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Estrogen Receptor Alpha Promotes Lupus in (NZB x NZW)F1 Mice in a B cell Intrinsic Manner

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Abstract

Lupus is a systemic autoimmune disease characterized by the production of autoreactive antibodies against nuclear antigens. Women are disproportionately affected by lupus, and this sex bias is thought to be due, in large part, to the ability of estrogens to promote lupus pathogenesis. Previously, we have shown that global deletion of estrogen receptor alpha (*ERα*) significantly attenuated loss of tolerance, immune cell activation, autoantibody production, and the development of lupus nephritis. Here we show that targeted deletion of *ERα* specifically in B cells retards production of pathogenic autoantibodies and the development of nephritis in lupus-prone (NZB x NZW)F1 mice. Furthermore, we observed that *ERα* deletion in B cells was associated with decreased B cell activation in young, pre-autoimmune (NZB x NZW)F1 females. Altogether, these data suggest that *ERα* acts in a B cell-intrinsic manner to control B cell activation, autoantibody production, and lupus nephritis.

Keywords

Lupus; estrogen receptor alpha; B cell; immunologic tolerance; immune cell activation

1. Introduction

Lupus is a systemic autoimmune disease in which patients lose immunologic tolerance and produce B and T cells reactive to nuclear antigens including double stranded DNA (dsDNA). Autoreactive B cells produce autoantibodies, and a subset of these autoantibodies, particularly the anti-dsDNA autoantibodies of the IgG isotype, have the potential to form immune complexes. Immunoglobulin-containing immune complexes that are deposited in tissues cause an inflammatory immune response through activation of complement and Fcγ receptors, which results in tissue damage [1]. Although lupus patients display abnormalities in multiple immune cell lineages, including B cells, T cells, dendritic cells and macrophages [2–9], lupus is considered to be a primarily B cell driven disease. Consistent with this

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premise, when lupus-prone mice are rendered unable to produce mature B cells, lupus is completely ameliorated [10]. However, when lupus-prone mice are genetically engineered to produce mature B cells that are unable secrete antibody, but are otherwise completely functional, lupus still developed, albeit in an attenuated manner [11]. Altogether, these data suggest that B cells contribute to lupus pathogenesis through both antibody-dependent and antibody-independent mechanisms.

Like most autoimmune diseases, lupus shows a significant sex bias; approximately 90% of lupus patients are female. Furthermore, lupus is primarily a disease that affects young and middle aged women. Female sex hormones, particularly estrogens, have long been thought to promote lupus and to be responsible for the profound sex bias in this disease. Indeed, exposure to both endogenous and exogenous estrogens is a risk factor for developing lupus [12, 13]. Cells of the immune system, including B cells, express the cellular receptors for estrogens, estrogen receptor α (ER α) and estrogen receptor β [14, 15].

Previously, we have shown that targeted disruption of the *ER α* gene attenuates the development of autoantibodies and lupus pathogenesis in female lupus-prone mice, whereas more modest effects are seen in male mice [16]. These studies were conducted using the (NZB x NZW)F1 mouse model of lupus, in which the development of pathogenic dsDNA IgG autoantibodies and fatal glomerulonephritis shows a strong female sex bias. These data indicate that although estrogen signaling through ER α promotes lupus in both sexes, the ability of ER α signaling to enhance autoantibody production and lupus is more pronounced in females than in males. We have also observed that estrogens and ER α signaling are responsible for the sex bias that is seen in mice carrying the lupus susceptibility locus *Sle1*, which controls loss of tolerance to nuclear antigens and immune cell activation. Targeted disruption of *ER α* attenuates the ability of *Sle1* to promote loss of tolerance, autoantibody production and B cell activation preferentially in females [17]. However, in these studies, the cell type or types responsible for these effects could not be identified because *ER α* was knocked out in all cells. Although it is hypothesized that estrogens influence lupus via actions within the immune lineage, there is little concrete evidence to support this hypothesis.

A growing body of literature suggests that estrogen, acting via ER α , can exert powerful effects on B cells. For example, sustained administration of a high level of the naturally occurring estrogen 17 β -estradiol (E2) allows high-affinity dsDNA-reactive B cells to escape mechanisms that maintain tolerance, including clonal deletion at the immature stage and anergy induction at the T2 stage [18]. Furthermore, continuous exposure to high levels of E2, leads to upregulation of *CD22* and *SHP-1* in B cells, which likely results in decreased B cell receptor (BCR) signaling, thereby increasing the concentration of antigen required for tolerization of autoreactive B cells, and protecting autoreactive B cells from receptor-mediated apoptosis [19, 20]. Importantly, the ability of estrogens to induce *CD22* and *SHP-1* expression is dependent upon *ER α* , although it is not known which cell type or types must express *ER α* to elicit this effect. High levels of E2 also cause increased expression of anti-apoptotic *Bcl-2* and the B cell survival factor *BAFF* [19, 20]. By decreasing the strength of BCR signaling and increasing the expression of pro-survival molecules, estrogens may enhance the survival of high-affinity dsDNA-reactive B cells. In addition to autoreactive B

cells that arise as the result of VDJ recombination, autoreactive B cells can be generated by somatic hypermutation in the periphery. Estrogens promote somatic hypermutation by stimulating the expression of activation-induced deaminase [21, 22]. Although these data suggest that estrogens can promote the development of autoreactive B cells at multiple stages of development, it is not known to what extent these various actions are mediated by B cell-intrinsic actions of ER α .

We hypothesize that ER α signaling in B cells promotes lupus. To test this hypothesis, we have generated lupus-prone (NZB x NZW)F1 mice in which *ER α* was deleted specifically in the B cell compartment. To generate (NZB x NZW)F1 mice with B cell specific deletion of *ER α* , we used the CD19-Cre driver strain and a floxed allele of *ER α* . In (NZB x NZW)F1 mice of both sexes, deletion of *ER α* in B cells significantly attenuated autoantibody production and extended survival. However, in female mice, B cell specific deletion of *ER α* also significantly reduced B cell activation, suggesting that *ER α* may enhance lupus in females by acting in a B cell-intrinsic fashion to promote B cell activation and thereby stimulate the production of autoantibodies.

2. Methods

2.1 Production of experimental animals

To produce NZB mice carrying the *CD19-Cre* knockin allele, B6.129P2(C)-*CD19^{tm1(Cre)Cgn/J}* female mice were purchased (The Jackson Laboratory, Bar Harbor, ME, USA) and crossed with NZB male mice. Genotyping for the *CD19-Cre* knockin allele was performed using primers that amplified the Cre gene (IMR1084 F: 5'-GCGGTCTGGCAGTAAAACTATC-3' and IMR1085 R: 5'-GTGAAACAGCATTGCTGTCACCT-3'). A pair of primers that amplified the IL-2 receptor gene (COO3IC F: 5'-CTAGGCCACAGAATTGAAAGATCT-3' and COO4IC R 5'-GTAGGTGGAAATTCTAGCATCATCC-3') were used as an internal positive control. Offspring carrying the *CD19-Cre* knockin allele (*CD19-Cre* or *CD19^{Cre/+}*) were serially backcrossed to NZB mice for 5 generations using simple sequence length polymorphism (SSLP) marker assisted selection as we have described previously [16, 23]. Briefly, at each backcross generation, mice were genotyped using 111 polymorphic markers spanning the 19 murine autosomes (detailed in Table 1). PCR genotyping was performed using oligonucleotide primers specific for SSLPs between the B6 and NZB strains. Primer sequences were obtained from the Mouse Genome Informatics database (www.informatics.jax.org). By the N5 generation, an average of only 7% of SSLP markers remained heterozygous in the NZB.*CD19^{Cre/+}* mice whereas 93% were homozygous for NZB allele (data not shown). At the N5 generation, the genetic background of the incipient congenic NZB.*CD19^{Cre/+}* strain was further confirmed at the DartMouse Mouse Speed Congenic Core Facility at Dartmouth Medical School (Supplemental Figure 1). DartMouse uses the Illumina, Inc. (San Diego, CA, USA) GoldenGate Genotyping Assay to interrogate 1449 possible SNPs spread throughout the genome. The raw SNP data were analyzed using DartMouse's SNaP-MapTM and Map-SynthTM software, to determine the SNP genotype, and thus strain of origin of SNP alleles, in each mouse. Analysis of 733 polymorphic SNPs indicated that the incipient congenic NZB.*CD19^{Cre/+}* strain was homozygous for NZB

alleles at 96% of markers evaluated (Supplementary Figure 1). A considerable fraction of markers remaining heterozygous were on distal chromosome 7 and thus linked to the *CD19-Cre* knockin allele. All areas of heterozygosity had been previously identified by our SSLP genotyping, and none of these areas of residual heterozygosity co-localized with known lupus susceptibility loci. Based upon these analyses, the NZB.*CD19-Cre* strain was determined to be extensively backcrossed onto the NZB background.

