AD_____

Award Number: W81XWH-07-1-0327

TITLE: DETERMINATION OF THE ROLE OF ESTROGEN RECEPTORS AND ESTROGEN REGULATED GENES IN B CELL AUTOREACTIVITY

PRINCIPAL INVESTIGATOR: DR. BETTY DIAMOND

CONTRACTING ORGANIZATION: The Feinstein Institute for Medical Research
Manhasset, NY 11030

REPORT DATE: July 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2011	Annual	1 JUL 2010 - 30 JUN 2011

4. TITLE AND SUBTITLE

DETERMINATION OF THE ROLE OF ESTROGEN RECEPTORS AND ESTROGEN REGULATED GENES IN B CELL AUTOREACTIVITY

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0327

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

DR. BETTY DIAMOND

E-Mail: bdiamond@nshs.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Feinstein Institute for Medical Research
Manhasset, NY 11030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Systemic lupus erythematosus is an autoimmune disease that occurs preferentially in women. We have developed a murine model in which BALB/c non-spontaneously autoimmune mice harbor a transgene encoding the heavy chain of an anti-DNA antibody. Using this model, we have shown that B cell expression of the estrogen receptor (ER)α mediates an estrogen-induced loss of B cell tolerance. This occurs through a reduced B cell receptor (BCR) signal strength in transitional B cells and the presence of DNA is required to mediate positive selection of the autoreactive B cells. Moreover, estrogen-induced autoimmunity depends on the genetic background. Exploiting the availability of an estrogen-responsive (BALB/c) strain and an estrogen-nonresponsive (C57Bl/6) strain, we have found that estrogen upregulates p202b, an anti-apoptotic factor, and itpkb, a molecular that limits the release of calcium stores, in BALB/c mice protecting autoreactive B cells from BCR-triggered apoptosis and impairing negative selection during B cell development.

15. SUBJECT TERMS

No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	32	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction:

There is abundant clinical data that estrogen can increase risk of developing systemic lupus erythematosus (SLE) and disease severity in some individuals. We have explored the hypothesis that this may be a consequence of the effects of estrogen on B cell function; the corollary is that protecting B cells from the effects of estrogen might ameliorate disease symptoms without altering bone health or interfering with other beneficial effects of estrogen.

We have shown that estrogen acts directly on B cells altering the survival and maturation pathway of developing B cells, and does so through engagement of both estrogen receptor (ER)α and ERβ. We have further shown that estrogen prevents antigen-induced deletion of autoreactive B cells through ERα but antigen is required to mediate their continued maturation. Finally, we have shown that this occurs when estrogen upregulate p202b, an anti-apoptotic molecule, and itpkb, a molecule which regulates cytosolic calcium concentration, in developing B cells. When B cells are resistant to these effects of estrogen, there is no estrogen-induced abrogation of B cell tolerance.

Body:

1) Determine which estrogen receptor is responsible for estrogen-induced alterations in BCR signaling.

We have shown that the estrogen-induced expansion of marginal zone B cells can be mediated through either ERα or ERβ, and contrary to expectation does not depend upon an attenuation of the BCR signal. In contrast, the estrogen-induced abrogation of negative selection is mediated by ERα only and involves attenuation of the BCR signal in transitional B cells. These data are presented in Hill, L., Venkatesh, J., Chinnasamy, P., Grimaldi, C and Diamond. B Differential roles of estrogen receptors α and β in control of B cell maturation and selection. Molecular Medicine 7:211-220 (2011).

We have further shown that the abrogation of B cell tolerance requires a decreased stringency of negative selection but also requires antigen-mediated positive selection. When estradiol-treated mice are given DNase to limit the availability of self antigen DNA-reactive B cells do not mature to immunocompetence. These data are published in Venkatesh, J., Yoshifuji, H., Kawabata, D., Chinnasamy, P., Stanevsky, A., Grimaldi, C., and Diamond, B. Antigen is required for maturation and activation of pathogenic anti-DNA antibodies and systemic inflammation. J Immunol. 186:5304-5312 (2011)

2) Analyze B cell maturation and selection in placebo or estrogen-treated C57B1/6 mice.
We are currently preparing a manuscript showing that estradiol affects B cell maturation but not B cell selection in C57Bl/6 mice. Estradiol induces an expansion of marginal zone B cells (Fig 1) but does not enhance survival of high affinity DNA-reactive B cells in C57B1/6 mice harboring a transgene encoding the heavy chain of a DNA-reactive antibody (Fig 2). This is not due to altered expression ERs or to altered metabolism of estradiol (Fig 3). Rather, we observed an estrogen-induced upregulation of p202b, an anti-apoptotic molecule, and itpkb, a molecule involved in the regulation of cytosolic calcium in transitional B cells of BALB/c, but not C57Bl/6, mice (Figs 4 and 5). These changes can account for the disparate effect of estradiol on BCR signal strength in the two strains (Fig 6). These studies may help us understand why

some women with SLE have a disease exacerbated by estrogen and others have a disease that is not altered by estrogen exposure. There would be significant clinical advantage to be able to subset SLE patients in this fashion.

3) Determine the genetic basis for an estrogen-responsive B cell compartment.
We have generated C57Bl/6 sle 1 mice that harbor the R4A transgene and will prepare a manuscript shortly.

Key Research Accomplishments:

1) The demonstration that loss of B cell tolerance is mediated through ERα.
2) The demonstration that estrogen must function in conjunction with antigen exposure to potentiate autoimmunity.
3) The identification of key molecules, p202b and itpkb, involved in estrogen-induced attenuation of negative selection.
4) The observation that genetic background regulates B cell susceptibility to estrogen.

Reportable Outcomes:

Publications:

Cohen-Solal, J. and Diamond, B. Lessons from an anti-DNA autoantibody. Molecular Immunology 32:130-2 (2011).

Hill, L., Venkatesh, J., Chinnasamy, P., Grimaldi, C and Diamond. B Differential roles of estrogen receptors α and β in control of B cell maturation and selection. Molecular Medicine 17:211-220 (2011) PMC3060981

Venkatesh, J., Yoshifuji, H., Kawabata, D., Chinnasamy, P., Stanevsky, A., Grimaldi, C., and Diamond, B. Antigen is required for maturation and activation of pathogenic anti-DNA antibodies and systemic inflammation. J Immunol. 186:5304-5312 (2011)

Degrees: PhD Latia Hill

Funding: Career Award SLE Foundation – Venkatesh J.

Presentations:

British Society for Rheumatology 2012

Invitation to AARDA 2011 symposium “Sex, Pregnancy, and Autoimmunity”, *Betty Diamond* – DATE: TBD – LOCATION: TBD.

Invited speaker at FoCIS Meeting “Selection of the B Cell Repertoire”, *Betty Diamond* – June 24-27, 2010 – Boston MA.

Invitation to 2010 symposium on hormones and the immune system

Speaker at 2009 Neuroimmunology symposium on immune system and hormones

Conclusion:

The studies supported by this award suggest that B cell specific blockade of ERα may be therapeutic in some patients with SLE. A bispecific molecule using antibody to target B cells and an ER modulator such as tamoxifen to target ERα might be of therapeutic benefit and is highly unlikely to be immunosuppressive or to have other untoward toxicities. These studies emphasize the potential of ER-related therapeutics in SLE.

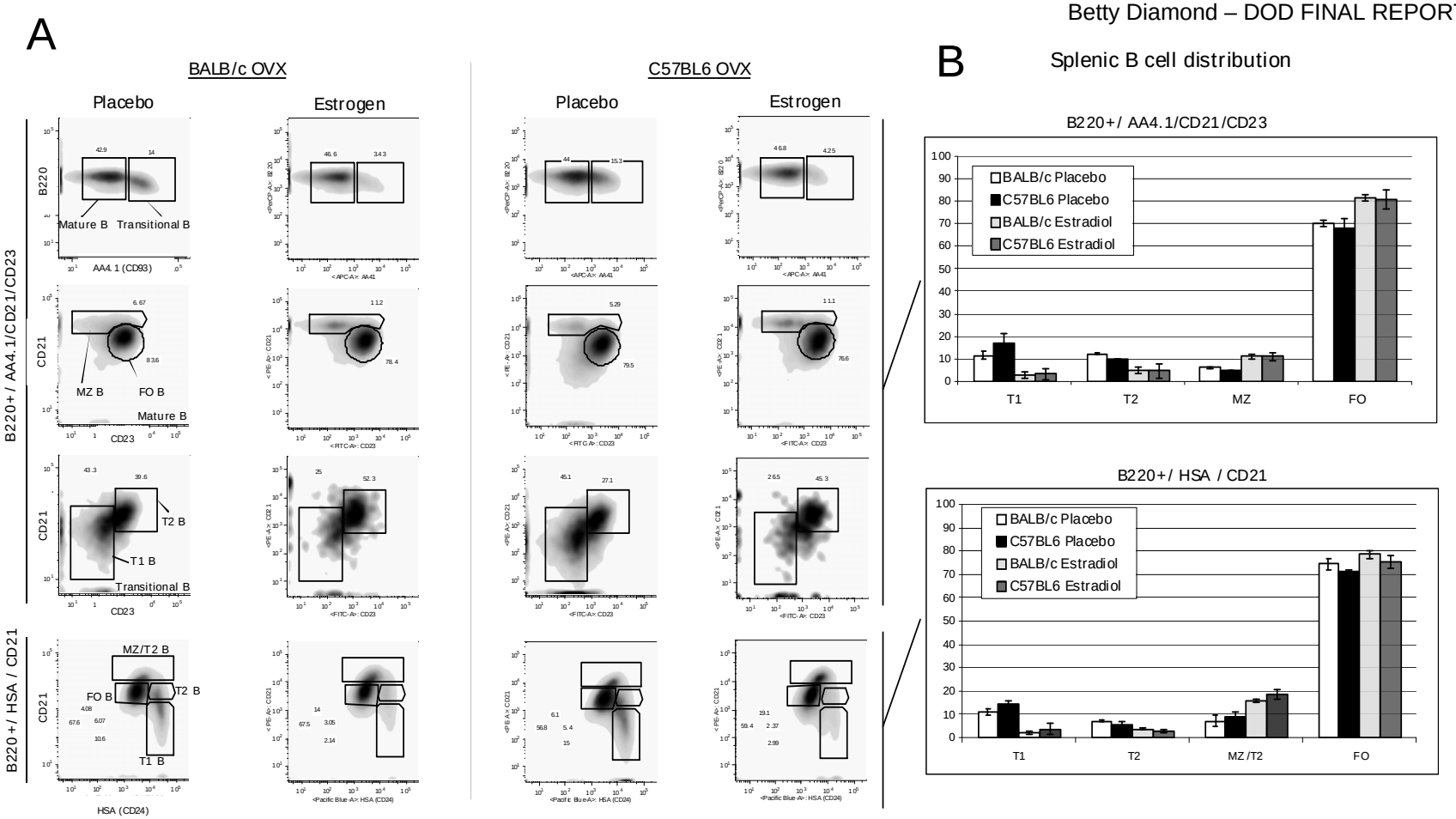
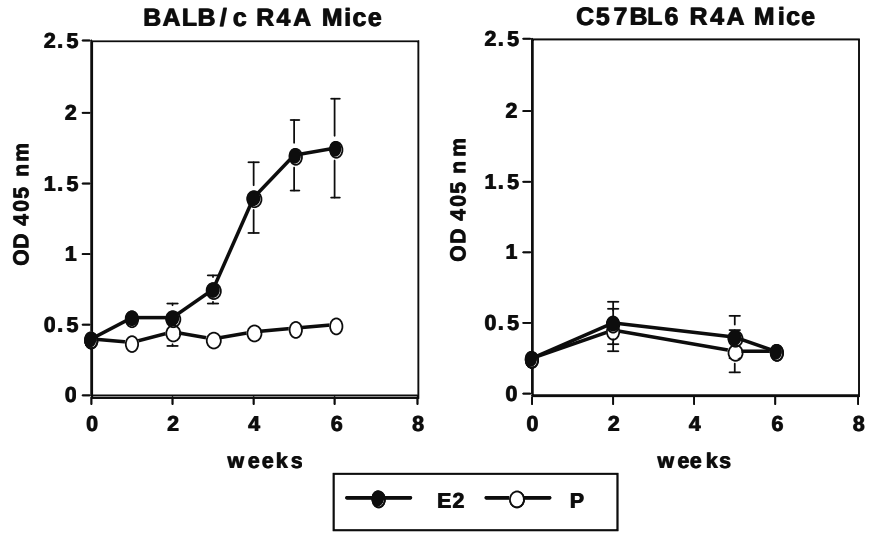


Figure 1 : Estrogen effects on splenic B cell development in wild type BALB/c and C57BL/6 ovariectomized (ovx) mice (A)Two strategies of gating to estimate B cell subtype distribution (AA4.1 and CD21, CD23 on B220+ cells versus HSA and CD21 on B220+ cells). (B) Chart of the splenic B cell distribution.



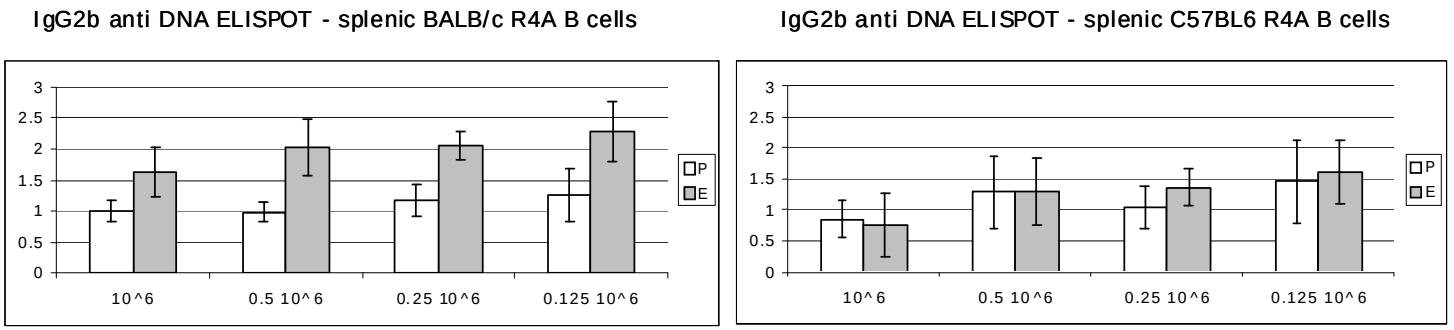


Figure 2 : A-ELISA for the detection of IgG2b anti DNA Antibodies
ovariectomized BALB/c R4A versus C57BL6 R4A mice were treated either by placebo or estradiol pellets for 6 weeks and bled repeatedly. Sera have been tested for IgG2b anti DNA auto-antibodies.

B-ELISPOT for the detection of splenic B cells secreting IgG2b anti DNA Antibodies
splenic B cells from ovariectomized BALB/c (A) versus C57BL6 (B) mice treated with placebo (P) or estradiol (E) pellets for 6 weeks have been tested for their ability to secrete anti-DNA autoantibodies encoded by the IgG2b R4A heavy chain transgene

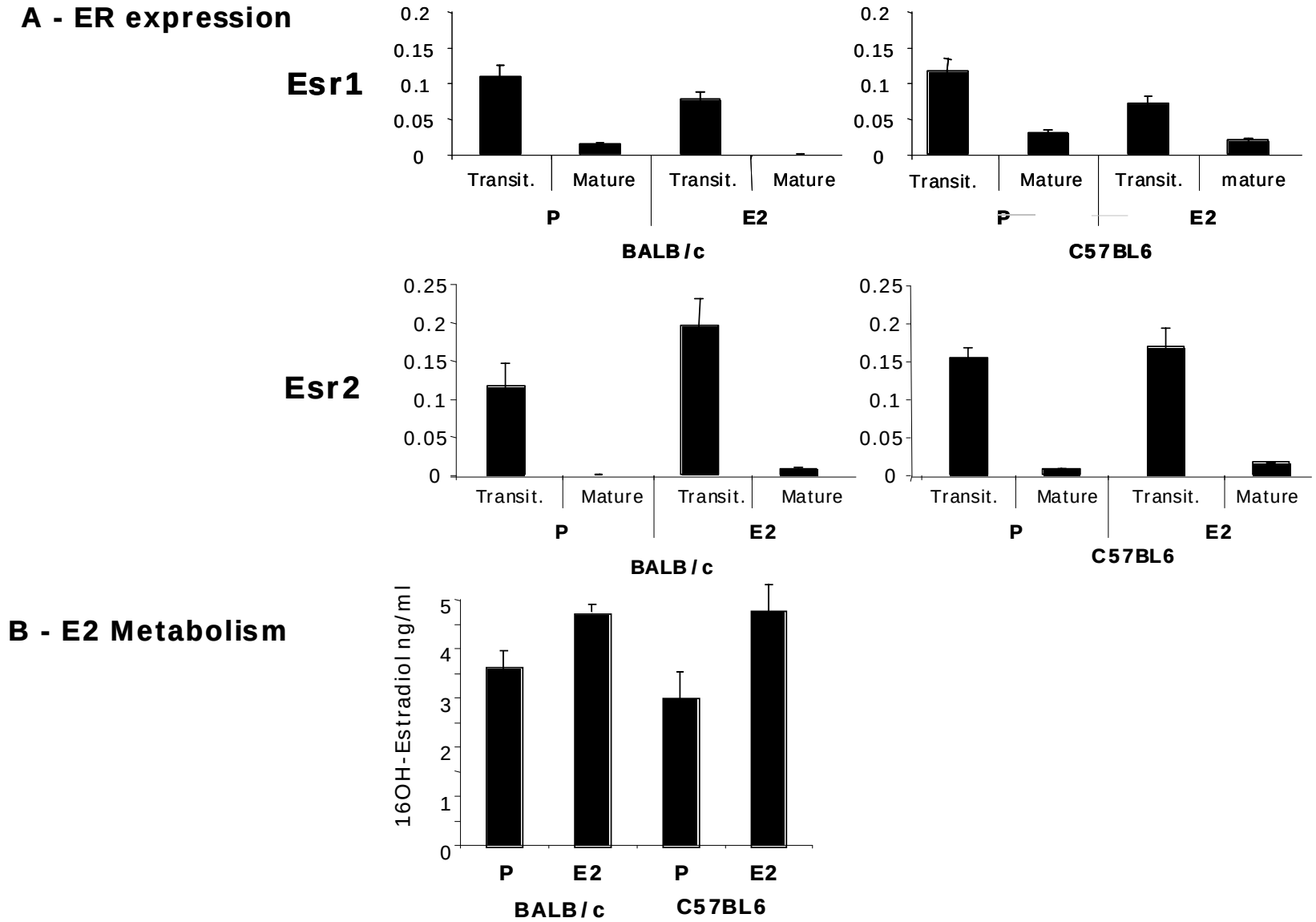
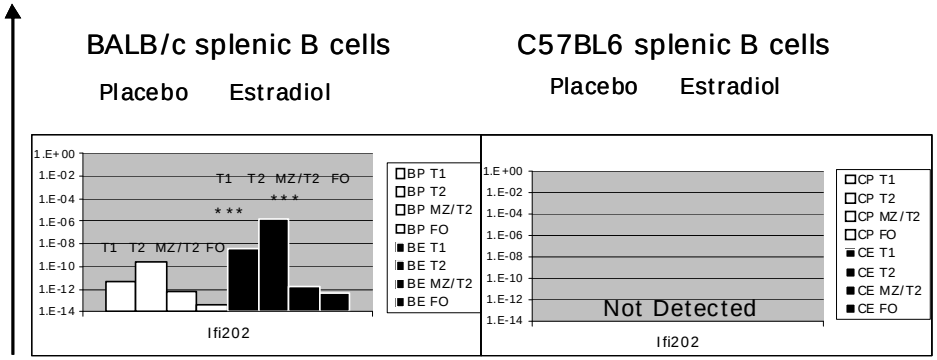
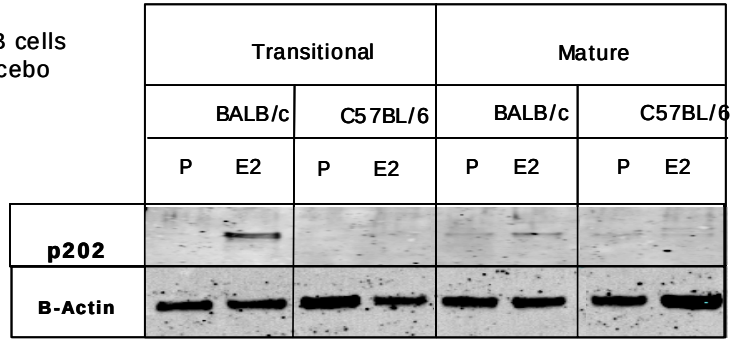


Figure 3: (A) expression of estrogen receptors ERalpha(Esr1) and ERbeta (Esr2) in splenic B cells and (B)Urinary 16 OH-Estradiol metabolite in BALB/c and C57BL6 mice.

