studies in B6.*Sle1* mice demonstrated that ERα signaling is responsible for the female sex bias and loss of tolerance in *Sle1* females, we hypothesize that estrogen signaling via ERα may synergize with genes underlying *Sle1a* to promote the loss to tolerance in female B6.*Sle1a* mice.

Sle1a is also associated with a unique immune cell phenotype, contributing to the overall T and B cell hyperactivation seen in B6.*Sle1* mice. Chen et al. (2005) found that, similar to that of B6.*Sle1*, B6.*Sle1a* mice have a significantly higher proportion of $CD4^+$ T cells display activation markers CD69 and CD134 compared to B6 control mice. Furthermore, $CD4^+$ T cells from B6.*Sle1a* showed increased proliferation and decreased apoptosis compared to control mice, at least in part due to a relative overexpression of the autoimmune immune isoform of *Pbx1*, *Pbx1-d* (Cuda et al, 2012). Our observations in *Sle1* mice suggest a relative increase in the expression of another *Pbx1* isoform, *Pbx1-a*, in B6.*Sle1* mice which may also contribute to autoreactivity in B6.*Sle1a* mice. Through the use of adoptive transfer into $Tcr\alpha^{-/-}$ mice, Chen and colleagues also found that *Sle1a* T cells induce activation of B cells from non-autoimmune mice, indicating that intrinsic defects in *Sle1a* T cells play a major role in increased activation and antibody production by B cells (2005). *Sle1a* T cells are also associated with higher cytokine expression, including IL-2, IL-4, and IFNγ (Chen et al. 2005). Finally, B6.*Sle1a* have a reduced population of T regulatory cells, which contributes to the generation of autoreactive T cells (Chen et al. 2005). Altogether, these data illustrate a major impact of *Sle1a* on the altered T cell phenotype displayed by *Sle1* mice. The impact of *Sle1a* on B cell activation has not been investigated in depth, although one study by Morel et al. (2001) demonstrated that B cells from B6.*Sle1a* mice do not differ in activation status from B6 controls. However, no additional studies have since determined the impact of *Sle1a* on *Sle1*-associated B cell hyperactivation, so further investigation is warranted.

Sle1b is identified as the most potent *Sle1* sublocus, with anti-chromatin autoantibody production penetrance reaching ~80% (Morel et al. 2001). The penetrance of loss of tolerance in *Sle1b* mice has been shown to be sex-biased, with *Sle1b* females developing anti-chromatin IgG antibodies earlier and with a higher penetrance. Despite a pronounced female sex bias, no studies to date have investigated the impact of hormones on B6.*Sle1b* lupus susceptibility. Furthermore, nothing is known about the impact of estrogen signaling on *Sle1b*-induced loss of tolerance.

In addition to promoting loss of tolerance, *Sle1b* is also associated with altered functions in both T and B cells (Sobel et al. 2002; Wandstrat et al. 2004; Wong et al. 2012). Unstimulated B cells from B6.*Sle1b* are hyperactive, and have an enhanced ability to present antigen compared to B cells from B6 controls (Jennings et al. 2008). It has been suggested that polymorphisms associated with *Sle1b* genes cause transcriptional and structural changes that alter B cell tolerance, contributing to the anti-chromatin IgG autoantibody production (Wang et al. 2010). Additionally, $CD4^+$ T cells from B6.*Sle1b* mice have been reported to exhibit hyperactivity, although this has not been shown consistently (Morel et al. 2001; Chen et al, 2005; Perry et al. 2012). Finally, consistent with the findings that *Sle1b* enhances loss of tolerance preferentially in females, Wong and colleagues identified a female sex bias in B cell activation and proliferation. It was also demonstrated that only female B6.*Sle1b* had an alteration in the germinal center tolerance checkpoint, leading to increased follicular T helper cell numbers and subsequent increase in autoreactive B cell autoantibody production (Wong et al. 2012). Despite identifying a strong female sex bias in the phenotype associated with *Sle1b*, no studies to date have investigated the impact of female sex hormones, or specifically ERα signaling, on *Sle1b*-induced loss of tolerance and immune cell hyperactivity.

Because our work in B6.*Sle1* mice showed that *ERα* deficiency attenuates *Sle1*-induced loss of tolerance in females, we predict that *ERα* deficiency will impact *Sle1b*-induced loss of tolerance, and to a lesser degree, *Sle1a*-induced loss of tolerance in female mice, as determined by anti-chromatin IgG production. Furthermore, we predict that ERα signaling cooperates with *Sle1a* and *Sle1b* to promote immune cell hyperactivity and alterations in immune cell populations in both male and female mice. We anticipate that *ERα* deficiency will attenuate *Sle1a*-associated $CD4^+$ T cell hyperactivation and cytokine production in females, and to a lesser degree, in males. We will also investigate the impact of *Sle1a* on B cell activation, as little is known about a possible impact of *Sle1a* on the B cell population. Finally, we hypothesize that *ERα* deficiency

will attenuate *Sle1b*-associated T and B cell hyperactivation, decrease the follicular T helper cell population size, and may alleviate the perturbation on the germinal center tolerance checkpoint in B6.*Sle1b* female mice.

Although the work cited above, from both our own lab and others, provide strong evidence that estrogen signaling synergizes with the *Sle1* interval to promote a female sex bias in loss of tolerance and immune cell activation, very little is known about the specific cellular/molecular pathways and processes that are modulated by ERα. To better understand the synergy between estrogens and lupus susceptibility loci, we will test our hypothesis that ERα signaling modulates the actions of *Sle1a* and *Sle1b* to enhance loss of tolerance in females. Identifying the role of ERα signaling in each *Sle1* sublocus will aid in the identification of molecular pathways impacted by both lupus susceptibility loci and hormone signaling pathways, the first step in developing more effective preventative and therapeutic agents for lupus treatment.

RESULTS

5.1 Impact of ERα deficiency on Sle1a-induced loss of tolerance in female and male B6.Sle1a mice.

Our previous studies in B6.*Sle1* mice demonstrate that ERα signaling promotes loss of tolerance in B6.*Sle1* females. Because *Sle1a*, a sublocus of *Sle1*, is associated with loss of tolerance preferentially affecting B6.*Sle1a* females, we hypothesized that *ERα* deficiency may attenuate *Sle1a*-induced loss of tolerance and may shed light on the genetic interval synergizing with ERα to promote loss of tolerance. To explicitly to test the hypothesis that ERα signaling synergizes with *Sle1a* to promote loss of tolerance, we intercrossed the B6.*ERα* knockout strain with B6.*Sle1a* and B6.*Sle1b* subcongenic strains to generate male and female mice that are $ERα^{+/+}$, $ERα^{+/-}$, and $ERα^{-/-}$ carrying an individual *Sle1* sublocus. We then evaluated the penetrance of anti-chromatin IgG autoantibody production via ELISA. As expected, ~38% of female B6.*Sle1a*.$ERα^{+/+}$ developed loss of tolerance (Figure 8). Heterozygosity and deficiency of *ERα* seemed to decrease the proportion of B6.*Sle1a* females that develop anti-chromatin IgG (~17% penetrance for each genotype), although this difference falls short of statistical significance ($P>0.05$).

Additionally, although our previous work investigating the impact of *ERα* deficiency on *Sle1*-induced loss of tolerance suggests that ERα signaling promotes autoantibody production preferentially in females, we are nevertheless considering any possible impact of estrogen on loss of tolerance in B6.*Sle1a* male mice. Similar to that seen in females, we observed that ~33% of B6.*Sle1a*.$ER^{+/+}$ male mice lose tolerance (Figure 9), suggesting a much less remarkable female sex bias in loss of tolerance compared to *Sle1* congenic mice (Figure 10). This observation is consistent with Morel and colleagues observation of a less robust impact of *Sle1a* alone on loss of tolerance, and a less significant impact of sex on *Sle1a*-induced loss of tolerance. Only ~13% of B6.*Sle1a*.$ERα^{+/-}$, and none (0%) of the B6.*Sle1a*.$ER^{-/-}$ male mice, develop anti-chromatin

