

# Raloxifene Modulates Estrogen-mediated B Cell Autoreactivity in NZB/W F1 Mice

YU ZHANG, SUBHRAJIT SAHA, GABRIEL ROSENFELD, JUANA GONZALEZ, KIRIL P. PEPELJUGOSKI, and ELENA PEEVA

**ABSTRACT. Objective.** Estrogen has been found to exacerbate disease activity in murine lupus and to induce a lupus-like syndrome in nonspontaneously autoimmune mice. This has led to the consideration that estrogen may be a risk factor for the development of systemic lupus erythematosus (SLE), and selective estrogen receptor modulators (SERM) may serve to ameliorate lupus activity. We evaluated the effects and mechanism of action of the SERM raloxifene in murine lupus.

**Methods.** Effects of raloxifene on the development of lupus in NZB/W F1 mice were evaluated in the presence and absence of estrogen by assessing the serum DNA reactivity, glomerular IgG deposition and kidney damage, B cell maturation and selection, and activation status of marginal zone and follicular B cells.

**Results.** Compared to estradiol-treated mice, mice treated with estradiol and raloxifene had significantly lower serum anti-DNA antibody levels and less kidney damage. These effects of raloxifene were due, at least in part, to antagonism of the influence of estrogen on DNA-reactive B cells. Raloxifene was found to prevent estrogen-mediated suppression of autoreactive B cell elimination at the T1/T2 selection checkpoint, to reduce estrogen-induced CD40 overexpression on follicular B cells, making them less responsive to T cell costimulation, and to ameliorate estrogen-mediated CD22 downregulation on marginal zone B cells, thereby decreasing their responsiveness to B cell antigen receptor-mediated stimuli.

**Conclusion.** Raloxifene suppressed estrogen-mediated effects on the survival, maturation, and activation of autoreactive B cells in NZB/W F1 mice. (J Rheumatol First Release June 15 2010; doi:10.3899/jrheum.090911)

*Key Indexing Terms:*

SYSTEMIC LUPUS ERYTHEMATOSUS

RALOXIFENE

ESTROGEN

B CELLS

NZB/W F1 MICE

Systemic lupus erythematosus (SLE), an autoimmune disease characterized by B cell hyperactivity and the production of pathogenic antinuclear antibodies, manifests itself in clinical presentations including rashes, serositis, psychosis, and glomerulonephritis. Like the majority of autoimmune diseases, SLE affects predominantly women. The main female sex hormone, estrogen, seems to play a crucial role in the pathogenesis of SLE, since the ratio of women to men developing SLE reaches 9:1 during peak childbearing years<sup>1</sup>. The link between estrogen and SLE is also supported by the association of SLE disease activity and specific phases of the menstrual cycle, pregnancy<sup>2</sup>, a high serum

estrogen/androgen ratio<sup>3</sup>, and alterations in estrogen metabolism that lead to increased concentrations of highly potent estrogenic compounds<sup>4</sup>. However, studies assessing the effects of exogenous estrogen in patients with SLE have provided variable results. The prospective Nurses' Health Study reported association between hormone replacement therapy (HRT) and increased risk for development of SLE<sup>5</sup>. Also, a study of the UK based General Practice Research Database found significantly increased risk of discoid lupus and SLE among women treated with HRT for 2 or more years<sup>6</sup>. However, in postmenopausal patients with established diagnosis of SLE, 2 small retrospective studies<sup>7,8</sup> and one prospective study with only 11 patients<sup>9</sup> reported no difference in the flare rate between HRT users and nonusers. By contrast, the multicenter randomized double-blind, placebo-controlled HRT-SELENA trial<sup>10</sup> found a significant increase in mild to moderate flares in SLE patients treated with conjugated estrogen and medroxyprogesterone. The severe flare rate, although numerically higher in the patients receiving HRT, did not reach statistical significance. In that study, the probability of any type of flare by 12 months was significantly higher for the HRT group than for the placebo group<sup>10</sup>.

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The notion that estrogen is implicated in the pathogenesis of lupus has raised the possibility that modulation of estrogenic activity may have a beneficial effect on the disease, but very limited data exist regarding the effects of estrogen modulators in patients with SLE. In a small clinical trial with 11 lupus patients, the selective estrogen receptor modulator (SERM) tamoxifen did not show beneficial effect on lupus disease indices<sup>11</sup>. In addition, a pilot study with the SERM raloxifene (16 patients taking raloxifene and 17 patients on placebo) did not find a difference in lupus activity between the raloxifene and placebo-treated patients with SLE<sup>12</sup>. The results of these 2 studies need to be interpreted with caution; given the small sample sizes, it is likely that both studies were inadequately powered.

Estrogen exerts its biological actions through 2 distinct intracellular estrogen receptors (ER), ER $\alpha$  and ER $\beta$ <sup>13</sup>. ER have discrete expression patterns and can engage an array of transcription cofactors that are functionally dissimilar and differentially expressed in a variety of cells. ER $\alpha$  is required for most of the estrogenic functions. ER $\beta$  activates the same genes as ER $\alpha$ , but it is less efficient. In cells that express both ER, ER $\beta$  functions as an inhibitor of ER $\alpha$  transcriptional activity. ER $\alpha$  contains 2 transactivation domains, AF-1 and AF-2, located within the NH<sub>2</sub> terminus and the ligand-binding terminus, respectively. ER $\alpha$ 's interactions with the transcription apparatus differs from tissue to tissue; in some tissues only AF1 or AF2 is required for transcriptional activity, whereas in others both AF are required<sup>14</sup>.

ER are widely distributed in the immune system. They are expressed in thymic and bone marrow stromal cells, dendritic cells, macrophages, T cells, and B cells. Also, both ER $\alpha$  and ER $\beta$  are expressed in autoimmunity-related B1 and CD4-CD8-B220-positive T lymphocytes in lupus-prone NZB/W F1 and *lpr/lpr* mice, respectively. A recent study in NZB/W F1 mice that utilized ER $\alpha$ -selective and ER $\beta$ -selective agonists indicated that the ER $\alpha$  activation plays an immunostimulatory role in murine lupus, whereas the ER $\beta$  activation has minor immunosuppressive effects<sup>15</sup>. The key role of ER $\alpha$ , but not ER $\beta$ , in the pathogenesis of lupus was further confirmed in the experiments with ER-deficient lupus-prone NZM and MRL/*lpr* mice. ER $\alpha$ -deficient female, but not male, mice had significantly less renal pathology and proteinuria than the intact females. ER $\beta$  deficiency had no effect on lupus activity in either mouse strain or sex<sup>16</sup>.

SERM are compounds that lack the steroid structure of estrogen but have a tertiary configuration that allows them to bind ER. After binding to the ER, each SERM induces a distinct ER ligand conformation that is recognized distinctly by the transcription machinery<sup>17</sup> and results in tissue-specific ER agonist or antagonist activities<sup>18</sup>. Currently, there are 2 classes of clinically available SERM: triphenylethylenes such as tamoxifen, and benzothiophenes such as raloxifene. Both tamoxifen and raloxifene have estrogenic activ-

ities in the bone and lipid metabolism<sup>19,20,21</sup> and anti-estrogenic effects in the breast<sup>22,23</sup>. However, they differ in their influence on the uterus; tamoxifen has estrogenic activity on the uterus while raloxifene lacks this effect<sup>23,24,25</sup>.

