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Estrogen Receptor Alpha Signaling Promotes *Sle1*-Induced Loss of Tolerance and Immune Cell Activation and is Responsible for Sex Bias in B6.*Sle1* Congenic Mice¹

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Abstract

Sex bias in lupus incidence is thought to be due, in part, to the ability of estrogens to promote loss of tolerance. Previously, we showed that estrogens promote lupus via estrogen receptor α (ER α). C57BL/6 (B6) mice carrying the *Sle1* lupus susceptibility locus (B6.*Sle1*) display loss of tolerance and develop anti-nuclear antibodies and immune cell hyperactivation. The incidence of loss of tolerance in B6.*Sle1* females is greater than in males. Here, we show that a deficiency of either estrogens or ER α attenuates loss of tolerance and autoantibody development in B6.*Sle1* females. Furthermore, we demonstrate that immune cell activation in B6.*Sle1* mice shows sex bias and that ER α deficiency diminishes this phenotype in B6.*Sle1* females. Thus, estrogens, acting via ER α , control sex bias in the *Sle1* phenotype. Furthermore, we show that ER α may impact the *Sle1* phenotype by modulating the expression *Pbx1*, one of genes that underlies the *Sle1* locus.

Keywords

Lupus; estrogen receptor alpha; sex bias; immunologic tolerance; immune cell activation

1. 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 [1-5], 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 \times NZW)F1 mice [6, 7]. Lupus development in these mice is influenced by sex; lupus incidence is higher and survival time is reduced in female (NZB \times NZW)F1 mice relative to

¹Abbreviations used: Estrogen receptor α (ER α)

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males [6, 7]. We demonstrated previously that disruption of estrogen receptor α ($ER\alpha$) in (NZB X NZW)F1 mice eliminated the sex bias in the development of lupus in these mice [12]. The decreased incidence of lupus and increased survival in $ER\alpha$ deficient (NZB X NZW)F1 females was associated with a dramatic attenuation of loss of tolerance to nuclear antigens and development of pathogenic autoantibodies[8]. These data indicate that the sex bias in lupus pathogenesis in this model is due, at least in part, to estrogens, acting via $ER\alpha$. These data also suggest that estrogens, acting through $ER\alpha$, 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 [9, 10]. In (NZB \times NZW)F1 mice, the loss of tolerance to chromatin is controlled by NZB- and NZW- derived lupus susceptibility alleles [11-13]. Among these loci, the NZW-derived *Sle1* locus is the best characterized [14-16]. 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 [12]. 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* [11, 17]. *Sle1* also leads to increased activation in B and T cells [18, 19], 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 [17]. 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 [17]. 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 [20]. 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 [17].

We postulate that estrogens, acting via $ER\alpha$ -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 $ER\alpha$ signaling. To test this hypothesis, we examined the impact of a targeted mutation in *ERa* on the phenotype in B6.*Sle1* congenic mice. We observed that $ER\alpha$ deficiency attenuated loss of tolerance and the development of anti-chromatin IgG in B6.*Sle1* congenic females but not males. *ERa* deficiency significantly decreased *Sle1*-induced immune cell hyperactivation in females, and to a lesser extent, in males. These effects of *ERa* 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 *ERa* deficiency. Furthermore, the phenotype of *ERa* deficient B6.*Sle1* congenic females was similar to that in $ER\alpha$ wildtype B6.*Sle1*

congenic males, suggesting that the sex bias in the phenotype of B6.*Sle1* congenic mice is both estrogen- and ER α -dependent.

2. Materials and Methods

2.1. 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 *ER α* knockout strain (B6.129-*Esr1^{tm1Ksk}* or B6.*ER α*) [21] was originally obtained from Dennis Lubahn. The B6.*Sle1* congenic strain [12, 22] was provided by Laurence Morel. 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 *ER α* gene (*ER α ^{+/-}*) were crossed to B6.*Sle1* congenic males. The resulting *ER α ^{+/-}* males were backcrossed to B6.*Sle1* females. Resulting *ER α ^{+/-}* 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 [23]. These mice were interbred to generate the experimental mice. PCR-based genotyping was performed as described previously [8]. 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).

2.2. Serological Analysis

Autoantibody levels were assessed by ELISA using serum isolated from blood collected monthly via the saphenous vein and stored at -80°C . 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 [8, 24]. 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 \times 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 [17]. 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.

2.3. 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), 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.

2.4. Cell isolation and Quantitative real-time PCR

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.

2.5. Immunoprecipitation and SDS-PAGE Analysis

CR1/CR2 expression and glycosylation were quantified using a modification of the method described previously by Boackle and colleagues [25]. 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)

2.6. 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 $P = 0.05$ was considered significant. Two-sided p-values are provided. Mean \pm standard error of the mean is presented.

3. Results

3.1. Estrogen receptor α deficiency attenuates *Sle1*-induced loss of tolerance and epitope spreading in females

To test the hypothesis that estrogens, acting via ER α , promote loss of tolerance in B6.*Sle1* female mice, we intercrossed mice from the B6.ER α knockout strain, which are heterozygous for a targeted disruption of ER α , with B6.*Sle1* congenic mice to produce B6.*Sle1* female mice that were wildtype (ER $\alpha^{+/+}$), heterozygous (ER $\alpha^{+/-}$) or homozygous null (ER $\alpha^{-/-}$) at ER α . As expected, a significant proportion (77%) of B6.*Sle1*;ER $\alpha^{+/-}$ females developed anti-chromatin antibodies (Figure 1A-B). Heterozygosity for ER α did not impact the development of anti-chromatin antibodies (Figure 1A-B). However, the proportion of B6.*Sle1*;ER $\alpha^{-/-}$ female mice that developed anti-chromatin antibodies (33%) was significantly lower than that observed in B6.*Sle1*;ER $\alpha^{+/+}$ females (Figure 1A-B; $P = 0.005$). These results suggest that ER α signaling promotes loss of tolerance in B6.*Sle1* females.