Incipient congenic NZB.*CD19^{Cre/+}* mice were then crossed to congenic NZB.*ERα^{+/-}* mice, which are NZB mice heterozygous for a targeted deletion of exon 2 of *ERα* [16, 24], thereby producing NZB mice heterozygous for both *CD19-Cre* and an *ERα* knockout allele. Genotyping for the exon 2 deletion of *ERα* was performed as described (www.jax.org/protocols). The common ERαEx2 F primer: 5'-TACGGCCAGTCGGGCATC-3' (0.5 uM/rxn) and the ERαEx2wtR: 5'-GTAGAAGGCGGGAGGGCCGGTGTC-3' (0.06 uM/rxn) or ERαEx2null R: 5'-GCTACTTCCATTTGTCACGTCC-3' (2 uM/rxn) primers were used to produce 234 bp (intact exon 2) and ~300 bp (deleted exon 2) products, respectively.

NZW.*ERα^{fl/fl}* mice, which are congenic NZW mice homozygous for an *ERα* allele in which exon 3 is flanked by loxP sites, were previously produced by backcrossing the floxed *ERα* allele from B6.*ERα^{fl/fl}* mice obtained from Ken Korach [25] to the NZW background using marker assisted selection [26]. Genotyping for the *ERα^{fl}* allele was done with the primers N6delK F: 5'-GACTCGCTACTGTGCCGTGTGC-3' and N6del3 R 5'-CTTCCCTGGCATTACCACTTCTCCT-3'. The *ERα⁺* allele produces a 275 bp product, while the *ERα^{fl}* allele produces a 475 bp product [27]. To produce experimental mice, NZB.*CD19^{Cre/+}; ERα^{+/-}* female mice were crossed with NZW.*ERα^{fl/fl}* males.

All animals were housed under conditions of controlled humidity, temperature, and lighting in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, operating in accordance with standards set by the *Guide for the Care and Use of Laboratory Animals*. Mice had *ad libitum* access to 7904 Teklad Irradiated S-2335 Mouse Breeder Diet (Harlan Teklad, Madison, WI, USA). All procedures involving live animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

2.2 Quantification of *ERα* deletion

To evaluate the efficiency of cre-mediated *ERα* deletion in B cells, CD19⁺ cells were isolated from the bone marrow and spleen of 2–6 month old (NZB x NZW)F1 *CD19^{Cre/+}; ERα^{fl/-}*, *CD19^{Cre/+}; ERα^{fl/+}*, *CD19^{+/+}; ERα^{fl/+}*, and *CD19^{+/+}; ERα^{fl/-}* mice. Briefly, bone marrow and spleen cells were isolated in MACS buffer, passed through a 70 μm cell strainer to create a single cell suspension, and red blood cells lysed with ACK Lysing Buffer (Gibco, Waltham, MA, USA). Cells were labeled using CD19 Microbeads, and CD19⁺ cells were isolated using an autoMACS Pro as per manufacturer protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). After isolation, DNA was isolated from CD19⁺ cells using a DNeasy Kit as per manufacturer protocol for cultured cells (Qiagen, Venlo, Netherlands). Quantitative PCR was performed on DNA isolated from CD19⁺ cells as previously described [26]. Briefly, a primer set was developed flanking the loxP sites around *ERα* Exon 3 (ERαDel F: 5'-TGGAATGAGACTTGTCTATCTTCG-3' and ERαDel R: 5'-

AACCAAGGAGAACAGAGAGACT-3'). These primers generate a 161 bp product from an *ERα^{fl}* allele that has successfully undergone cre-mediated recombination, but no product is generated from the wild type or intact floxed *ERα* alleles. A primer set in the unaffected *ERα* exon 5 served as a positive control in a separate reaction (*ERα*Ex5 F: 5'-GGAAGGCCGAAATGAAATGGG-3' and *ERα*Ex5 R: 5'-CCAACAAGGCACTGACCATC-3').

2.3 Survival studies

Survival of animals was monitored over one year. Animals were euthanized by CO₂ asphyxiation when they exhibited albuminuria of 4+ (>2000 mg/dl) or physical signs of distress. Albuminuria was initially measured monthly, and subsequently with increasing frequency after a positive measurement, using Albustix (Bayer Corporation, Elkhorn, IN, USA).

2.4 Histological analysis

Kidneys were collected from mice upon sacrifice. Tissues were fixed in 10% formalin, processed, paraffin embedded, and sectioned. Kidney sections were stained with Periodic Acid Schiff (Sigma-Aldrich, St. Louis, MO, USA) and 100 glomeruli per kidney were evaluated as previously described [16]. Kidney sections were stained with anti-mouse IgG to detect immune complexes and color developed with the DAB kit (Vector Laboratories, Burlingame, CA, USA). To quantify the amount of immune complex (IC) staining in glomeruli, the color density of 20 immune complex stained and unstained glomeruli was measured and averaged. A ratio of the two densities was calculated, and a higher number indicates more IC staining.

2.5 Serological analysis

Serum was collected from mice at monthly intervals beginning at 2 months of age. Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure serum antibody levels. Anti-dsDNA IgG ELISAs were done using Immulon 2 HB plates (Thermo Fisher Scientific, Waltham, MA, USA) coated with excess calf thymus dsDNA (Rockland Immunochemicals Inc, Limerick, PA, USA). Diluted serum samples were incubated on the plate, followed by anti-mouse IgG HRP conjugate, then TMB substrate, and Stop Solution (Alpha Diagnostic International, San Antonio, TX, USA) was added and plates read at 450 nm. Plates were washed with 0.05% Tween-20 in PBS. Quantitative measurements of autoantibody concentrations for experimental samples were obtained using a standard curve generated from serial dilution of a positive control that consisted of serum pooled from female (NZB × NZW)F1 mice with heavy albuminuria. Autoantibody data is expressed as arbitrary Units.

Total IgM, IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ ELISAs were done using Immulon 2 HB plates (Thermo Fisher Scientific) coated with 5 ng/ml capture antibody (Southern Biotech, Birmingham, AL, USA) and incubated overnight at 4°C. Briefly, wells were incubated in blocking solution, incubated with samples and standards, then incubated with isotype specific alkaline phosphatase-labeled antibody, after which, 1 mg/ml PNPP solution was applied for 20 minutes (Southern Biotech). Plates were read on a microplate reader at 415

nm. The concentrations of IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ were added together to calculate total IgG.

Anti-dsDNA Ig_{2a} and Ig_{2b} ELISAs were performed by applying standards and samples to dsDNA coated Immulon 2 HB plates (Thermo Fisher Scientific). Isotype specific alkaline phosphatase-labeled antibodies, and PNPP solution (Southern Biotech) were added and plates read as for isotype ELISAs.

2.6 Flow cytometry

To assess lymphocytes by flow cytometry, single cell suspensions were prepared and incubated with primary antibody for 30 minutes on ice. After staining for surface proteins, cells were incubated with Propidium Iodide (BD Biosciences, San Jose, CA, USA) for 10 minutes as a live/dead stain. After staining, cells were fixed with 0.6% formalin. The antibodies used were CD4-PE, CD5-PE, CD19-FITC, CD69-FITC, CD86-PE, B220-APC, CD93-BB515, CD279-APC, CXCR5-PECy7 (all BD Biosciences), IgM-FITC (Southern Biotech), IgD-APC-Cy7 (BioLegend, San Diego, CA, USA), CD21-eFlour450, and CD23-PE-Cy7 (eBioscience Inc., San Diego, CA, USA). Apoptosis was analyzed with Telford reagent. Flow cytometry was performed with a BD LSRII Flow Cytometer and analyzed with FACSDiva software (BD Biosciences, v.8.0).

