NIH Public Access
Author Manuscript
Clin Immunol. Author manuscript; available in PMC 2015 March 01.

Published in final edited form as:
Clin Immunol. 2014 March ; 151(1): 66–77. doi:10.1016/j.clim.2014.01.006.

Estrogen modulation of endosome-associated toll-like receptor 8: an IFNα-independent mechanism of sex-bias in systemic lupus erythematosus

Nicholas A. Young[1,2,6], **Lai-Chu Wu**[1,2,6], **Craig J. Burd**[3,6], **Alexandra K. Friedman**[1,2,6], **Benjamin H. Kaffenberger**[1,2,6], **Murugesan V.S. Rajaram**[4,5,6], **Larry S. Schlesinger**[4,5,6], **Hayley James**[7], **Margaret A. Shupnik**[7], and **Wael N. Jarjour**[1,2,6,8]

[1]Division of Rheumatology and Immunology, The Ohio State University, Columbus, Ohio, 43210, USA

[2]Department of Internal Medicine, The Ohio State University, Columbus, Ohio, 43210, USA

[3]Department of Molecular Genetics, The Ohio State University, Columbus, Ohio, 43210, USA

[4]Center for Microbial Interface Biology, The Ohio State University, Columbus, Ohio, 43210, USA

[5]Department of Microbial Infection and Immunity, The Ohio State University, Columbus, Ohio, 43210, USA

[6]Wexner Medical Center at The Ohio State University, Columbus, Ohio, 43210, USA

[7]University of Virginia Health System, Medicine/ Endocrinology, Charlottesville, VA 22908, USA

1. Introduction

From the time period of 1940-1998, mortality rates were significantly higher in males as opposed to females and disease susceptibility was the leading cause of death [1]. Furthermore, data on global infection rates of *Mycobacterium tuberculosis* show similar incidence in pre-adolescent males and females, but are markedly higher in adult males despite lower health care standards for many women in underdeveloped nations [2]. Sex differences in immune function making males less resilient to infection are well established and thought to account for this propensity [3]. Accordingly, antibody production [4] and immune responses [5] in humans challenged with infectious pathogens are significantly higher in females.

© 2014 Elsevier Inc. All rights reserved.

[8]Corresponding author and reprint requests: Wael Jarjour, MD, FACP Wael.Jarjour@osumc.edu Rm S2056 Davis Medical Research Center 480 Medical Center Dr Columbus, OH 43210 Phone: 614-571-1242 Fax: 614-293-5631.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication.As a service to our customers we are providing this early version of the manuscript.The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest and financial disclosure
There were no conflicts of interest to report in this study. Funding for this work provided through NIDDK K23DK059850, NIEHS R00ES019918, and the Wexner Medical Center at The Ohio State University. Funding for the Center for Clinical and Translational Science provided through CTSA grant number UL1TR000090.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Sex-bias is evident in many autoimmune diseases especially systemic lupus erythematosus (SLE) [6], but the basis for such bias is not clear. Many possible mechanisms have been considered, including microchimerism, X chromosome inactivation, and hormonal factors [7]. Evidence for the role of sex hormones in the pathogenesis of SLE has stemmed from the age at which the incidence of SLE peaks. Specifically, the adult premenopausal female to male ratio of SLE is 9:1 and is closer to 2:1 during childhood or post menopause [8]. Furthermore, clinical evidence shows that the incidence of flares in SLE is less in patients who develop ovarian failure due to cyclophosphamide compared to their counterparts who were treated under the same protocol, but maintained ovarian function [9].

Multiple studies have examined sex hormone levels in SLE and have shown a skewing of estrogen metabolism towards a more active and potent metabolite, 16α-hydroxyestrone, compared to healthy controls [10]. Also, an increase in aromatase activity was demonstrated in patients with SLE, which suggests that there is an increased peripheral conversion of testosterone to estrogen [11].