In a parallel study, we examined the impact of removing the ovaries, the primary source of estrogens, on loss of tolerance in B6.*Sle1* females. In sham-operated B6.*Sle1* females, the proportion of mice developing anti-chromatin IgG was 82% (Figure 1C-D). By contrast, just 33% of ovariectomized B6.*Sle1* females developed anti-chromatin IgG (Figure 1C-D; $P = 0.05$). Interestingly, the proportion of ovariectomized B6.*Sle1* females that developed anti-chromatin IgG was identical to that observed in B6.*Sle1*;ER $\alpha^{-/-}$ females. These data suggest that in terms of impact on *Sle1*-induced loss of tolerance, removal of endogenous estrogens was equivalent to loss of ER α signaling. The fact that genetic disruption of ER α signaling had essentially the same impact on *Sle1*-induced loss of tolerance as ovariectomy, which removes the primary source of endogenous estrogens, suggests that ER α -dependent estrogen action promotes *Sle1*-induced loss of tolerance in females.

Between 30% and 80% of B6.*Sle1* congenic mice develop anti-dsDNA IgG as a result of epitope spreading [12, 22]. However, the level of anti-dsDNA IgG in B6.*Sle1* congenic mice is low, and just 10% of these mice show evidence of mild nephritis [22]. To examine the

impact of *ERa* deficiency on epitope spreading, we evaluated the impact of *ERa* genotype on the development of anti-dsDNA IgG autoantibodies (Figure 1E-F). In our study, the proportion of B6.*Sle1*;*ERa*^{+/+} females with measurable levels of anti-dsDNA IgG was 46%, and this proportion was not significantly altered by heterozygosity for *ERa* (Figure 1E). By contrast, the proportion of B6.*Sle1*;*ERa*^{-/-} female mice that developed anti-dsDNA IgG autoantibodies was 9%, which is significantly lower than that observed in B6.*Sle1*;*ERa*^{+/+} females (Figure 1E-F; P = 0.05).

In addition to developing autoantibodies of the IgG isotype, B6.*Sle1* congenic mice also exhibit significantly greater total serum IgG levels compared to B6 controls [11]. Consistent with these previously published studies, we observed that total serum IgG levels were significantly elevated in B6.*Sle1*;*ERa*^{+/+} females compared to age- and sex-matched B6.*ERa*^{+/+} controls (Figure 1G; P < 0.05). Total serum IgG levels in B6.*Sle1*;*ERa*^{+/+} females was significantly different than that in B6.*ERa*^{+/+} controls (Figure 1G; P < 0.05) but not different from that in B6.*Sle1*;*ERa*^{+/+} females (Figure 1G; P = 0.09). This observation is consistent with the fact that the increase in total serum IgG in B6.*Sle1* mice shows no sex bias [11]. Furthermore, this result suggests that the attenuated development of anti-chromatin IgG in B6.*Sle1*;*ERa*^{-/-} females is not due a generalized defect in IgG production or maturation of the immune response. No significant differences were seen among the groups with respect to total serum IgM levels (Figure 1H), which are reported to be increased in B6.*Sle1* congenic mice in some, but not all, studies [11, 17].

3.2. *ERa* deficiency attenuates *Sle1*-induced B and T cell activation in females

Sle1 is associated with the hyperactivation of both B cells and T cells [17]. To determine the impact of *ERa* deficiency on immune cell activation in B6.*Sle1* female mice, flow cytometry was used to assess the expression of B and T cell activation markers (Table 1). As predicted, the proportion of B220⁺ CD86⁺ B cells was significantly greater in B6.*Sle1*;*ERa*^{+/+} congenic females compared to B6.*ERa*^{+/+} females (P < 0.05). Strikingly, in B6.*Sle1*;*ERa*^{-/-} females, the percentage of B220⁺CD86⁺ B cells was significantly less than that in B6.*Sle1*;*ERa*^{+/+} females (P < 0.01), but not different than that in either B6.*ERa*^{+/+} or B6.*ERa*^{-/-} controls (P > 0.05). These results indicate that *ERa* is required for *Sle1*-dependent B cell hyperactivation in females.

B6.*Sle1* mice also display a prominent T cell hyperactivation phenotype. Compared to B6.*ERa*^{+/+} females, a significantly smaller fraction of CD4⁺ T cells in B6.*Sle1*;*ERa*^{+/+} females expressed the naïve T cell marker CD62L whereas a significantly greater percentage of CD4⁺ T cells expressed the activation markers CD69 and CD134 (Table 1; P < 0.05). *Sle1*-induced T cell activation was largely abolished by *ERa* deficiency in females (Table 1); The proportion of activated CD4⁺ CD69⁺ and CD4⁺ CD134⁺ T cells in B6.*Sle1*;*ERa*^{-/-} females was significantly less than that in B6.*Sle1*;*ERa*^{+/+} females (P < 0.05). Consistent with this observation, the proportion of naïve CD4⁺CD62L⁺ T cells in B6.*Sle1*;*ERa*^{-/-} females was significantly greater than that in B6.*Sle1*;*ERa*^{+/+} females (P < 0.01). However, we noted that the fraction of CD4⁺ CD62L⁺ T cells in B6.*Sle1*;*ERa*^{-/-} females remained significantly less than that in B6.*ERa*^{+/+} or B6.*ERa*^{-/-} control females (P < 0.01), indicating that *ERa* deficiency did not completely impede *Sle1*-induced T cell activation in females.

Interestingly, the proportion of CD4⁺CD62L⁺ naïve T cells in B6.*ER*^{-/-} control females was significantly greater than that in B6.*ERα*^{+/+} control females, suggesting that *ERα* may promote T cell activation in females independently of *Sle1*. However, there was no significant difference between B6.*ERα*^{-/-} and B6.*ERα*^{+/+} control females with respect to the proportion of CD4⁺ T cells expressing CD69 or CD134 ($P > 0.05$). Altogether, these results suggest that *ERα* deficiency attenuates *Sle1*-induced T cell hyperactivation