2.7 Statistical Analysis

Statistical analysis was done using SPSS software (IBM, v. 22). P 0.05 was considered significant. Kaplan Meyer survival curves with log rank tests were used to evaluate survival, and T tests or ANOVA were used to analyze QPCR, ELISA, histology, and flow cytometry data.

3. Results

3.1 On the (NZB x NZW)F1 Genetic Background, the CD19-Cre Knockin Allele Causes Only Moderately Efficient Deletion of *ERα* and Increases B cell Apoptosis

To assess the impact of deleting *ERα* specifically in B cells in lupus-prone (NZB x NZW)F1 mice, NZB.*CD19^{Cre/+}; ERα^{+/-}* females were crossed with NZW.*ERα^{fl/fl}* males to produce (NZB x NZW)F1 *CD19^{Cre/+}; ERα^{fl/-}*, *CD19^{Cre/+}; ERα^{fl/+}*, *CD19^{+/+}; ERα^{fl/+}*, and *CD19^{+/+}; ERα^{fl/-}* mice. In the *CD19^{Cre/+}; ERα^{fl/-}* mice, cre-mediated deletion of the floxed allele of *ERα* in CD19⁺ B cells should result in B cell specific *ERα* deficiency. These *CD19^{Cre/+}; ERα^{fl/-}* mice remain heterozygous for *ERα* in all other cell lineages. In this regard, it is important to note that we have previously demonstrated that heterozygosity for *ERα* does not impact the development of autoantibodies or lupus in (NZB x NZW)F1 mice [16].

On the 129 genetic background, the *CD19-Cre* knockin allele has a deletion efficiency of 75–80% in bone marrow pre-B cells and 90–95% in splenic B cells [28]. To assess the efficiency of *CD19-Cre* mediated deletion of *ERα* on the (NZB x NZW)F1 genetic background, CD19⁺ cells from bone marrow and spleen were isolated from 2–6 month old mice, and DNA from these cells was analyzed for deletion efficiency. The efficiency of deletion was not different in mice at the various ages evaluated, suggesting that the relative

abundance of *ERa* deficient B cells did not change significantly over time (data not shown). Likewise, no sex-related differences in deletion efficiency were observed (data not shown). As expected, no deletion was detected in samples from *CD19^{+/+}; ERa^{fl/+}* or *CD19^{+/+}; ERa^{fl/-}* mice. However, to our surprise we found that in *CD19^{Cre/+}; ERa^{fl/+}* mice there was an average deletion efficiency of only 27% in *CD19⁺* bone marrow cells (Figure 1a). This poor deletion efficiency was not significantly different from that observed in *CD19^{Cre/+}; ERa^{fl/-}* mice, which had an average deletion efficiency of just 18%. *CD19⁺* spleen cells from *CD19^{Cre/+}; ERa^{fl/+}* mice had an average deletion efficiency of 53%, whereas those from *CD19^{Cre/+}; ERa^{fl/-}* mice had an average deletion efficiency of 54%. Our results affirm previous findings which showed that the efficiency of *CD19-Cre* mediated gene deletion is higher in splenic B cells than bone marrow B cells. However, deletion efficiency on the (NZB x NZW)F1 genetic background is significantly lower than that which has been previously reported on other strain backgrounds. Because there was no significant difference in the efficiency of cre-mediated deletion in *CD19^{Cre/+}; ERa^{fl/+}* and *CD19^{Cre/+}; ERa^{fl/-}* mice, the low to moderate efficiency of deletion of the *ERa^{fl}* allele in *CD19^{Cre/+}; ERa^{fl/-}* mice cannot be due to the resulting *ERa^{-/-}* B cells being at a competitive disadvantage. Rather, we posit that the lupus-prone genetic background of (NZB x NZW)F1 mice itself is the reason that only a moderate level of *ERa* deletion was observed.

Cre recombinase can cleave at loxP-like sites throughout the mammalian genome, causing double strand DNA (dsDNA) breaks [29]. dsDNA breaks are not effectively repaired in lupus patients [30, 31]. These unrepaired dsDNA breaks lead to cell death. This means that cells that express low levels of cre recombinase may have a survival advantage, and this could contribute to the decreased efficiency of cre-mediated deletion observed in (NZB x NZW)F1 mice. Our lab has previously observed inefficient *Lck-Cre* mediated deletion of *ERa* in T cells in (NZB x NZW)F1 mice, as well as an increase in T cell apoptosis caused by *Lck-Cre* [26]. To determine if the *CD19-Cre* allele was associated with increased B cell apoptosis, we examined apoptosis in young female (NZB x NZW)F1 *CD19^{+/+}* and *CD19^{Cre/+}* mice. We found that mice carrying the *CD19-Cre* knockin allele had significantly more apoptotic *CD19⁺* splenocytes than *CD19^{+/+}* mice (Figure 1b; $p=0.003$). However, in the bone marrow, no difference was observed in the frequency of apoptotic *CD19⁺* cells in (NZB x NZW)F1 *CD19^{+/+}* and *CD19^{Cre/+}* mice.

Thus, on the (NZB x NZW)F1 genetic background, deletion of *ERa* in B cells was only moderately efficient, resulting in a B cell population in *CD19^{Cre/+}; ERa^{fl/-}* mice which was composed of a mixture of *ERa^{+/-}* and *ERa^{-/-}* cells. Because of the residual *ERa* in the B cell compartment of *CD19^{Cre/+}; ERa^{fl/-}* mice, we anticipated that these mice might show a more modest attenuation of lupus than that seen in our previous study with the global *ERa* knockout, in which all B cells were *ERa* deficient [16].

3.2 The *CD19-Cre* Knockin Allele Causes Reduced Expression of *CD19*

The *CD19-Cre* knockin allele by itself has the potential to impact the development of lupus in (NZB x NZW)F1 mice. Mice that carry the *CD19-Cre* knockin allele have significantly lower levels of *CD19* expression on B cells compared to *CD19^{+/+}* littermates (representative image shown in Figure 1c). In the bone marrow of *CD19-Cre* mice, surface *CD19*

expression was ~65% of that in *CD19^{+/+}* mice, and in the spleen of *CD19-Cre* mice, surface CD19 expression was ~53% of that in *CD19^{+/+}* mice (Figure 1d; $p=6.0 \times 10^{-9}$, $p=3.3 \times 10^{-13}$). This decrease in CD19 on the cell membrane could impact the strength of BCR signaling and inhibit the negative selection of autoreactive B cells. The *CD19-Cre* knockin allele could also affect lupus through toxic off-target effects of cre recombinase expression. Debris from apoptotic cells is a source of autoantigen and accelerates the onset of lupus [32, 33]. Because of its potential to impact lupus, the effects of the *CD19-Cre* knockin allele alone were examined on disease development in (NZB x NZW)F1 mice.

3.3 The *CD19-Cre* Knockin Allele Accelerates Mortality on the (NZB x NZW)F1 Genetic Background

Comparison of *CD19^{+/+}; ERa^{fl/+}* and *CD19^{Cre/+}; ERa^{fl/+}* (NZB x NZW)F1 mice revealed that the *CD19-Cre* knockin allele itself significantly accelerated mortality in female and male mice. Female *CD19^{+/+}; ERa^{fl/+}* mice had a median survival time of 211 days, whereas *CD19^{Cre/+}; ERa^{fl/+}* females had a median survival time of 171 days (Figure 2a; $p=0.0003$). The 211 day median lifespan of *CD19^{+/+}; ERa^{fl/+}* (NZB x NZW)F1 females was similar to the 238 day median survival we have observed previously for *ERa^{+/+}* (NZB x NZW)F1 females [16]. *CD19-Cre* also accelerated mortality in (NZB x NZW)F1 males. Male *CD19^{+/+}; ERa^{fl/+}* mice had a median survival time of 301 days, whereas *CD19^{Cre/+}; ERa^{fl/+}* males had a median survival time of 257 days (Figure 2b; $p=0.0002$). The 301 day median survival of *CD19^{+/+}; ERa^{fl/+}* (NZB x NZW)F1 males is similar to the 321 day median survival we observed previously in *ERa^{+/+}* (NZB x NZW)F1 males [16]. Although we are unable to determine if the accelerated mortality in *CD19-Cre* (NZB x NZW)F1 mice is an effect of cre recombinase or reduced *CD19* expression, it is clear that the *CD19-Cre* knockin allele significantly accelerated mortality in (NZB x NZW)F1 mice. Therefore, the development of lupus in mice with B cell specific deletion of *ERa* must be compared to control mice also carrying the *CD19-Cre* knockin allele.