FIGURE 8

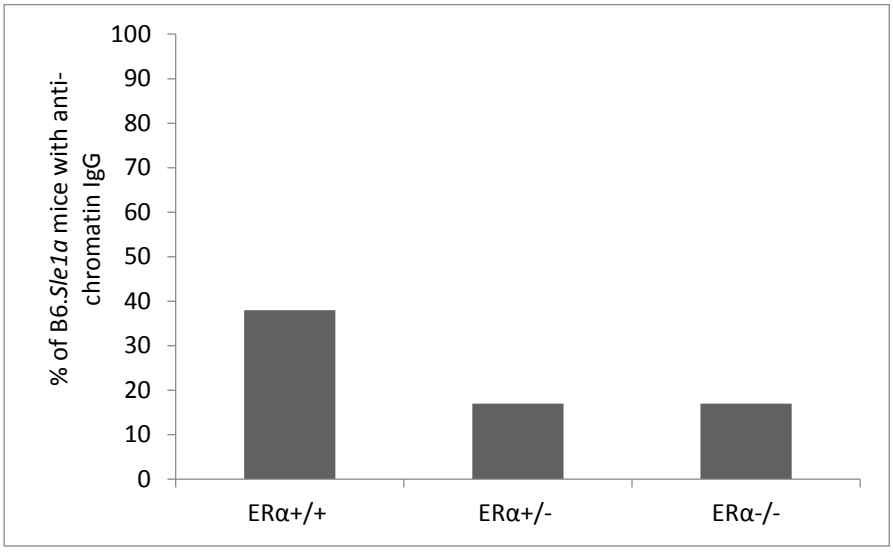

Figure 8. Impact of *ERα* deficiency on *Sle1a*-induced loss of tolerance in females.

ELISAs were used to evaluate the level of anti-chromatin IgG in 4-6 month old B6.*Sle1a* mice. N= 14-16 per *ERα* genotype. The threshold for a positive reading was set at 2 standard deviations above the mean of a group of control B6 mice as described previously by Morel et al. (2001).

antibodies (Figure 9). Interestingly, *ERα* deficiency completely eliminated *Sle1a*-induced loss of tolerance in males (33% in B6.*Sle1a*.*ERα*$^{+/+}$ males vs 0% in B6.*Sle1a*.*ERα*$^{-/-}$ males; P<0.05).

5.2 Impact of ERα deficiency on Sle1a-associated lymphocyte hyperactivation in female and male B6.Sle1a mice.

Sle1a is associated with T cell hyperactivation, as indicated by a greater proportion of CD4^{+} T cells expressing activation markers CD69 and CD134, and a reduced proportion of CD4^{+} T cells expressing naïve T cell marker CD62L (Morel et al. 2001). Because our initial work with B6.*Sle1* mice indicates that *ERα* deficiency attenuates T cell hyperactivity in B6.*Sle1* females, and to a lesser degree B6.*Sle1* males, we sought to determine the impact of *ERα* deficiency on T cell hyperactivation in B6.*Sle1a* mice. Furthermore, because relatively little is known about the impact of *Sle1a* on B cell hyperactivation, we evaluated the impact of *ERα* deficiency on B cell hyperactivation in B6.*Sle1a* mice. To do so, we used flow cytometry to assess the expression of surface markers on T and B cells isolated from B6.*Sle1a* mice.

As predicted from studies by Morel et al. (2001), *Sle1a* did not impact the proportion of B220^{+}CD86^{+} cells in B6.*Sle1a* female mice (Table 4). In contrast, the proportion of CD4^{+} T cells expressing activation markers CD69 and CD134 was significantly increased in T cells from female B6.*Sle1a*.*ERα*$^{+/+}$ mice (Table 4; p<0.01). Furthermore, the proportion of CD4^{+} T cells from B6.*Sle1*.*ERα*$^{+/+}$ female mice expressing naïve T cell marker CD62L was significantly reduced compared to B6 controls. However, in contrast to our findings in B6.*Sle1* T cells isolated from female mice, *ERα* deficiency had no significant impact on *Sle1a*-induced T cell hyperactivation (Table 4), as we observed no alteration in the proportions of CD4^{+} T cells expressing CD69, CD134, or CD62L from B6.*Sle1*.*ERα*$^{-/-}$ female mice compared to B6.*Sle1*.*ERα*$^{+/+}$ females. This finding strongly suggests that the attenuation of T cell hyperactivity by *ERα* deficiency in B6.*Sle1* females is likely dependent on molecular pathways altered by

FIGURE 9

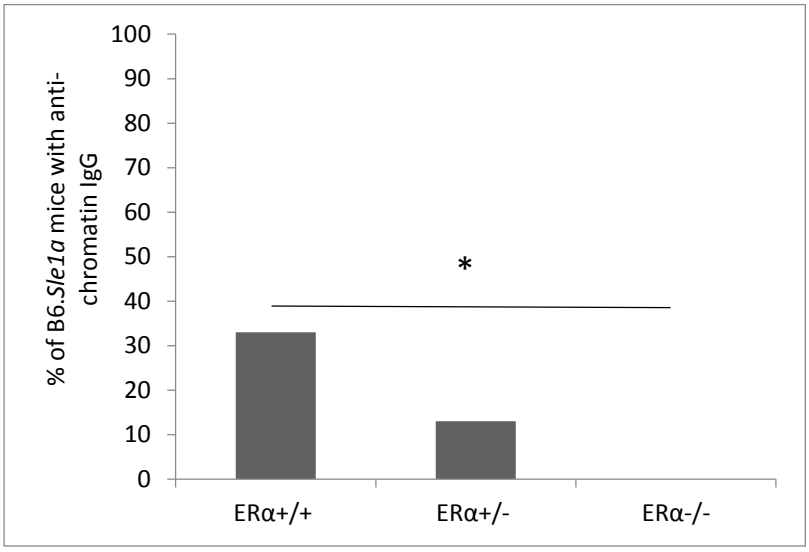

Figure 9. Impact of *ERα* deficiency on *Sle1a*-induced loss of tolerance in males.

ELISAs were used to evaluate the level of anti-chromatin IgG in 4-6 month old B6.*Sle1a* mice. N= 15-25 per *ERα* genotype. The threshold for a positive reading was set at 2 standard deviations above the mean of a group of control B6 mice as described previously by Morel et al. (2001). * denotes statistical significance ($P<0.05$).

FIGURE 10

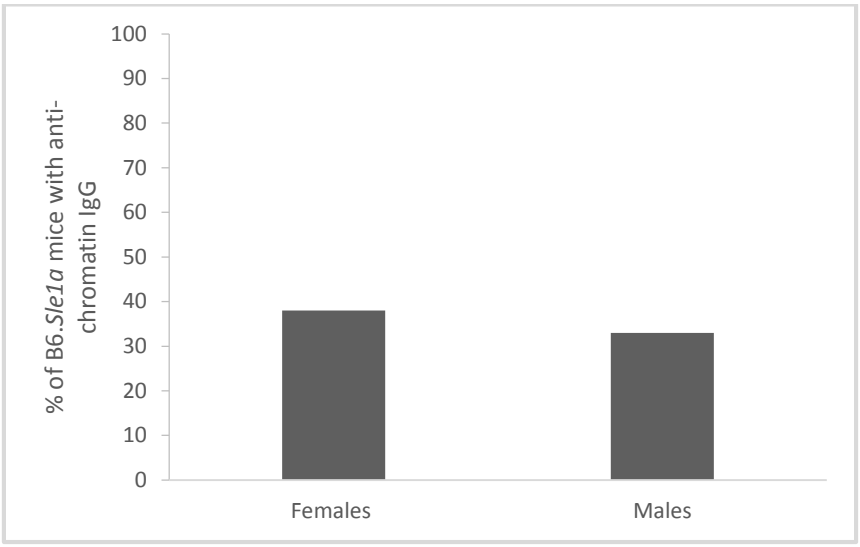

Figure 10. Impact of sex on *Sle1a*-induced loss of tolerance.

ELISAs were used to evaluate the level of anti-chromatin IgG in 4-6 month old B6.*Sle1a* mice. N= 15-16 per *ERα* genotype. The threshold for a positive reading was set at 2 standard deviations above the mean of a group of control B6 mice as described previously by Morel et al. (2001).

genes within other *Sle1* subloci, or at least requires synergy between *Sle1a* and genes with other subloci of *Sle1*. We also observed an increased proportion of follicular T helper cells in B6.*Sle1a* females (Table 4; $p<0.01$), however, *ERα* deficiency did not significantly impact this cell population.