Tamoxifen, a SERM widely used in the treatment of breast cancer, has been shown to ameliorate lupus activity in NZB/W F1 mice<sup>26,27</sup>. It achieves these effects, at least in part, by decreasing the production of specific pathogenic antibodies of the IgG3 subclass, thereby reducing renal inflammation<sup>27</sup>. We have demonstrated that tamoxifen prevents the development of estrogen-induced murine lupus by blocking the activation of DNA-reactive B cells<sup>28</sup>. Unfortunately, prolonged treatment with tamoxifen has been linked to development of uterine cancer<sup>29</sup>; this association creates a high risk:benefit ratio that makes tamoxifen unsuitable for the treatment of SLE.

The SERM raloxifene has a better safety profile than tamoxifen due to its anti-estrogenic effect on the uterus<sup>30</sup>. Raloxifene is widely used in the treatment of osteoporosis, which is of serious concern in patients with SLE treated with corticosteroid. Hence, if raloxifene is found to have a beneficial effect on lupus activity, it may provide double advantage to patients with SLE. Raloxifene binds both ER $\alpha$  and ER $\beta$ . Studies with ovariectomized mice have shown that raloxifene exerts estrogenic actions on stromal cells and normalizes the number of B220-positive B cells in the bone marrow<sup>25</sup>. These effects seem to be mediated by ER $\beta$  since ER $\alpha$  knockout mice do not display disturbances in B lymphopoiesis<sup>31</sup>. The effects of raloxifene on mature B cells differ from those of estrogen. Raloxifene does not increase the frequency of Ig-secreting splenic B cells and acts as an immunomodulator compared to estrogen, which acts as an immunostimulator of mature splenic B cells<sup>30</sup>.

The effects of raloxifene on lupus activity in NZB/W F1 mice have not been studied previously, although studies in other autoimmune mouse models suggest that raloxifene may have immunosuppressive effects<sup>16</sup>. We show that raloxifene counteracts the effects of estrogen on autoreactive B cell elimination at the T1/T2 maturation checkpoint, and decreases estrogen-mediated hyperactivity of both marginal zone and follicular B cells by reducing estrogen-induced overexpression of CD40 on follicular B cells and estrogen-induced downregulation of CD22 on marginal zone B cells.

## MATERIALS AND METHODS

*Mice and treatments.* Four- to 6-week-old NZB/W F1 female mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed in the barrier animal facility at the Albert Einstein College of Medicine. Mice were ovariectomized at 2 months of age, and then treated with placebo, estradiol, estradiol and raloxifene, or raloxifene for 5 months. Disease progression was assessed as the mice aged. Raloxifene (3 mg/kg in 0.1% DMSO; Sigma, St. Louis, MO, USA) was injected subcutaneously every day. Estradiol and placebo pellets (Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously. Estradiol pellets provide a constant serum 17 $\beta$ -estradiol concentration of 75 pg/ml, which is equivalent to

the serum estrogen level at the peak of the estrus cycle<sup>32</sup>. Serum and urine were collected before the removal of the ovaries and then monthly during the 5 months of treatment.

**Serum DNA reactivity.** ELISA for total IgG anti-DNA reactivity in serum were performed as described using plates coated with calf thymus dsDNA<sup>33</sup>. ELISA for individual IgG subclasses were performed by incubating serum samples at 1:1000 dilution overnight at 4°C, washing, and then incubation with horseradish peroxidase-conjugated goat antibodies reactive to mouse IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotech, Birmingham, AL, USA) at 1:2000 dilution. Plates were developed with ABTS Peroxidase Substrate (KPL Protein Research Products, South Gaithersburg, MD, USA) at room temperature and colorimetric change was quantified at 405 nm OD.

**Glomerular IgG deposition and pathology.** Formalin-fixed and paraffin-embedded kidney sections 5 µm thick were stained with PAS or biotin-conjugated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA), and then analyzed for PAS or total IgG deposition. Evaluation of IgG deposition was based on the percentage of IgG-stained glomeruli in 50 counted glomeruli. Kidneys with < 25% IgG-stained glomeruli were rated as (+), 25%–75% stained glomeruli as (++), and > 75% stained glomeruli as (+++). For determination of IgG subclass deposition in the glomeruli, kidneys were frozen in OCT medium and sectioned at a thickness of 5 µm. The frozen sections were fixed in acetone. Following a wash in phosphate buffered saline (PBS), kidney sections were blocked with 10% goat serum, and then stained with Alexa Fluor 488 conjugated anti-IgG2a and Alexa Fluor 594 conjugated anti-IgG3 antibodies at 1:1000 dilution (Molecular Probes, Eugene, OR, USA) followed by 3 washes in PBS. Stained kidney sections were mounted in ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA, USA).

**Proteinuria.** To assess the renal function, the concentration of protein in the urine was measured using Multistix 10SG<sup>®</sup> reagent strips for urinalysis (Bayer, Elkhart, IN, USA).

**Flow cytometry and cell sorting.** Splenocytes were depleted of red blood cells by ACK lysing buffer. Single cell suspensions were surface-stained with PerCp-Cy5.5, PeCy7, PE, APC, FITC- and biotin-conjugated antibodies to CD19, CD21, CD40, and CD69 (BD Pharmingen, San Diego, CA, USA), CD93 (eBioscience, San Diego, CA, USA), CD22 (Chemicon International, Temecula, CA, USA), and CD23 (Caltag, Burlingame, CA, USA) at 4°C for 30 min. Cells were washed and fixed with 2% paraformaldehyde. Samples were acquired on the LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with Flow Jo software, version 7.1.3 (Treestar, Ashland, OR, USA). CD19 and CD93 staining was used to distinguish between the immature transitional (CD19+CD93+) and mature (CD19+CD93-) B cells. CD21 and CD23 staining was used to identify the transitional T1 (CD19+CD93+CD21-CD23-) and T2 (CD19+CD93+CD21+CD23+) subsets and the mature follicular (CD19+CD93- CD21intermedCD23++) and marginal zone (CD19+CD93-CD21++CD23-) B cell subsets.

**Elispot assay.** Splenocytes were placed in serial dilutions on Immulon-2 plates coated with calf thymus dsDNA and incubated overnight in cell culture medium enriched with 10% fetal calf serum (FCS) at 37°C. Plates were washed and biotin-conjugated goat anti-mouse IgG antibody (Southern Biotech) at 1:500 dilution was added. Plates were incubated overnight at 4°C, then washed and incubated with SA-conjugated alkaline phosphatase (Southern Biotech) at 1:1000 dilution for 1 h at room temperature. Finally, the plates were developed with 5-bromo-4-chloro-3-indolyl phosphate substrate (Amresco, Solon, OH, USA) at room temperature for 2–4 h. DNA-reactive spots were counted under a dissecting microscope.

**Elispot assay after stimulation.** Splenocytes were cultured in RPMI medium supplemented with 10% FCS in the presence or absence of anti-CD40 antibody and F(abs)2 anti-IgG (10 µg/ml) antibody (Jackson ImmunoResearch, West Grove, PA, USA) at 37°C for 48 h. Cells were then washed and Elispot assay was performed as described above.

**Statistical analysis.** Data were analyzed by one-way ANOVA followed by Dunn's post test between groups. P values < 0.05 were considered statistically significant. Standard deviations of the means were reported where applicable.