The effects of estrogen on immune cell function are pleotropic and estrogen has been shown to significantly stimulate the proliferation of both lymphocytes and macrophages [12]. In SLE, it has been reported that T cells from lupus patients, but not controls, responded to estrogen by increasing the amounts of calcineurin transcripts, calcineurin phosphatase activity, and that this response can be blocked by an estrogen receptor antagonist [13].

We propose here that estrogen enhances immune system activation, which could contribute to inflammatory responses leading to autoimmune development. The treatment of PBMCs with 17β–estradiol (E2) resulted in the up-regulation of a unique set of genes and cytokines. Among this list was toll-like receptor 8 (TLR8), an X-linked mediator of innate immunity with a known association to SLE and expressed principally in macrophages. In addition to TLR8, our results also identified a novel, estrogen-mediated regulation of all remaining endosomal TLRs (TLR3,TLR7 and TLR9) and displayed estrogen induction of TLR8 in the macrophage subset. Examination of sex-biased responses revealed that while TLR8 agonist-induced stimulation of TLR8 expression was observed in both sexes, the response was more robust in females and enhanced in the presence of estrogen. Independent of IFNα influence, we demonstrated that E2 can induce ERα complex formation at a putative response element near TLR8. These results suggest a novel role for estrogen in innate immune stimulation through highlighting the sex-biased regulation of TLR8 expression, which may contribute to SLE pathogenesis.

2. Materials and Methods

2.1. Human samples

Female patients meeting the revised criteria of the American College of Rheumatology for SLE [14] and healthy volunteers were recruited for the study from the University of Virginia, The Ohio State University Wexner Medical Center (OSUWMC) clinics, local communities, and the American Red Cross. All female subjects were not currently taking hormonal medications and were pre-menopausal. Healthy age-matched males were used in comparative analysis. Participation was through approved Institutional Review Board

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

protocols at both universities. The samples obtained were either whole blood collected into heparinized tubes or filtered blood samples. Isolation of PBMCs was carried out using Ficoll-Paque centrifugation (GE Healthcare, Uppsala, Sweden) according to manufacturer's protocol.

2.2. Cell culture and siRNA transfection

Purified human PBMCs were cultured for 48 hours in phenol red-free RPMI tissue culture media (Gibco, Foster City CA) containing 5% charcoal-stripped FBS (Life Technologies, Grand Island, NY) with and without: E2 (Sigma-Aldrich, St. Louis, MO) at 10 nM, testosterone (Sigma-Aldrich) at 100nM, and agonists for the following TLRs: TLR3 (Poly I:C DNA, Enzolife Sciences, Farmingdale, NY) at 10 μg/μL; TLR4 (Ultrapurified LPS from E. coli Serotype EH 100, Alexis Biochemicals, Switzerland) at 1 μg/mL; TLR7 (imiquimod, Enzolife Sciences) at 0.6 μg/mL; TLR8 (R-848, Enzolife Sciences) at 0.3 μg/mL; or TLR9 (CpG rich DNA, Invitrogen, San Diego, CA) at 7.7 μg/mL. Hematopoietic cell lines (K562, Daudi, and THP-1) were cultured in complete RPMI medium containing 10% FBS (Life Technologies) and treated with E2 (10 nM) for 24 hours.

Transfection of THP-1 cells was performed using Lipofectamine® RNAiMAX™ transfection reagent and Opti-MEM® medium (Life Technologies) with SMARTpool® siRNA constructs targeting scramble (control), IFNα, or ERα (Dharmacon, Inc., Chicago, IL) according to manufacturer's protocol. Cells were cultured in X-VIVO™ 15 chemically defined, serum/phenol red-free hematopoietic cell medium (Lonza, Basel, Switzerland) and E2 (10 nM) was re-supplemented every 24 hours.