3.4 B cell Specific Deletion of *ERa* Attenuates Mortality in (NZB x NZW)F1 Mice

Our lab has previously shown that global deletion of *ERa* attenuates the development of lupus and extends survival in both female and male (NZB x NZW)F1 mice [16]. To determine the effect of B cell specific deletion of *ERa* (albeit incomplete deletion) on survival in lupus-prone mice, *CD19^{Cre/+}; ERa^{fl/+}* and *CD19^{Cre/+}; ERa^{fl/-}* (NZB x NZW)F1 mice were monitored for up to one year. Female *CD19^{Cre/+}; ERa^{fl/+}* control mice had a median survival time of 171 days, which was significantly shorter than that observed in *CD19^{Cre/+}; ERa^{fl/-}* mice, in which the median survival time was 239 days (Figure 2c; $p=0.001$). Although this difference in lifespan was statistically significant, the 68 day increase in median survival observed in female mice with *ERa* deletion in B cells was certainly less dramatic than the > 200 day difference in survival observed between *ERa^{-/-}* and *ERa^{+/+}* female mice in our previous study using the global *ERa* knockout [16]. This moderate extension of median lifespan in the *CD19^{Cre/+}; ERa^{fl/-}* mice was likely due, at least in part, to the fact that a significant proportion of B cells in *CD19^{Cre/+}; ERa^{fl/-}* mice retain *ERa* expression. It is also possible that *ERa* signaling in other cell types continued to promote lupus in these mice.

Male mice with B cell specific *ERa* deletion also experienced significantly delayed mortality. Male *CD19^{Cre/+}; ERa^{fl/+}* control mice had a median lifespan of 257 days, which is significantly shorter than that of *CD19^{Cre/+}; ERa^{fl/-}* males which had a median lifespan of 329 days (Figure 2d; $p=0.050$). The 72 day difference in the median survival of male mice in this study was similar to the 85 day difference in median survival observed between *ERa^{+/-}* and *ERa^{-/-}* male (NZB x NZW)F1 mice in our previous study using the global *ERa* knockout [16]. Thus, deletion of *ERa* from a moderate percentage of B cells results in attenuated mortality in male as well as female (NZB x NZW)F1 mice.

The primary cause of mortality in (NZB x NZW)F1 mice is severe glomerulonephritis. To assess the impact of *CD19-Cre* and B cell specific deletion of *ERa* on the development of glomerulonephritis, kidneys were collected from mice at the time of sacrifice, and Periodic Acid Schiff stained sections were analyzed to determine the extent of glomerular damage. (NZB x NZW)F1 mice with the *CD19-Cre* knockin allele developed glomerulonephritis that was histologically indistinguishable from *CD19^{+/+}* mice (data not shown). This indicates that mice with the *CD19-Cre* knockin allele died from lupus-related causes, not because of a separate *CD19-Cre* related effect. In kidneys of almost all female and male *CD19^{Cre/+}; ERa^{fl/+}* and *CD19^{Cre/+}; ERa^{fl/-}* mice, 50% of glomeruli showed evidence of wire loop lesions, matrix deposition, and/or hypercellularity, which is indicative of severe glomerulonephritis (Figure 3a). Thus, although deletion of *ERa* in approximately half of the B cells in *CD19^{Cre/+}; ERa^{fl/-}* mice was sufficient to extend the lifespan of these mice, the ultimate development of severe nephritis was not prevented (Figure 3b–e). Nevertheless, *CD19^{Cre/+}; ERa^{fl/-}* mice did reach this stage of disease at a more advanced age than their *CD19^{Cre/+}; ERa^{fl/+}* counterparts. The impact of B cell specific deletion of *ERa* on renal immune complex deposition was also evaluated. Interestingly, we found that the abundance of glomerular immune complexes in *CD19^{Cre/+}; ERa^{fl/-}* female mice was less than that in *CD19^{Cre/+}; ERa^{fl/+}* female mice, but this difference fell short of statistical significance (Figure 3f, $p=0.12$). B cell specific deletion of *ERa* had no impact on renal immune complex deposition in males. Therefore, although mice with B cell specific deletion of *ERa* survive significantly longer than control mice, both groups of mice ultimately developed immune complex mediated glomerulonephritis.

3.5 Mice with B cell Specific Deletion of *ERa* Produce Fewer Pathogenic Autoantibodies

The production of high levels of anti-dsDNA autoantibodies is highly specific to lupus [34]. In lupus patients, dsDNA autoantibodies correlate with disease activity and disease-associated damage [35, 36]. In (NZB x NZW)F1 mice, the levels of anti-dsDNA IgG antibodies in the serum increase significantly as mice age and lupus progresses [37]. The production of pathogenic autoantibodies is promoted by *ERa* [16, 38]. To determine the impact of B cell specific deletion of *ERa* on autoantibody production, serum anti-dsDNA IgG levels were measured by ELISA. Serum levels of anti-dsDNA IgG were significantly higher in control female *CD19^{Cre/+}; ERa^{fl/+}* mice at 5 months of age compared to female *CD19^{Cre/+}; ERa^{fl/-}* mice (Figure 4a; $p=0.03$). Similarly, male *CD19^{Cre/+}; ERa^{fl/+}* control mice had significantly higher levels of anti-dsDNA IgG antibodies compared to *CD19^{Cre/+}; ERa^{fl/-}* males at 7 months of age (Figure 4b; $p=0.02$). The lower levels of pathogenic autoantibodies in mice with B cell specific deletion of *ERa* is consistent with the attenuated

development of lupus and extended lifespan in *CD19^{Cre/+}; ERα^{fl/-}* mice. Furthermore, these observations are consistent with the hypothesis that ERα promotes lupus by stimulating the development of pathogenic autoantibodies in a B cell-intrinsic manner.

Different isotypes of anti-dsDNA IgG antibodies have different pathogenic potentials; dsDNA-reactive IgG_{2a} antibodies are more likely to bind to laminin and collagen, two proteins found abundantly in the kidney, resulting in the *in situ* formation of immune complexes in the kidney and glomerulonephritis [39]. Both IgG_{2a} and IgG_{2b} autoantibodies are particularly pathogenic because they activate the immune system through both complement and Fcγ receptors [1]. Additionally, global knockout of *ERα* in (NZB x NZW)F1 females caused a significant decrease in anti-dsDNA IgG_{2a} and anti-dsDNA IgG_{2b} [16]. To determine if these subclasses of anti-dsDNA IgG autoantibodies were affected by B cell-intrinsic actions of *ERα*, serum levels of anti-dsDNA IgG_{2a} and anti-dsDNA IgG_{2b} were measured by ELISA at 4 months of age in females and 7 months of age in males. Compared to age and sex matched *CD19^{Cre/+}; ERα^{fl/+}* controls, the levels of anti-dsDNA IgG_{2a} were significantly lower in both female and male *CD19^{Cre/+}; ERα^{fl/-}* mice (Figure 4c; p=0.04, p=0.02). Additionally, compared to *CD19^{Cre/+}; ERα^{fl/+}* controls, male *CD19^{Cre/+}; ERα^{fl/-}* mice had lower serum levels of anti-dsDNA IgG_{2b} antibodies (Figure 4d; p=0.002). However, B cell specific *ERα* deficiency had no impact on anti-dsDNA IgG_{2b} levels in female mice (Figure 4d).