## RESULTS

**Raloxifene ameliorated disease activity in NZB/W F1 mice.** Serum anti-dsDNA antibodies of IgG isotype, the hallmark of lupus<sup>34</sup>, were observed in ovariectomized NZB/W F1 mice treated with placebo, estradiol, estradiol/raloxifene, and raloxifene. By the fifth month of treatment (7 months of age), serum levels of IgG anti-dsDNA antibodies in estradiol/raloxifene, raloxifene, and placebo-treated mice were lower than the anti-dsDNA levels found in estradiol-treated mice ( $p < 0.01$ ,  $p < 0.01$ , and  $p < 0.05$ , respectively; Figure 1).

Since nephritogenic anti-dsDNA antibodies in NZB/W F1 mice are predominantly of IgG2a and IgG3 isotype<sup>35,36</sup>, we investigated whether raloxifene had any effects on the production of anti-dsDNA antibodies of these pathogenic IgG subclasses by analyzing their relative amounts in sera at specific timepoints in the disease progression. After 5 months of treatment, estradiol/raloxifene, placebo, and raloxifene-treated mice showed significantly lower serum levels of IgG2a and IgG3 anti-dsDNA antibodies in comparison to the mice treated with estradiol alone ( $p < 0.05$ ,  $p < 0.05$ , and  $p < 0.01$ , for both IgG subclasses; Figure 2A and 2B, respectively). This is in contrast to the observations made with tamoxifen, which induced reductions of serum levels of IgG3 but not IgG2a<sup>27</sup>.

To evaluate the effect of raloxifene on the development of lupus nephritis, the kidney sections from 7-month-old ovariectomized NZB/W F1 mice treated with placebo, estradiol, estradiol/raloxifene, and raloxifene for 5 months were stained with PAS. Raloxifene ameliorated the kidney injury induced by estradiol, which was characterized by gross renal enlargement and cyst formation, cortical atrophy, medullary hyperplasia, loss of glomeruli, and extensive PAS staining deposits (Figure 3A). Renal immunohistochemistry revealed that the mice treated with estradiol/raloxifene had significantly fewer glomerular IgG deposits than estradiol- and placebo-treated mice, and were similar, in this respect, to the mice treated with raloxifene alone. Estradiol/raloxifene-treated mice displayed IgG deposits in less than one-quarter of the glomeruli, with the majority of them (80%) being mild, in contrast to estradiol-treated mice, which had IgG deposits in two-thirds of the glomeruli, with more than 70% of them being moderate and severe (Figure 3B). Raloxifene specifically decreased the glomerular deposition of nephritogenic IgG2a and IgG3 antibodies compared to the mice treated with estradiol alone (Figure 3C), consistent with the lower serum anti-dsDNA levels of these pathogenic isotypes in the estradiol/raloxifene-treated mice. Finally, treatment with raloxifene decreased the amount of proteinuria induced by estradiol ( $p < 0.01$ ; Figure 3D).

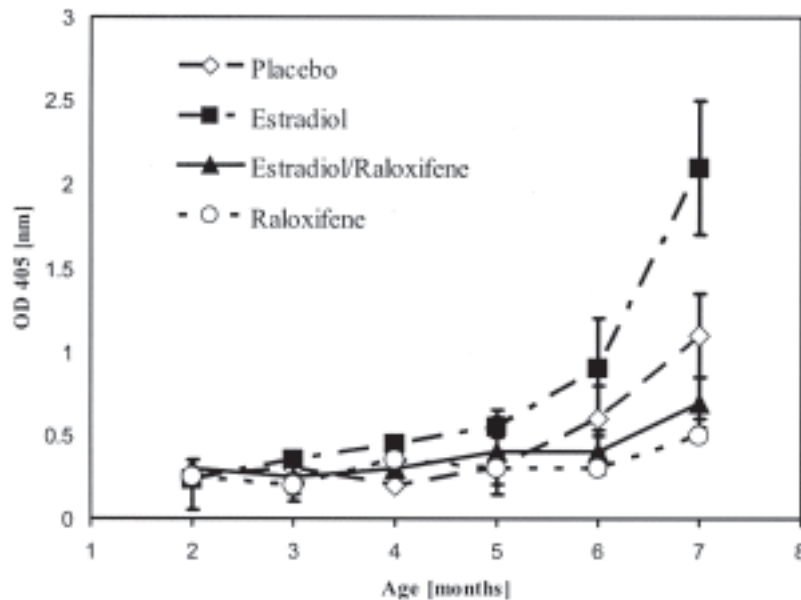


Figure 1. Serum anti-dsDNA reactivity. ELISA measuring anti-dsDNA IgG levels were performed on sera from ovariectomized NZB/W F1 mice treated with placebo (n = 3), estradiol (n = 5), estradiol/raloxifene (n = 5), and raloxifene alone (n = 5) from 2 to 7 months of age. Serum samples were taken before treatment and then monthly for 5 months. At the end of treatment, anti-dsDNA reactivity of serum was higher in estradiol-treated mice than in estradiol/raloxifene, raloxifene, or placebo-treated mice ( $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.05$ , respectively). Raloxifene decreased serum anti-dsDNA reactivity in comparison to placebo-treated mice ( $p < 0.05$ ), but there was no significant difference in serum anti-DNA levels between placebo and estradiol/raloxifene-treated mice. Experiment was repeated 3 times with similar results.