2.3. Proliferation assay

Healthy female PBMCs were isolated by Ficoll centrifugation and exposed to 2% phytohemagglutinin (Sigma-Aldrich) or Tdap/BOOSTRIX® (GlaxoSmithKline Biologicals, Rixensart, Belgium) with or without 10 nM E2 for the indicated time period. The fold change in proliferation was determined by measuring cellular viability using the AQueous One Solution cell proliferation assay according to the manufacturer's protocol (Promega Corporation, Madison, WI) with the Dynex MRX-TC Revelation microplate reader/ colorimeter (Dynex Technologies, Chantilly, VA) at an absorbance of 490 nM. Absorbance values in wells with only cells and media were designated +1 at each time point for each individual patient and the relative fold change in proliferation was determined for various treatments after normalizing to background absorbance levels.

2.4 RNA isolation and real-time-RT-PCR

RNA was isolated following our group's published protocol [15] and total RNA was quantitated using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE). Briefly, RNA was isolated from PBMCs using the RNeasy Mini Kit (Qiagen Sciences, Valencia, CA) and from whole blood using the Paxgene Blood RNA Kit (PreAnalytix, Qiagen Sciences). The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following manufacturer's protocol.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Real time RT-PCR was performed as described previously [15] with cDNA and primers using the TaqMan system (Applied Biosystems) and analyzed for the various genes of interest following manufacturer's protocol. All samples were normalized to beta-actin or OAZ1 internal positive controls using Applied Biosystems Assays-on-Demand gene expression assays and the TaqMan Universal PCR Master Mix in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative changes in gene expression were calculated as fold differences using the $2^{-\Delta\Delta Ct}$ method [16].

2.5. Western Blotting

Protein expression was measured by Western blotting according to previously described methods [15]. Briefly, protein samples were resolved on 10% Tris-HCl Bio-Rad Ready Gels (Life Science Research, Hercules, CA) and transferred onto PVDF membranes (GE Healthcare). Membranes were incubated with TLR8 (Rockland Immunochemicals Inc., Gilbertsvilla, PA) antibody and stripped using Millipore Re-Blot Plus (Millipore, Temecula, CA) for reprobing with monoclonal β-actin antibody (Sigma Aldrich) or hsp90 (Santa Cruz Biotechnologies Inc.). Image J software (v1.45s; NIH, Bethesda, MD) was used to determine signal intensities by normalization to the loading control. Fold differences in expression were determined relative to untreated samples.

2.6. Cytokine Analysis

Conditioned media was collected after 24 hours and cytokine analysis was performed using Bio-Plex Pro® single-plex magnetic beads for MIP-1β, IL-2, and IL-6 (Bio-Rad) on the Bio-Plex 200 system. Data analysis was performed using Bio-Plex Manager® 5.0 software and results were exported to Microsoft Excel (v2010) for further analysis. Cytokine levels were subtracted from background measurements and fold changes are expressed relative to untreated samples designated +1.

2.7. Estrogen treatment of mice

C57BL/6 mice obtained from The Jackson Laboratories were housed in the Biomedical Research Tower rodent research facility at OSUWMC in a BSL-3 barrier level. Animal maintenance and protocol was approved by the Institutional Animal Care and Use Committee through the University Laboratory Animal Resources. As previously described [15], females were given subcutaneous injections of E2 (0.5 μg) at a concentration of 1 μg/mL or PBS for 5 days. Mice were euthanized 24 hours after the fifth treatment and organs were harvested for RNA isolation using the RNeasy Mini Kit (Qiagen Sciences) following manufacturer's protocol.

2.8. Monocyte-derived-macrophage (MDM) isolation and siRNA nucleofection

Monolayers of attached MDM cells were prepared following previous protocol [17]. In brief, Ficoll-isolated PBMCs from human blood samples were cultured for 5 days in RPMI media supplemented with 20% autologous serum. Following siRNA nucleofection, the cells were plated in 10% autologous serum. Unattached lymphocytes were washed away and the remaining MDMs were cultured in RPMI media with 10% autologous serum overnight before 10 nM E2 treatment.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

siRNA nucleofection was carried out as described previously [18, 19] with either scramble (control), ERα, or IFNα SMARTpool® siRNA (Dharmacon) using the Amaxa Nucleofector system (Amaxa Biosystems, Gaithersburg, MD). Briefly, PBMCs were collected after 5 days in culture and re-suspended in 100 μL of nucleofector solution (Amaxa Biosystems) and 100 nMol siRNA. Nucleofection was carried out according to the manufacturer's protocol.