We also noted that the relative abundance of CD4⁺CD25⁺ T cells in B6.*Sle1*;*ERα*^{+/+} congenic females was significantly less than that in B6.*ERα*^{+/+} ($P > 0.05$). The proportion of CD4⁺CD25⁺ T cells in B6.*Sle1*;*ERα*^{-/-} females was significantly less than that in B6.*ERα*^{-/-} females ($P < 0.05$), but not different than that in B6.*Sle1*;*ERα*^{+/+} females. Because *Sle1* has been reported to decrease the relative abundance of CD4⁺CD25⁺ Treg cells [26, 27], we examined the impact of *ERα* deficiency on the relative abundance of this particular subset of CD4⁺CD25⁺ T cells. Consistent with the previous studies, the proportion of CD4⁺CD25⁺ T cells that were CD62L^{hi} (CD4⁺CD25⁺ CD62L^{hi} Tregs) in B6.*Sle1*;*ERα*^{+/+} females was significantly less than that in B6. *ERα*^{+/+} controls (Figure 2A; $P < 0.01$). However, the relative abundance of CD4⁺CD25⁺ CD62L^{hi} Tregs in B6.*Sle1*.*ERα*^{-/-} females did not differ from that in B6.*Sle1*.*ERα*^{+/+} females, indicating that *ERα* deficiency does not impact the *Sle1*-associated decrease in the size of the Treg population (Figure 2A). Consistent with this observation, *ERα* deficiency also did not impact the relative size of the CD4⁺CD25⁺FoxP3⁺ Treg population or FoxP3 expression level (Figure 2B-C). These observations suggest that *ERα* deficiency attenuates *Sle1*-induced T cell hyperactivation but does not remediate the *Sle1*-associated decrease in the Treg population.

3.3. Impact of *ERα* deficiency on expression of *Sle1*-associated genes in female mice

The *Sle1* subloci are associated with polymorphisms that impact the expression and/or splicing of one or more genes within the *Sle1* interval. Altered expression of these genes is responsible for the immune dysfunction and loss of tolerance in B6.*Sle1* congenic mice. Because ERα functions as a ligand-activated transcription factor, we postulated that ERα might promote *Sle1*-associated phenotypes in B6.*Sle1* congenic females by regulating the expression of the genes that underlie this lupus susceptibility locus. Therefore, we examined the effect of *ERα* deficiency on the expression of the genes that underlie *Sle1*.

The *Sle1a* sublocus is comprised of multiple genes, including the pre-B-cell leukemia homoeobox 1 (*Pbx1*) gene [28, 29]. *Sle1a* is associated with differential splicing of the *Pbx1* transcript (Figure 3A), leading to an increase in the relative abundance of the lupus-associated *Pbx1d* mRNA isoform compared to the *Pbx1b* mRNA isoform [29]. To evaluate the impact of *ERα* genotype on the relative abundance of *Pbx1d* and *Pbx1b* isoforms, we developed isoform-specific PCR primers (Figure 3B) and performed q-RT-PCR. Consistent with previous reports, the *Pbx1d*/*Pbx1b* ratio in CD4⁺ T cells from B6.*Sle1*;*ERα*^{+/+} female mice was significantly greater than that in B6.*ERα*^{+/+} females (Figure 4A; $P < 0.05$). The *Pbx1d*/*Pbx1b* ratio in CD4⁺ T cells derived from B6.*Sle1*;*ERα*^{-/-} female mice did not differ from that in B6.*Sle1*;*ERα*^{+/+} female mice (Figure 4A), indicating that *ERα* genotype did not impact the relative abundance of these transcripts. A third *Pbx1* mRNA isoform, *Pbx1a* (Figure 3A), has been reported to be expressed in human T cells but not T cells in the

B6.*Sle1a.1* subcongenic strain [29]. However, using q-RT-PCR and isoform-specific PCR primers (Figure 3B), we were able to detect the *Pbx1a* isoform in splenic CD4⁺T cells from B6.*Sle1* congenic mice, and thus we evaluated the impact of *Sle1* and *ERα* on the relative expression of *Pbx1a* in female mice. The *Pbx1a/Pbx1b* ratio in CD4⁺ T cells from B6.*Sle1*;*ERα*^{+/+} female mice was significantly greater than that in B6.*ERα*^{+/+} females, indicating that expression of this *Pbx1* isoform is also modulated by *Sle1* genotype (Figure 4A; P < 0.05). Interestingly, the *Pbx1a/Pbx1b* ratio in CD4⁺ T cells from B6.*Sle1*;*ERα*^{-/-} female mice was significantly less than that in B6.*Sle1*;*ERα*^{+/+} females, suggesting that the relative expression of this isoform is modulated by ERα signaling (Figure 4A; P < 0.05).

Sle1b consists of several linked polymorphisms in genes within the SLAM/CD2 cluster [30]. As a result of these polymorphisms, the expression of the *CD48* gene is significantly altered. Furthermore, there is differential splicing of the *Ly108* gene, leading to increased expression of the autoimmune *Ly108-1* isoform and decreased expression of the “normal” *Ly108-2* isoform. Consistent with previous reports, B cells derived from female B6.*Sle1*;*ERα*^{+/+} mice expressed significantly greater levels of *Ly108-1* and significantly lower levels of both *Ly108-2* and *CD48* compared to female B6.*ERα*^{+/+} mice (Figure 4B; P < 0.05). *ERα* deficiency did not significantly impact the expression of *Ly108-1*, *Ly108-2*, or *CD48* in female B6 mice (Figure 4B). Likewise, in female B6.*Sle1*;*ERα*^{-/-} mice, the level of expression of *Ly108-1* and *Ly108-2* did not differ significantly from that in B6.*Sle1*;*ERα*^{+/+} mice (Figure 4B). Although B cells from female B6.*Sle1*;*ERα*^{-/-} mice did express significantly more *CD48* than that in female B6.*Sle1*;*ERα*^{+/+} mice, the magnitude of this difference was quite small and *ERα* deficiency in B6.*Sle1* mice was insufficient to restore *CD48* levels to that seen in B6.*ERα*^{+/+} or B6.*ERα*^{-/-} controls (Figure 4B; P = 0.01). Expression of *CD48* and *Ly108-2* is also downregulated in CD4⁺ T cells from B6.*Sle1* mice [30]. The expression of *Ly108-2* and *CD48* in CD4⁺ T cells derived from female B6.*Sle1*;*ERα*^{-/-} mice did not differ from that in B6.*Sle1*;*ERα*^{+/+} females (data not shown). Although some previous studies indicate that *CD84* is also differentially expressed in B6.*Sle1* mice compared to B6 controls, we did not find that *Sle1* or *ERα* genotype had any impact on *CD84* expression (data not shown).