To evaluate the effect of B cell specific deletion of *ERα* on total antibody production, and to determine if the significant reduction in anti-dsDNA IgG autoantibodies in mice with B cell specific deletion of *ERα* was due to global changes in antibody production, we measured the total serum IgM, IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ levels by ELISA. Female *CD19^{Cre/+}; ERα^{fl/+}* mice produced significantly more IgG₃ than female *CD19^{Cre/+}; ERα^{fl/-}* mice (Figure 5a; p=0.005). No differences were observed in the level of IgM, total IgG, or any other IgG isotype in female *CD19^{Cre/+}; ERα^{fl/+}* and *CD19^{Cre/+}; ERα^{fl/-}* mice (Figure 5a). In male mice, there were no genotype dependent differences in the production of IgM, total IgG, or any IgG isotype (Figure 5b). Therefore, the lower levels of anti-dsDNA IgG autoantibodies produced by mice with B cell specific deletion of *ERα* were not due to overall suppression of antibody production, but were specific to anti-dsDNA autoantibodies. Thus, the attenuation of lupus in mice with B cell specific deletion of *ERα* is likely due to the reduction in anti-dsDNA IgG autoantibodies in these mice and not the consequence of systemic immunosuppression.

3.6 B cell Specific Deletion of *ERα* Leads to Decreased B cell Activation

Estrogens can potentially affect the development of B cells in the bone marrow. Specifically, it has been shown that E2 causes a reduction in the number of developing bone marrow B cells on a mixed C57BL/6/129 genetic background [40, 41]. There is some discrepancy in the literature regarding the impact of *ERα* knockout on B cell populations in the bone marrow; however, there is consensus that the effects of E2 on B cell populations are mainly mediated by signaling through ERα [40, 41]. It is not known if the effects of estrogens on B cell development in the bone marrow are B cell-intrinsic.

To evaluate the B cell-intrinsic effects of ER α on B cell populations in the bone marrow, cells from 3 month old pre-autoimmune (NZB x NZW)F1 mice were analyzed by flow cytometry. In female but not male mice, the *CD19-Cre* knockin allele was associated with a decrease in the percentage of pre-B cells and an increase in the percentage of immature B cells (Table 2). However, there were no changes in developing B cells in the bone marrow or spleen that could be attributed to *ERa* deletion in B cells in either female or male mice. These results indicate that in pre-autoimmune (NZB x NZW)F1 mice, deletion of *ERa* in B cells does not impact the relative abundance of bone marrow B cell populations.

The effect of global *ERa* knockout on B cell populations in the spleen has not previously been reported. We evaluated follicular and marginal zone B cell populations in 3 month old *ERa*^{-/-} and *ERa*^{+/+} (NZB x NZW)F1s to provide a comparison for mice with B cell specific *ERa* deletion. The follicular B cell population was not affected by *ERa* genotype (Table 3). However, the population of marginal zone B cells was significantly increased in *ERa*^{-/-} female mice (Table 3; p=0.00004). This was somewhat unexpected because the marginal zone B cell population is enriched for autoreactive B cells compared to follicular zone B cells, and *ERa*^{-/-} female mice produce less autoantibodies than *ERa*^{+/+} mice [16, 42]. However, the marginal zone B cell population also increases significantly in high-estrogen conditions or upon treatment with an ER α specific agonist [18, 20. Female *ERa*^{-/-} mice have significantly elevated serum E2 [43], and thus this may be the reason for the increase in marginal zone B cells in these mice.

The same analysis of splenic immune cell populations was performed in 3 month old (NZB x NZW)F1 *CD19-Cre* mice. The relative abundance of follicular and marginal zone B cells were similar in *ERa*^{+/+} and *CD19*^{+/+}; *ERa*^{fl/+} mice indicating that the floxed allele of *ERa* had no impact on these populations. *ERa* deletion in B cells did not have a significant impact on the follicular or marginal zone B cell populations (Table 4). However, the *CD19-Cre* knockin allele did significantly impact these populations. The *CD19-Cre* knockin allele caused a significant increase in the proportion of follicular B cells and a significant decrease in the proportion of marginal zone B cells (Table 4; females p=0.002, 0.02, males p=0.08, p=0.0009, respectively). Several other lymphocyte populations were examined in the spleen; no significant differences were observed in transitional B cell or T follicular helper cell populations (Table 4). These results suggest that *CD19-Cre* reduces the relative abundance of marginal zone B cells in the spleen, but that deletion of *ERa* in B cells does not affect the relative abundance of splenic B cell populations. Thus, the attenuated lupus development in *CD19*^{Cre/+}; *ERa*^{fl/-} mice is not the result of a shift in the relative abundance of splenic B cell subsets.

Our previous studies suggest that ER α promotes lupus by enhancing immune cell activation [17]. Therefore, the relative abundance of activated B and T cells in the spleen was also examined in these same groups of young (NZB x NZW)F1 mice. Female *CD19*^{Cre/+}; *ERa*^{fl/+} mice had significantly more activated B cells than *CD19*^{Cre/+}; *ERa*^{fl/-} mice (Table 5; p=0.006). Interestingly, no such difference was observed in male mice. B cell specific deletion of *ERa* had no impact on the proportion of T cells that were activated in mice of either sex. This result suggests that ER α promotes B cell activation in female lupus-prone mice in a B cell-intrinsic manner. Furthermore, the enhanced B cell activation in young, pre-

autoimmune *CD19^{Cre/+}; ERα^{fl/+}* female mice suggests that the ability of ERα to promote B cell activation may underlie the ability of ERα to promote lupus.

4. Discussion

To study the role of B cell-intrinsic actions of ERα on the development of lupus, we created (NZB x NZW)F1 mice with *ERα* deletion specifically in B cells. This was accomplished using an allele of *ERα* in which one exon is flanked by loxP sites in conjunction and the *CD19-Cre* knockin allele, which results in expression of cre recombinase in CD19⁺ B cells and thus B cell specific deletion of *ERα*. The cre-loxP system is widely used in mammalian systems to cause targeted deletion of DNA segments from the genome. Although this system is potentially very useful, experiments using the cre-loxP system can be complicated by the off target effects of cre recombinase [29, 44, 45].

In the present study, we found that the *CD19-Cre* knockin allele by itself significantly accelerated lupus development in (NZB x NZW)F1 mice. Cre recombinase can cleave endogenous pseudo-loxP sites that are found throughout the mouse genome, causing severe DNA damage [46, 47]. The accelerated development of lupus in *CD19-Cre* (NZB x NZW)F1 mice may be due, at least in part, to cre-induced DNA damage, leading to increased B cell apoptosis and increased nuclear antigen load. It is known that lupus-prone (NZB x NZW)F1 mice have impaired clearance of apoptotic debris [48, 49]. Thus, in young (NZB x NZW)F1 mice, enhanced B cell apoptosis would likely lead to the accumulation of apoptotic debris and an increase in the nuclear antigen load, which would, in turn, promote the earlier development of autoantibodies and thereby accelerate the development of lupus. Indeed, a recent study from our lab showed that the *Lck-Cre* allele significantly accelerated lupus in (NZB x NZW)F1 mice by causing increased apoptosis [26]. In the present study, we observed a small but significant increase in apoptotic CD19⁺ splenocytes in *CD19-Cre* mice, and this increase could be sufficient to accelerate lupus. However, the *CD19-Cre* knockin allele not only causes cre recombinase expression in B cells, but because it is a knockin allele, it also interrupts the coding region of *CD19*, resulting in a null allele of *CD19*. To our knowledge, the effects of *CD19* heterozygosity on B cell development, B cell function, and lupus pathogenesis have not been reported. It is known that homozygosity for the *CD19* knockout does not impact the development of conventional B2 cells; however, CD19 deficiency does decrease the B cell response to antigens requiring T cell help [50]. In (NZB x NZW)F1 mice, homozygosity for the *CD19* knockout allele does accelerate the development of lupus [51]. Thus, in our (NZB x NZW)F1 *CD19^{Cre/+}* mice, the accelerated development of lupus could be attributable to the decreased expression of *CD19* and/or the expression of cre recombinase.