2.9. Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as detailed formerly [15] using DNA fragments containing putative ERα binding sites labeled with [^{32}P] dCTP by Klenow. The sequences of the two potential ERE sites, ERα 1 and 2 (ERE sequences underlined), 25kb downstream of the TLR8 gene are: ERα 1:5'-GGGTCCCCTGTGACCTGCACGTACA -3', ERα 2: 5'-GGGGTGTGACCTGGCAATTTGTTTA -3'. ERα antibody (abcam, Cambridge, MA) and/or recombinant ERαprotein (Thermo Scientific, Rockford, IL) were incubated with DNA fragments prior to electrophoresis. Relative complex formation was determined using Image J software (NIH) and normalized to the untreated or baseline levels, both designated +1.

2.10. Chromatin Immunoprecipitation sequencing assay (ChIP-seq)

MCF7 cells were cultured in DMEM media containing 5% charcoal/dextran treated FBS for 72 hours prior to activation with 1 nM E2 for 45 minutes. Following stimulation, cells were cross-linked, harvested, and ChIP assays performed as previously described using an antibody specific for ERα (Santa Cruz Biotechnology) [20]. Sequencing libraries were prepared from enriched DNA using the Tru-seq Chip Sample Prep Kit (Illumina, San Diego, CA). Each library was sequenced on one lane of an Illumina GA IIx instrument generating 40 bp single end reads. Sequences were aligned to the hg19 human reference genome and graphical maps of aligned reads generated using HOMER [21]. Images shown were generated in the Integrative Genomics Viewer [22]. H3K4me1 data was generated by the ENCODE consortium (GSE29611).

2.11. Statistics

Data are expressed as mean values ± standard deviation. All p-values were determined in paired, 2-tailed Student's t-tests using Microsoft excel software (v2010). p-values ≤ 0.05 were considered statistically significant.

3. Results

3.1 Estrogen enhances antigenic responses in PBMCs

To examine our hypothesis that estrogen lowers the threshold of activation in the female immune system, PBMCs were isolated from healthy, pre-menopausal female volunteers and treated with mitogen or antigens to measure proliferative and cytokine responses. Exposure of PBMCs to phytohemagglutinin (PHA) induced a 4-fold ($p < 0.05$) increase in proliferation after 5 days when compared to untreated controls, while E2 treatment only mildly stimulated this response (1.3-fold; Fig.1 A). When cells were stimulated with Tdap/BOOSTRIX® (Tdap) containing diphtheria toxoid, tetanus toxoid, and an acellular pertussis

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

vaccine, the proliferative responses were similar to PHA, reaching 3.3-fold ($p < 0.05$) after 5 days (Fig. 1 A). These results were confirmed by cell counts performed with trypan blue staining for each condition and time point measured (data not shown). Further, a dose-dependent response was observed in cells treated with increasing amounts of Tdap for 24 hours and this proliferation was elevated in each circumstance in the presence of E2 (Fig. 1 B). Over time, the proliferation of PBMCs was also significantly increased with Tdap stimulation in an E2-supplemented environment relative to Tdap-alone and measured 4.9-fold ($p < 0.05$) after 5 days (Fig. 1 C).