Because the impact of sex has not yet been investigated on *Sle1a*-associated lymphocyte hyperactivation, we next compared immune cell activation phenotypes in female and male B6.*Sle1a*.*ERα*$^{+/+}$ mice. Similar to our observations in female B6.*Sle1a*.*ERα*$^{+/+}$ mice, we noted no difference in B cell activation between B6.*Sle1a*.*ERα*$^{+/+}$ males compared to male B6 controls (Table 5). However, unlike female B6.*Sle1a*.*ERα*$^{+/+}$ mice, male B6.*Sle1a*.*ERα*$^{+/+}$ mice did not differ in the proportion of $CD4^+CD69^+$ or $CD4^+CD134^+$ T cells from B6 controls, indicating a sex bias in *Sle1a*-induced T cell hyperactivation (Table 5). Similar to our findings in B6.*Sle1* mice, we did not observe a sex bias in the *Sle1*-induced reduction of $CD4^+$ T cells expressing naïve T cell marker CD62L between male and female B6.*Sle1a*.*ERα*$^{+/+}$ mice (Table 5). Finally, the proportion of follicular T helper cells did not differ between male and female B6.*Sle1a*.*ERα*$^{+/+}$ mice, indicating that there is no sex bias in the *Sle1a*-associated increase in this specific T cell population (Table 5).

Our studies evaluating the impact of *ERα* deficiency in B6.*Sle1* mice indicated that B6.*Sle1*.*ERα*$^{-/-}$ males had a modest attenuation of T cell hyperactivity. Therefore, we wanted to examine the effect of *ERα* deficiency on T cell hyperactivation in B6.*Sle1a* males. Overall, we observed little impact of *ERα* deficiency on B6.*Sle1a* males (Table 6). Interestingly, we actually noted a slight, but significant, increase in the proportion of $CD4^+CD69^+$ T cells from B6.*Sle1a*.*ERα*$^{-/-}$ males compared to B6.*Sle1a*.*ERα*$^{+/+}$ (Table 6; $p<0.01$). However, the percentage of total $CD4^+CD69^+$ T cells in these mice remains quite low; the functional significance, if any, of this slight increase remains to be determined.

Table 4.
Impact of *ERα* genotype on lymphocyte activation in female B6.*Sle1a* mice

		B6		B6.*Sle1a*	
Cell type	ERα	+/+	-/-	+/+	-/-
$B220^+$		48.0±2.0	45.6±1.9	50.3±1.9	49.6±1.9
$B220^+CD86^+$		3.7±0.4	3.3±0.2	4.4±0.3	3.9±0.3
$CD4^+$		17.9±0.9	20.2±1.3	15.1±0.5*	17.0±0.7 ŦŦ
$CD4^+CD69^+$		2.4±0.3	3.4±0.2 Ŧ	3.8±0.2**	4.2±0.2**
$CD4^+CD134^+$		1.1±0.1	2.4±0.3 ŦŦ	1.8±0.1**	2.9±0.3 ŦŦ
$CD4^+CD62L^+$		13.5±0.9	14.5±1.3	8.5±0.6**	9.0±0.8**
$CD4^+CD25^+$		2.0±0.1	2.7±0.1 ŦŦ	2.1±0.1	2.3±0.1**,Ŧ
$CD4^+CXCR5^{hi}PD1^{hi}$		19.0±4.3	19.4±4.7	35.4±2.1**	33.5±2.7**

*,** Significant difference compared to B6 mice of the same *ERα* genotype are denoted by * (P≤0.05) and ** (P≤0.01)

Ŧ, ŦŦ Significant difference compared to $ER\alpha^{+/+}$mice of the same *Sle1* genotype are denoted by Ŧ (P≤0.05) and ŦŦ (P≤0.01)

Table 4. Impact of *ERα* genotype on lymphocyte activation in female B6.*Sle1a* mice.

Splenocytes from each mouse (4-6 months of age) were analyzed individually by flow cytometry. The values presented represent the mean ± SEM for the samples of each group. N= 6-23 mice per genotype. Because we noted no significant difference between $ER\alpha^{+/+}$ and $ER\alpha^{+/-}$, these two genotypes are combined as $ER\alpha^{+/+}$.

Table 5.
Impact of sex on lymphocyte activation in B6.*Sle1a* mice

Cell type	Females		Males	
	B6	B6.*Sle1a*	B6	B6.*Sle1a*
$B220^+$	48.0±2.0	50.3±1.9	50.7±1.4	51.6±0.7
$B220^+CD86^+$	3.7±0.4	4.4±0.3	3.9±0.5	3.0±0.1 ŦŦ
$CD4^+$	17.9±0.9	15.1±0.5*	17.3±1.2	13.4±0.5**,ŦŦ
$CD4^+CD69^+$	2.4±0.3	3.8±0.2**	2.5±0.2	2.9±0.1 ŦŦ
$CD4^+CD134^+$	1.1±0.1	1.8±0.1**	1.8±0.2	2.0±0.1
$CD4^+CD62L^+$	13.5±0.9	8.5±0.6**	11.9±1.2	8.1±0.4**
$CD4^+CD25^+$	2.0±0.1	2.1±0.1	1.9±0.1	1.9±0.1
$CD4^+CXCR5^{hi}PD1^{hi}$	19.0±4.3	35.4±2.1**	27.8±5.6	45.0±2.4**,ŦŦ

*,** Significant difference compared to B6 mice of the same sex
are denoted by * (P≤0.05) and ** (P≤0.01)

Ŧ, ŦŦ Significant difference compared to female mice of the same *Sle1a* genotype
are denoted by Ŧ (P≤0.05) and ŦŦ (P≤0.01)

Table 5. Impact of sex on lymphocyte activation in B6.*Sle1a* mice.

Splenocytes from each mouse (4-6 months of age) were analyzed individually by flow cytometry. The values presented represent the mean ± SEM for the samples of each group. N= 6-45 mice per genotype. Because we noted no significant difference between $ER\alpha^{+/+}$ and $ER\alpha^{+/-}$, these two genotypes are combined as $ER\alpha^{+/+}$.

Table 6.
Impact of *ERα* genotype on lymphocyte activation in male B6.*Sle1a* mice

		B6		B6.*Sle1a*	
Cell type	ERα	+/+	-/-	+/+	-/-
$B220^+$		50.7±1.4	50.7±1.5	51.6±0.7	52.0±1.5
$B220^+CD86^+$		3.9±0.5	3.2±0.2	3.0±0.1	3.2±0.2
$CD4^+$		17.3±1.2	15.1±0.7	13.4±0.5**	15.2±0.7$^{\text{Ŧ}}$
$CD4^+CD69^+$		2.5±0.2	2.5±0.2	2.9±0.1	3.3±0.1**
$CD4^+CD134^+$		1.8±0.2	1.8±0.2	2.0±0.1	2.2±0.2
$CD4^+CD62L^+$		11.9±1.2	11.0±1.1	8.1±0.4**	8.3±0.8
$CD4^+CD25^+$		1.9±0.1	2.2±0.2	1.9±0.1	2.0±0.1
$CD4^+CXCR5^{hi}PD1^{hi}$		27.8±5.6	21.0±4.9	45.0±2.4**	38.7±2.9**

*,** Significant difference compared to B6 mice of the same *ERα* genotype are denoted by * (P≤0.05) and ** (P≤0.01)

Ŧ, ŦŦ Significant difference compared to $ER\alpha^{+/+}$mice of the same *Sle1* genotype are denoted by Ŧ (P≤0.05) and ŦŦ (P≤0.01)

Table 6. Impact of *ERα* genotype on lymphocyte activation in male B6.*Sle1a* mice.

Splenocytes from each mouse (4-6 months of age) were analyzed individually by flow cytometry. The values presented represent the mean ± SEM for the samples of each group. N= 8-45 mice per genotype. Because we noted no significant difference between $ER\alpha^{+/+}$ and $ER\alpha^{+/-}$, these two genotypes are combined as $ER\alpha^{+/+}$.

