Complex sex-biased antibody responses: estrogen receptors bind estrogen response elements centered within immunoglobulin heavy chain gene enhancers

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Abstract

Nuclear hormone receptors including the estrogen receptor (ER α) and the retinoic acid receptor regulate a plethora of biological functions including reproduction, circulation and immunity. To understand how estrogen and other nuclear hormones influence antibody production, we characterized total serum antibody isotypes in female and male mice of C57BL/6J, BALB/cJ and C3H/HeJ mouse strains. Antibody levels were higher in females compared to males in all strains and there was a female preference for IgG2b production. Sex-biased patterns were influenced by vitamin levels, and by antigen specificity toward influenza virus or pneumococcus antigens. To help explain sex biases, we examined the direct effects of estrogen on immunoglobulin heavy chain sterile transcript production among purified, lipopolysaccharide-stimulated B cells. Supplemental estrogen in B-cell cultures significantly increased immunoglobulin heavy chain sterile transcripts. Chromatin immunoprecipitation analyses of activated B cells identified significant ER α binding to estrogen response elements (EREs) centered within enhancer elements of the immunoglobulin heavy chain locus, including the E μ enhancer and hypersensitive site 1,2 (HS1,2) in the 3' regulatory region. The ERE in HS1,2 was conserved across animal species, and in humans marked a site of polymorphism associated with the estrogen-augmented autoimmune disease, lupus. Taken together, the results highlight: (i) the important targets of ER α in regulatory regions of the immunoglobulin heavy chain locus that influence antibody production, and (ii) the complexity of mechanisms by which estrogen instructs sex-biased antibody production profiles.

Keywords: female, ligand, male, nuclear hormone receptor, vaccine

Introduction

The nuclear hormone receptor superfamily comprises two classes of ligand-regulated transcription factors. Class I receptors include the homodimeric estrogen receptor, ER α , and class II receptors include the heterodimeric vitamin A and vitamin D receptors (RAR-RXR and VDR-RXR, respectively). These regulate a plethora of biological systems including reproduction, circulation, digestion, wound repair and inflammation (1–5).

Major domains of nuclear hormone receptors include the following: (i) an N-terminal domain with a site of activation function (AF-1), (ii) a DNA-binding domain (DBD) comprising two highly conserved zinc fingers and (iii) a C-terminal ligand-binding domain (LBD) with a second AF (AF-2). The consensus DNA binding site for ER α is a palindromic repeat with a 3 bp spacer (estrogen response element, ERE, GGTCAnnnTGACC). This sequence and the requirements for

ER α binding to DNA are highly promiscuous. Furthermore, ER α can bind DNA both directly and indirectly by tethering to other DNA-binding proteins (6–8).

While it is often assumed that the binding of ligand to receptor (e.g. estrogen to ER α) enhances gene expression, this is not always the case (9–16). ER α conformations are altered by the binding of estrogen ligands, and the variant configurations of ER α will influence its interactions with DNA and cofactors resulting in positive, negative or neutral effects on gene expression (17–20). Outcomes will depend on the sequence and context of the DNA target, the host cell type, the concentrations/forms of estrogen metabolites and the presence of other nuclear-co-repressors/co-activators in the environment. Cross-regulation can occur between the different nuclear hormone receptors (7, 12, 18, 20–34), possibly due to competition for DNA binding sites, ligands and co-activators.

Vitamins and sex hormones influence the immune response both *in vivo* and *in vitro* (32, 35–45), resulting in outcomes such as ‘man-flu’, the increased severity of influenza virus disease in men compared to women (46, 47). There are conflicting reports as to the mechanisms involved. Pauklin *et al.* previously reported that ER bound the activation-induced cytidine deaminase (*AICDA*) gene promoter in B cells to up-regulate the production of protein (AID) (48), an enzyme that supports somatic hypermutation and immunoglobulin heavy chain class-switch recombination (CSR). In contrast, Mai *et al.* suggested that the up-regulation of AID by estrogen was independent of ER binding to a putative ERE in the *AICDA* promoter. Rather, they proposed that ER up-regulated HOXC4 (a transcription factor and member of the homeobox family), which then up-regulated AID indirectly (49, 50). Mai *et al.* found no estrogen-induced increases in I μ -C μ or I γ 1-C γ 1 heavy chain gene sterile transcripts known to associate with CSR (49).

In this report, we revisit the mechanism of the sex-biased antibody response. We examined sex and strain effects on antibody production in mice, and examined consequences of estrogen addition to activated, purified B cells. Our results show that sex-biased antibody responses are influenced by mouse strain, vitamin levels and antigen specificity. When we examined purified, activated B cells in cultures supplemented with estrogen compared to unsupplemented cultures, we observed an estrogen-driven increase among sterile transcripts within the immunoglobulin heavy chain locus (51, 52). We further observed ER α binding to ERE centered within enhancer elements of the heavy chain locus. These activities show that estrogen does more than up-regulate AID. Rather, ER α and estrogen may be integral components of enhanceosomes (protein complexes associated with gene enhancers) and switchosomes (our term for the protein complexes associated with switch sites) that directly regulate antibody expression (6, 7, 21, 53–61).

Methods

Animals and tests for total serum antibodies and estrogen

Animal care practices followed the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC) guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC). Mice were C57BL/6J, C3H/HeJ

and BALB/cJ from Jackson laboratories. Mice were fed a control, grain-based diet (LabDiet®5013, Purina Mills, Inc.) unless otherwise stated. Blood was collected and allowed to clot, and sera were removed after a high-speed spin. For isotype testing, sera were diluted 1:25000 in assay buffer and evaluated for IgM, IgG3, IgG1, IgG2b, IgG2a and IgA using a MILLIPLEX MAP mouse immunoglobulin isotyping kit (Cat #MGAMMAG-300K), per manufacturer's instructions (note that the C57BL/6J mouse expresses IgG2c instead of IgG2a and that the MILLIPLEX MAP reagent was designed to score IgG2a). Samples were read on a Luminex 200 Multiplexing instrument using xPonent software. Data were processed using Milliplex Analyst software. Limits of detection (LOD) varied between experiments based on standard curves. For calculations, if samples were below or above an LOD, they were assigned that LOD score. Estrogen levels in mouse sera and cultures were tested with a Mouse/Rat Estradiol (E2) ELISA Kit (Calbiotech).