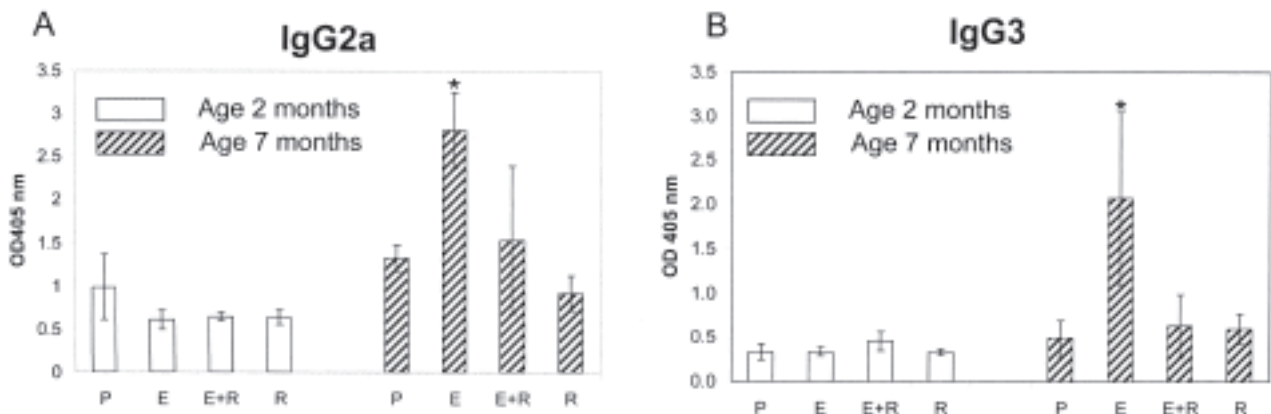
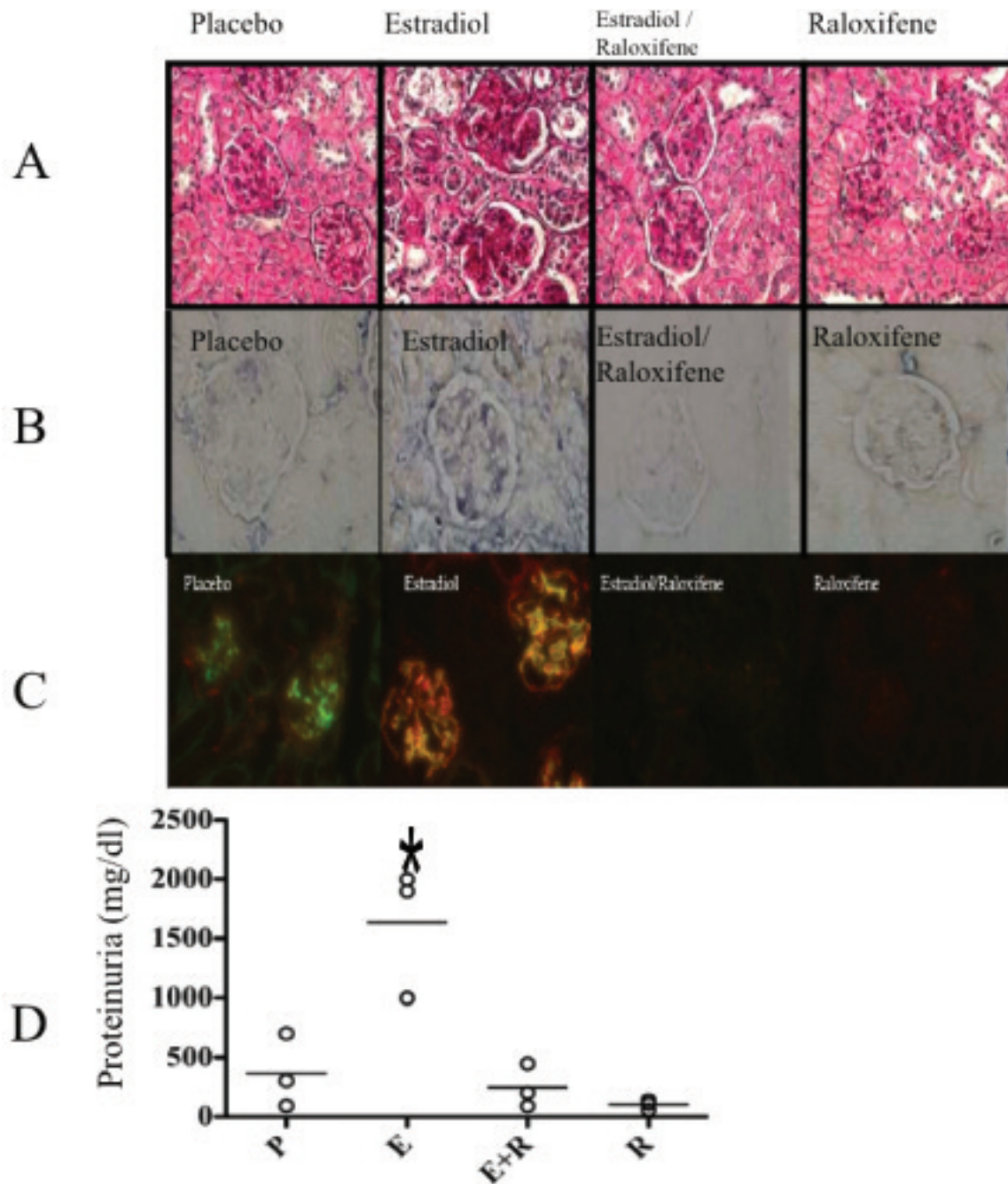


Figure 2. Serum anti-dsDNA IgG2a (A) and IgG3 (B) antibody levels were determined by ELISA in ovariectomized NZB/W F1 mice before treatment and after 5-month treatment with placebo (P, n = 4), estradiol (E, n = 5), estradiol/raloxifene (E+R, n = 5), or raloxifene (R, n = 5). At the end of treatment, estradiol/raloxifene-treated mice had lower levels of DNA-reactive IgG2a and IgG3 antibodies than mice treated with estradiol ( $p < 0.05$  for both). Placebo and raloxifene-treated mice also had less IgG2a and IgG3 antibodies than mice treated with estradiol ( $p < 0.05$  and  $p < 0.01$ , for both IgG subclasses). Experiment was repeated 3 times with similar results. \*  $p < 0.05$ .

*Raloxifene reversed the estradiol-mediated breach in B cell-negative selection.* To understand the effects of raloxifene on induction of B cell tolerance, we studied the effect of the SERM on the negative selection and maturation pattern of B cells in the spleen by analyzing the immature transitional T1 and T2 B cells and mature marginal zone and follicular B cells in 7-month-old NZB/W F1 mice that underwent treat-

ments with placebo, estradiol, estradiol/raloxifene, or raloxifene for 5 months. In nonautoimmune mice, the transitional T1 B cell subset is significantly larger than the T2 B cell subset due to the negative selection of autoreactive B cells that occurs at the T1/T2 interphase<sup>37</sup>. In contrast to the wild-type mice, in NZB/W F1 mice the T1/T2 ratio is close to 1<sup>38</sup>, reflecting the impairment of negative selection of autoreac-



**Figure 3.** Kidney histology and function. Kidney sections from ovariectomized NZB/W F1 mice treated from 2 to 7 months of age with placebo, estradiol, estradiol/raloxifene, and raloxifene ( $n = 5$  for each group) were stained with PAS (A) and anti-IgG antibody (B). A. Representative micrographs of PAS staining (original magnification 50 $\times$ ). Estradiol-treated mice displayed kidney damage and extensive PAS-positive deposits in glomeruli. Raloxifene prevented development of estrogen-induced kidney destruction and PAS staining. Kidney sections from estradiol/raloxifene, raloxifene, and placebo-treated mice showed no significant PAS staining. B. Representative renal sections show glomerular immunoglobulin deposition (original magnification 100 $\times$ ). Estradiol/raloxifene-treated mice had fewer IgG deposits than estradiol-treated mice ( $p < 0.01$ ), in an average  $23\% \pm 6\%$  of glomeruli, 80% of which were rated mild (+) and 20% moderate (++); mice treated with estradiol showed IgG deposits in an average  $66\% \pm 10\%$  of glomeruli, with 29% rated mild (+), 34% moderate (++), and 37% severe (+++). Estradiol/raloxifene-treated mice and raloxifene-treated mice also had fewer IgG deposits than placebo-treated mice ( $p < 0.05$ ). Mice treated with raloxifene alone had fewer glomerular IgG deposits than placebo-treated mice ( $p < 0.01$ ). C. Glomerular IgG2a and IgG3 antibody deposition in kidneys of ovariectomized NZB/W F1 mice treated with placebo ( $n = 3$ ), estradiol ( $n = 4$ ), estradiol/raloxifene ( $n = 4$ ), and raloxifene ( $n = 4$ ) for 5 months was examined by immunofluorescent microscopy. Representative micrographs for each group are shown (original magnification 50 $\times$ ). Overlapping glomerular deposition of both autoreactive IgG2a and IgG3 antibodies was observed in glomeruli of estradiol-treated mice. In estradiol/raloxifene-treated mice, IgG2a and IgG3 antibodies were barely detectable in the glomeruli. D. Proteinuria; level of protein in urine was measured in ovariectomized NZB/W F1 mice treated with placebo (P,  $n = 3$ ), estradiol (E,  $n = 4$ ), estradiol/raloxifene (E+R,  $n = 6$ ), and raloxifene (R,  $n = 6$ ) from 2 to 7 months of age. At the end of the 5-month treatment, the degree of proteinuria was higher in the estradiol-treated mice than in the mice treated with estradiol/raloxifene, raloxifene, or placebo (\* $p < 0.01$  for all). The experiment was repeated 3 times with similar results.

tive specificities at the T1/T2 junction. We found that ovariectomized NZB/W F1 mice treated with estradiol also have a T1/T2 B cell ratio of ~1. Estradiol-treated mice had a lower percentage of T1 B cells and higher percentage of T2 B cells than estradiol/raloxifene, raloxifene, and placebo-treated mice ( $p < 0.05$ , in all groups for T1 and T2; Table 1). Raloxifene exerted effects on B cell selection in the presence of estrogen; the spleens of estradiol/raloxifene-treated mice had fewer T2 B cells than those of estradiol-treated mice, indicating that raloxifene blocks the estradiol-mediated breach of negative selection at the transitional stage of B cell development. Estradiol-treated mice displayed higher absolute numbers of T1 B cells and lower absolute numbers of T2 B cells than the mice treated with estradiol/raloxifene, raloxifene, or placebo ( $p < 0.05$  for T1 in all groups, and  $p < 0.05$ ,  $p < 0.05$ , and  $p < 0.01$  for T2, respectively).