5.3 Impact of ERα deficiency on Sle1b-induced loss of tolerance in female and male B6.Sle1b mice

Sle1b is associated with highly penetrant loss of tolerance, and has been determined to show a significant female sex bias in this phenotype (Morel et al. 2001). Although it is evident that a greater proportion of B6.*Sle1b* females loss tolerance to chromatin than their male counterparts, the role of estrogen signaling via ERα in the female sex bias of *Sle1b*-associated loss of tolerance has not yet been investigated. In order to determine the impact of *ERα* deficiency on *Sle1b*-induced loss of tolerance, we evaluated the penetrance of anti-chromatin IgG autoantibody production in B6.*Sle1b*.*ERα*$^{+/+}$, B6.*Sle1b*.*ERα*$^{+/-}$, and B6.*Sle1b*.*ERα*$^{-/-}$ female and male mice via ELISA. As expected, a significant proportion (~64%) of B6.*Sle1b*.*ERα*$^{+/+}$ female mice develop anti-chromatin IgG autoantibodies (Figure 11). While heterozygosity for *ERα* had relatively little impact on loss of tolerance in female B6.*Sle1b* mice, only 15% of female B6.*Sle1b*.*ERα*$^{-/-}$ mice loss tolerance to chromatin (Figure 11; $P>0.05$). This significant reduction in penetrance in *ERα*-deficient B6.*Sle1b* female mice strongly suggests that ERα signaling promotes loss of tolerance in *Sle1b* females. Interestingly, *ERα* deficiency had no impact on the proportion of B6.*Sle1b* males that lose tolerance to chromatin (Figure 12; 31% vs 25%). Our findings strongly suggest a female sex bias in the penetrance of *Sle1b*-induced loss of tolerance (Figure 13; 64% in B6.*Sle1b* females vs. 31% in B6.*Sle1b* males), although this difference falls just short of statistical significance ($P>0.05$). We will continue to increase our sample sizes to determine if the *Sle1b*-induced loss of tolerance is in fact statistically significant.

Interesting, the proportion of B6.*Sle1b*.*ERα*$^{-/-}$ female mice that develop anti-chromatin IgG antibodies is less than that of B6.*Sle1b* males, regardless of *ERα* genotype (15% vs ~30%; Figure 12; 13). This suggests that ERα signaling may play a role in a female sex bias in *Sle1b*-associated loss of tolerance. Although *Sle1b* induces a basal loss of tolerance due to genetic

FIGURE 11

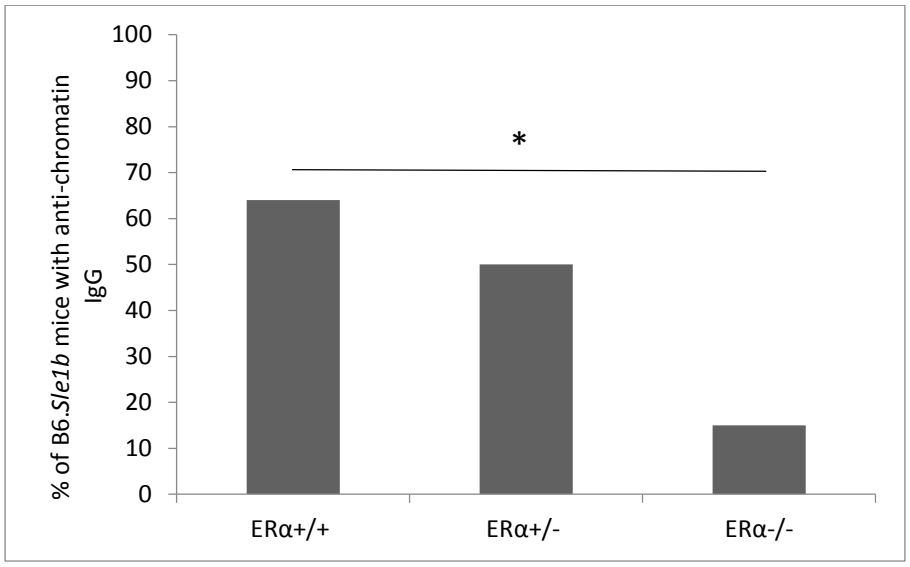

Figure 11. Impact of *ERα* deficiency on *Sle1b*-induced loss of tolerance in females.

ELISAs were used to evaluate the level of anti-chromatin IgG in 4-6 month old B6.*Sle1b* mice. N= 14-22 per *ERα* genotype. The threshold for a positive reading was set at 2 standard deviations above the mean of a group of control B6 mice as described previously by Morel et al. (2001). * denotes statistical significance ($P<0.05$).

FIGURE 12

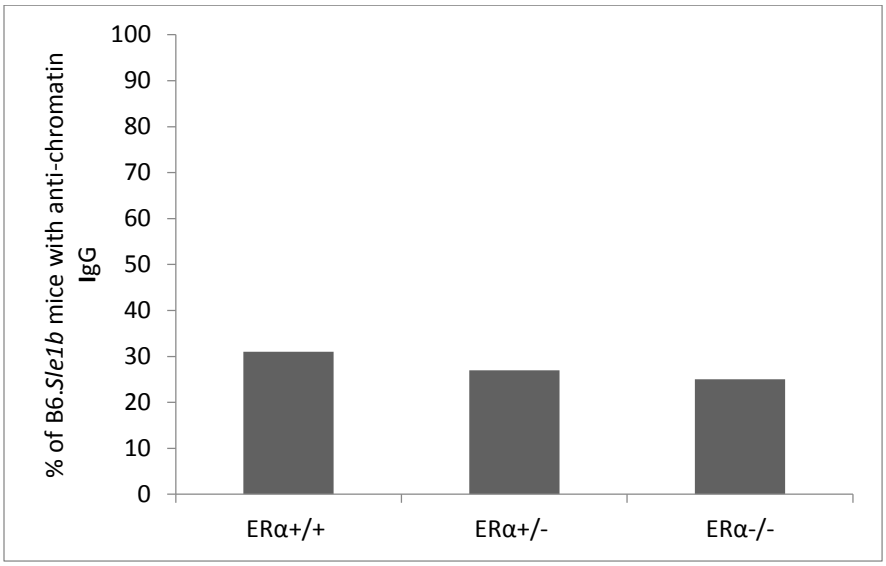

Figure 12. Impact of *ERα* deficiency on *Sle1b*-induced loss of tolerance in males.

ELISAs were used to evaluate the level of anti-chromatin IgG in 4-6 month old B6.*Sle1a* mice. N= 16-22 per *ERα* genotype. The threshold for a positive reading was set at 2 standard deviations above the mean of a group of control B6 mice as described previously by Morel et al.

FIGURE 13

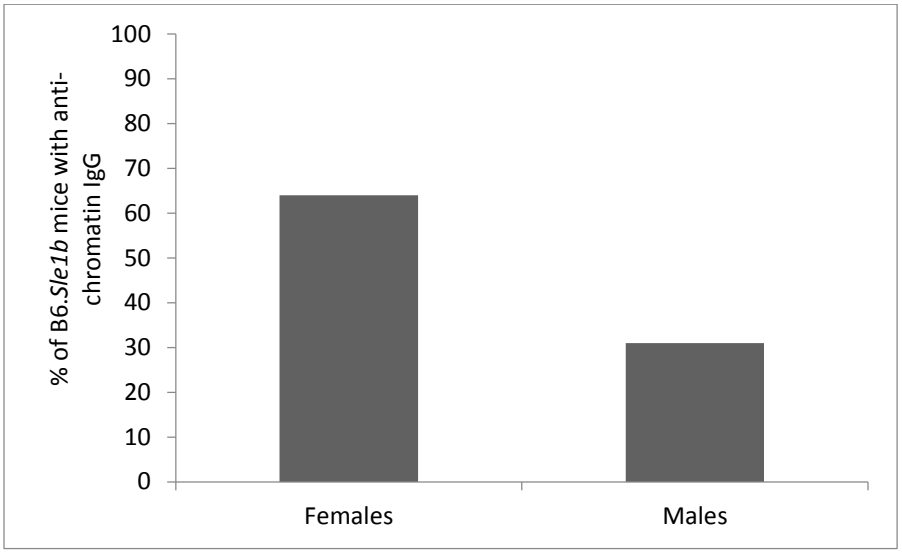

Figure 13. Impact of sex on *Sle1b*-induced loss of tolerance

ELISAs were used to evaluate the level of anti-chromatin IgG in 4-6 month old B6.*Sle1b* mice. N= 14-16 per *ERα* genotype. The threshold for a positive reading was set at 2 standard deviations above the mean of a group of control B6 mice as described previously by Morel et al. (2001).

alterations influencing tolerance, future studies may reveal a major role of ERα signaling in promoting the loss of tolerance seen in both B6.*Sle1b* females, as well as B6.*Sle1* female mice.