A- In vivo expression of P202b by splenic B cells from mice treated with estradiol or Placebo for 4 weeks (qPCR)



B- In vivo expression of p202 by splenic B cells from mice treated with estradiol or Placebo for 4 weeks (western Blot)



C- In vitro induction of P202b expression by exposure to E2, IFNa or ICI 182-780 / 18h (qPCR)

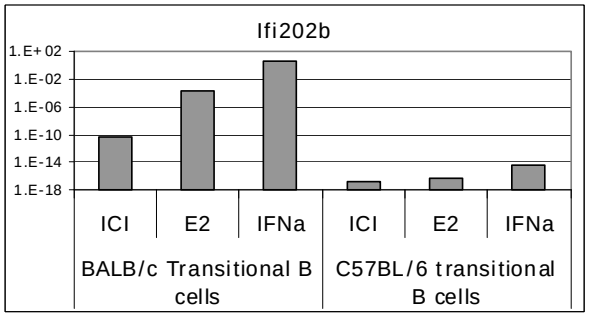
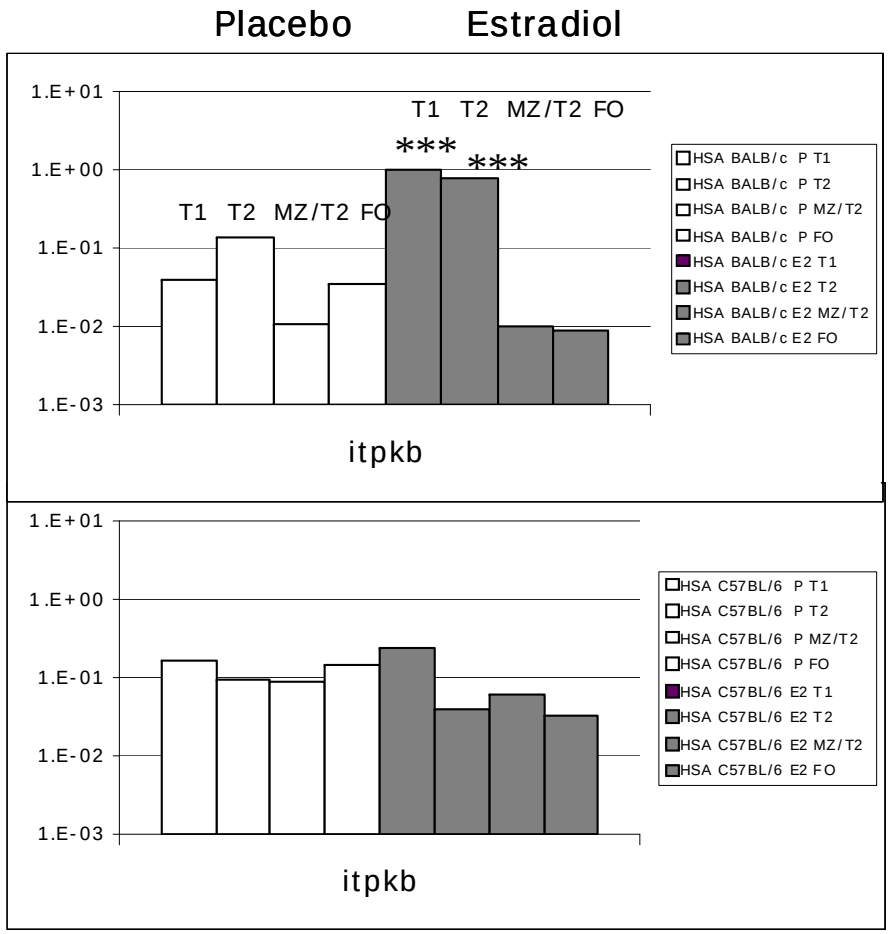


Figure 4 : Differential expression of p202 by transitional B cells from BALB/c and C57BL/6 mice treated with Estradiol in vivo or in vitro.



BALB/c

C57Bl/6

Figure 5: itpkb expression was measured by qPCR in B cell subsets from mice exposed to estradiol or placebo. Estradiol caused a significant increase in itpkb mRNA in T1 and T2 B cells of BALB/c but not C57Bl/6 mice.

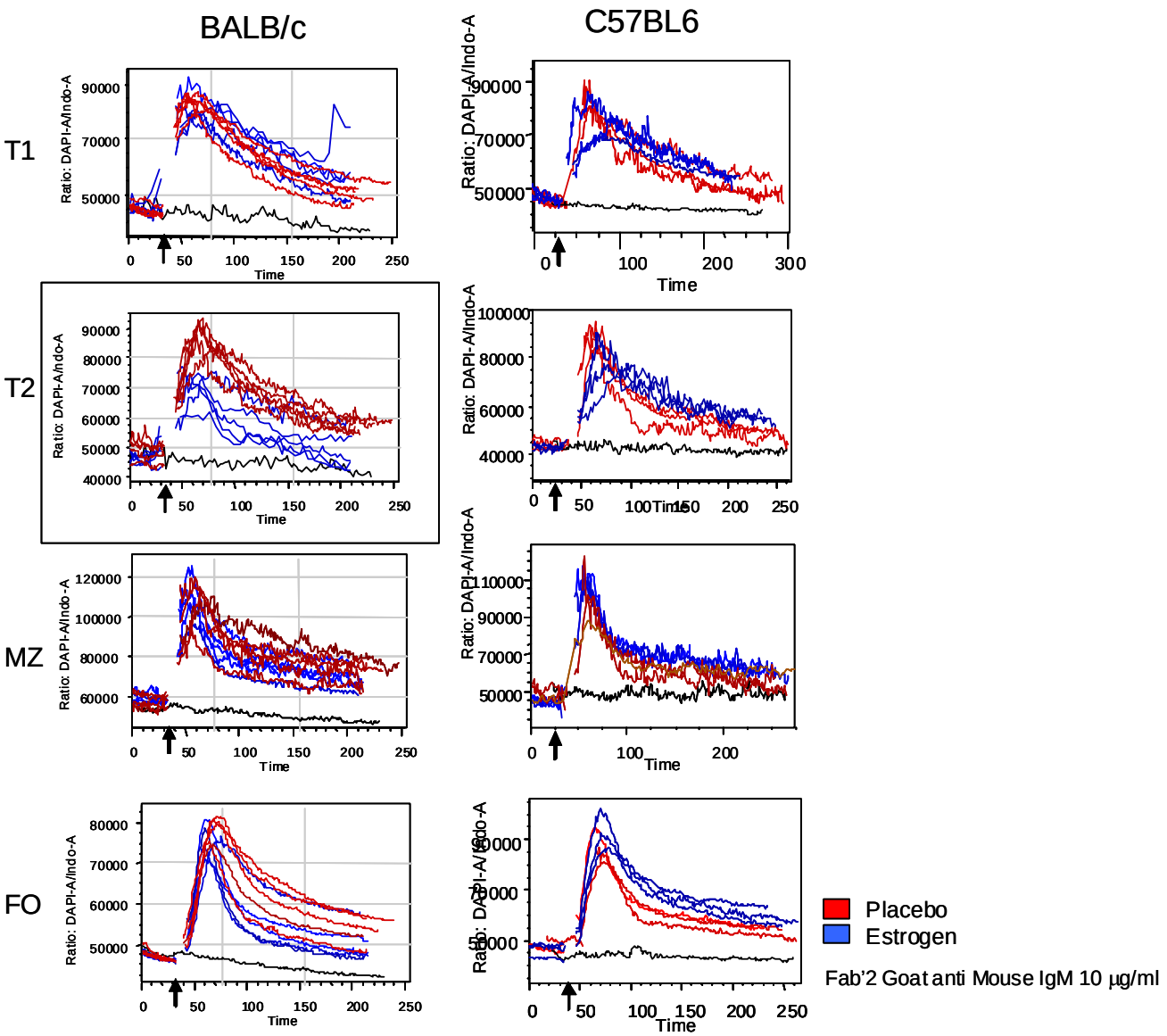


Figure 6 : Calcium flux is specifically impaired for BALB/c R4A E2 treated at the T2 stage.

Differential Roles of Estrogen Receptors α and β in Control of B-Cell Maturation and Selection

Latia Hill,[1,2] Venkatesh Jeganathan,[1] Prameladevi Chinnasamy,[1] Christine Grimaldi,[1,3] and Betty Diamond[1,2]

[1]Center for Autoimmune and Musculoskeletal Disease, The Feinstein Institute for Medical Research, Manhasset, New York, United States of America; [2]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, United States of America; and [3]current affiliation: Boehringer Ingelheim Pharmaceuticals, Ridgefield, Connecticut, United States of America

It is clear that estrogen can accelerate and exacerbate disease in some lupus-prone mouse strains. It also appears that estrogen can contribute to disease onset or flare in a subset of patients with lupus. We have previously shown estrogen alters B-cell development to decrease lymphopoiesis and increase the frequency of marginal zone B cells. Furthermore, estrogen diminishes B-cell receptor signaling and allows for the increased survival of high-affinity DNA-reactive B cells. Here, we analyze the contribution of estrogen receptor α or β engagement to the altered B-cell maturation and selection mediated by increased exposure to estrogen. We demonstrate that engagement of either estrogen receptor α or β can alter B-cell maturation, but only engagement of estrogen receptor α is a trigger for autoimmunity. Thus, maturation and selection are regulated differentially by estrogen. These observations have therapeutic implications.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2010.00172

INTRODUCTION

Developing and maintaining an antibody repertoire that protects an organism from the multiple pathogens in the environment begins with B-cell ontogeny in bone marrow. Antibodies against numerous antigens are generated during the formation of a B-cell repertoire, and processes are required to limit the survival and maturation of those B cells making autoantibodies (1,2). Tolerance checkpoints occur at multiple times throughout B-cell development; a breakdown in one or more of these checkpoints lies at the crux of systemic lupus erythematosus (SLE). SLE is characterized by an array of antibodies against self-antigens (3,4). Anti–double-stranded (ds) DNA antibodies are the most common and are essentially diagnostic of SLE. Additionally, they have been demonstrated to contribute to tissue damage in kidney and possibly in brain (5–9).

The etiology of SLE is currently unknown, but experimental evidence in mouse models and clinical evidence in patients implicate both genetic susceptibility and environmental triggers (10,11). SLE disproportionately affects women, with a 9x greater incidence in women than in men (12). Although this occurrence may be in part determined by sex, there are data to support the role of sex hormones as a trigger for disease and a modulator of disease severity (13,14). Patients with SLE have been reported to have increased metabolism of more mitogenic forms of estrogen (15). In several mouse models, exogenous estradiol (E2) can accelerate and exacerbate disease (16–19).

We developed a transgenic BALB/c mouse that harbors the heavy chain of an IgG2b anti-DNA antibody (20,21). Transgene-expressing B cells have been shown to develop normally in the bone marrow and spleen. The BALB/c mouse normally maintains B-cell tolerance, deleting high-affinity DNA-reactive B cells and permitting the maturation to immunocompetence of low-affinity DNA-reactive B cells. Serum titers of anti-DNA antibody remain low (22,23). In the mouse, E2 acts as an environmental trigger for an SLE-like serology. E2 administration breaks B-cell tolerance in this mouse and permits the survival and activation of high-affinity DNA-reactive B cells, leading to elevated serum levels of anti-DNA antibody (22). Altered B-cell selection occurs at the immature and T2 transitional stages of B-cell development; the autoreactive B cells mature as marginal zone (MZ) B cells (24).

There are two estrogen receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ) (25). These form homodimers and heterodimers and are expressed in many cells including T cells, B cells, monocytes and dendritic cells (26–28). ERα and ERβ regulate gene transcription, having both overlapping and distinct target genes (29,30). Some reports suggest that they can function antagonistically (25). ERα can also function at the cell membrane to activate certain signaling cascades. Polymorphisms in

Address correspondence and reprint requests to *Betty Diamond, Center for Autoimmune and Musculoskeletal Disease, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030. Phone: 516-562-3830; Fax: 516-562-2953; E-mail: bdiamond@nshs.edu.*
Submitted September 10, 2010; Accepted for publication November 16, 2010; Epub (www.molmed.org) ahead of print November 22, 2010.

ERα have been associated with SLE in studies of a small number of both Japanese and Swedish patients (31,32). Recently, it was shown that deletion of ERα in lupus-prone mice leads to reduced disease; the effect seems to be both a reduction in autoantibody production and an independent decrease in inflammation within the kidney itself (33,34).

Our interest has been the effect of E2 on B-cell maturation and selection. We chose to study the role of E2 on B-cell development and selection without the confounding factors present in an autoimmune background. E2 has been shown to decrease B-cell lymphopoiesis in the bone marrow at the pro–B-cell stage (35,36). We have previously shown that E2 alters B-cell subsets in the spleen. Because of the decreased lymphopoiesis in the bone marrow, there are fewer splenic transitional B cells. We also observed an E2-induced increase in the MZ B-cell compartment (24). Furthermore, E2 exposure causes a decrease in B-cell receptor (BCR) signaling in response to anti-IgM activation. This is accompanied by an E2-induced increase in expression of the negative regulator of the BCR, CD22 (24,37). These data led us to hypothesize that E2 dampens the BCR signal through an increased expression of CD22. We further hypothesized that the diminished BCR signal favored the generation of MZ B cells and allowed for survival of autoreactive B cells. Thus, we speculated that there was a relationship between the reduced BCR signal and the alteration in both B-cell maturation and selection.

Using BALB/c mice deficient in ERα or ERβ, we found that the decrease in transitional B cells and the expansion of the MZ B-cell compartment, which is seen in wild-type (WT) mice exposed to E2, was mediated by both ERα and ERβ. The E2-mediated reduction in BCR signal strength occurred in WT and ERβ-deficient mice, demonstrating that BCR signal strength is regulated by ERα. We further were able to demonstrate that ERα engagement led to a breakdown in B-cell tolerance, with increased survival to immunocompetence of high-affinity DNA-reactive B cells. Just as ERβ engagement did not alter BCR signal strength, ERβ engagement also did not alter B-cell selection. Thus, ERα may be a therapeutic target in some patients with SLE.

MATERIALS AND METHODS

Mice and *In Vivo* Treatment

All mice were housed in a specific pathogen-free barrier facility, and experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee. WT BALB/c mice, ERα-deficient (β-sufficient) and ERβ-deficient (α-sufficient) C57Bl/6 mice were obtained from Jackson Laboratories. ERα- and ERβ-deficient BALB/c mice were backcrossed to BALB/c mice for at least nine generations before homozygous ERα- or ERβ-deficient BALB/c mice were generated. ERα- and ERβ-deficient BALB/c mice were then mated to R4A-Tg BALB/c mice to produce ERα- or ERβ-deficient mice harboring the R4A transgene. Six- to ten-wk-old mice were ovariectomized, and time-release pellets, estradiol (E2) or placebo (P) (Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously for 3–6 wks as described (22). The E2 pellets maintain serum E2 concentrations of 75–100 pg/mL. In some studies, 100 μg 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) Tris-phenol (PPT), the ERα agonist; 100 μg diarylpropionitrile (DPN), the ERβ agonist; and 2 μg E2 or vehicle (DMSO) was given daily by subcutaneous injection for 3 or 6 wks (38).

Flow Cytometry and Antibodies

Fluorophore-coupled antibodies specific for B220, Erk1/2, CD21, CD23 and CD22 were purchased from BD Pharmingen (San Jose, CA, USA). Fluorophore-coupled antibody to CD23 and CD24 (M1/69) were obtained from Caltag Laboratories (Burlingame, CA, USA). Antibody to AA4.1 was purchased from eBioscience. Extracellular staining was performed by using a crystallizable fragment (Fc) receptor block for 20 min followed by incubation with antibody for 30 min in 0.2% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) at 4°C. For intracellular staining, cells were fixed and permeabilized with cytofix/cytoperm. Antibodies were diluted in cytoperm. Phosphoflow cytometry was performed using protocol from BD Biosciences (San Jose, CA, USA). Splenocytes were stimulated with 20 μg/mL anti-IgM $F(ab)'_2$ antibody for 5 min at 37°C. Cells were stained with B-cell surface markers, and intracellular staining was performed using antibodies to phospho Erk. Flow cytometry was performed on an LSR II (BD Biosciences) and analyzed using Flowjo software (Tree Star, Ashland, OR, USA).