In addition, estradiol-treated mice showed higher percentages of marginal zone B cells than estradiol/raloxifene, raloxifene, and placebo-treated mice ( $p < 0.01$  for all; Table 1). Treatment with raloxifene blocked estrogen-induced expansion of the marginal zone B cell subset ( $p < 0.01$ ); this B cell population has been shown to harbor autoreactivity in estrogen-induced murine lupus<sup>39</sup>. The percentages of follicular B cells were similar among all treatment groups. The absolute numbers of marginal zone and follicular B cells were higher in estradiol-treated mice compared to the mice treated with estradiol/raloxifene, raloxifene, and placebo ( $p < 0.01$  for marginal zone in all groups, and  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.05$  for follicular B cells, respectively).

*Raloxifene-mediated effects on the activation status of marginal zone and follicular B cells.* Marginal zone and follicular B cells are distinct mature splenic B cell populations; marginal zone B cells are early responders whose activation is T cell-independent, whereas follicular B cells will only initiate an antibody response if the necessary T cell signals are received<sup>40,41</sup>. As shown above, raloxifene reduced the number of activated B cells, thereby ameliorating the effect of estradiol, which increased the activation status of mature DNA-reactive B cells. In order to evaluate how raloxifene

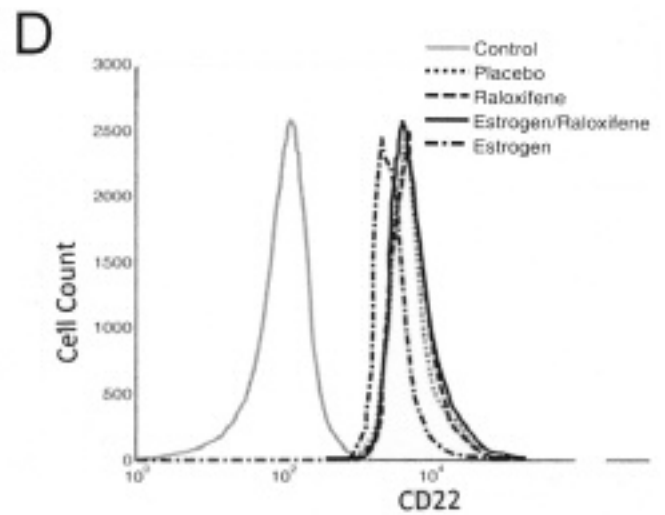
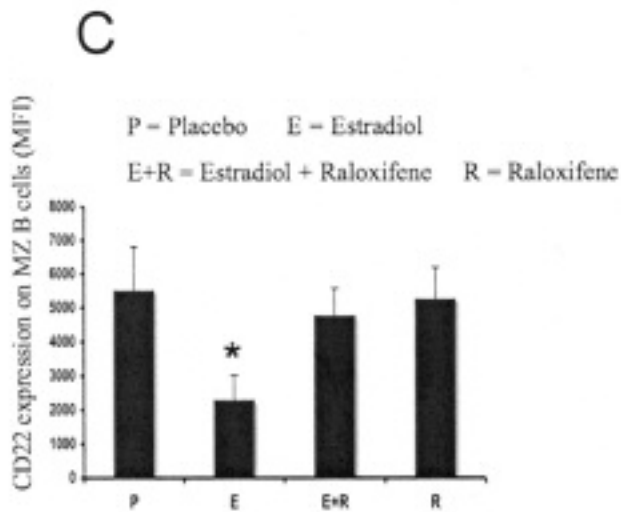
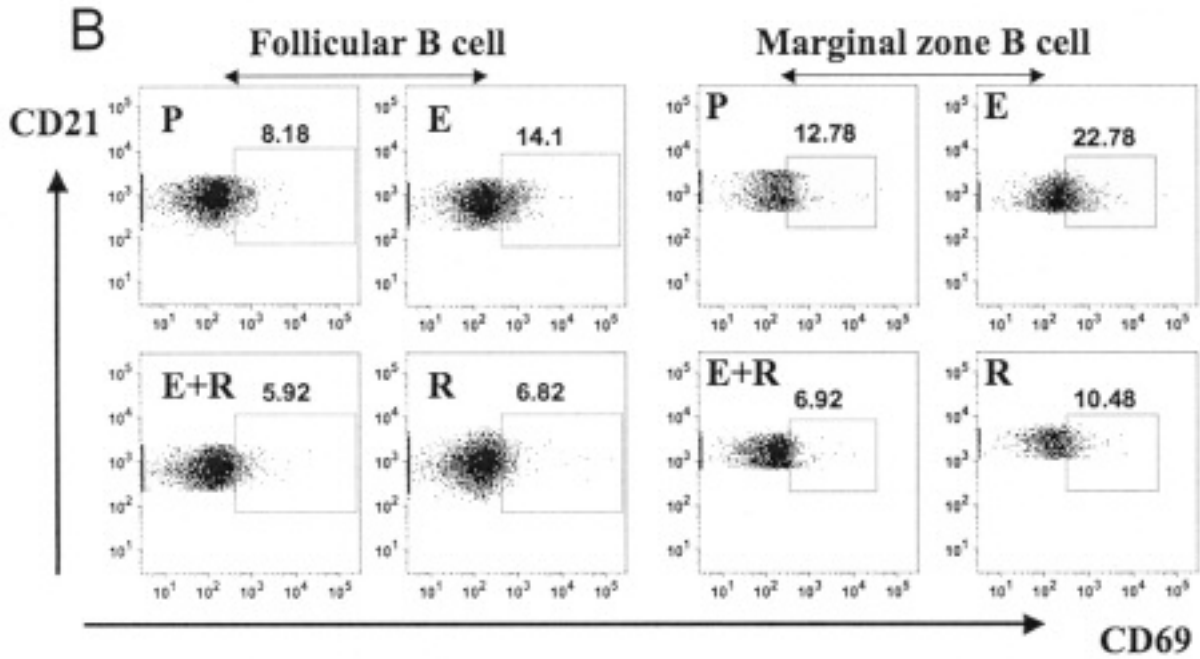
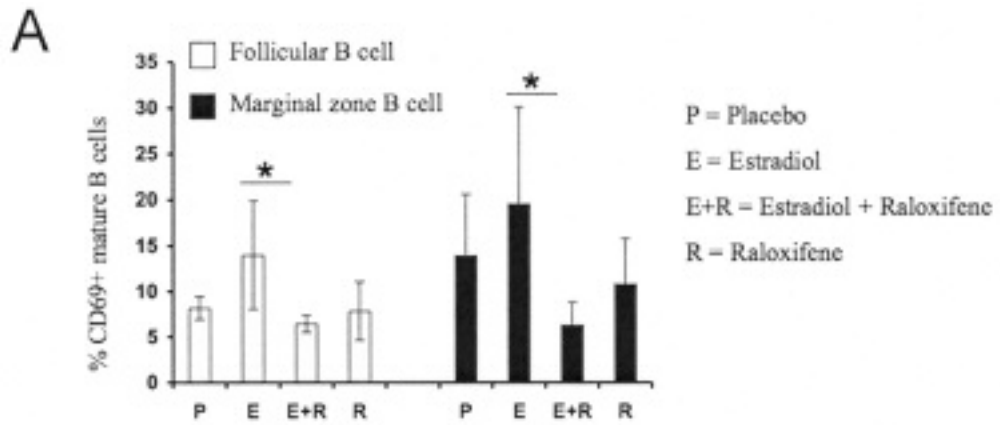
mediates these effects on the mature B cell subsets, we studied the expression of CD69 and CD22 in the various treatment groups. CD69, a C-type lectin, is expressed on activated lymphocytes and macrophages; increased CD69 expression on peripheral blood mononuclear cells from SLE patients has been associated with increased lupus activity<sup>42</sup>. Flow cytometric evaluation of mature B cell subsets from estradiol, estradiol/raloxifene, raloxifene, and placebo-treated NZB/W F1 mice ( $n = 5$  for each group) showed that estradiol/raloxifene-treated mice displayed fewer CD69-positive B cells with marginal zone or follicular phenotype than estradiol-treated mice ( $p < 0.01$  for both; Figure 4A and 4B). CD22, an ITIM (immunoreceptor tyrosine-based inhibitory motif)-containing molecule that dampens the signaling ability of the B cell antigen receptor<sup>43</sup>, has an important role in the development of autoimmunity inasmuch as deficiency of CD22 in mice leads to the emergence of high affinity anti-dsDNA antibodies<sup>44</sup>. As determined by flow cytometry, estradiol/raloxifene-treated mice displayed higher CD22 expression on marginal zone B cells than estradiol-treated mice ( $p < 0.05$ ; Figure 4C and 4D). Follicular B cells showed a variable expression of CD22 that did not significantly differ among the treatment groups.