Sle1c is associated with multiple genes, including *Estrogen Related Receptor γ* (*Esrrg*) and *Complement Receptor 1/2* (*Cr2*) [25, 31-33]. T cells from B6.*Sle1c* subcongenic mice have been reported to express lower levels of *Esrrg* compared to B6 CD4⁺ T cells [33]. Consistent with these reports, we found that B6.*Sle1* CD4⁺ T cells expressed lower levels of *Esrrg* compared to B6 in female mice, although this difference fell short of statistical significance (P = 0.1). *ERα* genotype had no impact on the abundance of the *Esrrg* transcript (Figure 4C). B6.*Sle1c* splenocytes also exhibit differential glycosylation of Complement receptor 2 (CR2) and Complement receptor 1 (CR1), which is produced by alternative splicing from the *Cr2* gene in mice. Similar to previous reports, the CR1 and CR2 expressed on splenocytes from B6.*Sle1* mice migrated at a higher molecular weight than CR1 and CR2 expressed on splenocytes from B6 mice (Figure 3D). CR1 and CR2 from B6.*Sle1*;*ERα*^{+/+} and B6.*Sle1*;*ERα*^{-/-} appear to have equivalent molecular weights, suggesting that ER genotype does not have a major impact on the glycosylation of CR1 or CR2 (Figure 4C). It has also been reported that the *Sle1c* allele of *Cr2* undergoes differential splicing, leading to

changes in the relative abundance of CR1 and CR2 [25]. Therefore, we examined the impact of ER α deficiency on the relative abundance of the CR1 and CR2 proteins. Consistent with what has been reported previously, we found that female B6.*Sle1*;*ER α ^{+/+}* had a significantly higher CR1/CR2 ratio compared to B6.*ER α ^{+/+}* mice (Figure 4E; $P < 0.05$). B6.*Sle1*;*ER α ^{+/+}* and B6.*Sle1*;*ER α ^{-/-}* females had similar ratios of CR1/CR2 expression, indicating that *ER α* genotype does not affect the splicing of *Cr2* (Figure 4E).

3.4. *ER α* deficiency does not impact loss of tolerance to chromatin in B6.*Sle1* congenic male mice

Our previous studies in lupus-prone (NZB \times NZW)F1 mice demonstrate that the low levels of endogenous estrogens, acting through ER α , promote loss of tolerance to chromatin and development of autoantibodies in male mice [8]. Although this effect in males was measurable, it was significantly less dramatic than that observed in female mice [8]. Based upon these results, we postulated that *ER α* deficiency might also attenuate to some degree the development of autoantibodies in B6.*Sle1* congenic males. As has been reported previously, we observed that the proportion of B6.*Sle1* congenic males that develop autoantibodies is significantly less than that in B6.*Sle1* congenic females (Figure 5A-D; $P < 0.05$). However, among those mice that had a positive anti-chromatin IgG titer, sex did not appear to impact the absolute levels of anti-chromatin IgG (Figure 5B); this observation is also consistent with previous studies [12]. Interestingly, *ER α* deficiency did not have a significant impact on the proportion of B6.*Sle1* males that developed anti-chromatin IgG (55% versus 43%; Figure 5E-F; $P > 0.05$) or anti-dsDNA IgG (6% versus 14%; Figure 5G-H; $P > 0.05$). Overall, these results, which indicated that *ER α* deficiency did not have a major impact on autoantibody development in B6.*Sle1* males, were in sharp contrast to what was observed in B6.*Sle1* females.

3.5. Estrogens and *ER α* control sex bias in *Sle1*-induced loss of tolerance

We noted that the proportion of B6.*Sle1* males, irrespective of *ER α* genotype, that developed anti-chromatin IgG, was not different than that observed in either B6.*Sle1*;*ER α ^{-/-}* females or ovariectomized B6.*Sle1* females ($P > 0.05$). These observations indicate that removing either estrogens or *ER α* eliminates the sex bias in *Sle1*-induced loss of tolerance and suggest that estrogens are the major drivers of sex bias in the *Sle1* phenotype. Nevertheless, because *ER α* deficiency leads to elevated serum testosterone levels [34, 35], we considered the possibility that androgens, which can also attenuate the development of autoantibodies in (NZB \times NZW)F1 mice [36, 37] might also contribute to the sex bias in B6.*Sle1* congenic mice. Similar to what has been reported previously in B6.*ER α ^{-/-}* female mice, we found that B6.*Sle1*;*ER α ^{-/-}* females have serum testosterone levels that are significantly higher than that B6.*Sle1*;*ER α ^{+/+}* female mice but similar to that seen in intact B6.*Sle1* male mice (Figure 5I). Thus, it seemed plausible that the attenuated loss of tolerance in B6.*Sle1*;*ER α ^{-/-}* females could be attributable, at least in part, to increased serum testosterone levels. However, the fact that the incidence of loss of tolerance in B6.*Sle1*;*ER α ^{-/-}* females is identical to that in ovariectomized B6.*Sle1* females, which have very low levels of serum androgen levels (Figure 5I), does not support this possibility. We also observed no evidence of an inverse correlation between serum anti-chromatin levels

and serum testosterone levels in these mice (Figure 5J). Altogether, these data suggest that estrogens, acting via *ERα* are responsible for the sex bias in *Sle1*-induced loss of tolerance.