Single-Cell PCR of Light Chain Genes

Splenocytes were stained with antibodies specific for B220, IgG2b and AA4.1, and mature ($B220^+/Tg^+/AA4.1^-$) cells were individually sorted into 96-well plates using a FACSAria (BD Biosciences). Single-cell RT-PCR was performed as described previously (39). Kappa light chain transcripts were amplified by two rounds of PCR. The following primers were used: universal Vκ 5′-GGCTGCAGSTTCAGTGGCAG TGGRTCWGGRAC-3′ + constant region primer (Cκ) (first round) 5-′TGGAT GGGTGGGAAGATG-3′; and Cκ (second round) 5′-AAGATGGATACAGTTGGT-3′. The PCR products were subjected to exo-SAP treatment (USB Biochemicals, Cleveland, OH, USA), and automated sequencing was performed using the second-round Cκ primer (Genewiz, South Plainfield, NJ, USA). Analysis of the DNA sequences was performed using the IgBLAST program (http://www.ncbi.nlm.nih.gov/igblast). The Fisher exact test was performed to assess statistical significance.

mRNA Analysis

Splenic B-cell RNA from $ER\alpha^{-/-}$, R4A-$ER\alpha^{-/-}$, $ER\beta^{-/-}$, R4A-$ER\beta^{-/-}$, WT BALB/c and R4A mice was prepared using an RNeasy plus kit (Qiagen). Reverse-transcriptase generation of cDNA was performed on 500 ng total RNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according

to the manufacturer's instructions. For the analysis of ERα mRNA, primers that amplify exon 2 of the *ER* gene were used: namely, 5′-GGGAGCCAGTCTGTA ACTCG-3′ and 5′-GGGCTCGTTCTCCAG GTAGT-3′. ERβ mRNA was analyzed using primers described by Krege *et al.* (40). Primers specific for β-actin cDNA were used as a positive control.

Real-Time PCR

Total splenocyte RNA from ERα$^{-/-}$, ERβ$^{-/-}$ and WT mice were isolated using the RNeasy plus kit (Qiagen, Valencia, CA, USA), and the cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed with a Roche 480 light cycler using a Roche 480 master mix (Roche Applied Science, Indianapolis, IN, USA) and Taqman primer/probe sets (Applied Biosystems, Carlsbad, CA, USA). The relative expression of B-cell–activating factor (BAFF); interferon (IFN)-α subunits 2, 4 and 12; IFNβ subunit 1; and IFNγ were determined by comparing the expression to polymerase (RNA) II (DNA directed) polypeptide A (Polr2a). Data were analyzed using the ΔΔCT method. Applied Biosystems primers used are as follows: Polr2A:Mm00839502_m1, BAFF:Mm00446347_m1, IFNα2:Mm00833961_s1, IFNα4:Mm00833969_s1, IFNα12:Mm00616656_s1, IFNβ1:Mm00439546_s1 and IFNγ:Mm00801778_m1.

DNA ELISA

Costar half-well plates were coated with 25 μl 100 μg/mL filtered calf thymus DNA (Sigma) in carbonate buffer, pH 8.6. DNA was dry coated overnight at 37°C. Plates were washed with water and blocked for 1 h at 37°C with 100 μl 2% BSA/PBS. Plates were washed twice with PBS/Tween. Sera were diluted in 0.2% BSA/PBS; 25 μL was added to each well and incubated at 37°C for 1 h. Plates were washed 5x. Alkaline phosphatase–coupled antibody to mouse IgG2b was diluted in 0.2% BSA/PBS and incubated for 1 h at 37°C. Plates were washed 5x with PBS/Tween and developed using phosphatase tablets according to the manufacturer's instructions (Sigma Aldrich, St. Louis, MO, USA). The optical density (OD) was measured at 405 nm.

ELISpot Assay

Immulon 2HD plates were coated with 50 μl 100 μg/mL filtered calf thymus DNA in PBS. To identify IgG2b-producing B cells, 50 μl anti-IgG2b antibody was adsorbed to the plate in PBS at a concentration of 10 μg/mL. To identify anti-DNA–producing B cells, 50 μl dsDNA (100 μg/mL) was adsorbed to the plate. Plates were blocked with tissue culture medium consisting of RPMI containing 10% fetal calf serum for 1 h at 37°C. Splenocytes in tissue culture medium were added to triplicate wells starting at 2×10^6 followed by two-fold serial dilutions. Plates were spun at 300*g* for 2 min, incubated for 16 h and washed 5x with PBS/Tween. Biotinylated anti-IgG2b antibody was diluted in tissue culture medium at a dilution of 1:600 and added to the plates for 2 h at 37°C. After washing, alkaline phosphatase–conjugated streptavidin was added at a dilution of 1:1,000 (Southern Biotechnology). The plates were further incubated for 2 h and developed using BCIP and AMP buffer. Protein plates were incubated at room temperature for 30 min. DNA plates were incubated at room temperature for 4 h, and the spots were counted under a dissecting microscope.

Western Blotting

Splenic B cells were purified by negative selection using Dynal Beads and resuspended in RPMI 1640 medium containing 5% fetal calf serum and 10 mmol/L HEPES. After incubation for 5 min at 37°C, the cells were left resting or were stimulated with $F(ab')_2$ anti-IgM (20 μg/mL) for 5 and 15 min at 37°C. The cells were then suspended in cold Dulbecco's PBS, pelleted and lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease (Roche Applied Science) and phosphatase inhibitors (Pierce). Protein cell lysates were quantitated using Coomassie Plus (Pierce, Rockford, IL, USA) and stored at –20°C until further use. A total of 20 μg protein was subjected to SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes. Direct immunoblotting for Erk tyrosine phosphorylation used phospho Erk1/2 or total Erk1/2 antibody followed by anti-rabbit IgG secondary antibody. The membranes were subsequently stripped and reprobed with the antibodies to hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Santa Cruz, Santa Cruz, CA, USA) to confirm equivalent Erk protein abundance between samples. Immunoblots were developed with an enhanced chemiluminescence kit (Pierce). Densitometry was performed to quantitate the levels of Erk phosphorylation compared with Erk1 and Erk2 total proteins individually. HPRT was used as a loading control.

Statistical Analysis

Statistical analysis was performed using an unpaired Student *t* test and the Fisher exact test as appropriate. A P value of <0.05 was considered statistically significant.

RESULTS

ERα and ERβ Alters B-Cell Maturation

Mice lacking ERα and ERβ have previously been reported and were generated

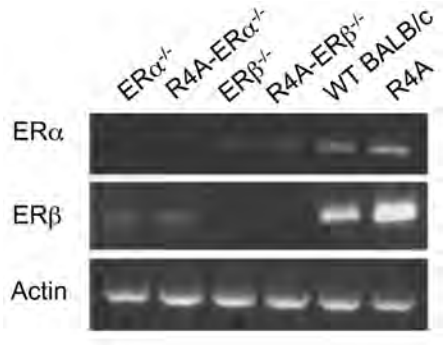


Figure 1. ERα and ERβ transcript in mouse B cells. ERα and ERβ mRNA levels were determined in purified splenic B cells from ERα$^{-/-}$, R4A-ERα$^{-/-}$, ERβ$^{-/-}$, R4A-ERβ$^{-/-}$, WT BALB/c and R4A mice. Actin mRNA was used as a control.

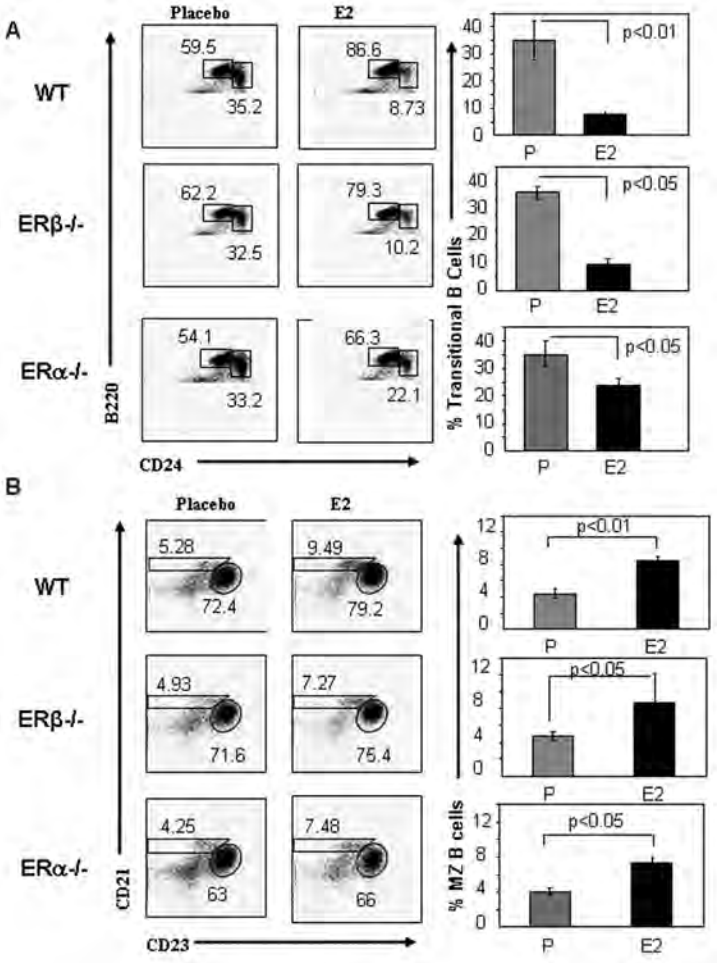


Figure 2. ERα B-cell subsets in WT, ERα-deficient and ERβ-deficient mice after administration of E2 or placebo. (A) Transitional B-cell subset in WT, ERα-deficient and ERβ-deficient mice. WT, ERα-deficient and ERβ-deficient mice were treated with E2 or placebo for 3 wks, and splenocytes were analyzed by flow cytometry. Transitional B cells were identified as $B220^{+}CD24^{high}$ cells. E2 but not placebo exposure reduced transitional B cells. (B) MZ B cells were identified as $B220^{+}CD24^{low}CD21^{high}CD23^{neg}$ cells. MZ B cells were expanded by E2 but not placebo exposure in WT, ERα-deficient and ERβ-deficient mice. At least five mice were included in each group, and representative dot blots are shown.

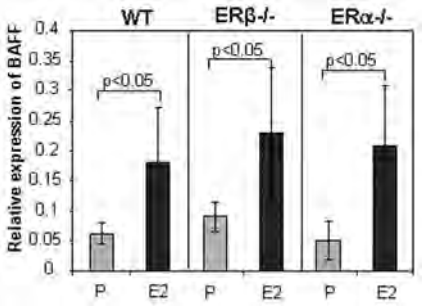


Figure 3. BAFF mRNA levels in splenocytes of WT, ERα-deficient and ERβ-deficient mice. Total splenocyte RNA from spleens of mice treated with E2 pellets for 6 wk was used to measure BAFF levels. BAFF was increased after administration of E2 compared with placebo in WT, ERα-deficient and ERβ-deficient mice (n = 5 per group). Student *t* test was used to analyze the significance between the groups.

by insertion of a neomycin resistance gene into exon 2 or 3, respectively, of the coding gene by homologous recombination (40,41). We determined the expression of ERα and ERβ in B cells by analyzing ERα and ERβ transcripts in total splenic B cells of $ERα^{-/-}$, $ERβ^{-/-}$ and WT mice (Figure 1). ERα transcripts were absent in ERα-deficient mice; ERβ transcripts were absent in ERβ-deficient mice. Interestingly, we saw no compensatory overexpression of ERα in ERβ-deficient B cells or ERβ in ERα-deficient B cells.

It has been shown that ERα or ERβ activation can inhibit B-cell maturation in the bone marrow. Studies have yet to elucidate the role of each ER on subsequent B-cell development. To this end, we analyzed B-cell maturation in WT, ERα-deficient and ERβ-deficient mice treated with E2 or placebo. Engagement of ERα in ERβ-deficient mice was sufficient to mediate a marked reduction in transitional B-cell number similar to that seen in WT mice (Figure 2A). These data confirmed the previously reported profound effect of ERα engagement on B-cell lymphopoiesis (42). Engagement of ERβ in ERα-deficient mice also led to a reduction in transitional B cells, although to a lesser degree (Figure 2A). In previous studies, we showed that E2 treatment leads to an expansion of the MZ B-cell subset ($CD21^{high}CD23^{neg}HSA^{low}$) in WT BALB/c mice (24). We confirmed this observation and observed an E2-induced expansion of MZ B cells in both ERα-deficient and ERβ-deficient mice similar to that seen in WT mice (Figure 2B).

Because diminished B-cell lymphopoiesis leads to elevated BAFF expression and increased BAFF enhances MZ B-cell development, we examined BAFF mRNA levels in WT, ERα-deficient and ERβ-deficient mice given E2 or placebo. We were able to detect a significant increase in BAFF in all strains after E2 treatment, probably contributing to the expansion in MZ B cells (Figure 3). The increase was approximately two-fold, similar to the increase in BAFF reported in some patients with SLE (43,44).

BCR Signaling

We previously hypothesized that both the expansion of MZ B cells and loss of B-cell tolerance in E2-treated mice was related to an observed reduction in BCR signal strength in the transitional B-cell

subset. Both elevated BAFF levels and reduced BCR signal strength can result in MZ B-cell expansion. Moreover, it was shown that BCR signal strength helps determine the threshold for apoptosis of developing B cells. Because we observed a significant expansion of MZ B cells when either ERα or ERβ was engaged by ligand, we asked whether BCR signaling was also modulated by engagement of both estrogen receptors. Figure 4A demonstrates a reduction in phosphorylation of Erk1/2 after BCR ligation by anti-IgM F(ab)′$_2$ in transitional B cells from WT and ERβ-deficient mice treated with E2 compared with placebo-treated mice, as detected by phosphoflow. In contrast, transitional B cells from ERα-deficient mice exhibited a significant increase in BCR signal strength after exposure to E2. We also performed Western blot analysis on total splenic B cells examining Erk phosphorylation after BCR engagement. Anti-IgM–induced Erk phosphorylation was greater in B cells from placebo-treated WT and ERβ-deficient mice than from the E2-treated mice. E2 treatment induced a modest decrease in BCR-mediated Erk2 phosphorylation in B cells from ERα-deficient mice (Figure 4B and C), but there was no effect of E2 treatment on Erk1 phosphorylation. Thus, ERα was the primary ER responsible for the E2-induced diminution in the BCR signaling pathways.

Expression of Molecules Regulating B-Cell Survival and Maturation

Previously, we demonstrated that E2 increased expression of CD22, a negative regulator of the BCR. We assumed that the altered expression of this molecule contributed to the E2-mediated change in BCR signaling (37). We, therefore, anticipated an E2-induced upregulation of CD22 in WT and ERβ-deficient mice but not in ERα-deficient mice after E2 exposure, consistent with the demonstration that only ERα engagement led to a reduced BCR signal strength. As shown in Figure 5, engagement of either ERα or ERβ led to an increase in CD22 expression, similar to that seen in WT BALB/c

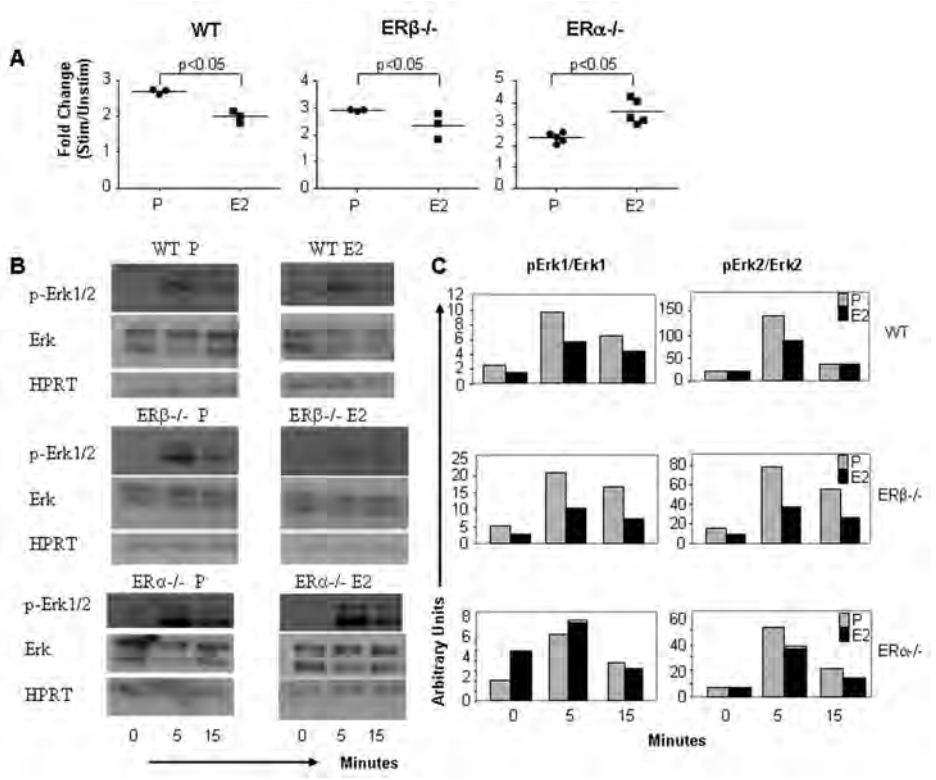


Figure 4. BCR signaling in B cells from WT, ERα-deficient and ERβ-deficient mice. (A) Splenic cells from WT, ERα-deficient and ERβ-deficient mice treated with E2 or placebo were incubated with or without 20 μg/mL anti-IgM F(ab)′$_2$ antibody, and Erk phosphorylation was determined by flow cytometry. Transitional B cells from E2-treated WT and ERβ-deficient mice displayed a decrease in pErk after anti-IgM stimulation compared with transitional B cells from placebo-treated mice (stimulated/unstimulated (Stim/Unstim)) as determined by flow cytometry. There was no reduction in anti-IgM–induced pErk in B cells of E2-treated ERα-deficient mice compared with B cells of placebo-treated mice. (B) Total splenic B cells from WT, ERα-deficient and ERβ-deficient mice were stimulated with 20 μg/mL anti-IgM F(ab)′$_2$ antibody for 0, 5 and 15 min at 37°C, and 20 μg protein at each time point was subjected to Western blotting. Erk phosphorylation was determined by probing the blots with antibodies to Erk and phospho Erk1/2. To normalize for protein levels, the blots were probed with antibodies to HPRT. (C) The blots were scanned to quantitate pErk1:Erk1 as well as pErk2:Erk2 ratio at 0, 5 and 15 min of stimulation with anti-IgM F(ab)′$_2$ antibody and were expressed as arbitrary units.

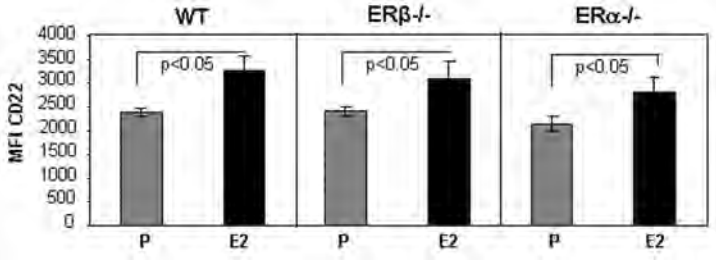


Figure 5. CD22 expression in transitional B cells. CD22 was significantly increased in transitional B cells of WT, ERα-deficient and ERβ-deficient mice administered E2 compared with placebo (n = 5 per group).