5.4 Impact of ERα genotype on lymphocyte activation in B6.Sle1b female and male mice.

Sle1b is associated with T and B cell hyperactivity (Sobel et al. 2002; Wandstrat et al. 2004; Wong et al. 2012). Although some aspects of *Sle1b*-induced lymphocyte alterations have been demonstrated to be more pronounced in female B6.*Sle1b* mice compared to males, the impact of estrogen signaling via ERα on the *Sle1b* phenotype has yet to be investigated. In order to explicitly test the impact of ERα on *Sle1b*-associated immune cell hyperactivation, we assessed T and B cells isolated from B6.*Sle1b* mice via flow cytometry. As expected, a significant proportion of B cells from B6.*Sle1b*.*ERα*$^{+/+}$ female mice express activation marker CD86 compared to B6 controls (Table 7; $p<0.01$). Additionally, the mean fluorescent intensity of $B220^{+}CD22$ cells was significantly increased in female B6.*Sle1b*.*ERα*$^{+/+}$ mice compared to B6 controls. Strikingly, both the increase in proportion of $B220^{+}CD86^{+}$ cell and MFI of $B220^{+}CD22^{+}$ cells were alleviated by *ERα* deficiency in B6.*Sle1b* female mice (Table 7; $p<0.01$). These data indicate *ERα* deficiency attenuates *Sle1b*-associated B cell hyperactivity in B6.*Sle1b* female mice.

As expected, female B6.*Sle1b*.*ERα*$^{+/+}$ mice also exhibited significant $CD4^{+}$ T cell hyperactivation, as demonstrated by a greater proportion of T cells expressing activation markers CD69 and CD134, and a decrease in expression of naïve T cell marker CD62L (Table 7; $p<0.01$). Importantly, the *Sle1b*-associated T cell hyperactivation was significantly attenuated by *ERα* deficiency in B6.*Sle1b* female mice (Table 7; $p<0.01$). Although *ERα* deficiency did not completely abrogate T cell hyperactivation in B6.*Sle1b* females, indicating a basal *Sle1b*-induced T cell hyperactivation, the significant reduction in T cell hyperactivity in *ERα*-deficient B6.*Sle1b* females strongly suggests that ERα signaling promotes this hyperactivation. Furthermore, we observed a significant increase in the population size of follicular T helper cells in female B6.*Sle1b*.*ERα*$^{+/+}$ mice, and this increase was also alleviated by *ERα* deficiency (Table 7; $p<0.01$).

Although the population size of follicular T helper cells remains elevated compared to B6 controls, the significant reduction in this population in B6.*Sle1b*.*ERα*$^{-/-}$ female mice indicates that ERα plays a role in expansion of this cell population. Altogether, these data strongly suggest a major role of ERα signaling in *Sle1b*-associated T and B cell hyperactivation.

Although it has been demonstrated that female B6.*Sle1b* mice exhibit increased B cell hyperactivation compared to their male counterparts (Wong et al. 2012), we sought to confirm previous reports of a sex bias in *Sle1b*-associated B cell hyperactivation, and determine if a similar sex bias is present to *Sle1b*-associated T cell hyperactivation. As expected, compared to female B6.*Sle1b*.*ERα*$^{+/+}$ mice, B cells isolated from male B6.*Sle1b*.*ERα*$^{+/+}$ mice do not differ in activation status compared to B6 controls (Table 8; $p<0.01$). These data confirm a female sex bias in B6.*Sle1b*-associated B cell hyperactivity. Furthermore, we identified a similar trend in T cell activation associated with *Sle1b*. Compared to B6 controls, B6.*Sle1b* male mice exhibit a greater proportion of activated T cells, as identified by cell surface markers CD69, CD134, and CD62L (Table 9; $p<0.01$). This indicates a basal level of T cell hyperactivation due to *Sle1b*. However, compared to T cells from female B6.*Sle1b* mice, male B6.*Sle1b* show a significantly smaller proportion of activated CD4^{+} T cells, indicating a sex bias in *Sle1b*-associated T cell hyperactivation (Table 8; $p<0.01$). Furthermore, B6.*Sle1b* males have a significantly smaller population size of follicular T helper cells compared to females (Table 8; $p<0.01$). Altogether, our findings confirm a significant sex bias in *Sle1b*-associated B cell hyperactivity, and demonstrate for the first time that *Sle1b*-associated T cell hyperactivation and alteration in follicular T helper cell population preferentially affects female *Sle1b* mice.

Because male B6.*Sle1b*.*ERα*$^{+/+}$ mice do not exhibit B cell hyperactivation, it is not surprising that *ERα* deficiency in these mice has no significant impact (Table 8). However, we observed a significant reduction in the proportion of CD4^{+} T cells from male B6.*Sle1b*.*ERα*$^{-/-}$

Table 7.
Impact of *ERα* genotype on lymphocyte activation in female B6.*Sle1b* mice

		B6		B6.*Sle1b*	
Cell type	ERα	+/+	-/-	+/+	-/-
$B220^+$		57.1±2.2	51.0±3.5	58.9±1.2	52.9±1.4††
$B220^+CD86^+$		4.6±0.4	4.7±1.1	9.6±0.6**	5.4±0.5 ††
$B220^+$CD22 MFI		5971±481	4586±167	8735±448**	6266±462**,††
$CD4^+$		14.9±1.1	18.4±1.5	21.4±1.2**	19.8±1.0
$CD4^+CD69^+$		3.2±0.2	3.4±0.2	12.9±0.8**	9.5±1.1**,†
$CD4^+CD134^+$		2.2±0.2	2.6±0.3	8.5±0.5**	5.1±0.5**,††
$CD4^+CD62L^+$		8.4±1.0	12.3±1.5†	2.2±0.2**	5.2±0.9**,††
$CD4^+CXCR5^{hi}PD1^{hi}$		34.3±2.9	21.9±5.7	63.4±2.1**	42.2±4.2**, ††

*,** Significant difference compared to B6 mice of the same *ERα* genotype are denoted by * (P≤0.05) and ** (P≤0.01)

$^{\dagger, \dagger\dagger}$ Significant difference compared to $ER\alpha^{+/+}$mice of the same *Sle1* genotype are denoted by † (P≤0.05) and †† (P≤0.01)

Table 7. Impact of *ERα* genotype on lymphocyte activation in female B6.*Sle1b* mice.

Splenocytes from each mouse (4-6 months of age) were analyzed individually by flow cytometry. The values presented represent the mean ± SEM for the samples of each group. N= 7-30 mice per genotype. Because we noted no significant difference between $ER\alpha^{+/+}$ and $ER\alpha^{+/-}$, these two genotypes are combined as $ER\alpha^{+/+}$.

mice that expressed activation marker CD134, and a significant increase in the expression of naïve T cell marker CD62L compared to their B6.*Sle1b*.*ERα*$^{+/+}$ counterparts (Table 8; $p<0.01$). This indicates that *ERα* deficiency also alleviates T cell hyperactivation in male B6.*Sle1b* mice. Again, the degree of T cell hyperactivation in B6.*Sle1b*.*ERα*$^{-/-}$ male mice remains elevated compared to B6 controls, indicating the impact of the *Sle1b* alone. However, this data illustrates that, similar to our findings in *Sle1*-induced T cell hyperactivation, ERα signaling modestly promotes T cell hyperactivation in B6.*Sle1b* male mice.

Table 8.
Impact of sex on lymphocyte activation in B6.*Sle1b* mice

	Females		Males	
Cell type	B6	B6.*Sle1b*	B6	B6.*Sle1b*
$B220^+$	57.1±2.2	58.9±1.2	57.6±1.7	58.4±0.8
$B220^+CD86^+$	4.6±0.4	9.6±0.6**	5.4±0.4	6.0±0.3 ŦŦ
$B220^+$CD22 MFI	5971±481	8735±448**	6738±431	7428±416 Ŧ
$CD4^+$	14.9±1.1	21.4±1.2**	13.5±0.8	15.6±0.5*,ŦŦ
$CD4^+CD69^+$	3.2±0.2	12.9±0.8**	2.4±0.2	7.3±0.4**,ŦŦ
$CD4^+CD134^+$	2.2±0.2	8.5±0.5**	2.0±0.1	4.8±0.3**,ŦŦ
$CD4^+CD62L^+$	8.4±1.0	2.2±0.2**	8.7±0.9	3.2±0.2**,ŦŦ
$CD4^+CXCR5^{hi}PD1^{hi}$	34.3±2.9	63.4±2.1**	22.1±2.4	51.6±1.9**,ŦŦ

*,** Significant difference compared to B6 mice of the same *ERα* genotype are denoted by * (P≤0.05) and ** (P≤0.01)

Ŧ, ŦŦ Significant difference compared to female mice of the same *Sle1b* genotype are denoted by Ŧ (P≤0.05) and ŦŦ (P≤0.01)

Table 8. Impact of sex on lymphocyte activation in B6.*Sle1b* mice.