3.6. *ERα* deficiency has a modest impact on *Sle1*-induced T cell activation in males

Although *ERα* deficiency did not impact *Sle1*-induced loss of tolerance and development of autoantibodies in males, we considered the possibility that *ERα* might nevertheless impact *Sle1*-associated immune cell hyperactivation in males [17]. Therefore, we wished to examine the impact of *ERα* deficiency on this aspect of the *Sle1* phenotype in B6.*Sle1* congenic males. Although no sex bias in *Sle1*-induced immune cell hyperactivation phenotype has been reported previously, we first performed some preliminary studies to explicitly examine this issue. We were surprised to find that in contrast to what was seen in B6.*Sle1* females, B6.*Sle1* males showed no evidence of B cell hyperactivation; there was no significant difference in the proportion of B220⁺CD86⁺ B cells in B6.*Sle1*;*ERα*^{+/+} males compared to B6.*ERα*^{+/+} males (Table 2). This observation was confirmed by analysis with CD25, a second marker of B cell activation (~12.9% B220⁺CD25⁺ in B6.*ERα*^{+/+} versus ~13.5% ±1.0 B220⁺CD25⁺ in B6.*ERα*^{+/+}; *P* > 0.05). Compared to B6.*ERα*^{+/+} males, B6.*Sle1* males possessed a higher proportion of CD4⁺CD69⁺ and CD4⁺CD134⁺ T cells and a lower proportion of CD4⁺CD62L⁺ T cells, indicating that B6.*Sle1* males do exhibit a T cell activation phenotype. Although B6.*Sle1* congenic males exhibited T cell hyperactivation, the relative increase in activation compared to sex-matched B6 mice was somewhat less than that observed in female mice. Furthermore, the absolute level of activation, as indicated by CD62L expression, in B6.*Sle1* males was somewhat less than that in B6.*Sle1* females (Table 2). Altogether, these data indicate that *Sle1*-induced immune cell hyperactivation is a sex-biased phenotype.

ERα deficiency had no significant impact on the proportion of activated B cells in B6.*Sle1* congenic males (Table 3). *Sle1*-induced T cell activation in males was somewhat attenuated by *ERα* deficiency. The proportion of CD4⁺CD69⁺ T cells in B6.*Sle1*;*ERα*^{-/-} males was significantly less than that in the B6.*Sle1*;*ERα*^{+/+} males (Table 3; *P* < 0.05). The proportion of CD4⁺CD134⁺ T cells was reduced in B6.*Sle1*;*ERα*^{-/-} males compared to B6.*Sle1*;*ERα*^{+/+} males, but this difference fell short of statistical significance (*P* = 0.1). Interestingly, *ERα* deficiency had no impact on the proportion of CD4⁺CD62L⁺ T cells (*P* > 0.05). These data indicate that *Sle1*-induced T cell hyperactivation in males is only modestly affected by *ERα* deficiency.

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

As we observed in females, *ERα* genotype did not have a major impact on the expression of *Ly108-1*, *Ly108-2*, or *CD48* in B6.*Sle1* male mice (Figure 6A). Consistent with this observation, we also found that sex did not impact the expression of these genes (Figure 6B). Likewise, the magnitude of the effect of *Sle1* on the expression of each of these genes was not different in females and males. *ERα* deficiency did not have a significant impact on the relative expression of either *Pbx1d* or *Pbx1a* in CD4⁺ T cells in B6.*Sle1* male mice (Figure 6C; *P* > 0.05). Interestingly, the relative expression of both *Pbx1d* and *Pbx1a* in CD4⁺ T cells was greater in B6.*Sle1* congenic females compared to congenic males, but this

difference achieved significance only for Pbx1d (Figure 6D). Consistent with our results in B6.*Sle1* congenic female mice, *ERα* genotype had no impact on *Esrrg* transcript level in CD4⁺ T cells or CR1/CR2 ratios in B6.*Sle1* congenic male mice (data not shown).

4. Discussion

It has been shown previously that the effects of the lupus susceptibility locus *Sle1* are more robust in female mice than male mice. However, the basis for this sex bias is not known. In the present study, we examined the impact of removing estrogens or *ERα* on the *Sle1* phenotype, and our results indicate that sex bias in the *Sle1* phenotype is due to estrogen-dependent, *ERα*-mediated processes. Our data do not provide any evidence to support the hypothesis that androgens, which are known to attenuate lupus in (NZB × NZW)F1 mice [36, 37], contribute to the 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 that 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 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 [20].

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 [20], 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 ER α 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*;ER α ^{-/-} females, indicating that the expression of this isoform is not ER α 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 [29]. However, the *Pbx1d*:*Pbx1b* ratio in our other set of non-autoimmune control mice, the B6.ER α ^{-/-} mice, was not significantly different from that in our B6.ER α ^{+/+} controls and was also very similar to that reported by Cuda et al [29]. 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 [29].

We also detected *Pbx1a*, another isoform of *Pbx1*, in T cells from both B6 and B6.*Sle1* congenic mice. Although *Pbx1a* was reported to be undetectable by conventional RT-PCR in both the B6 strain and the B6.*Sle1a.1* subcongenic strain [29], we were able to detect this isoform using an isoform specific Q-RT-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*;ER α ^{-/-} congenic females. These observations suggest that ER α signaling may impact the *Sle1* phenotype, in part, by modulating the expression of the *Pbx1a* isoform. It remains to be determined if the effects of ER α on *Pbx1* isoform expression are due to direct effects on *Pbx1* transcription and/or splicing or due to indirect effects. Neither sex nor ER α 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 ER α 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 [11, 12].

Pbx1 is a member of the three amino acid loop extension family of transcription factors ([38, 39]). 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 [40-43]. 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 [43-45]. In this context, it is of particular interest that Pbx1 has been shown to modulate the binding of ER α to specific estrogen response elements in ER⁺ breast cancers [46]. However, the impact of Pbx1 on either the accessibility of estrogen response

elements or ER α function in other cell types, including those relevant to lupus susceptibility, has not been examined.

Our results suggest that sex and ER α impact the relative expression of *Pbx1* isoforms. Differential expression of these isoforms has been shown previously to correlate with lupus susceptibility [29], 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 [45, 47-49]. The Pbx1b protein product has a c-terminal truncation that eliminates the c-terminal activation domain and co-repressor binding domains [45, 48]. 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 [29]. Pbx1d also lacks a portion of the c-terminal activation domain and co-repressor binding domain [29]. 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 [50-52], 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 ER α signaling of these processes.