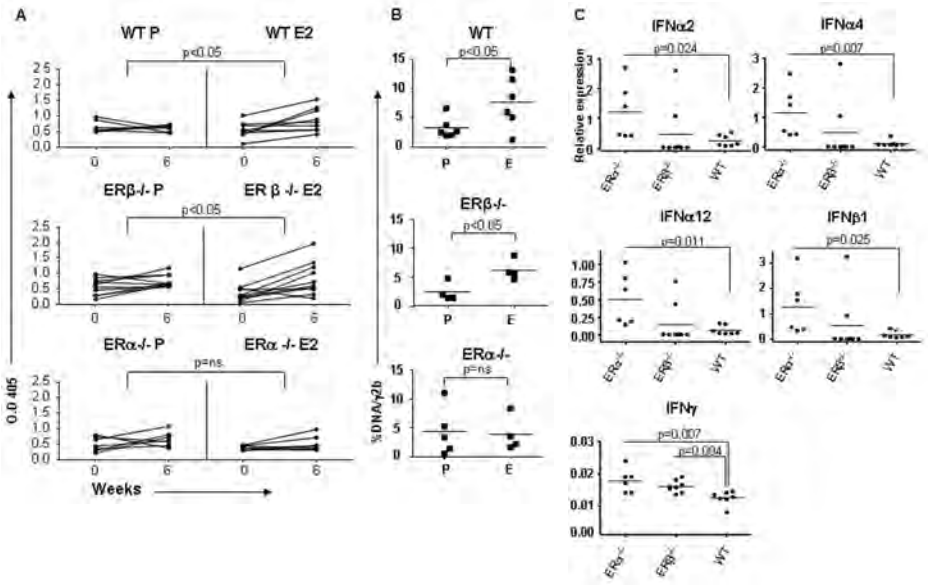


Figure 6. Serum anti-DNA antibody levels, DNA-reactive B cells and IFNγ transcripts in WT, ERα-deficient and ERβ-deficient mice. (A) Serum anti-DNA antibody levels in WT, ERα-deficient and ERβ-deficient mice. E2 or placebo (P) was administered to R4A-Tg WT, ERα-deficient and ERβ-deficient mice for 6 wk. Serum was obtained at several time points and analyzed for anti-dsDNA antibody levels. E2 induced increased anti-DNA antibody titers in WT and ERβ-deficient mice, whereas placebo treatment led to no change in antibody titer. ERα-deficient mice treated with E2 failed to display an increase in anti-DNA antibody titers compared with placebo. (B) Enumeration of DNA-reactive B cells in WT, ERα-deficient and ERβ-deficient mice. E2 or placebo was administered to WT, ERα-deficient and ERβ-deficient mice for 5–6 wk, and the total number of splenic B cells producing γ2b and B cells producing γ2b anti-dsDNA antibody was quantitated by ELISpot assay. The frequency of anti-dsDNA B cells among the γ2b-producing B cells was calculated as DNA spots/γ2b spots. Both WT and ERβ-deficient mice displayed an increased frequency of DNA-reactive B cells after E2 administration compared with placebo administration; ERα-deficient mice showed no E2-induced change in DNA-reactive B cells. Five mice were used per group for these studies. (C) Type 1 (IFNα, IFNβ) and type 2 (IFNγ) transcripts were measured in total splenocytes from $ER\alpha^{-/-}$, $ER\beta^{-/-}$ and WT mice. The relative expression in comparison to Polr2A is represented. Six or eight mice were used in each group for the studies.

mice. This increase was restricted to the transitional B-cell population and was not found in mature B cells (data not shown). Thus, CD22 overexpression was mediated by both ERα and ERβ and was not sufficient to cause a reduced BCR signal in B cells. Additional effects of E2 must be present in B cells of WT and ERβ-deficient E2-treated mice to mediate the change in BCR signal strength.

ERα Mediates an Increase in Anti-dsDNA Antibodies and an Altered B-Cell Repertoire

To analyze changes in autoantibody production and survival of autoreactive B cells, we studied mice that express the heavy chain of an anti-DNA antibody. We mated the R4A transgene onto ERα- or ERβ-deficient BALB/c mice. In the R4A-Tg mouse, a vast majority of the Tg-expressing B cells are allelically excluded and display normal maturation (45). Most express a non-DNA binding antibody or a low affinity DNA binding antibody. There is a small number of allelically included (IgM and IgG2b) anergic B cells that express an anti-DNA IgG2b antibody and a non-DNA reactive IgM antibody, but these cells can be detected only by generation of hybridomas of LPS-stimulated splenic B cells. We assayed serum anti-dsDNA levels after E2 treatment to determine whether B-cell tolerance is breached. DNA ELISAs confirmed the previously reported E2-mediated increase in anti-DNA antibody titers in WT R4A-Tg mice and demonstrated that ERα engagement in ERβ-deficient mice resulted in increased anti-DNA antibody production. ERβ engagement in ERα-deficient mice did not alter anti-DNA antibody levels (Figure 6A). Consistent with this observation, ELISpot analysis demonstrated an increased frequency of splenic B cells spontaneously secreting anti-DNA antibody in both WT and ERβ-deficient R4A-Tg mice after E2 exposure, but not in ERα-deficient R4A-Tg mice after E2 exposure (Figure 6B). The increase in serum anti-dsDNA antibody levels is not due to increased expression of type 1 IFN (IFNα, IFNβ) in splenocytes, since type 1 IFN was increased in $ER\alpha^{-/-}$ and not in WT or $ER\beta^{-/-}$ mice, which displayed an E2-mediated induction of anti-DNA antibodies. IFNγ mRNA levels were modestly increased in ER-deficient mice (Figure 6C). Thus, there was no significant evidence for an effect of IFN on antibody titer.

Because we know the light chains that associate with the R4A heavy chain to produce high-affinity or low-affinity anti-DNA antibodies, we were previously able to show that E2 alters the B-cell repertoire of WT mice by increasing survival of high-affinity DNA-reactive B cells. We, therefore, used single-cell PCR to determine light chain usage in Tg-expressing (γ2b) B cells in WT, ERα-deficient and ERβ-deficient mice with and without E2 exposure. Tg^{+} B cells were first analyzed to confirm that they expressed a γ2b heavy chain and not μ chain, thus maintaining allelic exclusion. The percent of B cells expressing a kappa light chain that associates with the R4A heavy chain to produce a high-affinity anti-dsDNA antibody was increased by E2 exposure in both WT and ERβ-deficient mice compared with placebo-treated mice (Table 1). ERβ engagement did not lead to an increased survival of high-affinity DNA-reactive B cells.

Table 1. Frequency of high- and low-affinity DNA-reactive B cells in WT, ERα-deficient, and ERβ-deficient R4A-Tg mice treated with P or E2.[a]

	WT		ERβ$^{-/-}$		ERα$^{-/-}$	
	P	E2	P	E2	P	E2
High-affinity	3/48 (6.3%)	14/54 (26%)[b]	6/58 (10%)	22/65 (33%)[b]	7/62 (11%)	14/70 (20%)
Low-affinity	7/48 (18%)	6/54 (11%)	6/58 (10%)	7/65 (11%)	8/62 (13%)	8/70 (9%)

[a]The Fisher exact test was performed to compare the frequency of high- and low-affinity DNA-reactive mature B cells between E2-treated and P-treated R4A-Tg WT, R4A-Tg ERβ-deficient, and R4A-Tg ERα-deficient mice.
[b]$P < 0.05$ (P value signifies a difference between P-treated and E2-treated mice).

Table 2. Frequency of high- and low-affinity DNA-reactive B cells in R4A-Tg mice treated with P, E2, or PPT.[a]

	P	E2	PPT
High-affinity	6/67 (9%)	15/65 (23.1%)[b]	14/70 (20%)[b]
Low-affinity	7/67 (10.5%)	5/65 (7.7%)	5/70 (7.1%)

[a]The Fisher exact test was performed to compare the frequency of high- and low-affinity DNA-reactive mature B cells in E2-treated or PPT-treated R4A-Tg WT mice, compared to P-treated mice.
[b]$P < 0.05$ (P value signifies a difference compared to P-treated mice).

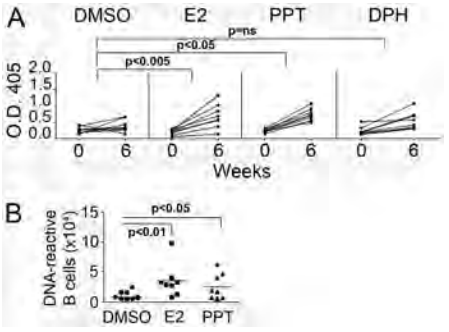


Figure 7. ERα agonists trigger autoimmunity. (A) Serum anti-DNA antibody levels in R4A-Tg mice given ER agonists. E2, PPT, DPN or vehicle were administered to R4A-Tg mice for 6 wks. Serum was obtained at several time points and analyzed for anti-dsDNA antibody levels. Mice administered E2 and PPT exhibited increased titers of anti-dsDNA antibody compared with mice administered vehicle. (B) DNA-reactive B cells in R4A-Tg mice given ERα agonists. E2, PPT, DPN or vehicle were administered to R4A-Tg mice for 6 wks. Total splenic IgG2b-producing B cells and IgG2b B cells producing anti-dsDNA antibody were enumerated. The frequency of anti-dsDNA B cells among the IgG2b-producing cells was calculated as DNA spots/IgG2b spots. Both E2 and PPT administration led to an increased frequency of DNA-reactive B cells compared with vehicle.

WT Mice Treated with an ERα Agonist

To confirm these observations on the importance of ERα in abrogating B-cell tolerance, we treated WT R4A-Tg mice with E2, the ERα agonist PPT, the ERβ agonist DPN or placebo. Administration of E2 and the ERα agonist PPT, but not placebo, led to an increase in anti-dsDNA antibody titers and an increased frequency of DNA-reactive B cells—but administration of DPN, the ERβ agonist, did not (Figure 7 and Table 2). We therefore focused subsequent studies on ERα and showed that both E2 and PPT caused a similar reduction in transitional B-cell number and expansion of MZ B cells compared with placebo in WT mice (Figure 8A, B). We were also able to demonstrate by flow cytometry a reduction in BCR-mediated Erk phosphorylation in WT mice administered E2 or PPT compared with placebo (Figure 9A, B). Moreover, administration of E2 or PPT led to an increase in CD22 expression (Figure 9C).

DISCUSSION

We have previously shown that continuous *in vivo* exposure to E2, at a concentration of 75–100 pg/mL, which is equivalent to a concentration at the high end of the estrus cycle, alters B-cell maturation, reduces BCR signaling strength and upregulates CD22 expression in WT BALB/c mice (24,37,39). In this study, we asked which ER was responsible for these changes. We addressed this question by studying WT mice with a specific deletion of ERα or ERβ. We also showed E2 exposure breaks tolerance in R4A-Tg mice; therefore, we studied survival and activation of autoreactive B cells in R4A-Tg mice with a deletion in ERα or ERβ. Our data demonstrate that the alterations in splenic B-cell maturation seen in ER-sufficient mice exposed to a continuous high, but physiologic, level of E2 can all be mediated by either ERα or ERβ. While the upregulation of CD22 was also seen after engagement of either ERα or ERβ, the E2-mediated change in BCR signal strength depended on engagement of ERα. Consistent with the role of BCR signaling in negative selection, an increase in autoreactive B cells was seen only after ERα engagement. Concordant results were obtained in ERβ-deficient mice and in WT mice exposed to high levels of an ERα-specific agonist.

This study confirms data from other investigators who have demonstrated that E2 decreases B-cell lymphopoiesis in the bone marrow and that this effect can be mediated through either ERα or ERβ (33,46). The decreased lymphopoiesis has been shown to reflect an E2-mediated decrease in IL-7 production by bone marrow stromal cells, although a B-cell intrinsic response to increased E2 at early stages of B-cell development has also been reported (35,36).

There are two possible mechanisms for the enhanced MZ B-cell population that was observed after either ERα or ERβ engagement. First, E2 induced an increase in BAFF levels in WT, ERα-deficient and ERβ-deficient mice two-fold similar to the increase in BAFF levels in SLE patients (43,44). It is now clear that low B-cell numbers, as occurs after increased

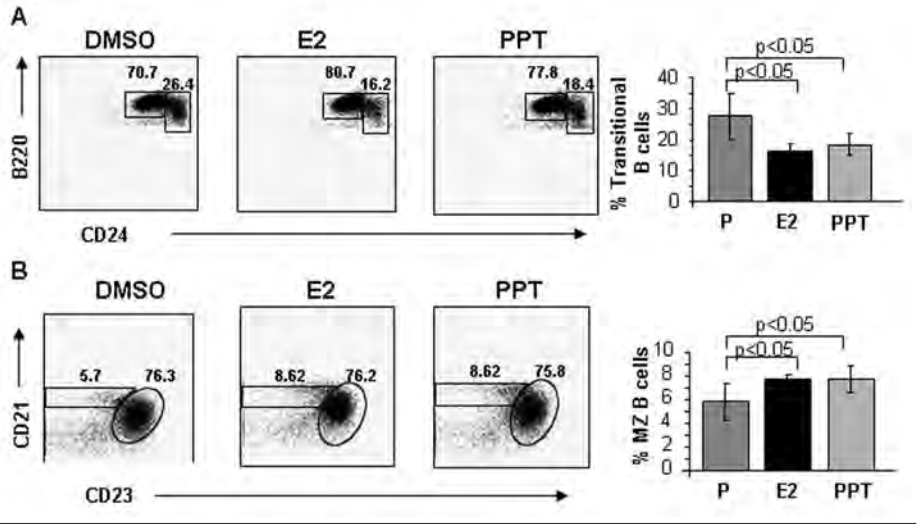


Figure 8. Splenic B-cell subsets in WT BALB/c mice given ER agonists or vehicle. (A) ERα regulates transitional B cells in the spleen. WT BALB/c mice were treated with E2, the ERα-specific agonist PPT or vehicle (DMSO) for 3 wks, and splenocytes were analyzed by flow cytometry. E2 and PPT reduced transitional B cells in WT mice compared with vehicle. O.D., optical density. (B) MZ B-cell subsets in E2 and PPT-treated WT mice. MZ B cells were expanded by E2 and PPT treatment compared with vehicle. At least five mice were analyzed in each group, and representative dot plots are shown.

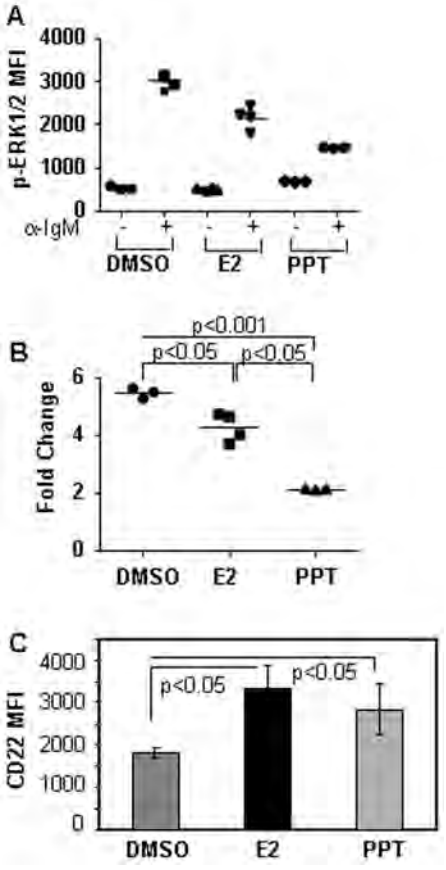


Figure 9. BCR signaling is altered by E2 and PPT. (A,B) BCR signaling in transitional B cells of BALB/c mice treated with E2, PPT or vehicle. B cells were incubated with or without 20 μg/mL anti-IgM F(ab)'$_2$, and Erk phosphorylation was determined by flow cytometry. B cells from E2 and PPT-treated B cells displayed a lesser increase in anti-IgM–induced Erk phosphorylation compared with B cells from vehicle-treated mice. (C) An increase in mean fluorescence intensity (MFI) of CD22 expression is evident in WT transitional B cells after E2 and PPT exposure compared with vehicle exposure.

E2 exposure due to reduced B-cell lymphopoeisis, always results in high serum BAFF levels (47). Studies of BAFF transgenic mice have shown that elevated BAFF causes an increase in MZ B cells (48). Although it was reported that B cells can express BAFF mRNA and perhaps BAFF protein (49), most BAFF protein is produced by other cell types (50). It may be, therefore, that the contribution of BAFF to the increase in MZ B cells occurs as an indirect effect of E2 on B cells.

The E2-mediated increase in CD22 expression seen in WT, ERα-deficient and ERβ-deficient mice might also contribute to the expansion of MZ B cells. CD22-deficient mice have a reduced MZ B-cell population. Moreover, mice deficient in ST6GALI, an enzyme involved in the generation of α2,6 sialic acid epitope, the ligand for CD22, exhibit a diminished MZ subset (51). Mice expressing a mutated CD22 that lacks the ligand binding domain also exhibit a diminished MZ B-cell subset; thus, MZ B-cell expansion may reflect a ligand-dependent consequence of increased CD22 expression (52).

Somewhat surprisingly, our studies demonstrate that the enhanced MZ B-cell population did not depend on a decreased BCR signal strength. Thus, if overexpression of CD22 contributed to the MZ B-cell expansion, it is not because of an inhibitory effect on the BCR signaling pathway. Interestingly, mice expressing a mutated CD22 which fails to bind ligand exhibit a reduced MZ subset but display no change in BCR signaling; thus, changes in CD22 function can lead to a change in MZ B-cell number without a change in BCR signaling (52).

In our studies, prolonged B-cell exposure to E2 reduced Erk phosphorylation after BCR ligation through ERα engagement, in particular, in transitional B cells. While we had previously believed the reduction in BCR signal strength was due to increased expression of CD22, our current data refute this hypothesis. We do not currently know the mechanisms for the reduced BCR signal strength that occurs after E2 engagement through ERα. Interestingly, T cells from SLE patients, exposed to E2, exhibit reduced Erk phosphorylation after TCR/CD3 stimulation (53). Additionally, reduced Erk phosphorylation was recently shown to associate with DNA hypomethylation, a trigger for the development of a lupus-like serology (54–56). Therefore, it is plausible that the E2-mediated reduction of phosphorylated Erk is associated with DNA hypomethylation in B cells. This point will need to be addressed in future studies.

We observed an E2-induced breakdown in B-cell tolerance in both WT and ERβ-deficient mice after E2 exposure, demonstrating that this effect is also me-

diated by ERα. In contrast, ERβ engagement did not alter B-cell selection. BCR signal strength correlated with stringency of negative selection of autoreactive B cells; thus, WT and ERβ-deficient mice exposed to E2 exhibited a reduced BCR signal and increased survival of high-affinity DNA-reactive B cells and elevated serum titers of anti-DNA antibody. In previous *in vitro* studies, we demonstrated that the effect of E2 on BCR signaling and BCR-mediated apoptosis in WT B cells was B-cell intrinsic (37). The change in BCR-mediated apoptosis may be sufficient to alter B-cell selection; it is possible, however, that increased BAFF levels contribute to this phenomenon also in the *in vivo* situation, since BAFF has been shown to alter the threshold for negative selection and permit survival of autoreactive B cells, even in the absence of an altered BCR signal (48). Finally, it is possible that ERα engagement affects other, as yet unknown, pathways to alter B-cell negative selection, although our data, in contrast to a study in NZB/W mice, do not demonstrate an E2-mediated decrease in IFNγ levels (33).