We also observed that the *CD19-Cre* allele modulated the relative abundance of B cell subsets in (NZB x NZW)F1 mice. In females, the *CD19-Cre* knockin allele lead to a decrease in pre-B cells and an increase in immature B cells in the bone marrow. In the spleen, we found that the *CD19-Cre* knockin allele increased the proportion of follicular B cells and decreased the proportion of marginal zone B cells in the spleen of both female and male (NZB x NZW)F1 mice. Because of these immunologic effects and the fact that the *CD19-Cre* allele accelerated the lupus phenotype in (NZB x NZW)F1 mice, it was

imperative to use *CD19^{Cre/+}; ERa^{fl/+}* mice as controls for the analysis of the *CD19^{Cre/+}; ERa^{fl/-}* mice that were generated to examine the impact of B cell specific deletion of *ERa*.

On the 129 genetic background, the *CD19-Cre* allele results in gene deletion in ~75–80% of bone marrow B cells and 90–95% of spleen B cells [28]. However, in our study, the observed efficiency of *CD19-Cre* mediated deletion on the (NZB x NZW)F1 genetic background was much lower. Only ~20% of *CD19⁺* bone marrow cells successfully deleted *ERa* whereas ~50% of *CD19⁺* B cells deleted *ERa*. These results indicate that the B cells in *CD19^{Cre/+}; ERa^{fl/-}* mice were a mixed population of *ERa^{+/-}* and *ERa^{-/-}* cells. Therefore, the effects that we have observed in these studies are the result of loss of *ERa* signaling in only a portion of the B cell population.

Despite the fact that only ~50% of B cells successfully deleted *ERa* in *CD19^{Cre/+}; ERa^{fl/-}* (NZB x NZW)F1 mice, this reduction in *ERa* was sufficient to significantly attenuate lupus in these mice. Female and male *CD19^{Cre/+}; ERa^{fl/-}* mice survived significantly longer than their *CD19^{Cre/+}; ERa^{fl/+}* counterparts. In terms of survival, the impact of deleting *ERa* in a fraction of B cells in male (NZB x NZW)F1 mice was roughly equivalent to the impact of global deletion of *ERa* deletion seen in our previous study [16]. By contrast, in female (NZB x NZW)F1 mice, the increased survival in mice with *ERa* deletion in a fraction of B cells was not as dramatic as the increase in survival we have seen previously in mice with global deletion of *ERa* [16]. There are several possible explanations for the observation that the impact of B cell specific *ERa* deletion on survival in (NZB x NZW)F1 female mice was less than that seen with global *ERa* deletion. One of the major factors likely to contribute to this difference is the fact that in *CD19^{Cre/+}; ERa^{fl/-}* mice, many, perhaps often the majority of B cells do not delete *ERa*; in these mice the efficiency of B cell specific deletion of *ERa* barely exceeds 50%. Thus, we postulate that the delay in the onset of lupus in *CD19^{Cre/+}; ERa^{fl/-}* female mice would have been greater if there had been deletion of *ERa* in a larger proportion of B cells. We also note that in female mice with a global *ERa* knockout, the hypothalamic-pituitary-gonadal axis is disrupted resulting in significant alterations in the serum levels of multiple hormones, including testosterone and prolactin, which could attenuate the development of lupus in *ERa* deficient (NZB x NZW)F1 females independently of the effects of *ERa*. Thus, it is possible that the more modest impact of B cell specific deletion of *ERa* on lupus development in (NZB x NZW)F1 females is a reflection of the fact that we have avoided disruption of the hypothalamic-pituitary-gonadal axis by using the cre-loxP system. Finally, in addition to B cells, other immune cells such as dendritic cells and T cells, which also express *ERa*, are also known to contribute to lupus. Thus, our observation that B cell specific deletion of *ERa* attenuates lupus in females to a lesser degree than global deletion of *ERa* may reflect the fact that *ERa* signaling in other immune cells also contributes significantly to lupus in females.

Higher levels of anti-dsDNA IgG autoantibodies are associated with increased disease severity in lupus-prone mice. In both female and male (NZB x NZW)F1 mice with B cell specific deletion of *ERa*, we observed significantly lower levels of pathogenic anti-dsDNA IgG antibodies as compared to sex and age matched control mice. Some isotypes of anti-dsDNA antibodies are particularly pathogenic, especially the IgG_{2a} and IgG_{2b} isotypes which cause immune activation through multiple pathways [1]. *CD19^{Cre/+}; ERa^{fl/+}* mice of

both sexes had higher levels of anti-dsDNA IgG_{2a} autoantibodies than sex and age matched *CD19^{Cre/+}; ERα^{fl/-}* mice. *CD19^{Cre/+}; ERα^{fl/+}* male mice also had higher levels of anti-dsDNA IgG_{2b} autoantibodies. By contrast, total serum levels of IgG and IgM antibodies were not different between *CD19^{Cre/+}; ERα^{fl/-}* and *CD19^{Cre/+}; ERα^{fl/+}* mice, clearly indicating that B cell specific deletion of *ERα* has no impact on overall IgG levels. Altogether, these data support the idea that ERα signaling specifically promotes pathogenic autoantibody production in a B cell-intrinsic manner.

Global deletion of *ERα* has not been reported to affect the relative abundance of B cell populations in the bone marrow of non-autoimmune mice [40]. However, there are no published reports in which the impact of global *ERα* deficiency on splenic B cell populations has been examined. Interestingly, in female (NZB x NZW)F1 mice homozygous for a global *ERα* knockout, we found that the marginal zone B cell population was significantly increased. By contrast, B cell specific deletion of *ERα* has no effect on the relative abundance of various subsets of splenic B cells. One possible explanation for this difference could be the relatively inefficient deletion of *ERα* in splenic B cells in (NZB x NZW)F1 mice. Alternatively, it is possible that the expansion of the marginal zone B cell compartment in female (NZB x NZW)F1 mice homozygous for the global *ERα* knockout allele could be the result of disruption of the hypothalamic-pituitary-gonadal axis, and the resulting high levels of serum estradiol in these mice. Indeed, an expansion of the marginal zone B cell compartment is also seen in transgenic mice expressing a DNA reactive heavy chain following continuous treatment with high levels of E2 [20, 42].

The deletion of *ERα* in B cells in (NZB x NZW)F1 mice did not affect T cell activation in the spleen. These observations suggest that *ERα* deficiency in B cells does not impair the ability of B cells to present antigen to T cells and thereby activate T cells. By contrast, *ERα* deletion in B cells did result in a significant decrease in the proportion of activated splenic B cells in female mice specifically. These observations are consistent with our previous finding that *ERα* deficiency attenuates B cell activation in female, but not male, mice carrying the lupus susceptibility locus *Sle1* [17]. However, the data presented here reveal that ERα acts in a B cell-intrinsic manner to promote B cell activation. Furthermore, this effect is seen in young, pre-autoimmune mice, which indicates that the ability of *ERα* to enhance B cell activation likely contributes to its ability to promote autoantibody production and the pathogenesis of lupus in females. Interestingly, *ERα* deficiency in B cells did not impact B cell activation in age matched male mice, suggesting that the enhanced B cell activation phenotype seen in female lupus-prone mice is dependent upon B cell-intrinsic actions of ERα. However, it is possible that ERα signaling might enhance B cell activation in older, pre-autoimmune male mice.

In conclusion, these studies show that loss of ERα signaling in a moderate proportion of B cells significantly attenuates lupus in (NZB x NZW)F1 mice. *ERα* deletion in B cells significantly decreased the production of autoantibodies, and prolonged survival in both female and male mice. These data indicate that ERα signaling in B cells specifically promotes lupus in both females and males. Of particular note, we observed a decrease in the proportion of activated B cells in young, pre-autoimmune female mice with B cell specific deletion of *ERα*, whereas no such effect was seen in males. These data indicate that ERα

acts in a B cell-intrinsic manner to promote B cell activation specifically in female mice. The ability of ER α to stimulate B cell activation in females likely contributes to the enhanced development of autoantibodies and more robust lupus observed in female (NZB x NZW)F1 mice compared to male mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

dsDNA	double stranded DNA
ERα	estrogen receptor alpha
E2	estradiol
BCR	B cell receptor
SSLP	simple sequence length polymorphism
IC	immune complex
ELISA	enzyme-linked immunosorbent assay

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Figure 1. *CD19-Cre* increased apoptosis and decreased *CD19* expression in (NZB x NZW)F1 mice
 The efficiency of *ERα* deletion in B cells was measured in (NZB x NZW)F1 mice. (a) There was moderate *ERα* deletion in *CD19⁺* bone marrow and spleen cells in both *CD19^{Cre/+}; ERα^{fl/+}* and *CD19^{Cre/+}; ERα^{fl/-}* mice (bone marrow n= 8–15; spleen n= 8–16). (b) The frequency of apoptotic cells in *CD19⁺* splenocytes was increased in female mice carrying the *CD19-Cre* knockin allele (p=0.003, bone marrow and spleen n= 5–6). (c) The fluorescence intensity of *CD19* staining on B cells was lower in mice carrying the *CD19-Cre* knockin allele. (d) Mean fluorescence intensity of *CD19* staining was consistently and significantly reduced in both bone marrow and spleen B cells of *CD19-Cre* mice (bone marrow p=6.0x10⁻⁹, spleen p=3.3x10⁻¹³; bone marrow and spleen n= 9–13).