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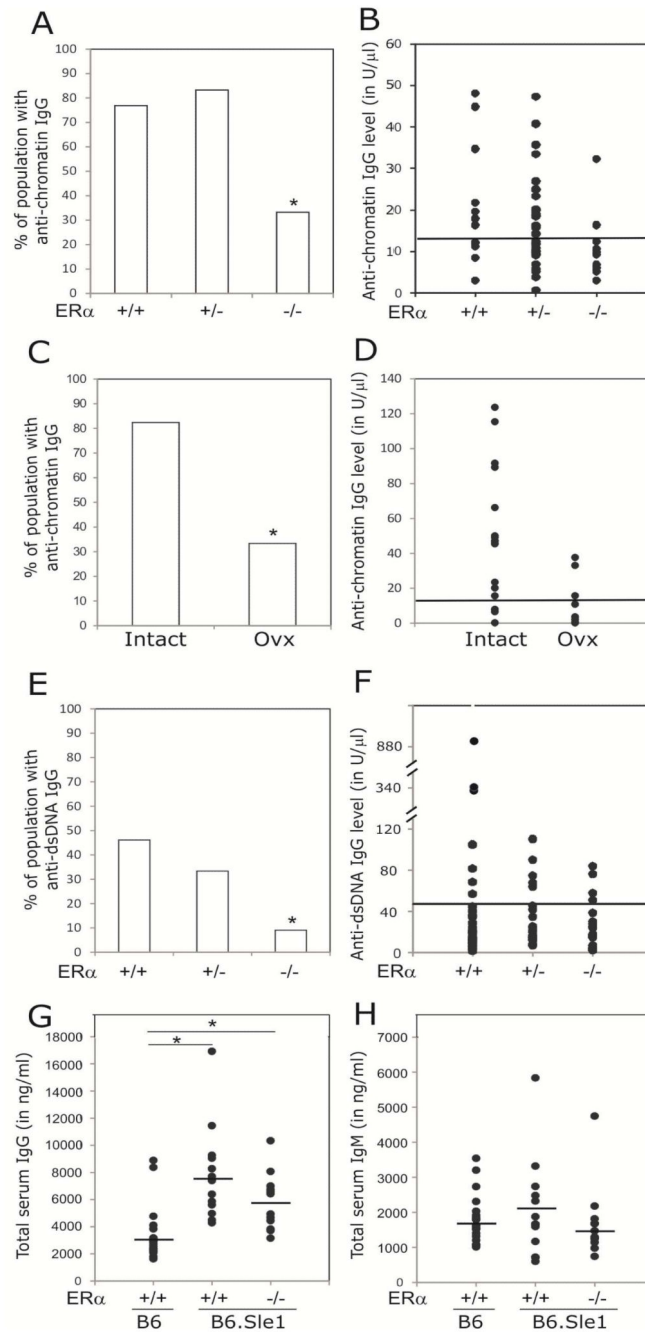


Figure 1. Attenuation of *Sle1*-induced loss of tolerance by ERα deficiency and ovariectomy
 ELISAs were used to assess the abundance of anti-chromatin IgG (A–D), anti-dsDNA IgG (E–F), total serum IgG (G) and IgM (H) in 5–9 month old female mice. The proportion of B6.*Sle1*;ERα^{+/+} (N=13), B6.*Sle1*;ERα^{+/-} (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 [17]. The mean (\pm standard error of the mean) abundance of total serum IgG (G) and IgM (H) in B6 (N=20), B6.*Sle1;ER $\alpha^{+/+}$* (N=13), and B6.*Sle1;ER $\alpha^{-/-}$* (N=12) females is shown. The * indicates a P<0.05 compared to B6.*Sle1;ER $\alpha^{+/+}$* females (A and E), intact females (C), and B6 females (G).

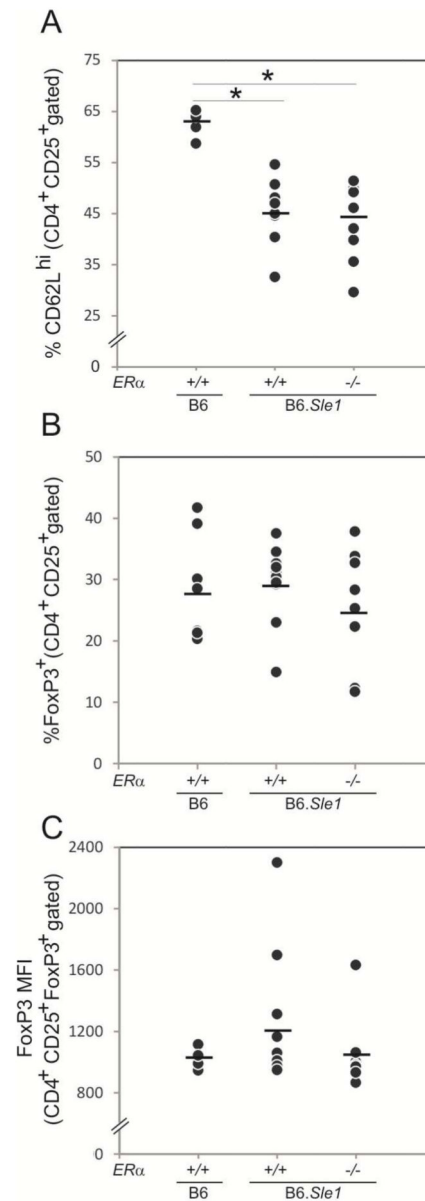


Figure 2. Impact of *ERα* genotype on the T regulatory population in *B6.Sle1* congenic mice
 Flow cytometry was used to assess the T regulatory cell pool in female mice at 3-4 months of age. Splenocytes from each mouse were analyzed individually by flow cytometry (N=4-12 per group). The percentage of 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.