Vitamin-deficient mice

To produce vitamin-deficient mice, pregnant C57BL/6 female mice were received at 4–5 days gestation from Jackson Laboratories (Bar Harbor, ME, USA) and were placed on characterized diets (Harlan Laboratories, Madison, WI, USA) in filter-top cages [as described previously (44)]. The vitamin A-deficient (VAD) diet (Harlan Cat #TD.10762) contained Casein, DL-Methionine, sucrose, corn starch, cotton seed oil, cellulose, mineral mix AIN-76 (170815), calcium carbonate, vitamin mix (lacking Vitamin A) plus choline and food coloring. The vitamin D-deficient (VDD) diet was matched, but lacked vitamin D rather than vitamin A (Harlan Cat #TD.10763). The control diet included vitamin A palmitate at 15 IU g⁻¹ and vitamin D at 1.5 IU g⁻¹ (Harlan Cat #TD.10764). The vitamin A+D-double-deficient diet was Harlan Cat #TD.10616. For animals depleted of vitamin D (VDD or VAD+VDD), cages were placed in dedicated cubicles with LED bulbs as the source of light to avoid UV-B irradiation. Pregnant females were maintained on diets throughout their pregnancies and weaned pups were maintained on diets throughout maturation and experimentation. Mice were adults when tested and had been administered intra-peritoneal injections with phosphate-buffered saline (PBS) to serve as experimental negative controls.

Flow cytometry

Blood was collected in ethylenediaminetetraacetic acid or acid citrate dextrose (ACD) buffer to avoid clotting and to allow cell separation by centrifugation. Red cells were lysed by lightly vortexing cells after the addition of ammonium chloride buffer (Cat #07850 Stem Cell), incubating for 10 min at room temperature, and washing cells by centrifugation. Cells were suspended in staining wash buffer [1% fetal calf serum (FCS) in PBS] in 96-well round bottomed plates. Fc receptor block (anti-CD16/CD32, BD Biosciences) was incubated with cells for 15 min at room temperature. Staining reagents were then incubated with cells for 30 min on ice and cells were washed, re-suspended in 7AAD buffer (Invitrogen) and analyzed on a FACS Calibur. Staining reagents were conjugated antibodies with specificity either for CD19 [FITC BD Biosciences 553785, Clone 1D3 (RUO)] or B220 [APC

BD Biosciences 553092, Clone RA3-6B2 (RUO)]. Cells were gated for live lymphocytes on the basis of forward scatter and side scatter parameters, and negative staining with 7AAD.

Antibody responses to an influenza virus vaccine in female and male mice

Female and male C57BL/6 mice were anesthetized with isoflurane and administered 30 μ l of a cold-adapted derivative of Influenza A/Puerto Rico/8/34 (PR8, kindly provided by Dr J. McCullers) by the intra-nasal route to mimic vaccination with the FluMist vaccine (62, 63). Virus was prepared by amplification in hens' eggs at 33°C. Virus vaccine was at a concentration of 1.25×10^9 EID₅₀ ml⁻¹. Sera were collected 3 weeks post-vaccination. Vaccine-induced antibodies in sera from individual mice were evaluated with an influenza virus-specific enzyme-linked immunosorbent assay (ELISA) using wild-type PR8 influenza virus as antigen. This reagent was produced in eggs and purified by sucrose gradient sedimentation. Purified influenza virus was lysed in disruption buffer (0.05% Triton X-100, 60 mM KCl, 10 mM Tris pH7.8) for 5 min at room temperature and diluted with PBS to 5 μ g ml⁻¹ for coating 96-well ELISA microtiter plates overnight at 4°C. Plates were washed and blocked with PBS containing 3% bovine serum albumin (BSA) for another overnight incubation, after which serially diluted (10 $\times$ dilutions starting at 1:1000 in 3% BSA, 0.1% Tween in PBS) test samples were plated and incubated for 1 h at 37°C. Assays were washed with 0.1% Tween in PBS and then incubated with alkaline phosphatase (AP)-conjugated anti-isotype antibodies diluted in 3% BSA and 0.1% Tween in PBS for 1 h at 37°C. AP-conjugated anti-isotype antibodies were specific for IgM [Southern Biotechnology Associates (SBA) Cat #1020-04], IgG3 (SBA Cat #1100-04), IgG1 (SBA Cat #1070-04), IgG2b (SBA Cat #1090-04) or IgG2c (SBA Cat #1077-04). Previous studies showed that IgM and IgG were the predominant isotype classes responsive to the influenza virus vaccine. Assays were washed again and developed with p-nitrophenyl phosphate (Sigma Aldrich) in diethanolamine buffer, and optical density (405 nm) measurements were taken. Titers were determined using non-linear regression software (GraphPad Prism) to identify the serum dilution required to give a reading of 0.1. When readings were below detection, a score of 100 was assigned.

Mouse vaccination with Prevnar-13

Pregnant C57BL/6 females at days 4–5 gestation were placed on a characterized, purified, control diet (TestDiet® 5W9M, Purina Mills, Inc.) upon arrival at St. Jude from Jackson Laboratories. Offspring remained on the diet until adulthood and throughout the course of the study. Animals were vaccinated with Prevnar-13, a pneumococcus vaccine (PCV, diluted 1:40 in PBS, 100 μ l per mouse) by the intra-peritoneal route. Mice were primed at 7 weeks of age and boosted at 10 weeks of age. Animals were bled 8–10 days after boosting. ELISAs were performed with a similar protocol to that described above for the influenza virus-specific ELISA, except that: (i) plates were coated with 5 μ g ml⁻¹ of T4 polysaccharide (from ATCC), (ii) plates were blocked for 4 h at room temperature, (iii) sera were diluted 1:100, 1:500, 1:2500

and plated for overnight incubation at 4°C and (iv) a subset of isotypes (IgM, IgG3, IgG1 and IgG2b) were tested by a 1-h incubation with anti-isotype antibodies at room temperature (previous experiments revealed that these were the predominant isotypes responsive to the PCV vaccine). Titers were determined using non-linear regression software (GraphPad Prism) to identify the serum dilution required to give a reading of 0.05. A score of 20 was assigned to samples that scored below detection.

B-cell purification

Single cell suspensions from female or male C57BL/6J mouse spleens were made by mechanically disrupting the tissues and passing cells through a 70- μ m cell strainer. Lymphocytes were separated from erythrocytes by centrifugation on a cushion of Lymphocyte Separation Medium (MP Biomedicals). B cells were purified by negative selection with anti-CD43 and anti-CD11b microbeads (Miltenyi Biotec) and passing through a MACS LD Column (Miltenyi Biotec) followed by the collection of the unbound B cells.

