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

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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.

No subject terms provided.
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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)\(\alpha\) and ER\(\beta\). We have further shown that estrogen prevents antigen-induced deletion of autoreactive B cells through ER\(\alpha\) 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 ER\(\alpha\) or ER\(\beta\), 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 ER\(\alpha\) 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 \(\alpha\) and \(\beta\) 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:**


**Degrees:** PhD Latia Hill

**Funding:** Career Award SLE Foundation – Venkatesh J.

**Presentations:**

British Society for Rheumatology 2012


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.
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 \( \alpha \) and \( \beta \) in Control of B-Cell Maturation and Selection

Latia Hill,1,2 Venkatesh Jeganathan,1 Prameladevi Chinnasamy,1 Christine Grimaldi,1,3 and Betty Diamond1,2

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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 \( \alpha \) or \( \beta \) engagement to the altered B-cell maturation and selection mediated by increased exposure to estrogen. We demonstrate that engagement of either estrogen receptor \( \alpha \) or \( \beta \) can alter B-cell maturation, but only engagement of estrogen receptor \( \alpha \) is a trigger for autoimmunity. Thus, maturation and selection are regulated differentially by estrogen. These observations have therapeutic implications.

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 other organs (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 \( \alpha \) (ER\( \alpha \)) and estrogen receptor \( \beta \) (ER\( \beta \)) (25). These form homodimers and heterodimers and are expressed in many cells including T cells, B cells, monocytes and dendritic cells (26–28). ER\( \alpha \) and ER\( \beta \) regulate gene transcription, having both overlapping and distinct target genes (29,30). Some reports suggest that they can function antagonistically (25). ER\( \alpha \) can also function at the cell membrane to activate certain signaling cascades. Polymorphisms in...
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 lymphopoesis 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 lymphopoesis 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. We therefore hypothesized 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-week-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, CD23 and CD22 were purchased from BD Pharminigen (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. Antibodies were diluted in cytoperm buffer (BD Biosciences). Single-cell RT-PCR was performed using protocol from BD Biosciences and analyzed using Flowjo software (Tree Star, Ashland, OR, USA).

Single-Cell PCR of Light Chain Genes

Splenic B cells were stained with antibodies specific for B220, IgG2b and AA4.1, and mature (B220+ / IgG2b+ / 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′-GGCTGCAGSTCTAGTGCCAG TGGRTCWGGRAC-3′ + constant region primer (Cκ) (first round) 5′-TGGAT GGTTGGAAGAT-3′; and Cκ (second round) 5′-AGAGATGGATACGTGGT-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 (Geneviz, 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 ERα−/−, R4A-ERα−/−, ERβ−/−, R4A-ERβ−/−, WT BALB/c and R4A mice was prepared using a 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 buffered saline (PBS) at 4°C. For intracellular staining, cells were fixed and permeabilized with cytotox/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).
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 primers described by Krege et al. (40). Primers specific for β-actin cDNA amplify exon 2 of the gene were used: namely, 5′-GGGAGCCAGTCTGTA ACTCG-3′ and 5′-GGGCTCGTTCTCCAG GTAGT-3′. Primers specific for IFNα subunits 2, and IFNβ subunit 1; and IFNβ–/– mice were isolated using Dynal Beads and subsequently transferred to polyvinylidene difluoride membranes. Direct immunoblotting for Erk tyrosine phosphorylation used phospho Erk1/2 or total Erk1/2 antibodies followed by anti-rabbit IgG secondary antibody. The membranes were subsequently stripped and reprobed with the antibodies to hypoxanthine-guanine phosphoribosyltransferase (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 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

ImmunoL 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 for 1 h at 37°C with 100 µL of blocking reagent (Biotec’s PBS, pelleted and lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease 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.

Western Blotting

Splenocyte B cells were purified by negative selection using Dynal Beads and resuspended in RPMI 1640 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 and phosphatase inhibitors (Pierce). Protein cell lysates were used as a control.
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 ER\textsubscript{α} and ER\textsubscript{β} in B cells by analyzing ER\textsubscript{α} and ER\textsubscript{β} transcripts in total splenic B cells of ER\textsubscript{α}–/–, ER\textsubscript{β}–/– and WT mice (Figure 1). ER\textsubscript{α} transcripts were absent in ER\textsubscript{α}-deficient mice; ER\textsubscript{β} transcripts were absent in ER\textsubscript{β}-deficient mice. Interestingly, we saw no compensatory overexpression of ER\textsubscript{α} in ER\textsubscript{β}-deficient B cells or ER\textsubscript{β} in ER\textsubscript{α}-deficient B cells.