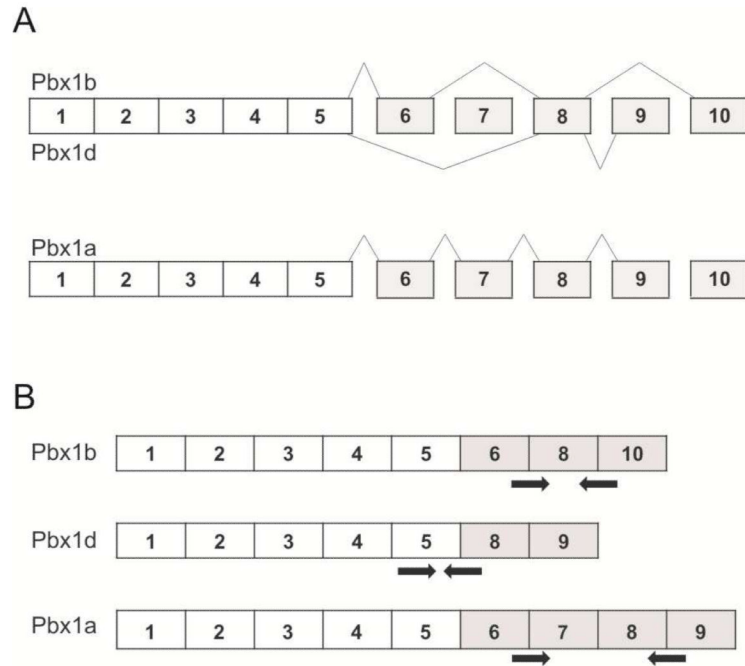


Figure 3. Alternative splicing of *Pbx1* yields multiple isoforms at the mRNA and protein level
 Alternative splicing of exons 6-10 of the *Pbx1* gene yields 3 unique isoforms, *Pbx1b*, *Pbx1d* and *Pbx1a* (A). PCR primers (indicated by arrows) were designed to independently quantify each transcript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

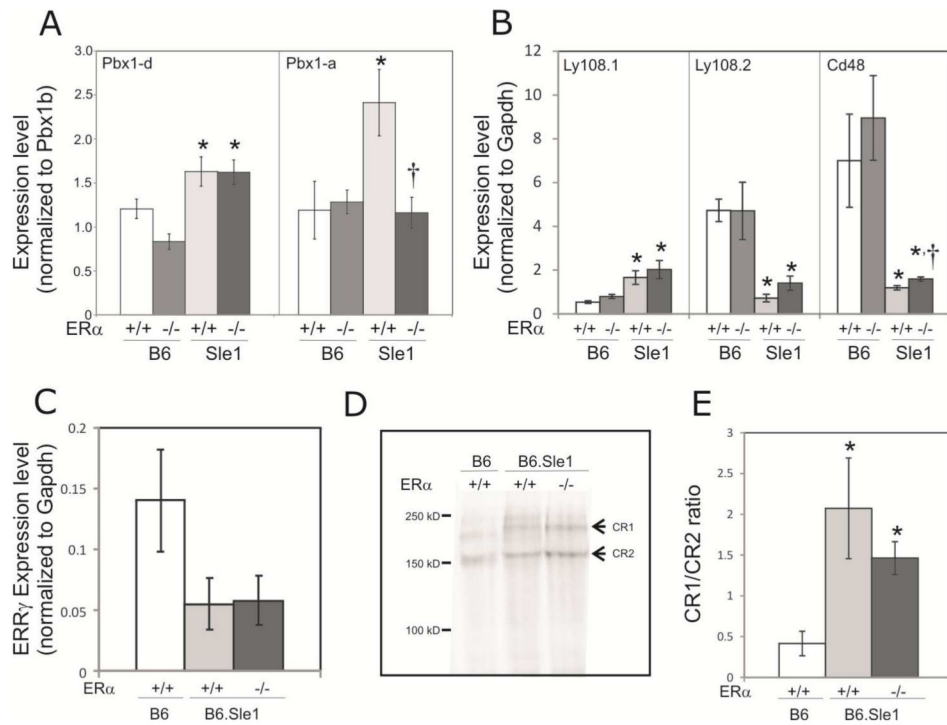


Figure 4. Impact of *ERα* genotype on the expression of *Sle1*-associated genes in female mice
 The relative abundance of transcripts produced by genes that underlie *Sle1* was assessed using quantitative RT-PCR. Data for panels A, B, C and E presented as mean \pm standard error of the mean. The expression 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 (\pm 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).

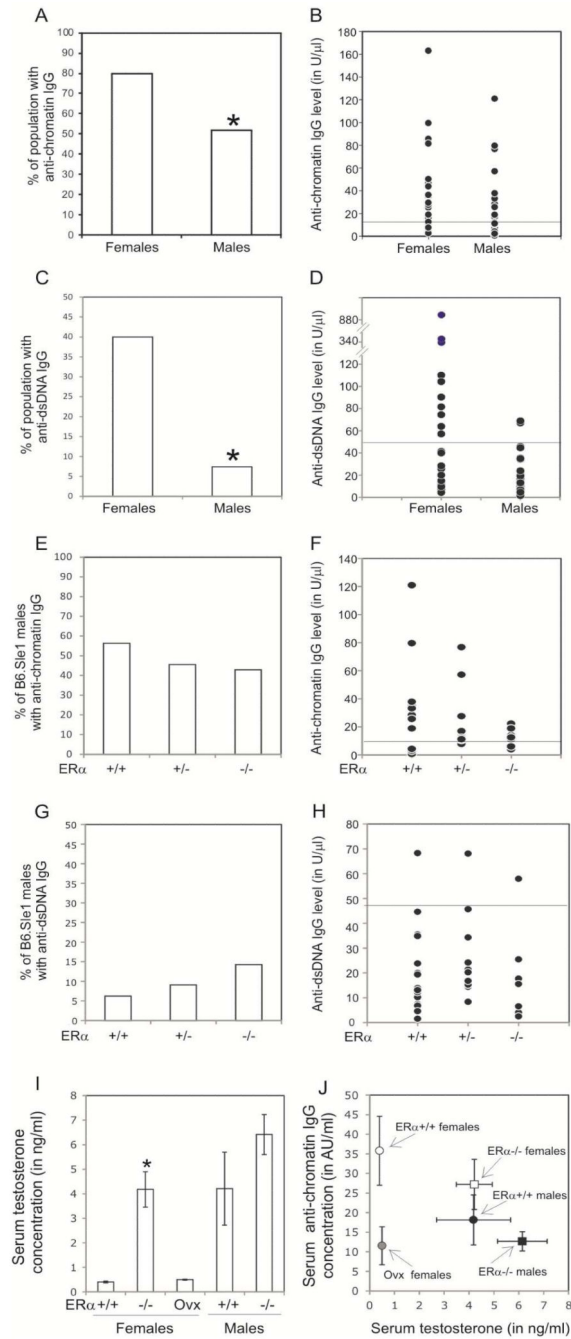


Figure 5. ERα deficiency does not impact loss tolerance in B6.Sle1 males
 ELISAs were used to assess the abundance of anti-chromatin IgG (A,B,E,F), anti-dsDNA IgG (C, D,G, H) in 5-9 month old mice. The proportion of B6.*Sle1*;ERα^{+/+} females (N=25) and B6.*Sle1*;ERα^{+/+} males (N=27) that develop 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 Mean serum testosterone levels (I) in mice of the indicated genotypes is shown (N=9-11 of each sex per

genotype). For each group shown in panel I, the mean concentration of anti-chromatin IgG (in arbitrary units/ml) was plotted in relation to the mean serum testosterone concentration (J) to determine if these two parameters were correlated. For panels I and J, the error bars represent the standard error of the mean. The * indicates a $P < 0.05$ compared to B6.*Sle1*;*ER α ^{+/+}* females (A, C, I).