B-cell cultures

Purified B cells were plated in 96-well flat-bottomed tissue culture plates (TPP Cat #92696) at a final concentration of 4×10^5 cells/200 μ l in freshly prepared RPMI medium (Life Technologies) containing 10% fetal bovine serum (Atlanta Biologicals), 2mM L-glutamine (Life Technologies), 50 μ g ml⁻¹ gentamicin (Lonza) and 55 μ M 2-mercaptoethanol (Life Technologies). Lipopolysaccharide (LPS; Sigma) was added to a final concentration of 5 μ g ml⁻¹ and cultures were incubated at 37°C in 5% CO₂. Estrogen (β -estradiol, Sigma Aldrich Cat #E2257) was added to some cultures to achieve a 100 nM concentration. FCS is often treated with charcoal to remove estrogen, but this procedure also depletes other factors. To avoid this, we used normal FCS that was pre-tested for estrogen by ELISA. The level was $\sim$ 30 pg ml⁻¹ ($\sim$ 0.1 nM). Female mice were also spot-checked for estrogen, and levels were consistently $<$ 75 pg ml⁻¹. Cultures were harvested on day 1 for chromatin immunoprecipitation (ChIP) analyses, day 2 for analyses of sterile immunoglobulin heavy chain transcripts and *AICDA* transcripts, and day 6 for analyses of secreted antibodies in culture supernatants.

ChIP-Seq

Cultured B cells were harvested and treated with 2 mM disuccinimidyl glutarate (DSG, ProteoChem) in Dulbecco's phosphate-buffered saline (DPBS; Lonza) with the following proteinase inhibitors (PIs); phenylmethylsulfonyl fluoride (Sigma), Pepstatin A (Sigma) and Leupeptin (Sigma), and incubated at room temperature with rotation for 30 min. Cells were washed and fixed in DPBS plus PIs and 1% paraformaldehyde (Sigma) for 5 min with rotation at room temperature. The reaction was quenched by adding glycine to achieve a 200 mM final concentration and rotating for an additional 5 min. The cell pellet was washed with DPBS plus PIs and then lysed in Covaris lysing buffer + PIs on ice for 10 min. Nuclei were centrifuged at 1500 $\times$ g for 5 min and subjected to a series of washes in Covaris wash buffer and shearing buffer with

Pls. The pellet was re-suspended in Covaris shearing buffer plus Pls at a concentration of 1 ml per initial 2×10^7 cells and sheared in the Covaris E210 (Covaris) in Covaris Milli Tubes under the following conditions: 200 cycles per burst, 20 W for 30 min. The Covaris shearing yielded reproducible DNA fragment sizes (~100–850 bp in size). Sheared chromatin was diluted 1:3 with Covaris ChIP dilution buffer and bound with anti-ER α antibody (Abcam #32063, monoclonal E115), 5 μ g/ 2×10^7 cell equivalents, overnight with rotation at 4°C. Protein A/G magnetic beads were added at 20 μ l ml⁻¹ and incubated with rotation at 4°C for at least 1 h. Magnetic beads were pelleted using a magnetic rack, and serially washed with a low-salt buffer, a high-salt buffer, a LiCl buffer and TE buffer. After washing, the beads were re-suspended in 130 μ l sterile water and heated to 95°C for 10 min. NaCl was added to 80 mM final concentration. Proteinase K (10 μ g; Ambion) was added per sample and samples were incubated at 56°C for at least 1 h. After incubation, the beads were heated to 95°C for 10 min and then allowed to cool to room temperature. The beads were pelleted with the magnetic rack and supernatant was transferred to a new tube. DNA was purified with a PCR Purification Kit (Qiagen) and eluted in 30 μ l RNA/DNase-free water. Samples were submitted to the Hartwell Center for completion of library preparation and sequence analysis.

Libraries were prepared from DNA using the NEBNext ChIP-Seq Library Prep Reagent Set for Illumina with NEBNext Q5 Hot Start HiFi PCR Master Mix according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA) with a modification: a second 1:1 Ampure cleanup was added after adaptor ligation. Completed libraries were analyzed for insert size distribution on a 2100 BioAnalyzer High Sensitivity kit (Agilent Technologies, Santa Clara, CA, USA) or Caliper LabChip GX DNA High Sensitivity Reagent Kit (PerkinElmer, Waltham, MA, USA). Libraries were quantified using the Quant-iT PicoGreen ds DNA assay (Life Technologies), Kapa Library Quantification kit (Kapa Biosystems, Wilmington, MA, USA) or low-pass sequencing on a MiSeq nano kit (Illumina, San Diego, CA, USA). Fifty-cycle single-end sequencing was performed on an Illumina HiSeq 2000 or 2500. For count comparisons between libraries, uniquely mapped reads were extracted using samtools (version 1.2 parameter '-F 1024 -q1') and extended by 200 bp 3'. Reads that overlapped regions of interest were counted using bedtools (version 2.24.0, intersect function).

ChIP-qPCR

Chromatin was prepared as described above for ChIP-Seq. Samples were diluted for immunoprecipitation. The sample was divided into equal parts representing $\sim 1.0 \times 10^7$ cells per immunoprecipitation. Two immunoprecipitations were with the mouse ChIP-IT qPCR Control Kit from Active Motif (Cat #53027), used per manufacturer's instructions. Included in the kit were the anti-RNA Pol II antibody (clone 4H8) and a negative control mouse IgG. The anti-ER α antibody (Cat #ab32063 Abcam, clone E115) was also used for immunoprecipitations. An equal amount of input chromatin was precipitated with ethanol. DNA was purified as described for ChIP analyses. PCR was performed with forward and reverse

primers in the hypersensitive site 1,2 (HS1,2) region, atcagtagcagaaacaaggc and ttgggtgaacctgcagc, yielding a 208 bp product (mm9 chr12:114483016–114483223) (64). PCR was also performed with primers in Sy1, cttatgccaccactgtcaatc and ctgtttgtatggcctcttcttgc (mm9 chr12:114577414–114577621) (65). The following qPCR conditions were used: 50°C for 2 min, 95°C for 10 min for 1 cycle, then 95°C for 15 s and 60°C for 1 min, for 40 cycles on a 7300 Realtime PCR (Applied Biosystems). Fold-over-control IgG values were calculated for each test antibody, for HS1,2 and Sy1 regions. HS1,2/Sy1 ratios were then determined.