The observations presented here have clinical implications. They suggest that selective antagonism of ERα may alter the threshold for negative selection during B-cell maturation to reduce autoreactivity in the naive, immunocompetent B-cell repertoire. It might be possible to design a drug that could specifically target ERα in B cells; this approach would not affect ERβ-regulated gene expression and would limit the effects of ERα antagonism in other tissues. This result would represent a nonimmunosuppressive approach to lupus therapy. Furthermore, understanding functional polymorphisms in ERα or other genes in ERα-regulated pathways may help explain why some but not all lupus patients may experience hormonally induced exacerbations of disease. Further studies to understand in detail the molecular pathways that underlie these changes in B-cell selection may identify important new therapeutic targets in autoimmune disease.

ACKNOWLEDGMENTS

We would like to thank Stella Stefanova and Alla Tashmukhamedova for technical assistance and Sylvia Jones for help with the preparation of the manuscript. This work was supported by grants from the DOD and the NIH.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

1. Ferry H, *et al.* (2006) B-cell tolerance. *Transplantation* 81:308–15.
2. Thomas MD, Srivastava B, Allman D. (2006) Regulation of peripheral B cell maturation. *Cell Immunol.* 239:92–102.
3. Mok CC, Lau CS. (2003) Pathogenesis of systemic lupus erythematosus. *J. Clin. Pathol.* 56:481–90.
4. Rahman A. (2004) Autoantibodies, lupus and the science of sabotage. *Rheumatology (Oxford).* 43:1326–36.
5. Tan EM, *et al.* (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 25:1271–7.
6. Alexander JJ, Quigg RJ. (2007) Systemic lupus erythematosus and the brain: what mice are telling us. *Neurochem Int.* 50:5–11.
7. Yung S, Chan TM. (2008) Anti-DNA antibodies in the pathogenesis of lupus nephritis: the emerging mechanisms. *Autoimmun. Rev.* 7:317–21.
8. Kowal C, Weinstein A, Diamond B. (1999) Molecular mimicry between bacterial and self antigen in a patient with systemic lupus erythematosus. *Eur. J. Immunol.* 29:1901–11.
9. Sanchez-Guerrero J, Aranow C, Mackay M, Volpe B, Diamond B. (2008) Neuropsychiatric systemic lupus erythematosus reconsidered. *Nat. Clin. Pract. Rheumatol.* 4:112–3.
10. Davidson A, Diamond B. (2001) Autoimmune diseases. *N. Engl. J. Med.* 345:340–50.
11. Wakeland EK, Liu K, Graham RR, Behrens TW. (2001) Delineating the genetic basis of systemic lupus erythematosus. *Immunity* 15:397–408.
12. Lahita RG. (1993) Sex hormones as immunomodulators of disease. *Ann. N. Y. Acad. Sci.* 685:278–87.
13. Ansar Ahmed S, Dauphinee MJ, Talal N. (1985) Effects of short-term administration of sex hormones on normal and autoimmune mice. *J. Immunol.* 134:204–10.
14. Cohen-Solal JF, *et al.* (2008) Hormonal regulation of B-cell function and systemic lupus erythematosus. *Lupus* 17:528–32.
15. Weidler C, Harle P, Schedel J, Schmidt M, Scholmerich J, Straub RH. (2004) Patients with rheumatoid arthritis and systemic lupus erythematosus have increased renal excretion of mitogenic estrogens in relation to endogenous antiestrogens. *J. Rheumatol.* 31:489–94.
16. Smith-Bouvier DL, *et al.* (2008) A role for sex chromosome complement in the female bias in autoimmune disease. *J. Exp. Med.* 205:1099–108.
17. Roubinian J, Talal N, Siiteri PK, Sadakian JA. (1979) Sex hormone modulation of autoimmunity in NZB/NZW mice. *Arthritis Rheum.* 22:1162–9.
18. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. (1978) Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J. Exp. Med.* 147:1568–83.
19. Carlsten H, Nilsson N, Jonsson R, Backman K, Holmdahl R, Tarkowski A. (1992) Estrogen accelerates immune complex glomerulonephritis but ameliorates T cell-mediated vasculitis and sialadenitis in autoimmune MRL lpr/lpr mice. *Cell Immunol.* 144:190–202.
20. Shefner R, Kleiner G, Turken A, Papazian L, Diamond B. (1991) A novel class of anti-DNA antibodies identified in BALB/c mice. *J. Exp. Med.* 173:287–96.
21. Offen D, Spatz L, Escowitz H, Factor S, Diamond B. (1992) Induction of tolerance to an IgG autoantibody. *Proc. Natl. Acad. Sci. U. S. A.* 89:8332–6.
22. Bynoe MS, Grimaldi CM, Diamond B. (1999) Estrogen up-regulates Bcl-2 and blocks tolerance induction of naïve B cells. *Proc. Natl. Acad. Sci. U. S. A.* 97:2703–8.
23. Spatz L, Saenko V, Iliev A, Jones L, Geskin L, Diamond B. (1997) Light chain usage in anti-double-stranded DNA B cell subsets: role in cell fate determination. *J. Exp. Med.* 185:1317–26.
24. Grimaldi CM, Michael DJ, Diamond B. (2001) Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *J. Immunol.* 167:1886–90.
25. Matthews J, Gustafsson JA. (2003) Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol. Interv.* 3:281–92.
26. Cutolo M, *et al.* (1996) Androgen and estrogen receptors are present in primary cultures of human synovial macrophages. *J. Clin. Endocrinol. Metab.* 81:820–7.
27. Sakazaki H, Ueno H, Nakamuro K. (2002) Estrogen receptor alpha in mouse splenic lymphocytes: possible involvement in immunity. *Toxicol. Lett.* 133:221–9.
28. Kovats S, Carreras E. (2008) Regulation of dendritic cell differentiation and function by estrogen receptor ligands. *Cell Immunol.* 252:81–90.
29. Lindberg MK, *et al.* (2003) Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. *Mol. Endocrinol.* 17:203–8.
30. Monroe DG, Secreto FJ, Subramaniam M, Getz BJ, Khosla S, Spelsberg TC. (2005) Estrogen receptor alpha and beta heterodimers exert unique

effects on estrogen- and tamoxifen-dependent gene expression in human U2OS osteosarcoma cells. *Mol. Endocrinol.* 19:1555–68.
31. Lee YJ, *et al.* (2004) Association of the oestrogen receptor alpha gene polymorphisms with disease onset in systemic lupus erythematosus. *Ann. Rheum. Dis.* 63:1244–9.
32. Kassi E, Vlachoyiannopoulos PG, Kominakis A, Kiaris H, Moutsopoulos HM, Moutsatsou P. (2005) Estrogen receptor alpha gene polymorphism and systemic lupus erythematosus: a possible risk? *Lupus* 14:391–8.
33. Bynote KK, Hackenberg JM, Korach KS, Lubahn DB, Lane PH, Gould KA. (2008) Estrogen receptor-alpha deficiency attenuates autoimmune disease in (NZB x NZW)F1 mice. *Genes Immun.* 9:137–52.
34. Svenson JL, EuDaly J, Ruiz P, Korach KS, Gilkeson GS. (2008) Impact of estrogen receptor deficiency on disease expression in the NZM2410 lupus prone mouse. *Clin. Immunol.* 128:259–68.
35. Medina KL, Garrett KP, Thompson LF, Rossi MI, Payne KJ, Kincade PW. (2001) Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. *Nat. Immunol.* 2:718–24.
36. Thurmond TS, Murante FG, Staples JE, Silverstone AE, Korach KS, Gasiewicz TA. (2000) Role of estrogen receptor alpha in hematopoietic stem cell development and B lymphocyte maturation in the male mouse. *Endocrinology* 141:2309–18.
37. Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. (2002) Estrogen alters thresholds for B cell apoptosis and activation. *J. Clin. Invest.* 109:1625–33.
38. Frasor J, Barnett DH, Danes JM, Hess R, Parlow AF, Katzenellenbogen BS. (2003) Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ER-beta in the uterus. *Endocrinology* 144:3159–66.
39. Venkatesh J, Peeva E, Xu X, Diamond B. (2006) Cutting edge: hormonal milieu, not antigenic specificity, determines the mature phenotype of autoreactive B cells. *J. Immunol.* 176:3311–4.
40. Krege JH, *et al.* (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *J. Biol. Chem.* 95:15677–82.
41. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. U. S. A.* 90:11162–6.
42. Smithson G, Couse JF, Lubahn DB, Korach KS, Kincade PW. (1998) The role of estrogen receptors and androgen receptors in sex steroid regulation of B lymphopoiesis. *J. Immunol.* 161:27–34.
43. Zhang J, *et al.* (2001) Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J. Immunol.* 166:6–10.
44. Cheema GS, Roschke V, Hilbert DM, Stohl W. (2001) Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. *Arthritis Rheum.* 44:3–9.
45. Iliev A, Spatz L, Ray S, Diamond B. (1994) Lack of allelic exclusion permits autoreactive B cells to escape deletion. *J. Immunol.* 153:3551–6.
46. Islander U, *et al.* (2003) Influence of oestrogen receptor alpha and beta on the immune system in aged female mice. *Immunology* 110:149–57.
47. Lesley R, *et al.* (2004) Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity* 20:441–53.
48. Thien M, *et al.* (2004) Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity* 20:785–98.
49. Chu VT, Enghard P, Riemekasten G, Berek C. (2007) In vitro and in vivo activation induces BAFF and APRIL expression in B cells. *J. Immunol.* 179:5947–57.
50. Brink R. (2006) Regulation of B cell self-tolerance by BAFF. *Semin. Immunol.* 18:276–83.
51. Ghosh S, Bandulet C, Nitschke L. (2006) Regulation of B cell development and B cell signalling by CD22 and its ligands alpha2,6-linked sialic acids. *Int. Immunol.* 18:603–11.
52. Poe JC, *et al.* (2004) CD22 regulates B lymphocyte function in vivo through both ligand-dependent and ligand-independent mechanisms. *Nat. Immunol.* 5:1078–87.
53. Cedeno S, *et al.* (2003) Defective activity of ERK-1 and ERK-2 mitogen-activated protein kinases in peripheral blood T lymphocytes from patients with systemic lupus erythematosus: potential role of altered coupling of Ras guanine nucleotide exchange factor hSos to adapter protein Grb2 in lupus T cells. *Clin. Immunol.* 106:41–9.
54. Lu LJ, Liehr JG, Sirbasku DA, Randerath E, Randerath K. (1988) Hypomethylation of DNA in estrogen-induced and -dependent hamster kidney tumors. *Carcinogenesis* 9:925–9.
55. Kovalchuk O, *et al.* (2007) Estrogen-induced rat breast carcinogenesis is characterized by alterations in DNA methylation, histone modifications and aberrant microRNA expression. *Cell Cycle* 6:2010–8.
56. Deng C, *et al.* (2001) Decreased Ras-mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients. *Arthritis Rheum.* 44:397–407.

Antigen Is Required for Maturation and Activation of Pathogenic Anti-DNA Antibodies and Systemic Inflammation

Jeganathan Venkatesh, Hajime Yoshifuji, Daisuke Kawabata, Prameladevi Chinnasamy, Anfisa Stanevsky, Christine M. Grimaldi, Joel Cohen-Solal and Betty Diamond

J Immunol 2011;186;5304-5312; Prepublished online 28 March 2011;
doi:10.4049/jimmunol.1000224
http://www.jimmunol.org/content/186/9/5304

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Antigen Is Required for Maturation and Activation of Pathogenic Anti-DNA Antibodies and Systemic Inflammation

Jeganathan Venkatesh, Hajime Yoshifuji,[1] Daisuke Kawabata,[1] Prameladevi Chinnasamy, Anfisa Stanevsky,[2] Christine M. Grimaldi,[3] Joel Cohen-Solal, and Betty Diamond

Systemic lupus erythematosus is an autoimmune disease characterized by autoantibodies and systemic inflammation that results in part from dendritic cell activation by nucleic acid containing immune complexes. There are many mouse models of lupus, some spontaneous and some induced. We have been interested in an induced model in which estrogen is the trigger for development of a lupus-like serology. The R4A transgenic mouse expresses a transgene-encoded H chain of an anti-DNA Ab. This mouse maintains normal B cell tolerance with deletion of high-affinity DNA-reactive B cells and maturation to immunocompetence of B cells making nonglomerulotropic, low-affinity DNA-reactive Abs. When this mouse is given estradiol, normal tolerance mechanisms are altered; high-affinity DNA-reactive B cells mature to a marginal zone phenotype, and the mice are induced to make high titers of anti-DNA Abs. We now show that estradiol administration also leads to systemic inflammation with increased B cell-activating factor and IFN levels and induction of an IFN signature. DNA must be accessible to B cells for both the production of high-affinity anti-DNA Abs and the generation of the proinflammatory milieu. When DNase is delivered to the mice at the same time as estradiol, there is no evidence for an abrogation of tolerance, no increased B cell-activating factor and IFN, and no IFN signature. Thus, the presence of autoantigen is required for positive selection of autoreactive B cells and for the subsequent positive feedback loop that occurs secondary to dendritic cell activation by DNA-containing immune complexes. ***The Journal of Immunology*****, 2011, 186: 5304–5312.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of multiple autoantibodies (1, 2). Anti-DNA Abs are among the most significant because they are common, they are essentially diagnostic of SLE, their titers fluctuate with disease activity (3), and they contribute to tissue injury in the kidney and probably brain as well (4–6). For these reasons, they have been studied extensively.

There are several ways to induce the production of anti-DNA Abs in nonspontaneously autoimmune mice. DNA has been coupled to a protein carrier to induce production of anti-DNA Abs (7–10). In these studies, the protein is foreign and, therefore, immunogenic to T cells and the DNA functions as a hapten to activate DNA-reactive B cells. Peptide mimetopes of DNA have been exploited to induce a T cell-dependent anti-DNA response (11–13). In some studies, apoptotic cells have been administered to mice and shown to induce production of an anti-DNA response (14–18). It has been demonstrated that many perturbations of the immune system that diminish the clearance of apoptotic debris can stimulate the production of anti-DNA Abs (19–23). The apoptotic material contains endogenous ligands for TLRs, and so may transform tolerogenic dendritic cells (DCs) into immunogenic DCs and trigger an immune response to self Ag (24–28). Once anti-DNA Abs are present, they form DNA-containing immune complexes that, when internalized by DCs, can activate TLR9 and excite production of B cell-activating factor (BAFF) and IFN-α (29, 30). When the immune complexes are internalized by DNA-specific B cells, the B cells can be activated through TLR9 engagement to secrete pathogenic Ab.

We have been studying the induction of anti-DNA Abs in the R4A transgene (Tg) BALB/c mouse that harbors an IgG2b H chain of an anti-DNA Ab (31, 32). Approximately 90% of B cells express an endogenous H and L chain, and 10% express the R4A Tg H chain with a spectrum of L chains. Tg-expressing B cells undergo a normal maturation program in the bone marrow (33). Under normal conditions, B cell tolerance is maintained (33–36). B cells expressing the R4A H chain along with a L chain that produces high-affinity DNA binding are deleted at both the immature stage in the bone marrow and the transitional stage in the spleen (35, 36). Many B cells expressing the R4A H chain along with a L chain producing a low-affinity DNA binding are allowed to mature to immunocompetence, as are B cells expressing the R4A H chain along with a L chain that confers no DNA binding (33, 34, 37).

When R4A Tg BALB/c mice are given estradiol (E2) pellets to achieve a sustained serum concentration of 75–100 pg/ml, the R4A-expressing high-affinity DNA-reactive B cells survive negative selection and mature to immunocompetence as marginal zone (MZ) B cells (38). Our studies have shown that E2 causes a decrease in BCR signaling strength and reduced BCR-mediated

Center for Autoimmune and Musculoskeletal Disease, Feinstein Institute for Medical Research, Manhasset, NY 11030

[1]Current address: Division of Rheumatology & Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan.

[2]Current address: Department of Medicine, Sheba Medical Center, Tel-Hashomer, Israel.

[3]Current address: Department of Toxicology, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT.

Received for publication January 25, 2010. Accepted for publication March 4, 2011.

This work was supported by grants from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (to C.M.G.) and the Department of Defense (to B.D.); the National Institutes of Health; a Career Development award from the Systemic Lupus Erythematosus Foundation (to J.V.); and a fellowship from the Arthritis Foundation (to D.K.).

Address correspondence and reprint requests to Dr. Betty Diamond, Center for Autoimmune and Musculoskeletal Disease, Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030. E-mail address: bdiamond@nshs.edu

Abbreviations used in this article: AP, alkaline phosphatase; BAFF, B cell-activating factor; DC, dendritic cell; E2, estradiol; HI, heat-inactivated; MZ, marginal zone; NZB/W, New Zealand Black/White; P, placebo; SLE, systemic lupus erythematosus; Tg, transgene.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000224

apoptosis of immature and transitional B cells (36, 39). In this study, we asked whether the E2-induced lupus-like serology was accompanied by other features of SLE, such as elevated serum BAFF levels and an IFN signature. We further asked whether Ag was needed for the proinflammatory milieu and the positive selection and activation of high-affinity DNA-reactive B cells. We demonstrate that Ag is critical to the generation of the proinflammatory milieu. It is also required for positive selection of pathogenic autoreactive B cells; the diminished negative selection alone that is secondary to reduced BCR signaling is not alone sufficient for the development of pathogenic autoreactivity. These observations have important clinical implications.

Materials and Methods

Mice, hormone treatment, and therapeutic regimens

R4A Tg BALB/c mice, described previously (31), were bred and maintained at the Feinstein Institute for Medical Research. All animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Sixty-day time-release pellets (Innovative Research of America) containing E2 (0.18 mg) or placebo (P; vehicle control) were implanted beneath the skin of 8- to 10-wk-old female mice. The E2 pellets maintain serum E2 concentrations of 75–100 pg/ml (34). To avoid the problem of fluctuations in the endogenous E2 levels that occur in P-treated mice, all mice were ovariectomized prior to implantation of pellets. For experimental studies, mice were divided into four groups, as follows: P, E2, E2 plus DNase, and E2 plus heat-inactivated (HI) DNase. DNase treatment of mice was performed as reported by Macanovic et al. (40). Briefly, mice were injected i.p. daily with 450 μg bovine pancreatic DNase (Sigma-Aldrich) or HI enzyme (68°C for 15 min) in 200 μl saline for 6 wk. Before the start of treatment, and at weekly intervals until 6 wk, animals were bled by retro-orbital puncture. Urine was collected at both the beginning and the end of the experiment to examine the level of proteinuria.

Flow cytometry

Splenocytes from R4A Tg mice treated with P, E2, E2 plus DNase, and E2 plus HI DNase were isolated, Fc blocked, and stained with PerCP-labeled anti-B220, FITC-labeled anti-CD21/CD35 Ab, PE-Cy7–labeled anti-CD23 Ab, Pacific blue-labeled anti-CD24 Ab, PE-labeled anti-IgG2b Ab (BD Pharmingen), and allophycocyanin-labeled anti-AA4.1 Ab (eBioscience) at 4°C for 30 min and washed with PBS. The stained cells were analyzed by flow cytometry using an LSRII instrument (BD Biosciences), and the data were analyzed using Flowjo software (Tree Star).