It has been shown that ER\textsubscript{α} or ER\textsubscript{β} 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, ER\textsubscript{α}-deficient and ER\textsubscript{β}-deficient mice treated with E2 or placebo. Engagement of ER\textsubscript{α} in ER\textsubscript{β}-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 ER\textsubscript{α} engagement on B-cell lymphopoiesis (42). Engagement of ER\textsubscript{β} in ER\textsubscript{α}-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\textsuperscript{high}CD23\textsuperscript{neg}HSAlow) in WT BALB/c mice (24). We confirmed this observation and observed an E2-induced expansion of MZ B cells in both ER\textsubscript{α}-deficient and ER\textsubscript{β}-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, ER\textsubscript{α}-deficient and ER\textsubscript{β}-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 quantify 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).
Polr2A is represented. Six or eight mice were used in each group for the studies. 


titers compared with placebo. (B) Enumeration of DNA-reactive B cells in WT, ERα-deficient and ERβ-deficient mice. ERα and ERβ were 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 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 ERα+/+, ERα−/− and WT mice. The relative expression in comparison to Pol2A is represented. Six or eight mice were used in each group for the studies.

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 ERα+/+, ERα−/− and WT mice. The relative expression in comparison to Pol2A is represented. Six or eight mice were used in each group for the studies.

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 ERα−/− and not in WT or ERβ−/− 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.

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 ERα−/− and not in WT or ERβ−/− 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.

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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

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ERβb−/−</th>
<th>ERα−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-affinity</td>
<td>3/48</td>
<td>14/54 (26%)</td>
<td>7/62 (11%)</td>
</tr>
<tr>
<td>Low-affinity</td>
<td>7/48</td>
<td>6/54 (10%)</td>
<td>8/62 (13%)</td>
</tr>
</tbody>
</table>

aThe 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−/−, and R4A-Tg ERα−/− deficient mice.

bP < 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

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>E2</th>
<th>PPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-affinity</td>
<td>6/67</td>
<td>15/65 (23.1%)</td>
<td>14/70 (20%)</td>
</tr>
<tr>
<td>Low-affinity</td>
<td>7/67</td>
<td>5/65 (7.7%)</td>
<td>5/70 (7.1%)</td>
</tr>
</tbody>
</table>

aThe 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.

bP < 0.05 (P value signifies a difference compared to P-treated mice).

WT Mice Treated with an ERα Agonist

To confirm these observations on the importance of ERs 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 up-regulates 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
E2 exposure due to reduced B-cell lymphopoiesis, 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 ST6GAL1, 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 ERβ antagonism in other tissues. This result would represent a nonimmunosuppressive approach to lupus therapy. Furthermore, understanding functional polymorphisms in ERs 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

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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.

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

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

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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

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 nonglomerulotrophic, 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.

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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 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 ovarioectomized 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-labeled anti-IgG2b Ab (BD Pharmingen), and allophycocyanin-labeled anti-CD23 plus HI DNase were isolated, Fc blocked, and stained with PerCP-labeled anti-mouse IgG, Pacific blue-labeled anti-CD24 Ab, PE-labeled anti-IgG2b Ab (BD Biosciences), and anti-DNase Abs in serum

Measurement of serum DNase

Serum samples at different dilutions were added to the serum treatment of DCs

with AP substrate, and measured at 405 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. Serial dilutions of mouse sera or mouse rBAFF (Apotech) were added to the wells, followed by 10 µg/ml antibiotinylated anti-mouse BAFF mAb (clone 5A8; Apotech) and HRP-labeled streptavidin. The plates were developed with HRP substrate, and the OD was measured at 450 nm.

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 kbp 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 × 10⁶ 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 antibiotinylated 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, 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. 1A), and increased serum BAFF levels (Fig. 1B). 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 10⁶ 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. 1C, 1D). 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. 1E, 1F).