Splenocytes from each mouse (4-6 months of age) were analyzed individually by flow cytometry. The values presented represent the mean ± SEM for the samples of each group. N= 6-32 mice per genotype. Because we noted no significant difference between $ER\alpha^{+/+}$ and $ER\alpha^{+/-}$, these two genotypes are combined as $ER\alpha^{+/+}$.

Table 9.
Impact of *ERα* genotype on lymphocyte activation in male B6.*Sle1b* mice

		B6		B6.*Sle1b*	
Cell type	**ERα**	**+/+**	**-/-**	**+/+**	**-/-**
B220$^+$		57.6±1.7	56.1±3.1	58.4±0.8	57.0±0.7
B220$^+$CD86$^+$		5.4±0.4	8.0±3.7	6.0±0.3	5.7±0.3
B220$^+$CD22 MFI		6738±431	5920±1239	7428±416	6890±629
CD4$^+$		13.5±0.8	15.2±0.8	15.6±0.5*	15.3±0.5
CD4$^+$CD69$^+$		2.4±0.2	2.5±0.3	7.3±0.4**	6.3±0.5**
CD4$^+$CD134$^+$		2.0±0.1	1.9±0.2	4.8±0.3**	3.8±0.2**,^{ŦŦ}
CD4$^+$CD62L$^+$		8.7±0.9	10.5±1.4	3.2±0.2**	5.0±0.6**,^{ŦŦ}
CD4$^+$CXCR5^{hi}PD1^{hi}		22.1±2.4	15.6±4.5	51.6±1.9**	43.7±3.5**

*,** Significant difference compared to B6 mice of the same *ERα* genotype are denoted by * (P≤0.05) and ** (P≤0.01)

Ŧ, ŦŦ Significant difference compared to *ERα*$^{+/+}$mice of the same *Sle1* genotype are denoted by Ŧ (P≤0.05) and ŦŦ (P≤0.01)

Table 9. Impact of *ERα* genotype on lymphocyte activation in male B6.*Sle1b* mice.

Splenocytes from each mouse (4-6 months of age) were analyzed individually by flow cytometry. The values presented represent the mean ± SEM for the samples of each group. N= 6-32 mice per genotype. Because we noted no significant difference between *ERα*$^{+/+}$ and *ERα*$^{+/-}$, these two genotypes are combined as *ERα*$^{+/+}$.

DISCUSSION

It has been demonstrated by work in our own lab and others that the impact of *Sle1* is more robust in females compared to males. Our initial work with B6.*Sle1* congenic mice revealed a significant sex bias in *Sle1*-induced loss of tolerance and lymphocyte hyperactivation. Furthermore, we showed that ERα signaling is responsible for this sex bias, and promotes the *Sle1*-associated lupus phenotype in female, and to a significantly lesser degree in male B6.*Sle1* mice. However, because *Sle1* is a complex locus consisting of individual subloci that each contribute uniquely to *Sle1*-induced loss of tolerance, it is necessary to continue our work investigating the synergy between ERα signaling and each *Sle1* sublocus to better understand how ERα modulates each individual sublocus. Our purpose for this work is to identify which *Sle1* subloci is/are modulated by ERα, with the goal of identifying the molecular pathways impacted by both lupus susceptibility loci and estrogens.

Our observations in this study are consistent with previous findings that *Sle1a* and *Sle1b* promote loss of tolerance in both female and male mice. *Sle1a* has been reported to induce a loss of tolerance to chromatin with ~30% penetrance (Morel et al. 2001). Consistent with these findings, we observed a penetrance of ~38% in female B6.*Sle1a* mice and ~30% in male B6.*Sle1a* mice. There does not appear to be a female sex bias in this *Sle1a* phenotype. Furthermore, our findings indicate that *ERα* deficiency may partially attenuate this loss of tolerance, as we see a trend in both female and male B6.*Sle1a*.*ERα*$^{-/-}$ mice of a lower penetrance of loss of tolerance (38% vs 17% in females, 33% vs 0% in males) due to *ERα* deficiency, although this difference only reaches statistical significance in males.

Similarly, we observed a penetrance of ~64% in *Sle1b*-induced loss of tolerance in B6.*Sle1b* female mice. As previously reported, *Sle1b* appears to be more potent in inducing anti-chromatin IgG autoantibody production compared to *Sle1a*. Importantly, this *Sle1b*-induced loss of tolerance shows sex bias in preferentially effecting B6.*Sle1b*.*ERα*$^{+/+}$ females compared to their

male counterparts (64% of B6.*Sle1b* females vs. 31% of B6.*Sle1b* males). Furthermore, our findings strongly suggest that ERα signaling promotes *Sle1b*-induced loss of tolerance in females, but not in males, as the proportion of B6.*Sle1b*.*ERα*$^{-/-}$ females that develop autoantibodies is significantly less than that in B6.*Sle1b*.*ERα*$^{+/+}$ females ($P<0.05$). In contrast, *ERα* deficiency had no impact on the proportion of B6.*Sle1b* males that lose tolerance to chromatin (31% vs 25%). This aligns with our findings in B6.*Sle1* mice, where ERα signaling promotes loss of tolerance in female B6.*Sle1* mice, but not in male B6.*Sle1* mice. It is tempting to speculate that both *Sle1a* and *Sle1b* induce a basal loss of tolerance in which both female and male B6.*Sle1a*.*ERα*$^{+/+}$ and just male B6.*Sle1b*.*ER*$^{+/+}$ mice exhibit a ~30% penetrance of loss of tolerance, and estrogen dependent, ERα-mediated processes synergize with *Sle1b* in increase the proportion of *Sle1b* females that lose tolerance to chromatin. Therefore, our observations strongly suggest that ERα signaling promotes *Sle1b*-induced loss of tolerance in females.

Sle1a also has been associated with T cell hyperactivation, however, the impact of sex or ERα signaling on *Sle1a*-induced T cell hyperactivation had not been investigated prior to our study. Our observations were consistent with previous reports as T cells isolated from female B6.*Sle1*.*ERα*$^{+/+}$ demonstrated hyperactivity as evident by increased proportions of activated T cells and decreased populations of naïve T cells. Because we did not observe a difference in the proportions of activated T cells between B6.*Sle1a*.*ERα*$^{+/+}$ and B6.*Sle1a*.*ERα*$^{-/-}$ female mice, it appears that ERα signaling does not significantly impact *Sle1a*-associated T cell hyperactivation. Additionally, we identified a significantly increased proportion of follicular T helper cells in B6.*Sle1a*.*ERα*$^{+/+}$ female mice, however this population of T cells was not affected by *ERα* deficiency. Interestingly, we did observe a moderate sex bias in *Sle1a*-induced T cell hyperactivation, as we observed no impact of *Sle1a* on the proportion of activated T cells in B6.*Sle1a* males. It appears the impact of *Sle1a* on the proportion of naïve T cells and follicular T cells is sex-independent, as male B6.*Sle1a*.*ERα*$^{+/+}$ mice also exhibit alterations in these cell

population sizes. Altogether, these data suggest the impact of *Sle1a* on T cell hyperactivation in female and male B6.*Sle1a* mice is due to alterations of genetic pathways influenced by *Sle1a* independent of ERα signaling.

Sle1b is associated with increased activation in both T and B cells. Although one study by Wong and colleagues (2012) suggested a female sex bias in *Sle1b*-associated B cell hyperactivation, nothing is known about the impact of ERα signaling on this phenotype. Furthermore, no studies prior have investigated the impact of sex or estrogens on *Sle1b*-associated T cell hyperactivation. In this study, we observed a significant increase in the proportion of activated B cells and $B220^{+}CD22$ MFI in female B6.*Sle1b*.*ERα*$^{+/+}$ mice. This *Sle1b*-induced B cell hyperactivity is strongly female sex-biased, as we observed no impact of *Sle1b* on B cell activation in male B6.*Sle1b* mice. Most importantly, we saw a complete attenuation of *Sle1b*-induced B cell hyperactivation due to *ERα* deficiency in B6.*Sle1b* female mice. This demonstrates a crucial role of ERα signaling in *Sle1b*-induced B cell hyperactivation in females.