Transcript analyses

RNA was isolated from day 2 cultures using a Qiagen AllPrep DNA/RNA Mini Kit (Cat #80204), and cDNA was prepared by using 8 μ g RNA pre-annealed to 2 μ g Oligo dT primer (Cat #S0131; Thermo Scientific) in 40 μ l total volume and reverse transcribing in the following reaction: 20 μ l 5 $\times$ AMV Reverse Transcriptase Buffer (Cat #M515A; Promega), 10 μ l dNTP 10 mM Mix (Cat #U151A; Promega), 40 μ l RNA/Oligo dT Primer, 2 μ l RNAsin Plus (Cat #N261A; Promega), 2 μ l AMV Reverse Transcriptase (Cat #M5101; Promega) and RNase-free Water to 100 μ l total volume. The reaction was conducted at 42°C for 1 h and then chilled on ice. qPCR was performed as described above using 5 μ l of a 1:4 dilution of the cDNA. Primers for transcripts were acctgggaatgtatggtgtggctt and tctgaacctcaaggatgctcttg for I μ -C μ , gcagaaatctgcagactaaca and accgaggatccagatgtgtc for I γ 3-C γ 3, ggccctccagatctttgag and atggagtagtttgggcagca for I γ 1-C γ 1, tgggcctttccagacctaata and gggctgatctgtcaactcct for I γ 2b-C γ 2b, cagcctgggatcaagcag and tggggctgtgttttggc for I γ 2c-C γ 2c, ctaccatagggaagatagcct and taatcgtgaatcaggcag for I α -C α , atgtgctctccccaattgt and attcgttgggcaaagcgtc for A/CDA, and tgggtgctgtcttccatgc and ttgctgtgaccggctcac for Ig β . Fold change of Δ CT(LPS) and Δ CT(LPS + Estrogen) were each standardized against Ig β (66).

B-cell culture supernatant analyses for secreted antibodies

Supernatants collected at day 6 following purified B-cell cultures were stored frozen, thawed and then tested for antibodies. The isotyping kit and procedures described above were used, except that supernatants were first mixed and diluted 1:5 in Millipore assay buffer. IgM values scored above the standard curve and were not reported.

Statistical evaluations

Statistical analyses were performed with GraphPad Prism software. Comparisons between two groups were with unpaired *T*-tests or Mann–Whitney tests. Analysis of variance (ANOVA) with Dunnett's test was used when several test groups were compared to the same control.

Results

Total antibody isotype profiles in male and female mice

To define antibody production patterns among male and female mice, we assessed serum antibodies using

bead-based assays. Results from C57BL/6 animals examined at various ages are shown in Fig. 1(A) and Supplementary Figure 1. We found that the concentration of serum antibodies increased with age, but regardless of age, females exhibited higher levels of antibodies than males. Statistically significant increases were observed for IgG subclasses in females compared to males (Supplementary Figure 1). We also evaluated male and female antibody levels in BALB/cJ and C3H/HeJ mouse strains (Figure 1B and Supplementary Figures 2 and 3). Similar to the situation for C57BL/6 mice, females of BALB/cJ and C3H/HeJ mouse strains exhibited higher serum antibody levels than males. IgG2b was 'female-preferred' in that it was significantly higher in females compared to males in all three mouse strains.

A preliminary test was conducted of purified B-cell cultures from C57BL/6 female spleens, stimulated with LPS in the presence or absence of supplemental estrogen. Supernatant samples were taken after 6 days to measure antibody levels (Figure 2). In the presence of supplemental estrogen, there was an increase in the relative production levels of IgG2b (e.g. increased IgG2b/IgG3 and IgG2b/IgA antibody ratios). The IgG2b preference observed in tissue culture on day 6 matched that observed in female mice, suggesting that estrogen may directly up-regulate B-cell production of IgG2b, both *in vitro* and *in vivo*.

Fig. 1. Female mice exhibit higher total antibody levels and altered isotype profiles compared to males. For each of the three experiments shown, male and female mice were evaluated for total antibody levels in blood samples (three to five males and three to five females per age group) using a magnetic bead platform. The ages of tested animals are shown in weeks (wk) or months (mo). (A) Experiments were conducted to compare total antibody and antibody isotype production among C57BL/6J (B6) male and female mice (ng ml⁻¹). As a preliminary analysis, mice from various age groups were also compared. Averages are shown. Note that the isotyping reagent was not designed to score the IgG2c isotype of C57BL/6 mice. (B) Experiments (and repeat experiments) compared male and female mice of the BALB/cJ (BALB/c) and C3H/HeJ (C3H) strains.

Blood lymphocytes were examined to determine if B-cell frequencies predicted changes in antibody levels. Supplementary Figure 4 shows results from C57BL/6 female and male mice compared for B-cell frequencies among blood lymphocytes. Supplementary Table 1 shows results from Supplementary Figure 4 in conjunction with results from additional experiments that tested different mouse strains and age groups. Although differences among lymphocyte frequencies were observed among strains and age groups (67, 68), there were not significant differences between males and females that could easily explain the sex-biased antibody levels (Supplementary Figure 4 and Supplementary Table 1).

Vitamin ligands for nuclear hormone receptors alter antibody levels in female and male mice

Given that cross-regulation can occur between ligands for the nuclear hormone receptors (53–55), we asked if vitamin levels influenced antibody production in female and male mice. We compared control ('++') C57BL/6 female and male mice with mice that were vitamin A deficient (VAD), vitamin D deficient (VDD), or double deficient for vitamins A+D ('--'). We found that vitamin deficiencies influenced antibody levels, causing changes in female:male antibody ratios (Figure 3). Most striking were the significant increases in IgG2b and trends toward increases in other IgG subtypes among males when mice were deficient in vitamins A or vitamins A+D. Data may have reflected cross-regulation between two nuclear hormone receptors, bound respectively by vitamins and sex hormones.

Antibody responses toward influenza virus and Plevnar-13 differ between female and male animals

To determine if the sex bias in total antibody levels predicted a sex bias in antibody responses to vaccines, we examined responses against viral and bacterial vaccines in female and male C57BL/6 mice. First, we analyzed C57BL/6 females and males three weeks after vaccination with an intra-nasal cold-adapted influenza virus vaccine (Figure 4A–E). Females generated significantly more influenza virus-specific antibodies than males. Significant increases in the female-preferred IgG2b isotype were reproducibly observed.

Experiments were next conducted in which mice were vaccinated with the pneumococcal vaccine Plevnar-13, a conjugated polysaccharide vaccine (Figure 4F–I). One might have expected that females would again respond better than males, but this was not the case. Rather, male mice responded better than females to the Plevnar-13 vaccination. Results showed that sex biases among antibody responses did not always favor females.