Figure 2. Both the *CD19-Cre* knockin allele and B cell specific deletion of *ERα* impacted the survival of (NZB x NZW)F1 mice

Survival was monitored for up to one year. (a) Female and (b) male $CD19^{+/+}; ER\alpha^{fl/+}$ mice had significantly longer median survival compared to $CD19^{Cre/+}; ER\alpha^{fl/+}$ mice (females $p=0.0003$, $n=62-73$; males $p=0.0002$, $n=51-63$). Compared to $CD19^{Cre/+}; ER\alpha^{fl/+}$ control mice, $CD19^{Cre/+}; ER\alpha^{fl/-}$ (c) female and (d) male mice had significantly longer median survival (females $p=0.001$, $n=22-73$; males $p=0.050$, $n=25-63$).

Figure 3. Deletion of $ER\alpha$ from B cells did not prevent the development of glomerulonephritis
 (a) The majority of female and male $CD19^{Cre/+}; ER\alpha^{fl/+}$ and $CD19^{Cre/+}; ER\alpha^{fl/-}$ mice developed severe glomerulonephritis by the end of the survival study (females n= 9–17; males n= 7–11). Representative histological sections from (b) female $CD19^{Cre/+}; ER\alpha^{fl/+}$, (c) female $CD19^{Cre/+}; ER\alpha^{fl/-}$, (d) male $CD19^{Cre/+}; ER\alpha^{fl/+}$, and (e) male $CD19^{Cre/+}; ER\alpha^{fl/-}$ mice showed abnormalities characteristic of glomerulonephritis. (f) Semi-quantitative analysis of immune complex staining showed immune complex deposition occurred equally in $CD19^{Cre/+}; ER\alpha^{fl/+}$ and $CD19^{Cre/+}; ER\alpha^{fl/-}$ mice of both sexes (females p=0.12, n=8–16; males n= 8–12).

Figure 4. *ERα* deletion in B cells reduced levels of pathogenic autoantibodies

(a) Female and (b) male $CD19^{Cre/+}; ER\alpha^{fl/+}$ mice produced significantly more anti-dsDNA IgG than $CD19^{Cre/+}; ER\alpha^{fl/-}$ mice (females $p=0.03$, $n=7-11$; males $p=0.02$, $n=5-10$). (c) More anti-dsDNA IgG_{2a} was produced by both female and male $CD19^{Cre/+}; ER\alpha^{fl/+}$ mice than $CD19^{Cre/+}; ER\alpha^{fl/-}$ mice (females $p=0.04$, $n=7-13$; males $p=0.02$, $n=5-12$). (d) Male $CD19^{Cre/+}; ER\alpha^{fl/+}$ mice produced more anti-dsDNA IgG_{2b} than $CD19^{Cre/+}; ER\alpha^{fl/-}$ males (females $n=7-13$; males $p=0.002$, $n=5-12$).

Figure 5. B cell specific deletion of *ERα* did not impact total IgM or IgG

There was no difference in total IgM, total IgG, or IgG₁, IgG_{2a}, or IgG_{2b} in (a) female and (b) male mice (females n= 7–15; males n=6–13). Female $CD19^{Cre/+}; ER\alpha^{fl/+}$ mice produced more total IgG₃ than $CD19^{Cre/+}; ER\alpha^{fl/-}$ controls (p=0.005).

Table 1

SSLP markers used for the production of NZB.CD19-Cre strain

Marker (location in cM, Mb)		
<i>D1Mit316</i> (2.46, 10.3)	<i>D6Mit123</i> (27.76, 56.9)	<i>D12Mit158</i> (38.14, 83.7)
<i>D1Mit169</i> (9.9, 24.1)	<i>D6Mit209</i> (32.56, 75.5)	<i>D12Mit7</i> (ND, 103)
<i>D1Mit123</i> (17.67, 39.2)	<i>D6Mit328</i> (52.62, 112.7)	<i>D12Nds2</i> (62.22, 115.1)
<i>D1Mit132</i> (39.51, 77.1)	<i>D6Mit14</i> (77.64, 145.6)	<i>D13Mit16</i> (7.26, 20.4)
<i>D1Mit440</i> (44.98, 90.7)	<i>D7Mit21</i> (1.91, 3.3)	<i>D13Mit275</i> (14.5, 37.4)
<i>D1Mit495</i> (55.79, 129.5)	<i>D7Mit267</i> (17.09, 30.3)	<i>D13Mit13</i> (30.06, 56.6)
<i>D1Mit159</i> (69.03, 161.6)	<i>D7Mit82</i> (32.76, 58.8)	<i>D13Mit126</i> (45.05, 85.5)
<i>D1Mit111</i> (76.73, 170.9)	<i>D7Mit248</i> (ND, 63.8)	<i>D13Mit74</i> (56.92, 106.7)
<i>D1Mit426</i> (84.32, 182.3)	<i>D7Mit323</i> (54.45, 108)	<i>D13Mit151</i> (64.72, 116.3)
<i>D1Mit209</i> (96.35, 193.3)	<i>D7Mit98</i> (60.49, 122.1)	<i>D14Mit126</i> (11.94, 22.7)
<i>D2Mit1</i> (2.33, 3.8)	<i>D7Mit358</i> (67.27, 129.9)	<i>D14Mit60</i> (24.6, 47.7)
<i>D2Mit83</i> (19.38, 28.8)	<i>D7Mit101</i> (69.01, 132.8)	<i>D14Mit5</i> (31.49, 60.3)
<i>D2Mit156</i> (31.66, 56.9)	<i>D7Mit332</i> (77.87, 141.2)	<i>D14Mit68</i> (37.61, 72.9)
<i>D2Mit327</i> (40.88, 69.3)	<i>D8Mit155</i> (2.14, 5)	<i>D14Mit106</i> (50.9, 100.6)
<i>D2Mit94</i> (47.93, 80)	<i>D8Mit289</i> (16.47, 29.9)	<i>D14Mit177</i> (60.21, 116.8)
<i>D2Mit395</i> (59.97, 119.4)	<i>D8Mit69</i> (29.7, 59.2)	<i>D15Mit252</i> (8.54, 22.6)
<i>D2Mit411</i> (80.04, 159.4)	<i>D8Mit178</i> (34.43, 73.6)	<i>D15Mit143</i> (19.62, 52)
<i>D2Mit145</i> (86.75, 166.2)	<i>D8Mit211</i> (52, 105.2)	<i>D15Mit67</i> (32.17, 70)
<i>D3Mit203</i> (10.82, 26.8)	<i>D8Mit49</i> (72.38, 126.6)	<i>D15Mit107</i> (39.79, 84.2)
<i>D3Mit51</i> (ND, 77.0)	<i>D9Mit90</i> (17.8, 32.3)	<i>D15Mit161</i> (52.78, 96.8)
<i>D3Mit26</i> (34.97, 79.5)	<i>D9Mit129</i> (24.45, 43.7)	<i>D16Mit131</i> (3.41, 7.3)
<i>D3Mit311</i> (40.14, 92.8)	<i>D9Mit123</i> (40.88, 73.4)	<i>D16Mit60</i> (23.27, 32.7)
<i>D3Mit320</i> (66.75, 143.2)	<i>D9Mit355</i> (51.41, 98.7)	<i>D16Mit139</i> (37.28, 65.7)
<i>D3Mit19</i> (ND, 164.4)	<i>D9Mit55</i> (65.28, 114.7)	<i>D16Mit52</i> (53.73, 92.7)
<i>D4Mit193</i> (13.99, 32.3)	<i>D10Mit213</i> (9.75, 20.1)	<i>D17Mit164</i> (2.11, 3.9)
<i>D4Mit17</i> (33.96, 63)	<i>D10Mit20</i> (34.83, 66.5)	<i>D17Mit51</i> (19.74, 43.6)
<i>D4Mit9</i> (43.34, 94.7)	<i>D10Mit230</i> (45.28, 89.7)	<i>D17Mit10</i> (ND, 51.0)
<i>D4Mit308</i> (57.66, 123.8)	<i>D10Mit233</i> (61.58, 113.8)	<i>D17Mit93</i> (45.2, 74.2)
<i>D4Mit42</i> (82.64, 150.9)	<i>D10Mit297</i> (72.31, 124.5)	<i>D17Mit122</i> (52.25, 83.5)
<i>D5Mit348</i> (11.97, 24.4)	<i>D11Mit71</i> (4.7, 6.8)	<i>D18Mit222</i> (8.08, 14.7)
<i>D5Mit352</i> (18.4, 36)	<i>D11Mit189</i> (27.39, 45.3)	<i>D18Mit177</i> (21.39, 41.1)
<i>D5Mit201</i> (39.55, 75.6)	<i>D11Mit5</i> (40.59, 67)	<i>D18Mit186</i> (45.63, 72.2)
<i>D5Mit314</i> (53.25, 110.1)	<i>D11Mit285</i> (54.64, 89.8)	<i>D18Mit144</i> (57.79, 85.7)
<i>D5Mit97</i> (76.1, 137.5)	<i>D11Mit333</i> (71.83, 108.6)	<i>D19Mit96</i> (15.54, 21.9)
<i>D5Mit143</i> (89.8, 151.8)	<i>D12Mit182</i> (5.52, 10.9)	<i>D19Mit88</i> (32.23, 37.3)
<i>D6Mit138</i> (1.81, 4.5)	<i>D12Mit60</i> (15.54, 35.5)	<i>D19Mit90</i> (35.97, 42.3)
<i>D6Mit116</i> (11.5, 21.1)	<i>D12Mit91</i> (30.06, 72.8)	<i>D19Mit103</i> (48.46, 53.8)