Furthermore, we observed a significant decrease in $B220^{+}CD22$ MFI in female B6.*Sle1b*.*ERα*$^{-/-}$ females compared to B6.*Sle1b*.*ERα*$^{+/+}$ female mice. Interestingly, Grimaldi and colleagues demonstrated that estrogen signaling via ERα causes an upregulation of *cd22* in B cells, and this overexpression decreased B cell receptor signaling allowing autoreactive B cells to escape deletion at tolerance checkpoints (2002). Additionally, the autoimmune variant of *Sle1b* gene *Ly108, Ly108-1*, has been shown to decrease signaling in B cells leading to a crippling of B cell tolerance in B6.*Sle1* mice (Wang et al. 2010). While the exact downstream signaling effects of *Ly108-1* remains to be elucidated, the tuning down of BCR signaling by *Ly108-1*, along with decreased BCR signaling due to ERα-induced *cd22* overexpression, sheds light on one mechanism by which ERα and *Sle1b* may be synergizing to impact B cell tolerance. Because *Sle1b* alters the germinal center checkpoint specifically in females, our results suggest that the ERα-associated upregulation of *cd22* in *Sle1b* B cells may synergize with the *Sle1b*-associated

Ly108-1 variant to allow autoreactive B cells to evade deletion and promotes anti-chromatin autoantibody production.

Because no previous studies has investigated the impact of sex or estrogen signaling via ERα on *Sle1b*-induced T cell hyperactivity, we next evaluated the T cell population in B6.*Sle1b* female and male mice. As expected, female B6.*Sle1b*.*ERα*$^{+/+}$ mice exhibit a significantly greater proportion of activated T cells compared to B6 controls. Additionally, B6.*Sle1b*.*ERα*$^{+/+}$ female mice show a significant reduction in the proportion of naïve T cells compared to B6 controls. Importantly, we saw a significantly reduced proportion of activated T cells in female B6.*Sle1b*.*ERα*$^{-/-}$ mice compared to female B6.*Sle1b*.*ERα*$^{-/-}$ mice, indicating that *ERα* deficiency attenuates *Sle1b*-induced T cell hyperactivation. Female B6.*Sle1b*.*ERα*$^{-/-}$ mice still exhibited a basal T cell hyperactivation compared to B6 controls, indicating that *Sle1b* alters the T cell activation phenotype independently, however, it appears that ERα signaling synergizes with *Sle1b* to promote T cell hyperactivity in female mice. Furthermore, we also observed a significant increase in the proportion of follicular T helper cells in female B6.*Sle1b*.*ERα*$^{+/+}$ mice compared to B6 controls, however, this *Sle1b*-induced increase in population size of this cell type was also partially attenuated by *ERα* deficiency. Altogether, it appears that ERα signaling plays a vital role in promoting T cell alterations associated with *Sle1b*.

Similar to that seen in B6.*Sle1b*.*ERα*$^{+/+}$ female mice, we observed a significant increase in the proportion of hyperactivated CD4^{+} T cells from B6.*Sle1b*.*ERα*$^{+/+}$ male mice compared to B6 controls. Additionally, we also noted a significant impact on T cell hyperactivation by *ERα* deficiency, as B6.*Sle1b*.*ERα*$^{-/-}$ male mice displayed a lesser proportion of activated T cells and a greater proportion of naïve T cells compared to B6.*Sle1b*.*ERα*$^{+/+}$ males. Although we again noted that these proportions were still increased compared to B6 controls, it appears ERα signaling promotes *Sle1b*-induced T cell hyperactivity. Finally, the *Sle1b*-associated increase in follicular T helper cells was not eliminated by *ERα* deficiency, as seen in female B6.*Sle1b* as well, indicating

that this *Sle1b* phenotype occurs independent of *ERα* genotype. While *Sle1b*-associated T cell phenotypes occur in both male and female B6.*Sle1b* mice, the overall impact on the T cell population is significantly female-biased, indicating that ERα signaling may play a large role in promoting *Sle1b*-associated T cell alterations, preferentially in female *Sle1b* mice. It is therefore of keen interest to further explore the impact of ERα signaling synergizing with the *Sle1b* subloci to better understand the interactions of genes/pathways associated with *Sle1b* and estrogens acting via ERα.

Altogether, our studies of *Sle1a*- and *Sle1b*-induced loss of tolerance and lymphocyte hyperactivation/alterations indicate that ERα signaling synergizes with *Sle1b*, and to a much lesser degree *Sle1a*, to promote autoreactivity. We have demonstrated a significant impact of *ERα* deficiency on loss of tolerance in *Sle1b* females, indicating that ERα signaling promotes the production of anti-chromatin IgG autoantibodies; future studies will aid in determining the significance of this finding. *Sle1a* appears to induce a basal loss of tolerance that in some phenotypes may be altered by ERα signaling. However, our findings suggest that ERα signaling may play a larger role in promoting *Sle1b*-induced loss of tolerance, as there appears to be a major impact of *ERα* deficiency on loss of tolerance and B and T cell hyperactivation preferentially in female B6.*Sle1b* mice.

Importantly, our observations reveal significant increases in the proportion of hyperactivated B cells and $B220^{+}CD22$ MFI in B6.*Sle1b* female mice due to ERα signaling. Because previous studies have demonstrated a significant alteration in the germinal center tolerance checkpoint in B6.*Sle1b* females, we speculate from our results that this alteration may be due in part to a synergy between the upregulation of *cd22* in B cells and autoimmune variant *Ly108-1* from *Sle1b* females, which may act together to downregulate BCR signaling and allows autoreactive B cells to escape deletion and produce autoantibodies. Additionally, the increase in follicular T helper cells due to presumably both *Sle1a* and *Sle1b* may aid in the development of

germinal center B cells, as suggested by Wong et al. (2012). These follicular T helper cells are crucial for the maturation of B cells within germinal centers, and previous studies have shown that increased populations of follicular T helper cells correspond to high titers of anti-nuclear autoantibodies (Wong et al. 2012). Because *ERα* deficiency significantly decreases B cell hyperactivation, CD22 MFI, and the proportion of follicular T helper cells in female B6.*Sle1b* mice, and we see a major trend in the reduction of loss of tolerance penetrance in female B6.*Sle1b*.*ERα*$^{-/-}$ mice, it is likely that ERα signaling has a major influence in the *Sle1b*-induced autoreactive phenotype, and therefore greatly contributes to the female sex bias, due to ERα signaling, we observed in B6.*Sle1* mice.

CHAPTER VI.

OVERALL SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Our previous studies investigating the role of ERα signaling in (NZBxNZW) F1 mice revealed a crucial role of ERα in promoting the female sex bias associated with the lupus phenotype of this strain. These studies prompted us to focus in on the NZW-derived *Sle1* locus, a lupus susceptibility locus associated with the initial breach in tolerance that leads to autoimmunity. Importantly, *Sle1* impacts female mice more significantly than their male counterparts, in that loss of tolerance occurs earlier and with a higher penetrance in female B6.*Sle1* mice compared to male B6.*Sle1* mice. Despite this finding, no previous studies have investigated the role of estrogens in *Sle1*-induced loss of tolerance. Because our previous observations in (NZBxNZW) F1 mice showed that *ERα* deficiency attenuates loss of tolerance and abolishes female-biased lupus in this strain, we hypothesized that ERα signaling may be synergizing with *Sle1* to induce loss of tolerance preferentially in female mice.

To explicitly test our hypothesis that ERα promotes female sex-biased loss of tolerance in B6.*Sle1* mice, we intercrossed the B6.*ERα*$^{-/-}$ strain with B6.*Sle1* congenic mice to produce mice that were *ERα* wildtype (*ERα*$^{+/+}$), heterozygous (*ERα*$^{+/-}$), or null (*ERα*$^{-/-}$). As expected, we observed that a significant proportion of female B6.*Sle1*.*ERα*$^{+/+}$ lose tolerance to chromatin. Furthermore, we confirmed previous observations that *Sle1*-induced loss of tolerance is female sex-biased, as we saw a significantly reduced penetrance of *Sle1*-induced loss of tolerance in male B6.*Sle1* mice. Importantly, we found that *ERα* deficiency attenuated the *Sle1*-induced loss of tolerance in female B6.*Sle1* mice, indicating that estrogen-dependent, ERα-mediated processes synergize with pathways affected by *Sle1* to promote lupus. Furthermore, we demonstrated for the first time that *Sle1*-induced lymphocyte activation also shows a female sex bias. We found that *Sle1*-induced B cell hyperactivation observed only in females was completely eliminated by *ERα* deficiency. This observation reveals a vital synergy between ERα signaling and *Sle1* that

promotes autoreactivity leading to loss of tolerance in B6.*Sle1* females. In contrast, we showed that *Sle1*-induced T cell hyperactivation affects both female and male B6.*Sle1* mice. We demonstrated that *ERα* deficiency attenuated, albeit incompletely, *Sle1*-induced T cell hyperactivation in female, and to a lesser degree male, B6.*Sle1* mice. Altogether, these data reveal a synergy between ERα signaling and the *Sle1* locus in promoting lupus.