Supplemental estrogen up-regulates sterile transcripts in the immunoglobulin heavy chain locus among purified, activated B cells

A previous study suggested that following stimulation with LPS plus cytokines, splenic B cells that received supplemental estrogen had enhanced levels of *AICDA* transcripts, whereas immunoglobulin heavy chain sterile transcripts $I\mu$ -C μ and $I\gamma$ 1-C γ 1 (precursors of CSR (51)) were unchanged (49). Using

Fig. 2. Estrogen-supplemented purified B-cell cultures. Purified B cells from eight female mouse spleens were cultured with LPS, with or without supplemental estrogen (100 nM), and supernatants were harvested after 6 days for antibody isotype analyses. Isotype levels and ratios are shown with SEs. Asterisks mark significant differences between B cells cultured with and without supplemental estrogen.

purified splenic B cells from female and male mice cultured with LPS and harvested 2 days after stimulation (Figure 5), we queried the same transcripts. We observed a trend toward increased *AICDA* mRNA in cultures supplemented with estrogen. More surprising and striking was our finding that supplemental estrogen significantly up-regulated sterile transcripts. Results revealed a new B-cell intrinsic regulatory function of estrogen, and a new explanation for the higher antibody levels in females compared to males.

ER α binding in the heavy chain locus of B cells stimulated with LPS or LPS + Estrogen

Previously, we discovered the presence of numerous retinoic acid response elements (RARE) and ERE in the immunoglobulin heavy chain locus (69, 70) encouraging a focused test of nuclear hormone receptor binding to the locus by ChIP assays. In follow-up experiments, we purified B cells from the spleens of female C57BL/6 mice stimulated with LPS and demonstrated significant binding of ER α to the heavy chain locus (69, 70). Here, we describe a study with B cells purified from female spleens and stimulated with LPS in the presence of supplemental estrogen. An alignment of ER α DNA binding peaks within the immunoglobulin heavy chain locus is shown in Fig. 6 for cultures stimulated with or without supplemental estrogen [Integrative Genomics Viewer (IGV) software]. For cells stimulated with LPS only (Fig. 6A, top graph), the four highest peaks were positioned at HS4 and HS1,2 enhancers of the 3' regulatory region (3'RR), the switch site S μ and the E μ enhancer (positioned from left to right in Fig. 6A). There were also peaks in immunoglobulin light chain and TCR

loci (Supplementary Figure 5) (71–74). HS4, HS1,2 and E μ enhancers have been studied extensively and shown to influence the chromatin looping/remodeling required for CSR, and immunoglobulin heavy chain gene expression (51, 75–78).

The B cells stimulated with LPS + Estrogen (Fig. 6A, bottom image) shared major peaks with LPS-stimulated B cells. The HS1,2 peak is magnified in Fig. 6(B) using IGV software and the autoscale function. Subtle pattern shifts, but not absolute on-off changes in ER α binding were evident. Read counts within regions of interest were tabulated for the two libraries as shown in Supplementary Table 2. Although preliminary, an intriguing observation was that when read ratios were calculated to compare cultures (LPS + Estrogen/LPS), the highest ratio among switch regions was for the switch region of the female-preferred IgG2b gene. Results suggest that the favored IgG2b production in female mice (Fig. 1 and 4; Supplementary Figures 1–3) and in our estrogen-supplemented B-cell culture (Fig. 2) was influenced by ER α binding within the immunoglobulin heavy chain locus.

We recognize that when there was a signal change in a DNA binding site in the ChIP studies, it may have reflected: (i) enhanced binding or release of ER α from a site, (ii) a conformational change in ER α driven by estrogen binding (24) that altered the ER α immunoprecipitation reaction and/or (iii) recruitment of cofactors to ER α that enhanced or inhibited ER α immunoprecipitation. Our ChIP-seq libraries were produced after DSG treatments to facilitate protein–protein interactions, meaning that the ER α associations with DNA may have been facilitated by the tethering of ER α to other factors.

Fig. 3. Vitamin levels differentially alter antibody production profiles in male and female mice. Antibody responses were evaluated in C57BL/6J animals that received a control diet (++), or that received diets lacking vitamin A (VAD), vitamin D (VDD) or vitamins A and D (– –). Results shown are from two combined experiments that included a total of 10 animals on the ++ diet (5 females and 5 males), 7 animals on the VAD diet (2 females and 5 males), 10 animals on the VDD diet (5 females and 5 males) and 10 animals on the double-deficient (– –) diet (2 females and 8 males). Animals were between 10 and 14 weeks of age, and had received intra-peritoneal injections with PBS (no vaccine was administered). Note: For isotypes, IgG2a and IgA, some values fell below the LOD, in which case they were set to the LOD. In the first experiment, the LOD for IgA was 193 750 ng ml⁻¹ and the LOD for IgG2a was 16 250 ng ml⁻¹. In the second experiment, the LOD for IgG2a was 23 500 ng ml⁻¹. Groups of VAD, VDD or VAD+VDD mice were compared to mice on control diets using ANOVA with Dunnett's test, evaluating males and females separately. (A) Averages for each isotype are shown. (B–G) Individual means and SEs for the different isotypes (indicated on Y-axes) are shown for mice on ++, VAD, VDD, and – – diets. *Significant difference between antibody levels when mice on a test diet were compared to sex-matched mice on the control (++) diet (ANOVA with Dunnett's test).

Fig. 4. Influenza virus vaccine-specific and Prevnar-13 vaccine-specific antibody response differences between male and female mice. (A–E) Male ($n = 5$) and female ($n = 5$) C57BL/6J mice were vaccinated with a cold-adapted influenza virus vaccine to compare vaccine-specific immune responses between sexes. The experiment was repeated. Mice were examined 3 weeks post-vaccination; 1:10 serial serum dilutions were made starting at 1:1000. Titers for naive mice were <1000. Test samples that showed no activity were assigned a titer of 100 to support calculations. (F–I) Male ($n = 5$) and female ($n = 5$) C57BL/6J mice were primed and boosted with the Prevnar-13 vaccine and tested 8–10 days after the last boost for antibody responses to pneumococcus polysaccharide type T4. The experiment was repeated; 1:5 serial dilutions were made of serum starting at 1:100. Titers for naive mice were <100. Test samples that showed no activity were assigned a titer of 20 to support calculations. Titers were defined by the inverse serum dilution that gave an optical density reading of 0.1 nm for influenza virus vaccine-specific responses and 0.05 nm for Prevnar-13 vaccine-specific responses. Means with SEM are shown. Mann–Whitney tests were used to compare male and female groups.*