Conditioned media was also collected from the proliferation assays above to measure cytokine production. E2 treatment alone stimulated a 1.3 fold ($p<0.05$) increase in macrophage inflammatory protein 1-β (MIP1-β) and an apparent but not significant increase in IL-2 (1.2 fold) and IL-6 (1.3 fold) (Fig. 1D). Although Tdap stimulation enhanced cytokine secretion as well, only in the presence of E2 was the production of MIP-1β(1.3-fold, $p < 0.05$), IL-2 (4.2-fold, $p < 0.05$), and IL-6 (1.7-fold, $p < 0.05$) significantly increased relative to untreated controls (Fig. 1 D). Taken together, our data display enhanced proliferation and secretion of several key pro-inflammatory cytokines in PBMCs stimulated with estrogen or antigen alone, but result in the most significant induction following antigen stimulation in an E2-supplemented environment.

3.2 TLR8 is induced by TLR8 agonist and is a novel estrogen-regulated gene over-expressed in SLE

To examine the effects of estrogen stimulation on gene expression, we performed gene array analysis on PBMCs isolated from healthy subjects and SLE patients after *in vitro* culture with or without 10 nM of E2 for 48 hours [15]. The untreated control array served as the internal baseline for each sample and was subtracted from the E2-treated expression values. Thus, only the estrogen mediated effect was reported for each individual sample. Total PBMC preparations were used to create an *in vitro* system biologically relevant to the *in vivo* interactions of all PBMC subtypes in the regulation of an estrogen-induced response. Furthermore, since the specific estrogen-regulated genes were yet to be determined, this predicated the inclusion of all subsets in the initial analysis. Interestingly, 988 genes were found to be significantly up-regulated by E2 in both healthy and SLE samples when compared to untreated levels; a result demonstrating a clear genome-wide estrogen effect over many genes. Ingenuity pathway analysis (IPA) software was then used to filter the genes most significantly up-regulated in PBMCs from SLE patients and select for those known to be associated with immunological disorders or immune function, as defined by Ingenuity® IPA Systems. This filtered collection of genes (supplemental table 1) included TLR8, a novel estrogen-regulated gene which has been previously linked to SLE pathogenesis [23, 24]. In concordance, we evaluated the expression of TLR8 in whole blood from SLE patients and found its levels to be 1.7-fold ($p < 0.005$) higher than in healthy subjects (Fig. 2 A).

To examine E2-induced TLR8 expression further, we treated hematopoietically derived human cell lines with E2 and measured protein expression of TLR8. Following E2 treatment, expression of TLR8 was increased 1.2-fold in K562 cells and 1.8-fold in Daudi

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

cells (Fig. 2 B). In addition, Jurkat (clone E6-1) cells did not show an observable difference in TLR8 expression with E2 stimulation under the same conditions (data not shown). To look at this response in primary human cells, PBMCs from healthy male and female subjects were isolated and treated with either E2 or testosterone. E2 induced TLR8 expression 1.3-fold ($p < 0.05$) at the RNA level and 2-fold at the protein level, while testosterone had no effect (Fig. 2 C). Data from both sexes were combined because similar levels of induction were observed in males and females under these conditions. Notably, further examination of the PBMC subset chiefly responsible for TLR8 expression, macrophages, is reserved until later in this work and presented concurrently with the data showing the mechanism of E2-regulated TLR8 expression. To examine E2-mediated induction of TLR8 in the immune system *in vivo*, wild-type mice were subcutaneously injected with E2 and lymphoid organs were harvested after six days. Treatment with E2 increased TLR8 mRNA expression 2-fold in the spleen, 2.1-fold ($p < 0.05$) in the thymus, 1.7-fold in the bone marrow, and 3.5-fold ($p < 0.05$) in the lymph nodes (Fig 2 D).

Recent work in human monocytic cell lines has demonstrated that stimulation with TLR8 agonist can also induce TLR8 expression [25]. To examine whether this signaling process is also present in primary cells, healthy PBMCs from male and female donors were stimulated with the synthetic TLR8 agonist, R-848. TLR8 transcript expression was significantly up-regulated 4.5-fold ($p < 0.05$) with agonist stimulation, while protein levels increased 2.3-fold (Fig 2 E). Since these experimental conditions yielded similar data between sexes, the results were again combined. Thus, these data demonstrate the positive effects of estrogen and TLR8 agonist-mediated induction of TLR8 expression.