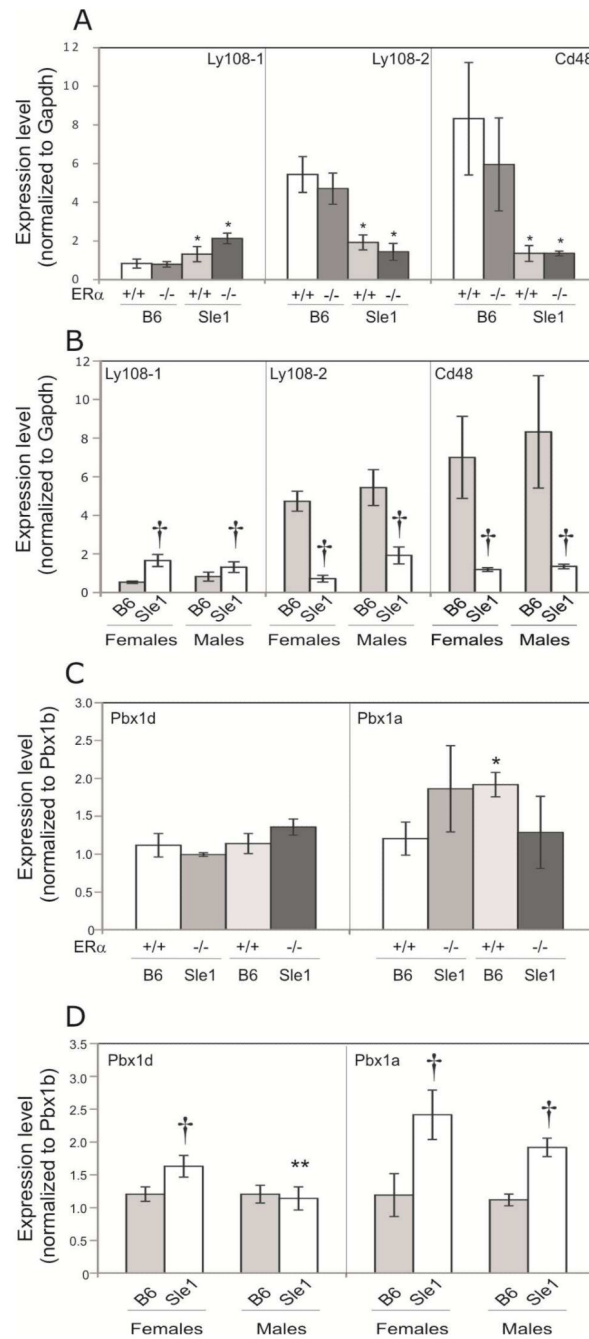


Figure 6. Impact of *ERA* genotype on the expression of *Sle1*-associated genes in male mice
 The relative abundance of transcripts produced by genes that underlie *Sle1* was assessed using quantitative RT-PCR. Data is presented as mean \pm standard error of the mean. The expression of *Ly108-1*, *Ly108-2* 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 *ERA* genotype on the expression of *Ly108-1*, *Ly108-2* and *CD48* is shown by comparing relative expression in B6.*ERA*^{+/+}, B6.*ERA*^{-/-}, B6.*Sle1*;*ERA*^{+/+} and B6.*Sle1*;*ERA*^{-/-} 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 *ERa* genotype on the expression of *Pbx1d* and *Pbx1a* is shown by comparing relative expression in B6.*ERa*^{+/+}, B6.*ERa*^{-/-}, B6.*Sle1*;*ERa*^{+/+} and B6.*Sle1*;*ERa*^{-/-} 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 *ERa* 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).

Table 1Impact of ER α genotype on lymphocyte activation in female B6.Sle1 mice^a

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

^aSplenocytes 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 \pm SEM for the samples in each group.

^bValues represent the mean percentage of total splenocytes.

^cValues represent the mean percentage of B220⁺ splenocyte

^dValues represent the mean percentage of CD4⁺ splenocytes.

^eSignificant difference compared to B6 mice of the same ER α genotype are denoted by (P 0.05)

^fSignificant difference compared to B6 mice of the same ER α genotype are denoted by (P 0.01)

^gSignificant difference compared to ER α ^{+/+} mice of the same Sle1 genotype are denoted by (P 0.01)

Table 2Impact gender on lymphocyte activation in B6.Sle1 mice^a

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

^aSplenocytes 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.

^bValues represent the mean percentage of total splenocytes.

^cValues represent the mean percentage of B220⁺ splenocytes

^dValues represent the mean percentage of CD4⁺ splenocytes.

^eSignificant difference compared to B6 mice of same sex are denoted by (P 0.05)

^fSignificant difference compared to B6 mice of same sex are denoted by (P 0.01)

^gSignificant difference compared to female mice of the same *Sle1* genotype are denoted by (P 0.05)

^hSignificant difference compared to female mice of the same *Sle1* genotype are denoted by (P 0.01)

Table 3Impact of ER α genotype on lymphocyte activation male B6.Sle1 mice^a

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

^aSplenocytes 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 \pm SEM for the samples in each group.

^bValues represent the mean percentage of total splenocytes.

^cValues represent the mean percentage of B220⁺ splenocytes

^dValues represent the mean percentage of CD4⁺ splenocytes.

^eSignificant differences compared to *Sle1*^{B/B} mice of same gender and *ER α* genotype are denoted by (P 0.01)

^fSignificant difference compared to *ER α* ^{+/+} mice of same gender and *Sle1* genotype are denoted by (P 0.05)