ChIP-qPCR was used to confirm the binding of ER α to the HS1,2 region. In this study, we evaluated a 208 bp PCR product that marked the highest HS1,2 peak shown in Fig. 6(B) for cells stimulated with LPS + Estrogen (mm9 chr12:114483016–114483223). We also evaluated a 208 bp PCR product in Sy1, where there was relatively little ER α binding (mm9 chr12:114577414–114577621). In repeat experiments with unstimulated or day 1 stimulated purified B cells, there was increased binding of anti-ER α and anti-RNA Pol II antibodies to the HS1,2 site compared to the control IgG (data not shown). Ratios (HS1,2/Sy1) for ER α and RNA Pol II fold-over-IgG values are shown in Fig. 7 to illustrate the relative repositioning of ER α and RNA Pol II binding in favor of the HS1,2 site when supplementary estrogen was added to cultures. Factor repositioning may license the enhanceosome to increase activities [e.g. to support sterile transcription, CSR

and gene expression (51, 79–81)] within the immunoglobulin heavy chain locus.

EREs define peaks of ER α binding in immunoglobulin heavy chain enhancer regions

Using IGV software, we searched for the sequence GGYANNNTGAYY to determine if EREs were present near the highest peaks of ER α binding in enhancer elements [a hotspot for ERE was previously identified in Sp (70)]. We found isolated EREs central to two ER α peaks (marked by asterisks in Fig. 6). Within a 30 000 bp sequence encompassing the HS1,2 site (mm9 chr12:114460000–114490000), there was just one ERE (114483000–114483012), and this was positioned precisely at the peak of ER α activity in the LPS cultures. In the E μ region, a similar region of 30 000 bp (chr12:114640000–114670000)

Fig. 5. Sterile transcripts increase among purified B cells stimulated with supplemental estrogen. B cells were purified from C57BL/6 female (A and B, $n = 10$) or male (C and D, $n = 10$) mouse spleens and stimulated with LPS for 2 days, either with or without supplemental estrogen, to examine *AICDA* mRNA (A and C) and sterile transcripts (B and D). Mean differences in *AICDA* and sterile transcript results from cultures stimulated with and without supplemental estrogen are shown with SEM. *AICDA* expression values trended higher in estrogen-supplemented cultures. Significant increases in $I\mu$ -C μ and $I\gamma$ -C γ transcript expression (labeled with asterisks) were demonstrated in estrogen supplemented cultures. Statistical analyses were with ANOVA and Dunnett's test for multiple comparisons, using Ig β as the control.

was queried. Again, there was only one ERE in this region (ch12:114665825–114665837), but in reverse orientation compared to that in HS1,2. A peak of ER α binding to E μ in both cultures was precisely coincident with the ERE. Results are compelling, as they position an ERE and an ER α binding peak at sites critical for (i) the juxtaposition of enhancers, promoters and switch regions, (ii) CSR and (iii) antibody gene expression (82).

Fig. (8) illustrates a working hypothesis to explain the influence of estrogen and ER α on antibody production. We propose that when estrogen is at limiting concentrations (Fig. 8A), ER α binds regulatory elements including E μ , HS1,2 and HS4 (80, 81, 83, 84). Binding in this case may be partially inhibitory in that ER α may block sites that could otherwise be occupied by activation factors. In the presence of supplemental estrogen, ER α and RNA Pol II [a factor recognized for its complex influences on antibody expression (51, 79, 85)] are repositioned within the locus. The HS1,2 enhancer is now licensed to support DNA looping/remodeling (portrayed by dotted lines in Figure 8B), and interactions among enhancers, promoters and switch sites to modulate CSR, and antibody production.

The sequence labeled 'intermediate anchor' just upstream of S γ 2B in Fig. 8 has not been well studied in mice, but in humans, the position upstream of S γ 2 contains a well-defined regulatory region (86, 87). As shown in Fig. 6, ER α binds to the intermediate anchor in mouse B cells. We propose that the binding of ER α to the intermediate anchor, along with the increased binding of ER α to S γ 2B in the presence of supplemental estrogen (Supplementary Table 2) assists the association of S γ 2b with enhanceosomes. Such associations may increase CSR within S γ 2b and thereby increase the production of the female-preferred IgG2b. The overall outcome is that supplemental estrogen alters antibody responses both quantitatively and qualitatively; estrogen repositions ER α and RNA Pol II within the immunoglobulin heavy chain locus to increase antibody production, while biasing production toward IgG2b.

We emphasize that other mechanisms are also at play when estrogen levels increase *in vivo* or *in vitro*. For example, the migration and functions of non-B cells may be impacted. However, the inclusion of ER α in enhanceosomes and switchosomes within the B-cell immunoglobulin heavy chain locus may provide the most direct and immediate influence of ER α on antibody expression patterns.

Fig. 6. ER α binding among activated B-cell cultures with and without supplemental estrogen. (A) ChIP-seq was performed with purified splenic B cells from female C57BL/6 mice stimulated for 1 day with LPS or with LPS and supplemental estrogen (100 nM). The library from LPS-stimulated cells has been described previously and used purified B cells combined from 7 mouse spleens (70). The library from cells stimulated with LPS and supplemental estrogen used purified B cells combined from 10 mouse spleens. Methods for DNA shearing and library preparation were similar for both B-cell cultures. Libraries were evaluated using IGV software and the two sets of results were aligned. IGV images were set on autoscale. The data scale for the LPS culture was ~0–130 and the data scale for the LPS + Estrogen culture was ~0–70. Control input values were <30 throughout the region. Gene positions are marked below profiles (markings are from the UCSC annotation file refFlat.txt.gz). Constant region genes are marked and switch regions are indicated immediately above constant regions. Two EREs that are central to peaks of ER α binding activity are indicated by asterisks. Read counts are shown in Supplementary Table 2. (B) A close-up of the HS1,2 region is shown for cultures treated with LPS (top graph) or LPS + Estrogen (bottom graph). IGV software and autoscale settings were used.

ERE sequences in HS1,2 and associations with lupus

Nucleotides adjacent to the HS1,2 ERE are shown in Fig. 9(A). Here, mouse and primate sequences are aligned at a position immediately downstream of the conserved HS1,2 enhancer core sequence, described previously (87, 88). The two half-sites of the ERE are shown in yellow and the spacer is highlighted in green. As demonstrated, the ERE is conserved. It is followed by an RRY repeat that may influence palindrome formation (shown in purple). A position of potential NF- κ B binding is shown in blue. CpG sequences (shown in red) are sites of potential methylation, additional epigenetic marks that may alter gene activity.