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 levels of E2 mice was previously shown (Fig. 1A), and increased serum BAFF levels (Fig. 1B). 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 the type 1 IFNs (IFN-α,β), as well as the IFN-inducible genes ifi202b 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, ifi202b, and mx1 transcription to levels comparable to P-treated mice. RNA from splenocytes was analyzed for expression of BAFF and the IFN-inducible genes ifi202b 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, ifi202b, 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.
levels was observed throughout the treatment period, stabilizing by 3 wk (Fig. 2A). 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. 2B). 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. 2C). 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 HI DNase, demonstrating that E2 did not by itself regulate expression of IFNs as well as IFN-inducible genes. Thus, E2 does not directly and by itself upregulate BAFF. DNase treatment also caused a decline in the expression of IFN-inducible genes to baseline levels. Therefore, E2 does not directly and by itself 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.

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 Vk1A-Jk1, Vk1A-Jk4, and Vk10-Jk5 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 Vk1A-Jk5, Vk21-Jk1, and Vk21-Jk2 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...
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- or E2- or 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 Vx-Jk L chains in all experimental groups, 10 of which were commonly expressed in all the groups. In R4A mice, Vx4/5 L chains predominated (40%), followed by Vx1 (22%) and Vx21 (12%) L chains. In contrast, E2 treatment resulted in a predominant Vx1 L chain usage (45%), followed by Vx21 (21%) and Vx9/10 (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 Vx4/5 L chains, followed by Vx1 and Vx21 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). 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. 4A). 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 ∼3.6 × 10⁻⁸ M. Sera from E2-treated R4A Tg mice have an apparent affinity of 2.6–5.4 × 10⁻⁸ M. Interestingly, sera from E2 plus DNase-treated R4A Tg mice displayed an apparent affinity of 1.4–3.2 × 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 × 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. 4B, 4C) that correlated with a decrease

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

<table>
<thead>
<tr>
<th>Vx-Jk Usage</th>
<th>Relative Affinity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vx1A-Jk1</td>
<td>9.1 × 10⁻⁸</td>
<td>33, 37</td>
</tr>
<tr>
<td>Vx1A-Jk5</td>
<td>4.5 × 10⁻⁹</td>
<td>33</td>
</tr>
<tr>
<td>Vx1A-Jk4</td>
<td>4.2 × 10⁻⁶</td>
<td>37</td>
</tr>
<tr>
<td>Vx10-Jk5</td>
<td>6.6 × 10⁻⁸</td>
<td>34</td>
</tr>
<tr>
<td>Vx21-Jk1</td>
<td>2.2 × 10⁻⁸</td>
<td>33</td>
</tr>
<tr>
<td>Vx21-Jk2</td>
<td>9 × 10⁻⁶</td>
<td>33</td>
</tr>
<tr>
<td>Vx4/5-Jk5</td>
<td>ND</td>
<td>34</td>
</tr>
<tr>
<td>Vx19-Jk5</td>
<td>ND</td>
<td>34</td>
</tr>
<tr>
<td>Vx24/25-Jk2</td>
<td>ND</td>
<td>34</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Placebo (%)</th>
<th>E2 (%)</th>
<th>E2 + DNase (%)</th>
<th>E2 + HI DNase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transitional</td>
<td>6/50 (12)</td>
<td>12/52 (23)</td>
<td>8/63 (12.6)²²</td>
</tr>
<tr>
<td>Mature</td>
<td>1/9 (11)</td>
<td>4/41 (10)</td>
<td>3/46 (6.5)</td>
</tr>
<tr>
<td>High affinity</td>
<td>5/60 (8.3)</td>
<td>18/62 (27.7)</td>
<td>8/72 (11.1)²²</td>
</tr>
<tr>
<td>Low affinity</td>
<td>11/60 (18)</td>
<td>5/62 (8.3)</td>
<td>18/72 (25)</td>
</tr>
</tbody>
</table>

²²Significant 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 placebo group.

³³p < 0.05.

ns, Not significant.
in proteinuria (Fig. 4D, 4E); HI DNase failed to affect immune complex deposition (Fig. 4B, 4C). 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 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<sup>hi</sup>CD23<sup>lo</sup>CD24<sup>lo</sup>, and transitional B cells were identified as CD21<sup>lo</sup>CD24<sup>hi</sup> (T1) and CD21<sup>hi</sup>CD24<sup>hi</sup> (T2). Five mice were used in each group.

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.
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 increase 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 nonspecifically 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 (25–26, 59). 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 DNase-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.

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Disclosures
The authors have no financial conflicts of interest.

References