Measurement of dsDNA in plasma

dsDNA was measured in the plasma of the experimental mice using picogreen dsDNA reagent (Invitrogen), according to the manufacturer's instructions. Different dilutions of plasma in 100 μl Tris-EDTA buffer were incubated with 100 μl picogreen assay reagent. The mixture was incubated for 5 min at room temperature, the fluorescence was measured using a fluorescence microplate reader, and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm) were used. The dsDNA in the samples was quantitated by means of a standard curve using lambda DNA (25 pg/ml to 25 ng/ml) as the standard. Appropriate controls such as plasma from BALB/c mice and plasma from New Zealand Black/White (NZB/W) young and sick mice were used to validate the picogreen assay.

Measurement of serum DNase

The levels of DNase protein in the serum of mice were measured by a sandwich ELISA. ELISA plates (Costar) were coated with anti-DNase Ab (1:1000 dilution). Serum samples at different dilutions were added to the plates subsequent to blocking with 1.0% BSA/PBS, followed by the addition of biotinylated anti-DNase Ab. DNase protein levels were detected using streptavidin coupled to alkaline phosphatase (AP; 1:1000 dilution). A standard curve with purified DNase was generated to calculate the concentration of DNase in serum.

Anti-DNase Abs in serum

Abs to DNase in mice sera were assayed by ELISA after 6 wk of treatment with P, E2, E2 plus DNase, or E2 plus HI DNase. Bovine pancreatic DNase-coated plates (10 μg/ml) were blocked with 1.0% BSA/PBS, followed by the addition of serum (1:250 dilution) from R4A Tg mice treated with P, E2, E2 plus DNase, and E2 plus HI DNase for 6 wk. The plates were probed with AP-conjugated anti-mouse IgG (1:1000 dilution), developed with AP substrate, and measured at 405 nm.

Analysis of Vκ-Jκ L chain genes by single-cell RT-PCR

Splenocytes from three mice in each experimental group were stained with Abs specific for B220, IgG2b, and AA4.1, and the mature ($B220^+IgG2b^+AA4.1^-$) and immature ($B220^+IgG2b^+AA4.1^+$) Tg^+ B cells were sorted as single cells into 96-well plates using a FACS Aria cell sorter (BD Biosciences). cDNA was prepared from the single cells, and the Vκ-Jκ L chain genes were amplified by two rounds of PCR, as previously described (41), using the following primers: universal Vκ, 5′-GGCTGCAGSTTCAGTGGCAGTGGRTCWGGRAC-3′ plus C region primer (Cκ) (first round), 5′-TGGATGGGTGGGAAGATG-3′ and Cκ (second round), 5′-AAGATGGATACAGTTGGT-3′. Sequence analysis of the PCR products was performed using the second-round Cκ primer (Genewiz) subsequent to exo-shrimp alkaline phosphatase treatment (USB Biochemicals).

Real-time PCR

Splenocyte total RNA was isolated using the RNeasy kit from Qiagen, and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed with a Roche 480 light cycler using Roche 480 master mix (Roche Applied Science) and TaqMan primer/probe sets (Applied Biosystems). The relative expression of BAFF, type I IFNs α and β, and the IFN-regulated genes *mx-1* and *ifi202b* was determined in comparison with polymerase (RNA) II (DNA-directed) polypeptide A (*polr2a*). Data were analyzed using the Pfaffl method (42).

Measurement of serum BAFF

An ELISA was performed to determine serum BAFF levels, as described previously (41). Ninety-six–well plates were coated with 5 μg/ml anti-mouse BAFF mAb (clone 5A8; Apotech) overnight at 4°C and blocked with 5% BSA/PBS. Serial dilutions of mouse sera or mouse rBAFF (Apotech) were added to the wells, followed by 10 μg/ml biotinylated anti-mouse BAFF mAb (clone 1C9; Apotech) and HRP-labeled streptavidin. The plates were developed with HRP substrate, and the OD was measured at 450 nm.

Anti-dsDNA Ab ELISA

Serum anti-DNA Ab levels were determined, as previously described (43). Immulon 2HB 96-well plates (Thermo LabSystems) were coated with 100 μl/ml sonicated calf thymus DNA that had been passed through a nitrocellulose filter to remove ssDNA. Mouse serum at different dilutions was added to plates after blocking with 1.0% BSA/PBS. $IgG2b^+$ anti-DNA Abs were detected using AP-labeled anti-mouse IgG2b Ab (Southern Biotechnology). Purified R4A mAb was used (1–50 μg/ml) to generate a standard curve for calculating the concentration of anti-dsDNA Abs in the sera.

DNA inhibition ELISAs

The linear range of DNA reactivity was determined by the generation of dilution curves for serum samples from three each E2-, E2 plus DNase-, and E2 plus HI-inactivated DNase-treated R4A Tg mice. Serum samples were diluted (1:100) and preincubated with various concentrations of sonicated DNA (~2.0 kb in length) for 2 h at 37°C, and the remaining DNA reactivity was measured by DNA ELISA. The range of relative affinities of the anti-DNA Abs present in the sera was calculated, as previously described (44).

Serum treatment of DCs

Splenic DCs isolated from 20 10-wk-old BALB/c mice using CD11c-coated microbeads (Miltenyi Biotec) were resuspended in RPMI 1640 complete medium containing 10% FBS. The cell purity was >85%, as assessed by flow cytometry. A total of 7.5×10^5 cells was plated in 48-well tissue culture plates (Costar) containing 500 μl RPMI 1640 complete medium and stimulated with 5 μl (1% final concentration) serum from R4A Tg mice treated with P, E2, E2 plus DNase, or E2 plus HI DNase (three in each group) for 16 h. The cells were harvested and RNA isolated using RNeasy kit (Qiagen).

Renal pathology

Kidneys from the different experimental groups of R4A Tg mice (three per group) described above were fixed in formalin, paraffin embedded, sectioned (10 μm thickness), stained with biotinylated anti-mouse IgG, and developed with an AP ABC detection kit (Vector Laboratories).

Glomerular IgG deposition in kidney sections was visualized under a Zeiss microscope at original magnifications ×5 and ×20. The number of glomeruli present in three different microscopic fields for each sample was determined. Three mice in each group were analyzed, and the mean percentage of positive glomeruli is shown. The investigator was blinded to the origin of the kidneys.

Analysis of proteinuria

Proteinuria was measured using Bayer reagent strips (Bayer), according to the manufacturer's instructions, as well as by measuring total protein in the urine using the Coomassie blue reagent (Pierce).

Studies of renal pathogenicity of anti-dsDNA Ab-containing serum

Sera (100 μl) from R4A Tg mice treated with P, E2, E2 plus DNase, or E2 plus HI DNase were administered i.p. to 8-wk-old SCID mice (Jackson ImmunoResearch Laboratories). After 24 h, kidneys from the SCID mice were analyzed for glomerular IgG deposition, as described above. Purified R4A (75 μg), which has previously been demonstrated to deposit in kidneys of SCID mice, was used as a positive control (45).

Statistical analysis

Statistical analysis was performed using unpaired Student's *t* test, the exact Kruskal-Wallis test, and Fisher's exact tests, used as appropriate. A p value <0.05 was considered statistically significant.

Results

Generation of a proinflammatory milieu by E2 administration

R4A Tg mice harbor the H chain of the nephritogenic R4A anti-DNA Ab (31). These mice normally maintain B cell tolerance; upon exposure to increased levels of E2, they display an altered B cell repertoire with enhanced survival and activation of high-affinity DNA-reactive B cells. Elevated anti-dsDNA Ab levels, immune complex deposition in kidneys, and subsequent proteinuria can be observed, peaking ~6 wk after initiation of treatment and remaining high for months thereafter (33) (J. Venkatesh, E. Peeva, and B. Diamond, unpublished observations). The mice exhibit minimal inflammation in the kidney despite the presence of IgG deposition, presumably because they lack the genetic background necessary for renal inflammation. Hence, the R4A Tg mouse model is a useful model system to study some downstream effects of anti-DNA Abs in a host devoid of pre-existing immunologic abnormalities.

Studies in SLE have shown that DNA-containing immune complexes can activate DCs in vitro to produce both BAFF and IFN-α (29, 30), leading to the increased expression of multiple IFN-inducible genes, termed the IFN signature (46–49). Other studies have suggested that RNA-containing immune complexes are more contributory to DC activation and the induction of an IFN signature (50). Still another study performed in humans has suggested a genetic predisposition to increased type 1 IFN production that may precede autoantibody production (51). We have previously shown increased BAFF mRNA in E2-treated R4A Tg mice (36). In this study, we asked whether the induction of R4A-encoded anti-DNA Abs was sufficient to induce inflammatory features of SLE.

Serum BAFF levels were measured by ELISA. Mice receiving E2 pellets exhibited an increase in BAFF mRNA in splenocytes, as previously shown (Fig. 1*A*), and increased serum BAFF levels (Fig. 1*B*). It is known that B cell lymphopenia leads to increased BAFF levels (52). We, therefore, ascertained that there was no decrease in total B cell number secondary to the E2 administration (P, 586,352 ± 56,301; E2, 509,414 ± 14,049 B cells per 10^6 splenocytes), although we have previously shown a decrease in transitional B cells in the spleen (38) and others have shown an E2-induced decrease in B cell lymphopoiesis in the bone marrow (53). We also assayed for expression of type 1 IFNs (IFN-α,β) in splenic DCs treated with serum from P- and E2-treated R4A Tg mice, as well as ifi202b and mx-1, two prominent genes in the IFN signature. An increase in the mRNA of the IFN-inducible genes ifi202b and mx-1 in splenocytes from E2-exposed R4A Tg mice was observed (Fig. 1*C*, 1*D*). Cultured splenic DCs treated with serum from E2-exposed R4A Tg mice displayed an upregulation in the transcription of IFN-α and IFN-β genes (Fig. 1*E*, 1*F*).

To determine whether E2 was directly responsible for the induction of a proinflammatory milieu or whether the production of proinflammatory cytokines was secondary to the presence of DNA-containing immune complexes, R4A Tg mice were injected with 450 μg bovine pancreatic DNase daily i.p. for 5 wk during the period of treatment with E2 to reduce the availability of DNA. To confirm that the exogenous DNase altered serum levels of DNase, a DNase ELISA was performed. An increase in serum DNase

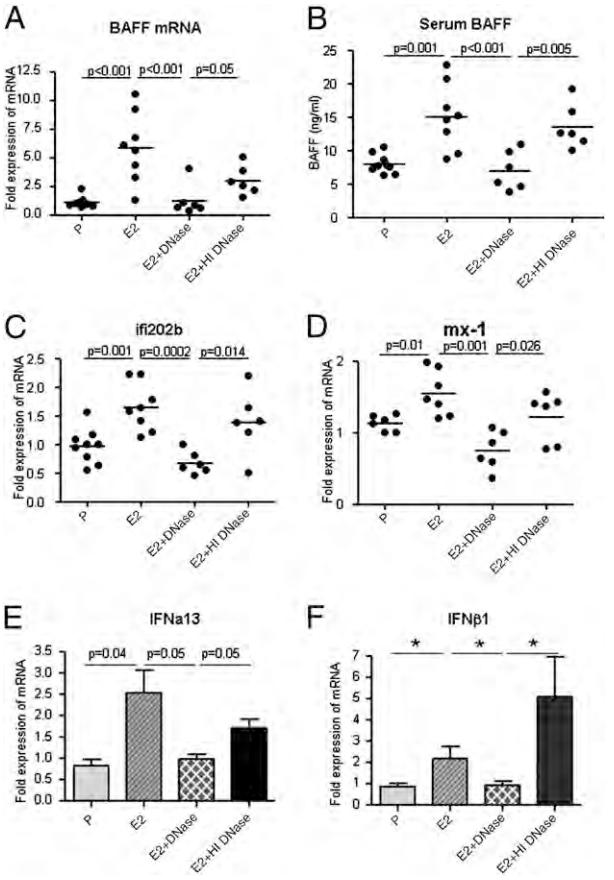


FIGURE 1. Administration of DNase diminishes BAFF induction and leads to abrogation of an E2-induced increase in type 1 IFNs and IFN-inducible genes in R4A Tg mice. DNase treatment of R4A Tg mice restores E2-induced BAFF mRNA levels (*A*) as well as serum BAFF levels (*B*) to that of levels observed in P-treated mice after 5 wk of treatment, whereas treatment with HI DNase did not affect E2-induced BAFF levels. *C–F*, An upregulation of type 1 IFNs (IFN-α,β), as well as the IFN-inducible genes *ifi202* and *mx1*, was observed in E2-treated R4A Tg mice. Administration of DNase, but not HI DNase, resulted in diminution of IFN-α, IFN-β, ifi202, and mx1 transcription to levels comparable to P-treated mice. RNA from splenocytes was analyzed for expression of BAFF and the IFN-inducible genes *ifi202* and *mx1*, whereas RNA from mouse splenic DCs treated with serum from P, E2, E2 plus DNase, or E2 plus HI DNase R4A Tg mice was analyzed for IFN-α and IFN-β expression by real-time PCR. Unpaired *t* test was used to analyze the statistical differences in BAFF, *ifi202*, *mx1*, and IFN-α, and the exact Kruskal-Wallis test to determine the statistical significance in IFN-β between groups (*$p < 0.04$). Six to nine mice were studied per treatment group. For IFN-α and IFN-β assay, $n = 3–4$.

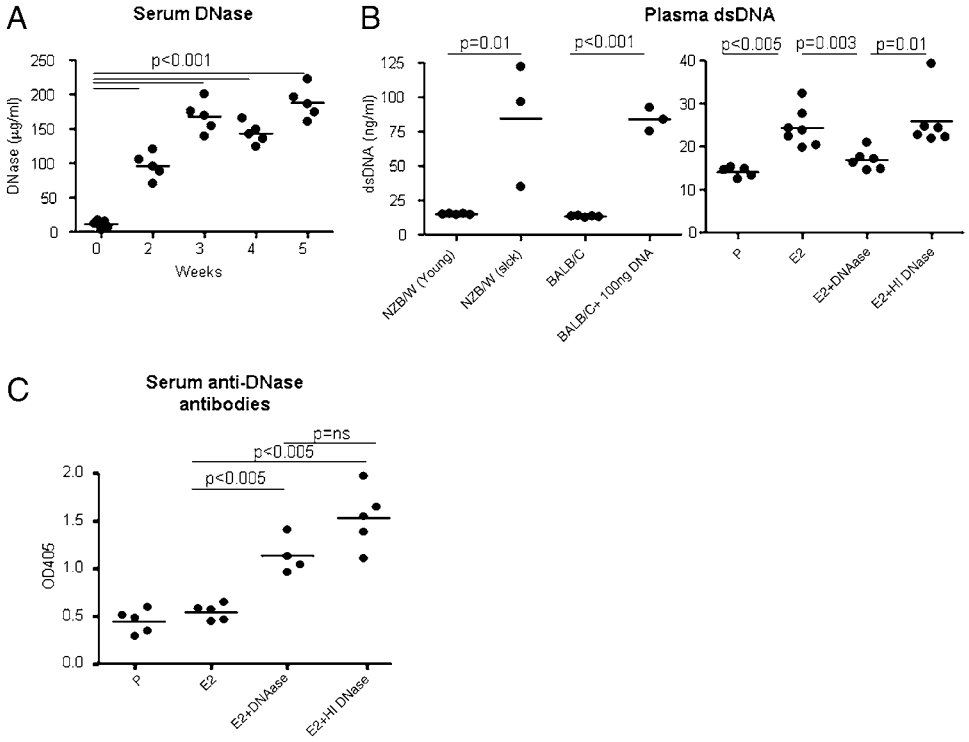


FIGURE 2. Bovine pancreatic DNase is biologically active in vivo. *A*, A detectable increase in serum DNase levels was observed upon administration of 450 μg bovine pancreatic DNase in R4A Tg mice as measured by ELISA. *B*, A significant decrease in circulating plasma dsDNA levels was observed in E2-treated R4A Tg mice administered DNase, but not HI DNase compared with E2-treated R4A Tg mice. As controls for the picogreen assay, plasma dsDNA levels were measured in BALB/c mice and young and sick NZB/W mice. *C*, Treatment with bovine pancreatic DNase resulted in the generation of anti-DNase Abs in R4A Tg mice. R4A Tg mice were treated with P, E2, E2 plus DNase, and E2 plus HI DNase for 6 wk. After 6 wk, anti-DNase Abs were detected in serum by ELISA using serum at 1:250 dilution. Four to seven mice per group were used for the studies.

levels was observed throughout the treatment period, stabilizing by 3 wk (Fig. 2*A*). Furthermore, bovine pancreatic DNase was biologically active in mouse circulation, as determined by a decrease in plasma DNA detectable by the dsDNA-specific picogreen assay (Fig. 2*B*). Administration of HI DNase did not increase plasma dsDNA levels. This was observed as early as 2 wk following initiation of treatment. Because the DNase was active, we could investigate the effects of lowering the concentration of endogenous DNA in B cell selection and development of a lupus-like serology. As the source of DNase was bovine pancreas, there was a possibility that mice would mount an Ab response to DNase itself. Treatment with bovine pancreatic DNase, both native and HI, induced an anti-DNase response in R4A mice (Fig. 2*C*). However, bovine pancreatic DNase decreased endogenous DNA levels in mouse serum; thus, there was residual DNase activity not neutralized by anti-DNase Abs.

Both the increased BAFF and the induction of type 1 and type 2 IFNs as well as IFN signature were reversed by administration of DNase, but not by HI DNase, demonstrating that E2 did not by itself cause these effects (Fig. 1). The elimination of BAFF overexpression by exogenous DNase strongly suggests that DNA-containing immune complexes were responsible for this feature of SLE and that E2 does not directly upregulate BAFF. DNase treatment also caused a decline in the expression of IFN-inducible genes to baseline levels. Thus, E2 does not directly and by itself regulate expression of IFNs as well as IFN-inducible genes ifi202b and mx-1. Rather, DNA, presumably in TLR9-activating immune complexes, is responsible for establishing the proinflammatory milieu induced by E2 exposure. These observations suggest that DNA-containing immune complexes can activate DCs sufficiently in vivo to establish a proinflammatory milieu.

DNase treatment results in preferential elimination of high-affinity anti-DNA MZ B cells induced by E2

Previously, we demonstrated that the strength of the BCR signaling is diminished by E2 exposure (36). Engagement of the BCR following E2 exposure results in a lower calcium flux and decreased ERK phosphorylation (36). This is associated with an expansion of transgene-expressing B cells and more high-affinity DNA-reactive B cells with a MZ phenotype (35, 38). Because we know the repertoire of L chains that fails to generate DNA binding, the repertoire that generates low-affinity DNA binding, and the repertoire that generates high-affinity DNA binding (Table I) (33, 34), we can examine L chain usage in transgene-expressing B cells and determine how different manipulations of the mice alter B selection. Usually, B cells expressing L chains such as Vκ1A-Jκ1, Vκ1A-Jκ4, and Vκ10-Jκ5 that generate high-affinity DNA-reactive B cells, with apparent affinities of 10^{-8} to 10^{-9} M, are eliminated by deletion in R4A mice as immature B cells in the bone marrow and at the transitional stage of B cell maturation in the spleen, whereas B cells expressing L chains such as Vκ1A-Jκ5, Vκ21-Jκ1, and Vκ21-Jκ2 L chains that generate low-affinity DNA-reactive B cells (Table I) are less susceptible to negative selection, and many of these latter B cells, with apparent affinities of 10^{-6} to 10^{-7} M, survive and are selected into the mature, immunocompetent repertoire (35).