3.3 Expression of intracellular TLRs is increased in SLE patients and induced by estrogen or agonist stimulation

Given the distinct relationship between functionality and cellular localization within the TLR family [26], all other intracellular TLRs were also examined; TLR3, TLR7, TLR8, and TLR9 exclusively bind nucleic acids associated with various pathogens and are expressed with endosomal subcellular localization [26]. Additionally, representative cell membrane-associated members, TLR2 and TLR4, were examined. Analysis of the *mRNA*from peripheral blood showed increased expression of TLR3 (1.5-fold), TLR4 (1.8-fold, $p < 0.005$), TLR7 (1.2-fold, $p < 0.05$), and TLR9 (1.2-fold) in SLE patients compared to healthy controls (Fig. 3 A). This correlation underscores the potential role of TLR over-expression and innate immunity in SLE.

To determine if any of these TLRs respond to estrogen and agonist similar to TLR8, PBMCs from healthy subjects were analyzed by RT-PCR analysis. TLR2 and TLR4 did not respond to E2 treatment (Fig 3 B). In contrast, endosome-localized TLR3, TLR7, and TLR9 expression was induced with E2 stimulation 1.4-fold ($p < 0.05$), 1.2-fold ($p < 0.05$), and 1.6-fold ($p < 0.05$), respectively (Fig 3 C). Testosterone was used as a control hormonal treatment and had no effect over TLR expression (Fig 3 B-C). Similarly, while agonist treatment had no impact on TLR4 expression (Fig 3 D), TLR3 expression was induced 3.4-fold ($p < 0.05$) and TLR7 stimulation reached 1.4-fold ($p < 0.005$; Fig 3 E). This demonstrates agonist-induced stimulation of receptor expression in these TLRs as well. The

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

same trend was observed in TLR9, but did not reach statistical significance using two commercially available agonists developed against this receptor (Fig. 3 E). Therefore, both E2-mediated and positive ligand-induced regulation of TLR expression appears evident in all endosomal TLRs.

3.4. Sex-biased stimulation of TLR8 expression by ligand is more robust in females and further enhanced in the presence of estrogen

Upon immune system activation, gene array data has shown differential expression of sex-biased genes in female T cells, indicating that a significantly stronger response is elicited [27]. In contrast, the responses thus far in our PBMC stimulations did not differ between males and females at 48 hours; both sexes induced endosomal TLR expression significantly with estrogen or agonist stimulation (Fig 2-3). To determine if a sex-biased response was indeed present in our system, PBMCs from healthy males and females were stimulated over time to analyze the effect on TLR8 expression. Although no time point studied reached a statistical significance and no difference was observed with E2 stimulation at 48 hours, female PBMCs displayed enhanced TLR8 expression by 1.4-fold after 6 hours and 1.9-fold after 24 hours relative to males (data not shown). Agonist-induced TLR8 expression was also enhanced in females compared to males, showing a significant 4.7-fold ($p < 0.01$) and 4.2-fold ($p < 0.05$) induction at 18 and 24 hours, respectively (Figure 4 A). When TLR8 agonist was incubated with cells in the presence of E2, TLR8 expression was further enhanced and again more robust in female samples. Here, TLR8 expression was induced 7-fold ($p < 0.05$) at 24 hours and 6.3-fold ($p < 0.05$) at 36 hours (Fig 4 B). Protein analysis at 24 hours showed a 2.4-fold increase in TLR8 expression in males with E2 and R-848 treatment, while a 4.2-fold increase was observed in females under the same conditions (Fig 4 C). These results establish a sex-biased and additive influence of E2 and TLR8 agonist in inducing female TLR8 expression.