Because ERα functions as a ligand-dependent transcription factor, we next sought to determine if ERα may be impacting the *Sle1* phenotype by altering the expression or splicing of the genes underlying this interval. Interestingly, we did not observe any sex bias nor impact of *ERα* deficiency on the expression of the SLAM/CD2 cluster genes that underlie *Sle1*. Because this cluster of genes is known to play a role in immune cell tolerance, and the sublocus in which these genes are located is known to preferentially induce loss of tolerance in females, it is a bit surprising that we observed no difference due to sex or *ERα* deficiency in the expression or splicing of these genes. We did, however, observe an alteration in the relative expression of *Pbx1a*, an isoform of a gene that lies within the *Sle1a* interval. We reported for the first time that *Pbx1a* is in fact expressed in mouse $CD4^+$ T cells, and that *ERα* deficiency impacts the expression of this isoform. Although the precise impact of this *Sle1*-induced alteration in *Pbx1* isoform expression remains to be elucidated, the known role of *Pbx1* in lupus susceptibility leads us to hypothesize that this differential expression of *Pbx1* isoforms in concert with ERα signaling may promote autoreactivity in female B6.*Sle1* mice. Future studies will aid in determining the impact of the *Sle1*-, and ERα-, induced overexpression of *Pbx1a*, with the intent of uncovering mechanisms through with the different *Pbx1* isoforms modulate lupus susceptibility, and the role of ERα in these processes.

Because *Sle1* is comprised of individual subloci that each promote loss of tolerance in unique ways, we next focused our efforts in discerning the possible impact of ERα signaling on *Sle1a* and *Sle1b*. These *Sle1* subloci have been shown to preferentially impact loss of tolerance in

female mice, although the exact mechanisms through which this occurs are unknown. Our intentions with these studies in B6.*Sle1a* and B6.*Sle1b* strains are to better understand the impact of ERα signaling on the female-biased *Sle1*-induced loss of tolerance. Our findings will thereby facilitate the identification of molecular pathways impacted by both estrogens and lupus susceptibility loci, and will shed light on potential preventative and therapeutic strategies for lupus.

To test our hypothesis that ERα signaling synergizes with *Sle1a* and *Sle1b* to promote loss of tolerance and lymphocyte hyperactivation, we intercrossed the B6.*ERα* knockout strain with B6.*Sle1a* and B6.*Sle1b* subcongenics. We then evaluated the impact of *ERα* deficiency on loss of tolerance and *Sle1a*- and *Sle1b*-associated immune cell hyperactivity. We found that *Sle1a*-induced loss of tolerance is relatively mild compared to that of the entire *Sle1* interval, with only ~30% of female and male *Sle1a* developing anti-chromatin IgG autoantibodies. Importantly, we do not observe a female sex bias in *Sle1a*-induced loss of tolerance. We did, however, note a trend in decreased penetrance of *Sle1a*-induced loss of tolerance in B6.*Sle1a*.*ERα*$^{-/-}$ male and female mice, so it is likely that this is through a *Sle1a*-mediated, ERα-independent mechanism.

Because no previous studies have investigated the impact of sex or estrogen signaling on the *Sle1a*-induced lymphocyte hyperactivity, we next assessed *Sle1a* T and B cell populations via flow cytometry. Our findings were consistent with previous work by others, in that we observed no *Sle1a*-induced B cell hyperactivity in male or female mice. In contrast, we observed a significant impact of *Sle1a* on T cell hyperactivity and cell population size alterations in both female and male mice. This *Sle1a*-induced T cell hyperactivation is more robust in females, as we saw greater proportions of activated CD4^{+} T cells in female B6.*Sle1a* mice compared to male B6.*Sle1a* mice. However, we did not observe any attenuation of T cell hypereactivity due to *ERα* deficiency in either male or female mice. Therefore, it appears as though *Sle1a*-induced loss of tolerance is not significantly promoted by ERα signaling.

In contrast, ERα signaling appears to significantly impact *Sle1b*-induced loss of tolerance and immune cell hyperactivation. Our initial work reveals a relatively high penetrance of loss of tolerance in B6.*Sle1b* females, but not B6.*Sle1b* males, and this phenotype is significantly attenuated by *ERα* deficiency. Furthermore, we noted a significant sex bias in *Sle1b*-induced B cell hyperactivity, as female B6.*Sle1b* but not male B6.*Sle1b* mice display greater proportions of activated B cells compared to controls. Strikingly, this *Sle1b*-induced B cell hyperactivity is completely eliminated due to *ERα* deficiency in B6.*Sle1b* females, indicating that ERα signaling promotes the B cell hyperactivity in these mice. We observed a similar trend in *Sle1b*-induced T cell hyperactivation. Although both male and female *Sle1b* display greater proportions of activated T cells, there is a significant female sex bias in this phenotype. Furthermore, we observed a significant attenuation of *Sle1b*-induced T cell hyperactivation in *ERα* deficient females, and to a lesser degree, male mice. This indicates that, similar to *Sle1b*-induced B cell hyperactivation, ERα signaling promotes *Sle1b*-induced T cell hyperactivation.

Finally, we observed increased population sizes of follicular T helper cells in *Sle1b* female and male mice. This alteration is more robust in females, but does not appear to be significantly impacted by *ERα* deficiency. Additionally, we saw a significant increase in $B220^{+}CD22$ MFI in female, but not male, B6.*Sle1b* mice. Importantly, this *Sle1b* phenotype was largely eliminated by *ERα* deficiency. Because estrogens are known to cause the overexpression of *cd22*, our findings indicate that there may be synergy between ERα signaling and *Sle1b*-induced increases in $B220^{+}CD22$. Because *Sle1b* is associated with alterations in the germinal center checkpoint and crippled B cell tolerance, it is likely that estrogen-induced CD22 upregulation, known to impair B cell signaling, promotes this alteration in B cell tolerance. Future studies investigating the possible impact of ERα signaling at the germinal center tolerance checkpoint in *Sle1b* female mice are warranted.

Altogether, our studies have shed light on a crucial role of ERα signaling on *Sle1*-induced loss of tolerance, the first and necessary step in lupus pathogenesis. Our initial work using B6.*Sle1a* and B6.*Sle1b* subcongenic mice have begun to elucidate mechanisms through which estrogens may synergize with lupus susceptibility loci to promote autoimmunity. We will continue to pursue our studies in B6.*Sle1a* and B6.*Sle1b* mice, to further identify ways in which ERα signaling impact the lupus phenotype. Furthermore, we will continue our investigation on the impact of *ERα* deficiency on *Sle1*-induced *Pbx1a* overexpression and *Sle1b*-induced B cell tolerance checkpoint alterations to better understand the impact of each on *Sle1*-induced loss of tolerance. Because significant alterations in the germinal center checkpoint have been observed specifically in female *Sle1b* mice, we plan to further investigate how polymorphisms associated with the *Sle1b* locus may synergize with the estrogen-induced alterations in crucial B cell signaling molecules to allow autoreactive B cells to escape deletion and produce autoantibodies. Our next step may be to determine which cell type(s) must be expressing ERα to induce the *Sle1* phenotype as demonstrated by our preliminary studies. Understanding how ERα signaling influences immune cell tolerance will shed light on potential therapeutic targets for the treatment of lupus.

While there have been significant advances in the treatment of lupus over the past decade, specifically the targeting B cells to reduce autoantibody production, much is still unknown about the effectiveness of these treatments in human patients. Fulvestrant, a new anti-estrogen treatment for lupus patients, has shown promise in relieving some aspects of the lupus phenotype in female patients. Therefore, as our studies indicate that ERα signaling synergizes with B cell signaling in female *Sle1b* mice, understanding the impact of ERα signaling specifically in B cell signaling and tolerance may shed light on more effective preventative and therapeutic agents to treat lupus by targeting pathways modulated by both estrogens and *Sle1*. Although the effectiveness of lupus treatments is inherently different between experimental

mouse models and human patients, understanding a universal hallmark of lupus pathogenesis such as loss of tolerance will certainly provide guidance in our quest for more effective treatment options for lupus patients.

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