Because essentially all IgG2b-producing B cells express the R4A transgene (41), we can analyze L chain expression in transgene-expressing B cells by focusing on IgG2b$^+$ B cells. Using single-cell PCR analysis, we have demonstrated in earlier studies that E2 treatment of R4A Tg mice leads to a shift in the DNA-reactive B cell repertoire, with an increase in high-affinity DNA-reactive B cells in both the transitional and mature B cell repertoire and

Table I. Relative affinities of DNA-reactive B cells from R4A Tg mice

Vκ-Jκ Usage	Relative Affinity[a]	Ref.
Vκ1A-Jκ1	9.1×10^{-8}	33, 37
Vκ1A-Jκ5	4.5×10^{-9}	33
Vκ1A-Jκ4	4.2×10^{-6}	37
Vκ10-Jκ5	6.6×10^{-8}	34
Vκ21-Jκ1	2.2×10^{-6}	33
Vκ21-Jκ2	9×10^{-6}	33
Vκ4/5-Jκ5	ND	34
Vκ19-Jκ5	ND	34
Vκ24/25-Jκ2	ND	34

[a]The relative affinity of the R4A H chain paired with each L chain was determined in previous studies by inhibition ELISA (33, 34, 37).

a decrease in low-affinity DNA-reactive B cells in the mature B cell subset (35). Because we demonstrated that DNase led to an elimination of the effects of DNA-containing immune complexes, we were interested in ascertaining whether DNase treatment also led to an alteration in the E2-induced shift in the B cell repertoire in R4A Tg mice or merely led to a reduction in the amount of Ag available to form immune complexes. L chain sequences were, therefore, determined in both transitional and mature Tg^+ B cells isolated from P-, E2-, E2 plus DNase-, or E2 plus HI DNase-treated R4A Tg mice (three in each group, yielding a total of 110, 114, 135, and 114 sequences). We identified 23 different Vκ-Jκ L chains in all experimental groups, 10 of which were commonly expressed in all the groups. In R4A mice, Vκ4/5 L chains predominated (40%), followed by Vκ1 (22%) and Vκ21 (12%) L chains. In contrast, E2 treatment resulted in a predominant Vκ1 L chain usage (45%), followed by Vκ21 (21%) and Vκ9/10 (10%) L chains, as identified in our previous studies (35). Interestingly, DNase treatment shifted the B cell repertoire toward that observed in P-treated mice with predominant usage of Vκ4/5 L chains, followed by Vκ1 and Vκ21 L chains.

The transitional and mature R4A-expressing B cells in E2-treated mice expressed L chains that generate high-affinity DNA reactivity at a frequency of 23 and 29%, respectively, whereas only 12 and 8%, respectively, of immature and transitional Tg^+ B cells in P-treated mice expressed L chains that give rise to high-affinity DNA-reactive B cells. Upon DNase administration, E2-treated R4A Tg mice expressed L chains that confer high-affinity DNA reactivity in 13 and 11% of transitional and mature B cells, respectively (Table II). HI DNase treatment did not alter the E2-induced repertoire, demonstrating that active DNase was required for the reversion of the B cell repertoire to that present in P-treated mice.

As previously reported, there was no effect of E2 on the frequency of low-affinity transitional DNA-reactive B cells. In E2-treated mice, there were fewer mature Tg-expressing B cells with L chains that generate low-affinity DNA-reactive B cells than in P-treated mice (Table II). We have previously reported this and believe it reflects a competition for Ag with fewer low-affinity DNA-reactive B cells when high-affinity B cells are present. DNase treatment, but not HI DNase treatment, resulted in a restoration of low-affinity DNA-reactive B cells in the mature Tg^+ B cells. Just as the decrease in the frequency of low-affinity DNA-reactive B cells in E2-treated R4A Tg mice probably reflects a failure of these low-affinity DNA-reactive B cells to compete for entrance into follicular niches when high-affinity DNA-reactive B cells escape tolerance, the increase in low-affinity DNA-reactive B cells probably occurs when high-affinity B cells do not survive (35). Taken together, these data suggest that DNase treatment of E2-exposed R4A Tg mice causes preferential elimination of high-affinity DNA-reactive B cells and restoration of low-affinity DNA-reactive B cell population. Moreover, it suggests that the low BCR signal strength is not by itself sufficient to change the B cell repertoire; rather, Ag is required to mediate positive selection.

We have demonstrated previously that E2 exposure of R4A Tg mice displayed decreased number of transitional B cells and a shift in T1:T2 ratio, with more T2 cells (38). An increase in the mature B cell population that is comprised of MZ and follicular B cells was observed and the percentage of MZ B cells was doubled (38). We wanted to determine whether DNase treatment could abrogate E2-induced changes in peripheral B cell development. Interestingly, treatment with DNase, but not HI DNase, diminished the 2-fold increase in the MZ B cells seen in E2-treated R4A Tg mice and the transitional T1 and T2 B cells were restored to that observed in the placebo group (Fig. 3).

Serum titers of anti-DNA Abs

Surprisingly, administration of DNase to E2-treated R4A Tg mice did not decrease the serum titers of anti-DNA Ab to baseline levels (Fig. 4*A*). Because we knew that the DNase-treated mice harbored few high-affinity DNA-reactive B cells, we reasoned that the Ab titers reflected low-affinity Abs that were not bound to DNA in serum. To further ascertain that administration of DNase to E2-treated R4A mice led to a loss of high-affinity DNA binding, we measured the apparent affinities of the anti-dsDNA Abs in the sera of E2-, E2 plus DNase-, and E2 plus HI DNase-treated R4A Tg mice. The parental R4A mAb has an affinity of $\sim 3.6 \times 10^{-8}$ M. Sera from E2-treated R4A Tg mice have an apparent affinity of $2.6–5.4 \times 10^{-8}$ M. Interestingly, sera from E2 plus DNase-treated R4A Tg mice displayed an apparent affinity of $1.4–3.2 \times 10^{-7}$ M. However, the apparent affinity of sera from R4A Tg mice administered E2 plus HI DNase was comparable to that observed in sera from E2-treated R4A Tg mice ($3.7–6.3 \times 10^{-8}$ M). Thus, high-affinity DNA-reactive B cells present in E2-treated mice were secreting Ab into serum.

Previously, we have shown that E2-treated R4A Tg mice display immune complex deposition in the kidneys and that only high-affinity Abs deposit in the kidney (33). DNase treatment of E2-treated R4A Tg mice resulted in a marked decrease in Ab deposition in the kidney (Fig. 4*B*, 4*C*) that correlated with a decrease

Table II. Frequency of high-affinity and low-affinity DNA-reactive B cells in R4A Tg mice treated with E2 with or without DNase

	Placebo (%)	E2 (%)	E2 + DNase (%)	E2 + HI DNase (%)
Transitional	6/50 (12)	12/52 (23)*	8/63 (12.6)[ns]	13/47 (29.7)*
Mature				
High affinity	5/60 (8.3)	18/62 (27.7)*	8/72 (11.1)[ns]	13/57 (22.8)*
Low affinity	11/60 (18)	5/62 (8)*	18/72 (25)	4/57 (7)*

A significant increase in high-affinity anti-DNA B cells in R4A Tg mice treated with E2 was observed and was abrogated by treatment with DNase, but not HI DNase. Fisher's exact test was used to analyze significance between the various treatment groups compared with the P-treated group.

*$p < 0.05$.

ns, Not significant.

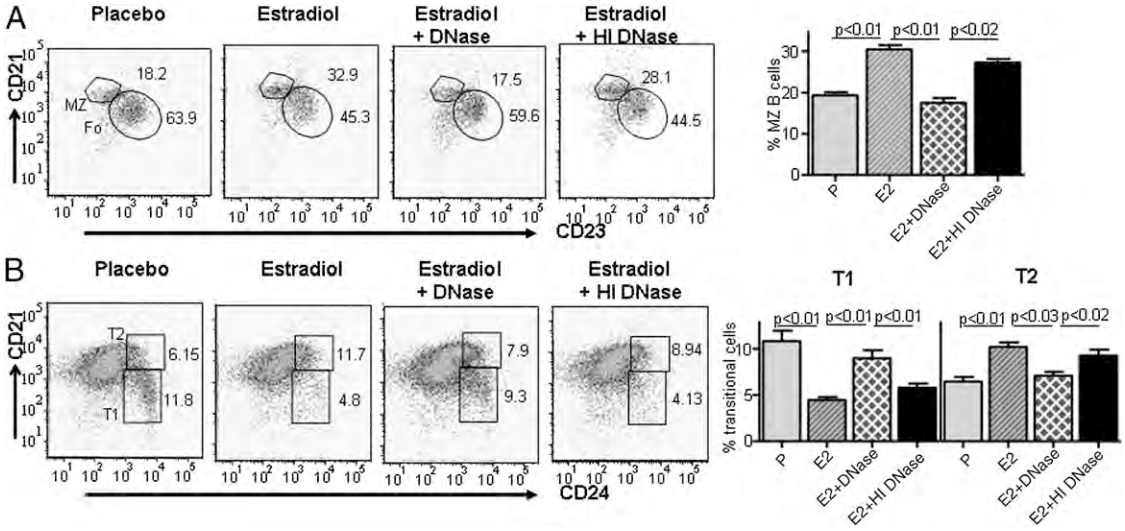


FIGURE 3. DNase treatment abrogates E2-induced changes in B cell development in R4A Tg mice. Administration of DNase resulted in reversal of E2-induced increase in MZ B cells (*A*) and transitional B cells (*B*). However, HI DNase did not affect E2-induced changes in MZ and transitional B cells. MZ B cells were identified as $CD21^{high}CD23^{neg}CD24^{low}$, and transitional B cells were identified as $CD21^{low}CD24^{high}$ (T1) and $CD21^{high}CD24^{hgh}$ (T2). Five mice were used in each group.

in proteinuria (Fig. 4*D*, 4*E*); HI DNase failed to affect immune complex deposition (Fig. 4*B*, 4*C*). Whereas these data were consistent with the observation that the anti-DNA Abs in E2 plus DNase-treated mice were of low affinity, they might also reflect an enzymatic removal of accessible Ag from the glomeruli. We, therefore, asked directly whether the serum of E2-exposed DNase-treated mice had glomerulotropic potential.

The parental R4A Ab with an affinity of 10^{-8} has been shown to deposit in the glomeruli of the kidneys in SCID mice when administered i.p. (44). This approach permits a study of the potential pathogenicity of anti-DNA Abs. To confirm that the administration of DNase to E2-treated R4A Tg mice resulted in the accumulation of low-affinity anti-DNA Abs that are nonglomerulotropic, the serum from E2-treated R4A Tg mice given DNase was assayed for glomerular deposition in SCID mice. As shown in Fig. 5, serum from E2-treated R4A Tg mice bound strongly to glomeruli; however, the serum from E2 plus DNase-treated R4A Tg mice did not deposit in kidneys of SCID mice. IgG in serum

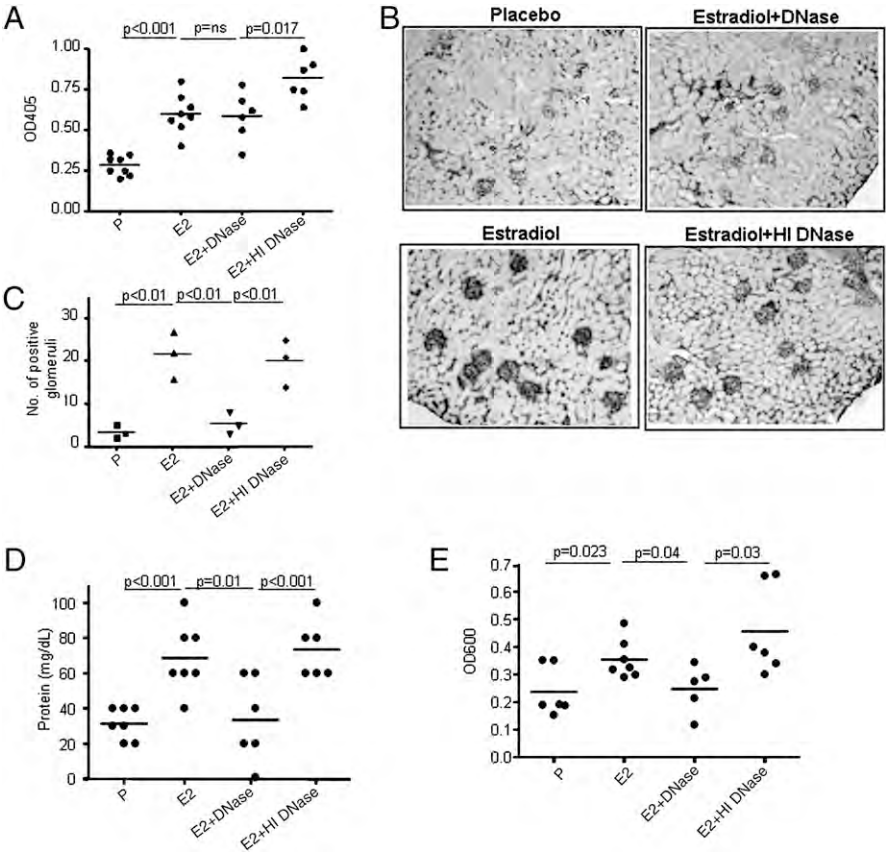


FIGURE 4. Treatment with DNase alleviates E2-induced target organ damage despite persistently elevated serum anti-dsDNA Ab titers. *A*, Serum anti-dsDNA Ab levels in R4A Tg mice treated with E2, E2 plus DNase, and E2 plus HI DNase for 6 wk. A significant increase in anti-dsDNA Ab levels in sera of R4A Tg mice was observed after implantation with E2 pellets and was unaltered by DNase administration. *B*, Glomerular IgG deposition in R4A Tg mice following administration of E2, E2 plus DNase, or E2 plus HI DNase. *C*, The number of positive glomeruli was counted in three different microscopic fields in each section. The average number of positive glomeruli in three individual mice in each group is represented. DNase treatment, but not HI DNase treatment of E2-treated R4A Tg mice resulted in a marked decrease in Ab deposition in the kidney. A representative of five mice per group is shown at original magnification ×5. Proteinuria was measured in five P-, E2-, E2 plus DNase-, and E2 plus HI DNase-treated R4A Tg mice using reagent strips (*D*) and by the Coomassie blue reagent (*E*). Proteinuria was increased in E2-treated R4A Tg mice and was diminished upon administration of DNase. Treatment with HI DNase did not affect the E2-induced increase in proteinuria levels.

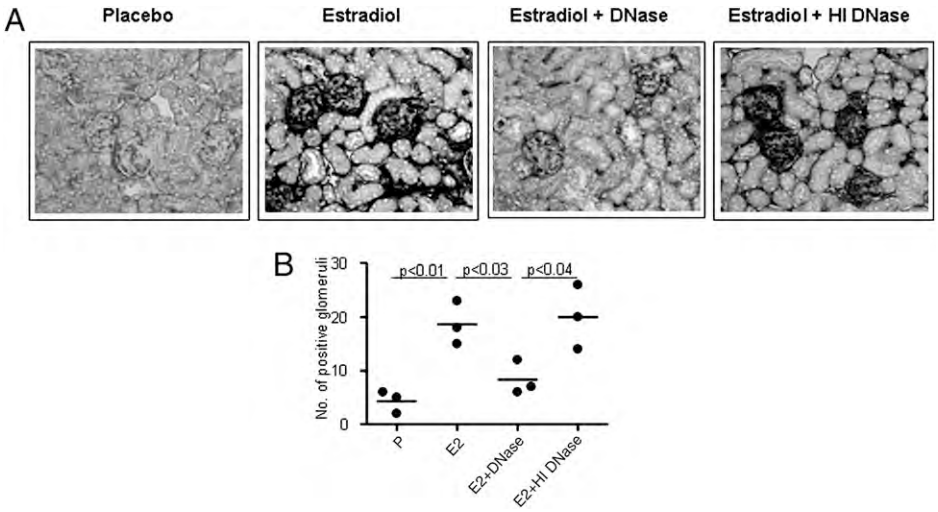


FIGURE 5. Serum from E2-treated, but not E2 plus DNase-treated R4A Tg mice leads to glomerular IgG deposition in SCID mice. SCID mice were injected i.p. with 100 μl serum from P-, E2-, E2 plus DNase-, or E2 plus HI DNase-treated R4A Tg mice. Mice were sacrificed 24 h later, and the kidneys were stained for IgG deposition (*A*). The IgG-positive glomeruli were counted in three different microscopic fields for each section. The average number of IgG-positive glomeruli in three individual mice in each group is represented (*B*). Serum from E2-treated, but not E2 plus DNase-treated R4A Tg mice resulted in glomerular deposition. A representative section from each group is shown at original magnification ×20. IgG from E2-treated R4A Tg mice deposits in kidneys; however, IgG from E2 plus DNase-treated mice does not deposit in glomeruli. HI DNase does not prevent IgG deposition in E2-treated R4A Tg mice.

from E2 plus HI DNase-treated R4A Tg mice bound to glomeruli similar to IgG in serum from E2-treated R4A Tg mice (Fig. 5). These data corroborate the repertoire analysis and the analysis of serum Ab affinity.

Discussion

It is of considerable interest that the presence of anti-DNA Abs is sufficient to increase BAFF levels in the serum and to induce an IFN signature in splenocytes. That DNA-containing immune complexes can induce increased BAFF expression in DCs has been shown in in vitro studies (54). The data reported in this study demonstrate that a proinflammatory milieu can be generated in a nonspontaneously autoimmune host just by virtue of inducing high-affinity anti-DNA Abs. From our studies it is apparent that E2 alone did not directly cause the proinflammatory milieu, as administration of DNase to E2-treated mice totally abrogated the inflammatory response. This observation also confirms that some anti-DNA Abs exist in immune complexes and that the availability of DNA in a mouse with no apparent defect in clearance of apoptotic debris is sufficient to form proinflammatory immune complexes. Furthermore, it supports the hypothesis that TLR9 activation can lead to the upregulation of inflammatory cytokines. This is a contentious issue, as some, but not all, lupus-prone strains of mouse display improvement with a deletion of TLR9 (55–58). Similarly, studies of human SLE have been contradictory, with some investigators suggesting that DNA-containing immune complexes induce the IFN signature and others arguing that R4A-containing immune complexes, which activate TLR7, are more important (25, 26, 59). It has also been shown that relatives of patients with SLE exhibit high serum levels of type 1 IFN, suggesting that there is a predisposition to enhanced IFN production in SLE patients (51). This is consistent with an IFN regulatory factor 5 susceptibility allele in this disease, which has been identified in genome-wide scans (60). This study, however, shows that elevated serum titers of high-affinity anti-DNA Abs are necessary to induce DC activation in a host with unimpaired clearance of apoptotic debris and no pre-existing overexpression of inflammatory cytokines. Because it now seems that the systemic immune activation present in SLE may contribute to accelerated atherosclerosis (61), the fact that anti-DNA Abs alone can initiate an inflammatory cascade may inform therapeutic strategies. It should be noted that E2-treated mice given either DNase or HI DNase might have circulating immune complexes composed of enzyme and anti-DNase Ab. These complexes did not appreciably alter the inflammatory milieu as E2- and E2 plus HI DNase-treated mice appear similar.