3.5 Estrogen-induced TLR8 expression requires ERα but not IFNα

Since TLR8 expression has been shown to be primarily observed in mononuclear phagocytes [28], we also isolated monocyte-derived-macrophages (MDMs) from this PBMC subset to examine the mechanism of estrogen-mediated stimulation of TLR8 expression using siRNA. ERα was targeted with siRNA because animal models have shown this to be the primary estrogenic subtype [29]. With E2 stimulation for 48 hours, MDMs showed enhanced expression of ERα 1.7-fold ($p < 0.05$), IFNα 2.1-fold, and TLR8 2.4-fold ($p < 0.01$; Fig 5 A). Although enhanced expression of IFNα was not statistically significant, this E2-mediated response was investigated further since IFNα has been shown to mediate some estrogenic effects and has a strong association with SLE pathobiology [30]. Using siRNA to knock down expression of ERα and IFNα by 40% [15], only blocking ERα prevented the E2-mediated induction of TLR8 expression (Fig 5 B). Here, TLR8 expression was reduced by 30% ($p < 0.01$) in the presence of E2 (Fig 5 B). In contrast, knockdown of IFNα by siRNA did not affect the E2-induced expression of TLR8 (Fig 5 B). These data suggest that ERα-mediated TLR8 expression by E2 treatment is IFNα-independent.

The THP-1 cell line is of monocytic origin and therefore of the same hematopoietic lineage as the cell subset principally expressing TLR8. Since we observed E2-stimulated up-

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

regulation in both sexes and THP-1 cells do not express detectable IFNα levels via real time-RT-PCR analysis at baseline (data not shown), we selected this cell line for further mechanistic analysis. E2 treatment of THP-1 cells induced TLR8 expression 1.4-fold at the transcript ($p < 0.05$) and protein level after 24 hours (Fig 5 C). To examine the effects of E2-mediated stimulation of TLR8 expression through ERα in an environment devoid of IFNα influence, THP-1 cells were treated with E2 and transfected with siRNA targeting ERα. After transfection with scrambled control siRNA, E2 stimulation resulted in a significant increase in ERα(2.6-fold, $p < 0.01$) and TLR8 (1.3-fold, $p < 0.05$) levels (data not shown). Furthermore, siRNA targeting ERα decreased its expression levels by 96% ($p< 0.01$) at 48 hours and 43% ($p < 0.05$) at 72 hours (Fig 5 D); thus displaying the transient nature of the inhibition. With ERα expression repressed by siRNA, E2-mediated stimulation of TLR8 expression was significantly reduced 40% ($p < 0.05$) at 24 hours and 30% ($p < 0.01$) at the 48 hour time point (Fig 5 E). Collectively, these results indicate that E2 induction of TLR8 is mediated through ERα and is not dependent on IFNα.

3.6 ERα binds directly to a putative estrogen responsive element near the TLR8 locus

Chromatin immunoprecipition sequencing (ChIP-seq) data from a breast cancer cell line (MCF-7) stimulated with E2 for 45 minutes identified two putative ERα-binding regions (ERα 1 and 2) for the TLR8 locus 25 kb downstream from the 3' end of the TLR8 gene (supplemental figure 1). Examination of the region upstream of TLR8 did not identify any ERβ-binding regions within 100 kb of the transcriptional start site. Furthermore, the same E2 stimulation of a hematopoietically-derived cell line, K562, revealed open chromatin marks of transcriptional enhancers that overlapped with the location of these two ERα-binding regions (supplemental figure 1). To determine whether ERα directly binds to these putative estrogen response elements (EREs), EMSA analysis was performed on THP-1 cells to measure ERαbinding. After E2 stimulation and incubation of nuclear extracts with radio-labeled probes for these response elements, enhanced DNA-protein complex formation was observed only using probes corresponding to the ERα 2 sequence. This response was observed over time beginning at one hour (Fig 6 A). The relative extent of complex formation was quantified and displayed graphically to show enhanced DNA-protein complex formation over time (Fig 6 B). To confirm ERα binding, a dose-dependent enhancement of complex formation was demonstrated with increasing amounts of recombinant human ERαprotein incubated with ERE probes (Fig 6 C). Further, the addition of ERα antibody successfully shifted DNA-protein complexes to display a retarded migration pattern (Fig 6 C). The dose-dependent response of complex formation was also quantified for graphical representation (Fig 6 D). These results suggest that E2 induces TLR8 expression through ERα by directly binding to a putative downstream ERE.