Costimulatory ligation of CD40 on B cells by CD40L on activated T cells induces the upregulation of surface antigen-presenting molecules on follicular B cells, as well as their proliferation and maturation into plasma cells with the ability to secrete isotype-switched antibodies<sup>45,46</sup>. As determined by flow cytometry, follicular B cells from estradiol-treated mice overexpressed CD40 on their surface; this effect was blocked by simultaneous treatment with raloxifene ( $p < 0.01$ ; Figure 5A and 5B). Raloxifene-mediated antagonism of estrogen-induced CD40 overexpression correlates with its ability to reduce the production of pathogenic IgG subclasses and glomerular deposition of autoreactive IgG2a and IgG3 antibodies. Additionally, treatment with raloxifene decreased B cell response to costimulation as measured by the lower number of DNA-reactive spots after stimulation with anti-CD40 and anti-IgG antibodies ( $p < 0.01$ ; Figure 5C).

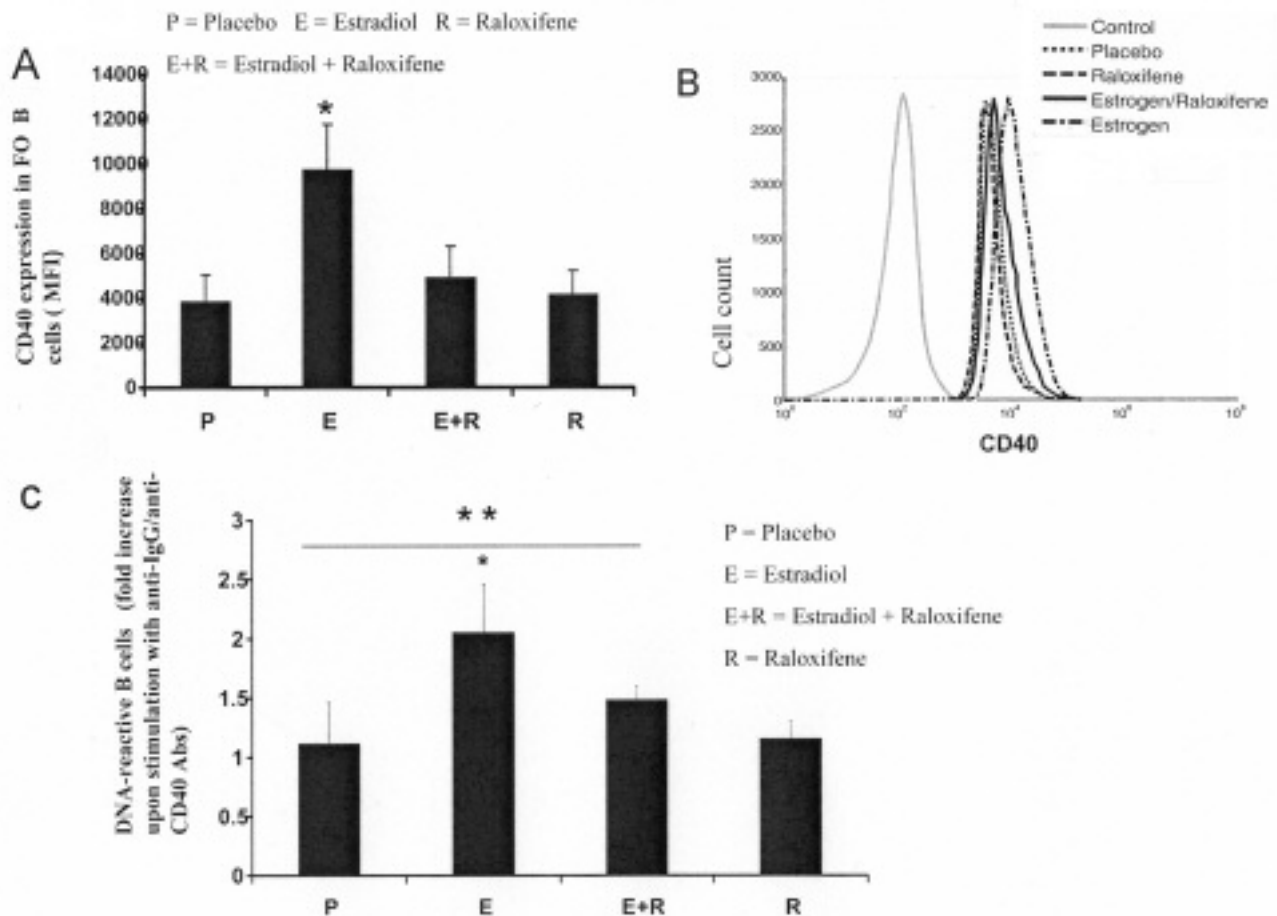
Table 1. Frequency of splenic B cell subsets. Values are represented as average percentage  $\pm$  SD and absolute cell numbers  $\pm$  SD of transitional T1 and T2 B cells and mature follicular (Fo) and marginal zone (MZ) B cells.