Table 2Bone marrow B cell populations in (NZB x NZW)F1 *CD19-Cre* mice

	CD19^{+/+}; ERα^{fl/+}	CD19^{Cre/+}; ERα^{fl/+}	CD19^{Cre/+}; ERα^{fl/-}
Females			
Pre-B cells (IgM ⁻ IgD ⁻)	53.0 ± 1.5	42.6 ± 2.8 [*]	40.6 ± 1.9 [*]
Immature B cells (IgM ⁺ IgD ⁻)	18.4 ± 1.6	31.9 ± 1.6 [*]	27.7 ± 1.4 [*]
Recirculating B cells (IgM ^{+/+} IgD ⁺)	26.6 ± 1.2	23.2 ± 1.8	29.3 ± 1.2 [†]
Males			
Pre-B cells (IgM ⁻ IgD ⁻)	50.9 ± 5.3	48.9 ± 3.1	50.1 ± 5.7
Immature B cells (IgM ⁺ IgD ⁻)	25.3 ± 2.9	24.4 ± 1.8	24.5 ± 3.0
Recirculating B cells (IgM ^{+/+} IgD ⁺)	21.8 ± 3.2	24.9 ± 2.5	24.0 ± 4.3

Data presented is the % of live B220+ cells. For these experiments, 3 month old mice were used, with n= 5–9 per group.

^{*} Indicates p = 0.05 compared to *CD19^{+/+}; ERα^{fl/+}*

[†] indicates p = 0.05 compared to *CD19^{Cre/+}; ERα^{fl/+}*

Table 3

Splenic B cell populations in (NZB x NZW)F1 mice

	ER α ^{+/+}	ER α ^{-/-}
Females		
Follicular B cells (CD21 ⁺ CD23 ⁺)	55.4 $\pm$ 1.4	54.5 $\pm$ 0.9
Marginal zone B cells (CD21 ⁺ CD23 ⁻)	26.6 $\pm$ 1.1	36.4 $\pm$ 0.9 *
Males		
Follicular B cells (CD21 ⁺ CD23 ⁺)	57.2 $\pm$ 2.0	53.9 $\pm$ 5.3
Marginal zone B cells (CD21 ⁺ CD23 ⁻)	28.6 $\pm$ 2.1	31.5 $\pm$ 2.2

Data present are the % of live B220⁺ CD5⁻ cells. 3 month old mice were used for this experiment, with n= 3–10 per group

* indicates p = 0.05 compared to ER α ^{+/+}

Table 4Splenic lymphocyte populations in (NZB x NZW)F1 *CD19-Cre* mice

	<i>CD19</i> ^{+/+} ; <i>ERα</i> ^{fl/+}	<i>CD19</i> ^{Cre/+} ; <i>ERα</i> ^{fl/+}	<i>CD19</i> ^{Cre/+} ; <i>ERα</i> ^{fl/-}
Females			
Follicular B cells (CD21 ⁺ CD23 ⁺)	59.8 ± 1.2	68.1 ± 1.6 *	66.0 ± 1.0 *
Marginal zone B cells (CD21 ⁺ CD23 ⁻)	25.2 ± 2.0	18.0 ± 1.1 *	18.9 ± 1.6 ^
Transitional B cells (AA4.1 ⁺)	5.6 ± 0.5	6.0 ± 0.5	5.6 ± 0.6
T follicular helper cells (% of live CD4 ⁺ CXCR5 ⁺ PD1 ⁺)	46.0 ± 1.4	49.3 ± 2.4	51.9 ± 3.9
Males			
Follicular B cells (CD21 ⁺ CD23 ⁺)	60.4 ± 1.9	65.0 ± 1.3 ^	67.7 ± 1.6 *
Marginal zone B cells (CD21 ⁺ CD23 ⁻)	25.0 ± 1.4	19.2 ± 0.4 *	20.8 ± 0.3 *
Transitional B cells (AA4.1 ⁺)	4.8 ± 0.3	5.3 ± 0.5	4.2 ± 0.3
T follicular helper cells (% of live CD4 ⁺ CXCR5 ⁺ PD1 ⁺)	42.5 ± 1.3	46.9 ± 1.5	45.8 ± 2.8

B cells-% of live B220⁺ CD5⁻ cells. 3 month old mice were used for this experiment. n= 5-9 per group* indicates p = 0.05 compared to *CD19*^{+/+}; *ERα*^{fl/+}^ indicates p = 0.1 compared to *CD19*^{+/+}; *ERα*^{fl/+}

Table 5B and T cell activation in (NZB x NZW)F1 *CD19-Cre* mice

	<i>CD19^{+/+};ERα^{fl/+}</i>	<i>CD19^{Cre/+};ERα^{fl/+}</i>	<i>CD19^{Cre/+};ERα^{fl/-}</i>
Females			
Activated B cells (% live of B220 ⁺ CD86 ⁺)	3.0 ± 0.6	5.7 ± 0.8 [*]	1.9 ± 0.3 [†]
Activated T cells (% live of CD4 ⁺ CD69 ⁺)	6.2 ± 0.8	9.2 ± 2.3	5.5 ± 0.9
Males			
Activated B cells (% live of B220 ⁺ CD86 ⁺)	3.5 ± 0.5	2.7 ± 0.4	2.3 ± 0.4 [^]
Activated T cells (% live of CD4 ⁺ CD69 ⁺)	5.4 ± 0.8	5.2 ± 0.9	4.7 ± 0.6

3 month old mice were used for this experiment with n= 5–13 per group.

^{*} Indicates p = 0.05 compared to *CD19^{+/+};ERα^{fl/+}*

[^] indicates p = 0.1 compared to *CD19^{+/+};ERα^{fl/+}*

[†] indicates p = 0.05 compared to *CD19^{Cre/+};ERα^{fl/+}*