It is apparent from these studies that the production of high-affinity DNA-reactive Abs triggers a positive feedback loop. The ensuing elevation in BAFF can function to facilitate the survival and maturation to immunocompetence of more autoreactive B cells. Such a model has clearly been demonstrated in mouse studies and is highly likely to apply in humans as well (62). This would explain the high BAFF levels even in patients who are not B cell lymphopenic.

It was perhaps most surprising that the enhanced number of high-affinity DNA-reactive B cells in the transitional and mature B cell compartments of E2-treated mice depends on the presence of DNA. Having previously shown that E2 exposure diminishes the strength of BCR signaling (36), we assumed that there would be less negative selection of high-affinity DNA-reactive B cells independent of the presence of Ag. The data reported in this study, however, strongly suggest that differentiation to a mature state requires positive selection. Thus, in the absence of an adequate exposure to DNA, high-affinity DNA-reactive B cells did not mature to immunocompetence, despite a lower BCR signaling capacity and reduced negative selection.

Low-affinity anti-DNA Abs do not initiate renal inflammation and do not form immune complexes that activate TLR9. It is also possible that the low-affinity Abs are not present in immune complexes, whereas the high-affinity Abs form immune complexes in plasma, and that these differences may contribute to the difference in glomerular deposition. In parallel, it is clear that some individuals with high titers of anti-DNA Abs do not develop renal disease (63). These individuals may have primarily low-affinity Abs. These data suggest that it may be important to screen patients for the presence of high-affinity anti-DNA Abs to determine

appropriate treatment and response to therapy. The current ELISAs used in most clinical assays do not distinguish between high- and low-affinity Abs.

These studies also suggest that DNase might be an effective therapy in SLE. Whereas we cannot know that active DNase functions only to decrease Ag load, it clearly led to a reduction in plasma DNA. The DNase1-deficient mouse develops a SLE-like disease (64). A few patients with SLE have been shown to be DNase1 deficient (65), and some patients have been reported to have Abs to DNase (66). One study in NZB/W mice showed a short-term delay in disease onset when DNase treatment was begun prior to disease onset and even showed reduced renal pathology if therapy is begun after onset of disease (39). A second study failed to replicate a reduction in renal disease, but did show a decrease in DNA-reactive B cells, similar to what was seen in the data reported in this study (67). It is possible that the limited success of DNase treatment reflected the production of Abs to exogenous DNase.

The first trial of DNase in patients with SLE was reported in 1961 (68). Eight patients were treated with bovine enzyme, which was highly immunogenic and led to some severe Arthus reactions and early termination of the study. Almost 40 years later, a second clinical trial was initiated (69). Clinical measurements included serum cytokines, serum anti-DNA Abs, and anti-DNA–secreting B cells in peripheral blood. None of these parameters was significantly affected, but there was no detectable increase in serum DNase activity in the patients studied. Thus, it remains unresolved whether DNase therapy might be a nonimmunosuppressive therapeutic strategy in SLE. The studies reported in this work strongly support the need to revisit this question.

Acknowledgments

We thank Sylvia Jones for expert secretarial assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Plotz, P. H. 2003. The autoantibody repertoire: searching for order. *Nat. Rev. Immunol.* 3: 73–78.
2. Davidson, A., and B. Diamond. 2001. Autoimmune diseases. *N. Engl. J. Med.* 345: 340–350.
3. ter Borg, E. J., G. Horst, E. J. Hummel, P. C. Limburg, and C. G. Kallenberg. 1990. Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus: a long-term, prospective study. *Arthritis Rheum.* 33: 634–643.
4. Lipsky, P. E. 2001. Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. *Nat. Immunol.* 2: 764–766.
5. Waldman, M., and M. P. Madaio. 2005. Pathogenic autoantibodies in lupus nephritis. *Lupus* 14: 19–24.
6. DeGiorgio, L. A., K. N. Konstantinov, S. C. Lee, J. A. Hardin, B. T. Volpe, and B. Diamond. 2001. A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. *Nat. Med.* 7: 1189–1193.
7. Desai, D. D., M. R. Krishnan, J. T. Swindle, and T. N. Marion. 1993. Antigen-specific induction of antibodies against native mammalian DNA in non-autoimmune mice. *J. Immunol.* 151: 1614–1626.
8. Petrakova, N., L. Gudmundsdotter, M. Yermalovich, S. Belikov, L. Eriksson, P. Pyakurel, O. Johansson, P. Biberfeld, S. Andersson, and M. Isaguliants. 2009. Autoimmunogenicity of the helix-loop-helix DNA-binding domain. *Mol. Immunol.* 46: 1467–1480.
9. Cerutti, M. L., L. M. Zarebski, G. de Prat Gay, and F. A. Goldbaum. 2005. A viral DNA-binding domain elicits anti-DNA antibodies of different specificities. *Mol. Immunol.* 42: 327–333.
10. Moens, U., I. Mathiesen, M. V. Ghelue, and O. P. Rekvig. 2002. Green fluorescent protein modified to bind DNA initiates production of anti-DNA antibodies when expressed in vivo. *Mol. Immunol.* 38: 505–514.
11. Putterman, C., and B. Diamond. 1998. Immunization with a peptide surrogate for double-stranded DNA (dsDNA) induces autoantibody production and renal immunoglobulin deposition. *J. Exp. Med.* 188: 29–38.
12. Dryden, D. T., and M. R. Tock. 2006. DNA mimicry by proteins. *Biochem. Soc. Trans.* 34: 317–319.
13. Sibille, P., T. Ternynck, F. Nato, G. Buttin, D. Strosberg, and A. Avrameas. 1997. Mimotopes of polyreactive anti-DNA antibodies identified using phage-display peptide libraries. *Eur. J. Immunol.* 27: 1221–1228.
14. Bach, J. F., S. Koutouzov, and P. M. van Endert. 1998. Are there unique autoantigens triggering autoimmune diseases? *Immunol. Rev.* 164: 139–155.
15. Isenberg, D., M. A. Rahman, C. T. Ravirajan, and J. K. Kalsi. 1997. Anti-DNA antibodies: from gene usage to crystal structures. *Immunol. Today* 18: 149–153.
16. van Venrooij, W. J., and G. J. Pruijn. 1995. Ribonucleoprotein complexes as autoantigens. *Curr. Opin. Immunol.* 7: 819–824.
17. Desai, D. D., and T. N. Marion. 2000. Induction of anti-DNA antibody with DNA-peptide complexes. *Int. Immunol.* 12: 1569–1578.
18. Voynova, E. N., A. I. Tchorbanov, T. A. Todorov, and T. L. Vassilev. 2005. Breaking of tolerance to native DNA in nonautoimmune mice by immunization with natural protein/DNA complexes. *Lupus* 14: 543–550.
19. Taylor, P. R., A. Carugati, V. A. Fadok, H. T. Cook, M. Andrews, M. C. Carroll, J. S. Savill, P. M. Henson, M. Botto, and M. J. Walport. 2000. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells in vivo. *J. Exp. Med.* 192: 359–366.
20. Cohen, P. L., R. Caricchio, V. Abraham, T. D. Camenisch, J. C. Jennette, R. A. Roubey, H. S. Earp, G. Matsushima, and E. A. Reap. 2002. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. *J. Exp. Med.* 196: 135–140.
21. A-Gonzalez, N., S. J. Bensinger, C. Hong, S. Beceiro, M. N. Bradley, N. Zelcer, J. Deniz, C. Ramirez, M. Díaz, G. Gallardo, et al. 2009. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity* 31: 245–258.
22. Hanayama, R., M. Tanaka, K. Miyasaka, K. Aozasa, M. Koike, Y. Uchiyama, and S. Nagata. 2004. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 304: 1147–1150.
23. Gaipl, U. S., R. E. Voll, A. Sheriff, S. Franz, J. R. Kalden, and M. Herrmann. 2005. Impaired clearance of dying cells in systemic lupus erythematosus. *Autoimmun. Rev.* 4: 189–194.
24. Lövgren, T., M. L. Eloranta, U. Båve, G. V. Alm, and L. Rönnblom. 2004. Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum.* 50: 1861–1872.
25. Lövgren, T., M. L. Eloranta, B. Kastner, M. Wahren-Herlenius, G. V. Alm, and L. Rönnblom. 2006. Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen- and Sjögren's syndrome autoantigen-associated RNA. *Arthritis Rheum.* 54: 1917–1927.
26. Eloranta, M. L., T. Lövgren, D. Finke, L. Mathsson, J. Rönnelid, B. Kastner, G. V. Alm, and L. Rönnblom. 2009. Regulation of the interferon-alpha production induced by RNA-containing immune complexes in plasmacytoid dendritic cells. *Arthritis Rheum.* 60: 2418–2427.
27. Tian, J., A. M. Avalos, S. Y. Mao, B. Chen, K. Senthil, H. Wu, P. Parroche, S. Drabic, D. Golenbock, C. Sirois, et al. 2007. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat. Immunol.* 8: 487–496.
28. Rönnblom, L., and V. Pascual. 2008. The innate immune system in SLE: type I interferons and dendritic cells. *Lupus* 17: 394–399.
29. Boulé, M. W., C. Broughton, F. Mackay, S. Akira, A. Marshak-Rothstein, and I. R. Rifkin. 2004. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J. Exp. Med.* 199: 1631–1640.
30. He, B., X. Qiao, and A. Cerutti. 2004. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. *J. Immunol.* 173: 4479–4491.
31. Offen, D., L. Spatz, H. Escowitz, S. Factor, and B. Diamond. 1992. Induction of tolerance to an IgG autoantibody. *Proc. Natl. Acad. Sci. USA* 89: 8332–8336.
32. Shefner, R., G. Kleiner, A. Turken, L. Papazian, and B. Diamond. 1991. A novel class of anti-DNA antibodies identified in BALB/c mice. *J. Exp. Med.* 173: 287–296.
33. Bynoe, M. S., C. M. Grimaldi, and B. Diamond. 2000. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc. Natl. Acad. Sci. USA* 97: 2703–2708.
34. Spatz, L., V. Saenko, A. Iliev, L. Jones, L. Geskin, and B. Diamond. 1997. Light chain usage in anti-double-stranded DNA B cell subsets: role in cell fate determination. *J. Exp. Med.* 185: 1317–1326.
35. Grimaldi, C. M., V. Jeganathan, and B. Diamond. 2006. Hormonal regulation of B cell development: 17 beta-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint. *J. Immunol.* 176: 2703–2710.
36. Venkatesh, J., E. Peeva, X. Xu, and B. Diamond. 2006. Cutting edge: hormonal milieu, not antigenic specificity, determines the mature phenotype of autoreactive B cells. *J. Immunol.* 176: 3311–3314.
37. Bynoe, M. S., L. Spatz, and B. Diamond. 1999. Characterization of anti-DNA B cells that escape negative selection. *Eur. J. Immunol.* 29: 1304–1313.
38. Grimaldi, C. M., D. J. Michael, and B. Diamond. 2001. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *J. Immunol.* 167: 1886–1890.
39. Grimaldi, C. M., J. Cleary, A. S. Dagtas, D. Moussai, and B. Diamond. 2002. Estrogen alters thresholds for B cell apoptosis and activation. *J. Clin. Invest.* 109: 1625–1633.
40. Macanovic, M., D. Sinicropi, S. Shak, S. Baughman, S. Thiru, and P. J. Lachmann. 1996. The treatment of systemic lupus erythematosus (SLE) in NZB/W F_1 hybrid mice; studies with recombinant murine DNase and with dexamethasone. *Clin. Exp. Immunol.* 106: 243–252.

41. Kawabata, D., J. Venkatesh, M. Ramanujam, A. Davidson, C. M. Grimaldi, and B. Diamond. 2010. Enhanced selection of high affinity DNA-reactive B cells following cyclophosphamide treatment in mice. *PLoS One* 5: e8418.
42. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29: e45.
43. Ray, S. K., C. Putterman, and B. Diamond. 1996. Pathogenic autoantibodies are routinely generated during the response to foreign antigen: a paradigm for autoimmune disease. *Proc. Natl. Acad. Sci. USA* 93: 2019–2024.
44. Nieto, A., A. Gaya, M. Jansa, C. Moreno, and J. Vives. 1984. Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. *Mol. Immunol.* 21: 537–543.
45. Gaynor, B., C. Putterman, P. Valadon, L. Spatz, M. D. Scharff, and B. Diamond. 1997. Peptide inhibition of glomerular deposition of an anti-DNA antibody. *Proc. Natl. Acad. Sci. USA* 94: 1955–1960.
46. Baechler, E. C., F. M. Batliwalla, G. Karypis, P. M. Gaffney, W. A. Ortmann, K. J. Espe, K. B. Shark, W. J. Grande, K. M. Hughes, V. Kapur, et al. 2003. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc. Natl. Acad. Sci. USA* 100: 2610–2615.
47. Bennett, L., A. K. Palucka, E. Arce, V. Cantrell, J. Borvak, J. Banchereau, and V. Pascual. 2003. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *J. Exp. Med.* 197: 711–723.
48. Crow, M. K., and J. Wohlgemuth. 2003. Microarray analysis of gene expression in lupus. *Arthritis Res. Ther.* 5: 279–287.
49. Bauer, J. W., M. Petri, F. M. Batliwalla, T. Koeuth, J. Wilson, C. Slattery, A. Panoskaltsis-Mortari, P. K. Gregersen, T. W. Behrens, and E. C. Baechler. 2009. Interferon-regulated chemokines as biomarkers of systemic lupus erythematosus disease activity: a validation study. *Arthritis Rheum.* 60: 3098–3107.
50. Båve, U., G. V. Alm, and L. Rönnblom. 2000. The combination of apoptotic U937 cells and lupus IgG is a potent IFN-alpha inducer. *J. Immunol.* 165: 3519–3526.
51. Niewold, T. B., J. Hua, T. J. Lehman, J. B. Harley, and M. K. Crow. 2007. High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus. *Genes Immun.* 8: 492–502.
52. Hoek, K. L., G. Carlesso, E. S. Clark, and W. N. Khan. 2009. Absence of mature peripheral B cell populations in mice with concomitant defects in B cell receptor and BAFF-R signaling. *J. Immunol.* 183: 5630–5643.
53. Erlandsson, M. C., C. A. Jonsson, U. Islander, C. Ohlsson, and H. Carlsten. 2003. Oestrogen receptor specificity in oestradiol-mediated effects on B lymphopoiesis and immunoglobulin production in male mice. *Immunology* 108: 346–351.
54. Hanada, T., H. Yoshida, S. Kato, K. Tanaka, K. Masutani, J. Tsukada, Y. Nomura, H. Mimata, M. Kubo, and A. Yoshimura. 2003. Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. *Immunity* 19: 437–450.
55. Wu, X., and S. L. Peng. 2006. Toll-like receptor 9 signaling protects against murine lupus. *Arthritis Rheum.* 54: 336–342.
56. Ehlers, M., H. Fukuyama, T. L. McGaha, A. Aderem, and J. V. Ravetch. 2006. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J. Exp. Med.* 203: 553–561.
57. Christensen, S. R., M. Kashgarian, L. Alexopoulou, R. A. Flavell, S. Akira, and M. J. Shlomchik. 2005. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J. Exp. Med.* 202: 321–331.
58. Herlands, R. A., S. R. Christensen, R. A. Sweet, U. Hershberg, and M. J. Shlomchik. 2008. T cell-independent and Toll-like receptor-dependent antigen-driven activation of autoreactive B cells. *Immunity* 29: 249–260.
59. Vollmer, J., S. Tluk, C. Schmitz, S. Hamm, M. Jurk, A. Forsbach, S. Akira, K. M. Kelly, W. H. Reeves, S. Bauer, and A. M. Krieg. 2005. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. *J. Exp. Med.* 202: 1575–1585.
60. Harley, J. B., M. E. Alarcón-Riquelme, L. A. Criswell, C. O. Jacob, R. P. Kimberly, K. L. Moser, B. P. Tsao, T. J. Vyse, C. D. Langefeld, S. K. Nath, et al; International Consortium for Systemic Lupus Erythematosus Genetics (SLEGEN). 2008. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat. Genet.* 40: 204–210.
61. Sherer, Y., H. Zinger, and Y. Shoenfeld. 2010. Atherosclerosis in systemic lupus erythematosus. *Autoimmunity* 43: 98–102.
62. Kalled, S. L. 2005. The role of BAFF in immune function and implications for autoimmunity. *Immunol. Rev.* 204: 43–54.
63. Manson, J. J., A. Ma, P. Rogers, L. J. Mason, J. H. Berden, J. van der Vlag, D. P. D'Cruz, D. A. Isenberg, and A. Rahman. 2009. Relationship between anti-dsDNA, anti-nucleosome and anti-alpha-actinin antibodies and markers of renal disease in patients with lupus nephritis: a prospective longitudinal study. *Arthritis Res. Ther.* 11: R154.
64. Napirei, M., H. Karsunky, B. Zevnik, H. Stephan, H. G. Mannherz, and T. Möröy. 2000. Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat. Genet.* 25: 177–181.
65. Yasutomo, K., T. Horiuchi, S. Kagami, H. Tsukamoto, C. Hashimura, M. Urushihara, and Y. Kuroda. 2001. Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat. Genet.* 28: 313–314.
66. Yeh, T. M., H. C. Chang, C. C. Liang, J. J. Wu, and M. F. Liu. 2003. Deoxyribonuclease-inhibitory antibodies in systemic lupus erythematosus. *J. Biomed. Sci.* 10: 544–551.
67. Jacob, M., M. Napirei, A. Ricken, C. Dixkens, and H. G. Mannherz. 2002. Histopathology of lupus-like nephritis in Dnase1-deficient mice in comparison to NZB/W F_1 mice. *Lupus* 11: 514–527.
68. Lachmann, P. J. 1967. Allergic reactions, connective tissue, and disease. *Sci. Basis Med. Annu. Rev.* 1967: 36–58.
69. Davis, J. C., Jr., S. Manzi, C. Yarboro, J. Rairie, I. Mcinnes, D. Averthelyi, D. Sinicropi, V. G. Hale, J. Balow, H. Austin, et al. 1999. Recombinant human Dnase I (rhDNase) in patients with lupus nephritis. *Lupus* 8: 68–76.