B Cell Subset <sup>†</sup>	Placebo, n = 10	Estradiol, n = 10	Estradiol/Raloxifene, n = 12	Raloxifene, n = 12
MZ	9.6 ( $\pm$ 3.4)	19.3 ( $\pm$ 5.1)**	11.4 ( $\pm$ 3.2)	11.5 ( $\pm$ 2.7)
	5.1 ( $\pm$ 1.7)	9.4 ( $\pm$ 3.9)**	4.9 ( $\pm$ 2.2)	5 ( $\pm$ 1.1)
Fo	59.0 ( $\pm$ 5.3)	56.7 ( $\pm$ 4.7)	58.7 ( $\pm$ 6.4)	58.2 ( $\pm$ 4.8)
	32.1 ( $\pm$ 5.1)	40.3 ( $\pm$ 9.6)*	29.2 ( $\pm$ 9.4)	30.5 ( $\pm$ 8.2)
T1	10.9 ( $\pm$ 1.8)	7.6 ( $\pm$ 1.4)*	11.5 ( $\pm$ 2.3)	10.4 ( $\pm$ 2.2)
	5.3 ( $\pm$ 1.2)	3.85 ( $\pm$ 1.7)*	5.4 ( $\pm$ 1.5)	5.5 ( $\pm$ 1.9)
T2	4.5 ( $\pm$ 0.6)	6.5 ( $\pm$ 1.1)*	5.1 ( $\pm$ 1.2)	5.1 ( $\pm$ 1.1)
	2.5 ( $\pm$ 0.8)	3.5 ( $\pm$ 1.2)*	2.4 ( $\pm$ 0.6)	2.1 ( $\pm$ 1.1)

<sup>†</sup> %/no. cells  $\times 10^6$ . \*  $p < 0.05$ ; \*\*  $p < 0.01$ .



**Figure 4.** A. CD69 expression on mature B cell subsets. Splenocytes from ovariectomized NZB/W F1 mice treated with estradiol, estradiol/raloxifene, raloxifene, and placebo (n = 5 for each group) for 5 months were stained with fluorochrome-labeled antibodies for flow cytometric identification of marginal zone (MZ) (CD19+CD93–CD21++CD23–) and follicular (CD19+CD93–CD21intermedCD23++) B cells, which were then analyzed for percentage of activated CD69+ B cells. Estradiol/raloxifene-treated mice had fewer CD69+ MZ B cells and fewer CD69+ follicular B cells than estradiol-treated mice (\*p < 0.01 for both). B. Representative flow cytometric dot plots of percentage CD69+ follicular and MZ B cells in placebo, estradiol, estradiol/raloxifene, and raloxifene-treated mice. C. CD22 expression. MZ and follicular B cells from ovariectomized mice treated for 5 months with placebo (n = 6), estradiol (n = 6), estradiol/raloxifene (n = 6), and raloxifene (n = 5) were evaluated for CD22 expression by flow cytometry. Compared to estradiol/raloxifene-treated mice, mice treated with estradiol showed decreased expression of CD22 on MZ B cells (\*p < 0.05). Also, MZ B cells from estradiol-treated mice expressed less CD22 than placebo-treated mice (\*p < 0.05) and showed a lower average CD22 expression than MZ B cells from raloxifene-treated mice, but the latter value did not reach statistical significance. D. Representative histograms of CD22 expression on MZ B cells in placebo, estradiol, estradiol/raloxifene, and raloxifene-treated mice. All treatment groups showed similar expression of CD22 on follicular B cells (data not shown). The experiment was repeated 3 times with similar results. MFI: mean fluorescence intensity.



**Figure 5.** A. CD40 expression on follicular B cells (CD19+CD93–CD21intCD23+) identified by flow cytometry in ovariectomized mice treated with placebo (n = 5), estradiol (n = 5), estradiol/raloxifene (n = 5), and raloxifene (n = 4) for 5 months. Raloxifene prevented development of estradiol-mediated over-expression of CD40 on follicular B cells (\*p < 0.01). Follicular B cells from estradiol-treated mice expressed more CD40 than follicular B cells from raloxifene and placebo-treated mice (p < 0.01 for both). There was no difference in the level of CD40 expression on follicular B cells of estradiol/raloxifene and raloxifene-treated mice. B. Representative histograms of CD40 expression on follicular B cells in placebo, estradiol, estradiol/raloxifene, and raloxifene-treated mice. Experiment was repeated 3 times with similar results. C. Enumeration of DNA-reactive B cells responsive to T cell help. Splenocytes from ovariectomized NZB/W F1 mice that underwent 5-month treatment with placebo (n = 4), estradiol (n = 4), estradiol/raloxifene (n = 5), and raloxifene (n = 5) were cultured in the presence or absence of anti-CD40 and anti-IgG antibodies for 48 h at 37°C, and then enumerated by Elispot assay. Raloxifene/estradiol-treated mice displayed fewer B cells responsive to anti-IgG and anti-CD40 antibodies than estradiol-treated mice (\*p < 0.01). Mice treated with estradiol had more anti-IgG/anti-CD40-reactive B cells than raloxifene and placebo-treated mice (\*\*p < 0.05 for both). Although raloxifene decreased estradiol-induced B cell responsiveness, it was not able to bring the response back to the baseline level of placebo-treated mice (p < 0.05). There was no significant difference in B cell response to anti-CD40 and anti-IgG between mice treated with raloxifene and those treated with placebo. Experiment was repeated twice with similar results. MFI: mean fluorescence intensity.



## DISCUSSION

The NZB/W F1 murine model of lupus is similar to human SLE in its manifestations and gender predominance. Female NZB/W F1 mice begin to produce autoantibodies at 4 months of age and develop increased serum autoantibody levels and diffuse glomerulonephritis at about 5–7 months of age, 6 months earlier than the onset of disease in their male counterparts<sup>47</sup>. Removal of endogenous estrogen via ovariectomy causes a decrease in serum autoantibody levels and delayed onset of disease, whereas administration of estrogen to ovariectomized NZB/W F1 female mice results in accelerated lupus activity. In contrast, castration of male NZB/W F1 mice results in early lupus onset and treatment with estrogen exacerbates disease activity<sup>31,32,33</sup>.

The exact mechanism of estrogen-mediated effects in lupus has not been fully elucidated, although a role for B cells has been suggested by experimental evidence. Ovariectomy has been shown to increase the generation of immature B cells in the bone marrow of mice<sup>48</sup>, whereas treatment with estrogen reduced the generation of bone marrow B cells<sup>25,49</sup> and stimulated B cell maturation in the periphery<sup>32</sup>. Ovariectomized lupus-prone Mrl.lpr/lpr mice respond to estrogen treatment with enhanced basal immunoglobulin production<sup>50</sup>. In addition, treatment with estrogen increased the autoantibody levels in lupus-prone NZB/W F1 mice<sup>51</sup> and induced autoantibodies in wild-type C57BL/6J mice<sup>52</sup>. In our studies, we found that the number of DNA-reactive B cells in NZB/W F1 mice rose in response to estradiol treatment, indicating that estrogen promoted the development and survival of these autoreactive cells. This is in keeping with earlier findings that in BALB/c mice expressing an autoreactive heavy-chain transgene for anti-dsDNA antibody, treatment with estradiol impaired the negative selection of autoreactive specificities in both the immature and transitional B cell compartments and induced a lupus-like syndrome<sup>53</sup>. Lupus-prone NZB/W F1 mice have a T1:T2 B cell ratio  $\sim 1$ <sup>29</sup>, indicative of defective negative selection of immature B cells at the T1-T2 junction. We found that ovariectomized NZB/W F1 mice had a T1:T2 B cell ratio  $> 1$ , and that estradiol treatment of these mice caused a decrease in the T1:T2 ratio to  $\sim 1$ , in part explaining the hormone-induced acceleration of disease activity in this lupus-prone mouse strain.