The region shown in Fig. 9 identifies a position of HS1,2 polymorphism. Two common alleles in HS1,2 among European populations are termed 1A or *1 and 2A or *2. Frezza *et al.* described the association of the *2 allele with the autoimmune disease lupus (88) and Napolioni *et al.* described a negative association with life expectancy (89). Lupus is a sex-biased disease that is influenced by estrogen and is characterized by high antibody levels (87, 90–102). In Fig. 9(B), the *2 sequence in HS1,2 is shown. We found that this allele contains tandem duplications of the potential ER α and NF- κ B binding sites. Based on these data, we

hypothesize that (i) estrogen influences ER α binding to the ERE in HS1,2, (ii) ER binding to the HS1,2 enhancer directly modifies antibody expression, and (iii) when the HS1,2 ERE is duplicated, there are estrogen-induced increases in antibody levels that exacerbate autoimmune disease in humans.

Discussion

Results in this report illustrate differences among antibody production patterns between male and female mice. We found that female mice generally exhibited higher levels of antibodies than males. However, changes in nuclear hormones other than estrogen (vitamins) altered results, suggesting nuclear hormones co-regulated antibody levels. Although previous explanations for estrogen up-regulation of B-cell responses focused on non-B-cell populations or AID up-regulation (49), we found that estrogen also influenced B cells by up-regulating sterile transcripts (particularly $I\mu$ -C μ transcripts when B cells were evaluated 2 days after stimulation). Our studies further identified ER α binding to ERE centered within key regulatory elements in the heavy chain locus (E μ , and HS1,2). The ERE in HS1,2 coincided precisely with a human polymorphism that was associated with enhanced vulnerability to the female-biased autoimmune disease, lupus. In total, results

revealed similarities between ER α and other nuclear factors that directly influence antibody expression patterns (e.g. AID, Mediator and Ikaros) (65, 78). We hypothesize that by binding enhancers in the immunoglobulin heavy chain locus, ER α

directly impacts antibody responses and thereby influences sex-biased vulnerabilities to pathogens and autoimmune disease.

Differences in total and antigen-specific antibody levels in females and males

There were a number of variables found to be instrumental in immune response regulation among females and males. We observed that age, strain, vitamin levels and antigen specificities all affected antibody output. Of particular interest were the significant increases in IgG2b among male mice in the context of vitamin A deficiency. One explanation for this finding may be that vitamin A competes with estrogen and can thereby inhibit estrogen's influence on antibody production (e.g. the IgG2b preference). Inhibition by vitamin A may be more prominent in males versus females because of the relatively low estrogen levels in males, but when vitamin A is removed, estrogen's influence on antibody production in males may be observed.

The regulation of antibody expression by both sex and vitamin hormones is not surprising, given that hotspots for EREs and RAREs, respectively, overlap in the immunoglobulin heavy chain locus (69). Published studies describe nuclear factor interactions in B cells, other lymphoid cells and non-lymphoid cells (103–106). Cross-talk among sex hormones and other nuclear hormone receptor ligands [e.g. thyroxine (53)] likely lead to the variability observed in clinical studies when the immune responses of males and females are compared.

The data from influenza virus vaccination experiments help to explain 'man-flu' (47), because males may generate a weaker immune response to influenza virus compared to females. Of interest was the opposite sex bias that we observed in the response to PCV, the Prevnar-13 vaccine. The change in ratios may have reflected differences in the

Fig. 7. ChIP-qPCR with anti-ER α and anti-RNA Pol II. ChIP-qPCR was performed to confirm the binding of ER α to the HS1,2 region among purified B cells. Experiments were conducted with anti-ER α antibody, anti-RNA Pol II antibody and a control IgG. A 208 bp HS1,2 PCR product marked the peak of highest ER α binding to HS1,2 shown in Fig. 6(B) for B cells stimulated with LPS + Estrogen (mm9 chr12:114483016–114483223). A second 208 bp PCR product marked a position in Sy1 where ER α binding was relatively low. Fold-over-IgG results were first determined for each antibody at each site. HS1,2 scores were then normalized by calculating ratios: HS1,2/average Sy1. Asterisks identify significant differences between normalized values for cultures stimulated with (A) LPS and (B) LPS + Estrogen. In five of six experiments using either male or female purified, cultured B cells, normalized values for ER α and RNA Pol II both trended higher or were significantly higher when LPS + Estrogen cultures were compared to cultures stimulated with LPS alone.

Fig. 8. How estrogen up-regulates antibody expression. One hypothesis to explain estrogen-induced up-regulation of antibody production is illustrated. Gene elements are shown below the line and proteins are shown above the line. (A) In the context of limited estrogen, ER α binds the immunoglobulin heavy chain locus in enhancer elements and switch sites. The binding may be inhibitory in that it may block binding by other activating factors. (B) In the presence of supplementary estrogen, ER α and RNA Pol II are repositioned within the locus. Repositioning licenses the HS1,2 enhanceosome to up-regulate looping/remodeling (indicated by dotted lines; not shown are additional interactions involving E μ , downstream S sites and HS4), and CSR. ER α binding to the intermediate anchor, and Sy2b may promote CSR at Sy2b to bias antibody production toward the female-preferred IgG2b isotype.

Fig. 9. Conserved ERE in the HS1,2 region of mice and primates. (A) The ERE in HS1,2 is shown for mouse and primate species. Sequences for the mouse were from the C56BL/6 mm9 sequence (IGV). Additional sequences were reported by Giambra *et al.* (87). (B) The *2 human polymorphism associated with increased vulnerability to lupus was reported by Frezza *et al.* (88). EREs are marked in yellow (1/2 sites) and green (spacer). A repeat RRY sequence is marked in purple. Positions of potential NF- κ B binding are marked in blue. CpG sequences are marked in red.

environment and phenotype of activated B cells. Animals in both experiments were on control diets, but diets were not identically formulated. The cold-adapted influenza virus vaccine was administered by the intra-nasal route with a single immunization and likely triggered conventional B cells in draining lymph node follicles. IgG2b was a prominent isotype in the response to this vaccine. In contrast, PCV was administered systemically by priming and booster immunizations, and may have preferentially triggered splenic marginal zone B cells, characterized by a very different isotype profile. Given that B-cell subsets, locations and environments differed between the two experiments, it is not surprising that nuclear hormone influences and sex biases were also different.