SERM combine with the estrogen receptor and induce estrogen agonistic activity in some tissues and anti-estrogenic activity in others. SERM have been studied in animals and humans, and data derived from these studies have shown that not all SERM are created equal. For example, tamoxifen induces adverse effects on the endometrium in humans<sup>54</sup>, primates<sup>55</sup>, and possibly mice<sup>56</sup>. In contrast, raloxifene, a SERM known to prevent bone loss in human<sup>57</sup> and murine<sup>58</sup> osteoporosis, has not shown tamoxifen-like effects on the uterus in humans<sup>59</sup> or rodents<sup>60</sup>.

Raloxifene and tamoxifen affect the immune system.

Tamoxifen has been shown to alleviate inflammatory responses<sup>61</sup>, modify cytokine concentrations<sup>62,63</sup>, inhibit interleukin 2-mediated proliferation of lymph node T cells, and reduce the weights of spleen and lymph node<sup>64</sup>. Further, tamoxifen has been proven to have a beneficial effect on the renal disease of NZB/W F1 mice<sup>26</sup> by inhibiting the generation of the pathogenic IgG3 autoantibodies that deposit in the glomeruli<sup>27</sup>. We have previously demonstrated that tamoxifen prevents the expansion of estrogen-induced autoreactive marginal zone B cells in BALB/c mice expressing a transgene for an autoreactive heavy-chain of a pathogenic anti-dsDNA antibody<sup>28</sup>. Both tamoxifen and raloxifene maintain dendritic cells in an immature state by causing hyporesponsiveness to inflammatory stimuli<sup>65</sup>. Like tamoxifen, raloxifene may also dampen immune responses. In murine studies, raloxifene negatively regulated B cell lymphopoiesis<sup>25</sup>, and compared to estrogen, raloxifene did not increase the frequency of antibody-secreting mature splenic B cells<sup>30</sup>. In agreement with these data, raloxifene showed immunosuppressive and therapeutic effects in mouse models of nephritis<sup>66</sup>.

We hypothesized that because of its immunomodulatory effects raloxifene might have an ameliorative effect on lupus activity. We found that raloxifene reduced the number of DNA-reactive B cells and decreased serum anti-DNA antibody levels in estrogen-treated ovariectomized NZB/W F1 mice. Raloxifene was also found to lower the levels of both IgG2a and IgG3 anti-dsDNA antibodies, IgG subclasses known for their nephritogenic potential, whereas tamoxifen specifically lowers the level of autoantibodies of the IgG3 subclass<sup>27</sup>, suggesting that raloxifene may inhibit the activation of Th1 cells independently of its antagonism of the action of estrogen. Raloxifene modulated the development of renal injury and proteinuria induced by estrogen treatment.

As noted, NZB/W F1 mice have abnormal B cell selection at maturational checkpoints, allowing the survival of autoreactive B cells<sup>29</sup>. We have found that ovariectomized NZB/W F1 mice have an increased T1/T2 B cell ratio compared to the intact NZB/W F1 mice (Zhang Y, *et al*, unpublished data). The raloxifene treatment of ovariectomized NZB/W F1 mice did not affect the T1/T2 ratio, but ameliorated estrogen-induced increase of T2 B cells and consequently decreased the T1/T2 ratio in estradiol/raloxifene-treated mice. Raloxifene also prevented estrogen-induced expansion of the marginal zone B cell subset that has been shown to harbor autoreactive specificities in an estrogen-induced model of lupus<sup>32,39</sup>. Raloxifene blocked an estrogen-mediated increase in the number of activated CD69-positive B cells with a marginal zone phenotype. This effect of the SERM appears to be a consequence of modulating the estrogen-mediated downregulation of CD22. CD22 is an inhibitory receptor on B cells that attenuates Ca<sup>++</sup> influx upon B cell receptor engagement<sup>43</sup>; decreased CD22

expression has been shown to promote B cell hyperactivity and autoantibody secretion<sup>67</sup>. We found that raloxifene blocked the estradiol-induced downregulation of CD22 on marginal zone B cells; compared to mice treated with estradiol alone, marginal zone B cells from estradiol/raloxifene-treated mice expressed more CD22. Raloxifene also reduced the estrogen-induced increase of activated B cells with follicular phenotype as determined by the expression of CD69, and downregulated the estrogen-induced overexpression of CD40 on these B cells. Estrogen/raloxifene-treated mice displayed fewer B cells responsive to costimulatory signals upon B cell receptor engagement than the estradiol-treated mice, indicating that raloxifene abrogates estradiol-mediated hyperresponsiveness of B cells to costimulatory signals. Hyperactive T cell-B cell costimulatory interactions have been implicated in the pathogenesis of lupus<sup>46</sup>, and blockade of the CD40-CD40L pathway has been shown to have a therapeutic effect in murine<sup>68</sup> and human lupus<sup>69</sup>.

The mechanism of raloxifene's tissue selectivity has been studied through experiments with the interactions between ER and raloxifene<sup>70</sup>, but the relations between raloxifene and ER in mature B cells have not been analyzed in detail. This latter set of interactions is most likely very complex because B cells express both ER $\alpha$  and ER $\beta$ , and raloxifene binds both ER. ER $\alpha$  is the predominant receptor expressed in immune cells, and the effects of estrogen in lupus are mainly mediated by this receptor<sup>71</sup>. A recent study demonstrated a key role for the AF-1 domain of the ER $\alpha$  receptor in mediating lupus nephritis and mortality in female NZM2410 and MRL/lpr mice<sup>16</sup>. These findings are of specific interest to our study because the NZM2410 strain is a congenic recombinant derivative of the NZB/W F1 strain utilized in our experiments. In the presence of estrogen, ER $\alpha$  activates transcription in the AP1 domain, whereas ER $\beta$  inhibits it. Raloxifene is a transcriptional activator with ER $\beta$  at an AP1 site<sup>72</sup>. ER $\beta$  acts as an inhibitor of ER $\alpha$  transcriptional activity in cells that express both ER<sup>73</sup>. Thus, based on these data, one can hypothesize that raloxifene's immunomodulatory effects in the presence of estradiol in NZB/W F1 mice are mediated predominantly by ER $\beta$ . Further studies are needed to elucidate the exact interactions of raloxifene and ER in B cells. To that end, ER $\alpha$  and ER $\beta$  knockout mice can provide a suitable setting for investigating the effects of raloxifene mediated by each of the ER subtypes.

Our findings indicate that raloxifene antagonizes the effects of estrogen on the development and activation status of autoreactive B cells. Raloxifene has been shown to counter estrogen-induced suppression of autoreactive B cell elimination at the T1/T2 maturation checkpoint, to reduce estrogen-induced enhancement of CD40 expression on follicular B cells, making them less responsive to T cell costimulation, and to block estrogen-induced downregulation of CD22 on marginal zone B cells, thereby damping their responsiveness

to B cell receptor-mediated stimuli. Human SLE appears to be influenced by gender and by estrogen, and raloxifene has proven to have a reasonable safety profile over many years of use in treatment of osteoporosis. Our findings indicate that raloxifene may have therapeutic value for murine lupus and suggest that modulation of immune effects of estrogen may be a useful therapeutic approach in SLE. Further studies are needed to evaluate the effect of raloxifene on fully developed murine lupus in order to determine the therapeutic potential of raloxifene for human SLE.

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