Previous literature further demonstrates the complexities of estrogen and other nuclear hormone impacts on antibody production (32, 36–39). Estrogen's capacity to up-regulate antibody was previously demonstrated in that treatment of male guinea pigs with diethylstilboestrol increased serum gamma-globulin levels (107), and treatment of male Swiss Webster mice with 17- β -estradiol increased numbers of splenic anti-*E.coli*-specific antibody-producing cells (108). Walker and Bole (101) found that over one-half New Zealand Black (NZB)/New Zealand White (NZW) male mice developed anti-nuclear antibodies after 6 weeks of treatment with mestranol. More variable influences of estrogen were demonstrated by Kenny *et al.* in that effects on *E.coli*-specific antibody-producing cells *in vitro* were both positive and negative depending on estrogen concentration (108). They further showed that a portion of NZB/NZW females exhibited hypergammaglobulinemia when administered 17- β -estradiol for 6 weeks, but that neither male NZB/NZW mice nor Swiss Webster mice of either sex were significantly affected (101, 102). Such variations encourage focused attention on host background, host age, environment, hormone metabolite, and hormone concentration

when estrogen supplements are tested in basic and clinical research studies.

B-cell intrinsic influences of estrogen include $I\mu$ -C μ and $I\gamma$ -C γ sterile transcript up-regulation

Unlike Mai *et al.* (48, 49), we observed significant increases in $I\mu$ -C μ and $I\gamma$ -C γ sterile transcripts when B cells were activated for 2 days in the presence of supplemental estrogen. Differences between the cultures described here and those of Mai *et al.* included our B-cell purification method, our use of higher estrogen concentrations and our use of LPS without cytokines. The finding that estrogen increases sterile transcripts suggests a direct influence of estrogen on antibody expression and CSR, and encourages renewed attention to ER binding sites and functions (70) within the heavy chain locus.

ER binds ERE within key enhancer regions of the heavy chain locus

Our ChIP studies identified ER α binding to enhancer elements in the immunoglobulin heavy chain locus. We focused on the HS1,2 region, because this is well known to influence antibody quantity and isotype quality (80, 81, 83, 84, 88, 109–113); numerous nuclear factors bind to the 3'RR including Pax5 and NF- κ B [see encode entries, e.g. ChIP-pol II-CH12, ChIP-Smc3 and ChIP-AID-activated B cells (114–116)]. Deletion of HS1,2 can associate with significant reductions in sterile transcripts and CSR at C γ 3, C γ 1 and C γ 2a loci. The regulation of ϵ and γ 2b expression by promoter-HS1,2 interactions has also been described (117). The patterns of ER α binding described here are reminiscent of other factors known to regulate antibody production (e.g. the zinc finger transcription factor Ikaros, the transcription co-activator Mediator and the histone reader bromodomain-family member BRWD1 that bind immunoglobulin regulatory elements before and after

B-cell stimulation) (65, 78, 118). There were generally subtle pattern shifts in binding rather than absolute on-off changes.

One hypothesis to explain the estrogen-induced changes in antibody profiles is that supplemental estrogen repositions ER α and RNA Pol II in the immunoglobulin heavy chain locus to license HS1,2 enhanceosome activities (e.g. up-regulation of DNA looping and CSR, see Fig. 8). Indeed, we observed that when estrogen was added to B-cell cultures, there was repositioning of ER α and RNA Pol II within the locus (Fig. 6 and 7), an up-regulation of sterile transcription (Fig. 5, day 2), and a biased representation of the female-preferred IgG2b in tissue culture supernatants (Fig. 2, day 6, preliminary results). Direct influences of estrogen and ER α on antibody activities can help explain, at least in part, why males and females are differentially vulnerable to infectious pathogens and autoimmune disease.

Estrogen, ERE and autoimmune disease

Antibody responses and autoimmune diseases are often more prevalent in females compared to males. For example, lupus has a natural female bias (9:1 female:male ratio), and during pregnancy when estrogen levels soar, there is high risk of morbidity and mortality for both mother and baby (92, 119–121). Perhaps antibody up-regulation during pregnancy provides necessary protection to the fetus, but at the same time, increases responses to ‘self’ and organ damage. Our research showed that ER α exhibited significant binding in HS1,2 precisely at a site of polymorphism associated with lupus. The position is central to a switch-like region in HS1,2 proposed to assist full deletion of C genes and consequent suicide of antibody production, another potential mechanism of autoimmune disease control (64). The polymorphic site associated with lupus exhibited a sequence duplication such that two EREs and associated putative NF- κ B sites were positioned in tandem. It makes sense that if ER and estrogen up-regulate antibody responses at this site, a duplicated ERE could exacerbate estrogen-induced antibody responses and autoimmune disease consequences.

The complexity of estrogen influences on autoimmune disease is illustrated by studies with ER α knock-out mice. With one mouse model (B6/129 mouse model for immune complex-type glomerulonephritis), Shim *et al.* described the increase of autoimmune disease upon knock-out of normal ER α expression. In contrast, using another mouse model (female NZM2410 mouse model for lupus), Svenson *et al.* described protection against autoimmune disease upon knock-out of normal ER α expression (60, 122). Results illustrate the difficulties of designing strategies for autoimmune disease control in humans, and caution that the best clinical treatments may differ between males and females. A closer examination of nuclear hormone receptor binding in immunoglobulin loci among males and females with autoimmune disease symptoms may help explain discrepancies and assist the future design of improved treatment strategies.

Conclusions, hypotheses and future research

Altogether, results encourage further experimentation to examine ER functions as they relate to CSR and antibody

expression. An improved understanding of the complex mechanisms by which hormones regulate (and cross-regulate) antibody activities will assist the development of better vaccines and therapies in the clinical arena. We consider a working hypothesis to guide further research, based on the understanding that estrogen levels can vary from ≤ 10 pg ml $^{-1}$ in some males to ≥ 7000 pg ml $^{-1}$ in some pregnant females. We hypothesize that ER α is an integral member of enhanceosomes and switchosomes in the immunoglobulin heavy chain locus. Depending on estrogen levels, ER may change its conformation and/or shift position, to alter the binding of other factors (e.g. RNA polymerase II and AID) to the locus and thereby up- or down-regulate antibody expression. When the ERE in HS1,2 is duplicated (*2) (88), and when ER α is bound by estrogen, enhancer activities may heighten, consequently heightening antibody levels. While advantageous for the control of virus-specific responses (e.g. influenza virus-specific responses), this condition may up-regulate antibodies specific for ‘self’ and thereby render individuals highly vulnerable to autoimmune disease.

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