4. Discussion

Females are generally more resistant to infection than males, which can be reflected by significantly longer life spans in many female species ranging from nematodes to mammals [31]. Thus, it has been suggested that estrogenic effects could lead to a heightened immuno-reactive state, which would have a survival advantage in the defense against infection, but may contribute to autoimmune inflammation [32]. While many advances have been made

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

delineating possible pathogenic mechanisms of SLE, female predominance in this disease remains largely unexplained. We hypothesized that estrogen could lower the threshold of immune activation and that this effect could contribute to SLE pathogenesis.

Upon antigenic challenge, PBMCs from healthy females responded with significantly higher proliferative and cytokine production responses in the presence of estrogen. In addition, E2 treatment in physiologic doses increased the production of multiple cytokines, including stimulation of MIP1-β Considering that patients with active SLE have significantly elevated levels of pro-inflammatory cytokines [33] and that MIP1-βhas previously been shown to be elevated in the serum of SLE subjects when compared to healthy individuals [34], estrogen-regulated secretion of this inflammatory mediator could contribute to the autoimmune inflammation observed in SLE.

In recent years, studies have shown several genes to be strongly associated with SLE development and severity [35], including TLR8 [36, 37]. In this study, we show that *in vitro* stimulation with E2 significantly up-regulated the expression of hundreds of genes. TLR8 was identified in this regard both in PBMCs and primary human macrophages; and was also expressed at higher levels in peripheral blood of premenopausal SLE patients when compared to age-matched, healthy females. Further, our results indicate that E2-mediated induction of TLR8 expression requires ERα and occurs through direct DNA binding of ERα to an ERE just downstream of the TLR8 gene. Interestingly, activation of ERα enhanced SLE pathogenesis through immunomodulatory influence in a lupus mouse model, while no effect was observed through ERβ [38]. Moreover, E2-mediated regulation of TLR8 was also shown to be independent of IFNα. Given the focus on IFNα-mediated pathogenesis in SLE by up-regulating IFNα signature gene expression (reviewed in [39]), our results display a novel area of therapeutic intervention; specifically, blocking ERα to prevent the induction of estrogenic genes, such as TLR8, in female SLE patients.

Enhanced expression of TLR subytpes has been associated with disease activity and observed in various lymphocyte subsets of SLE patients [40, 41]. Our results have identified a novel relationship among SLE, estrogen, and TLR expression. The TLR family can be separated into two main groups based on subcellular localization. TLRs 1, 2, 4-6, and 10-11 are expressed on the cell surface, while TLRs 3 and 7-9 are expressed on intracellular compartments such as endosomes [42]. TLR expression in the peripheral blood of SLE patients revealed significantly increased levels of TLR4, TLR7, and TLR8 when compared to healthy subjects, with TLR3 and TLR9 also displaying a similar trend. Further, E2 treatment also resulted in significant stimulation of all the endosome-associated receptors (TLR3, TLR7, TLR8, and TLR9). This is particularly intriguing since endosomal TLR signaling is required for the production of anti-nucleic acid autoantibodies in mice [43], which is a clinical hallmark of SLE. Additionally, TLR7 overexpression in mice has been shown to lead to anti-nucleic acid autoantibody secretion and accelerate autoimmune pathology in a lupus model [44, 45]. Considering that antimalarial drugs including hydroxychloroquine and chloroquine which specifically inhibit endosome-associated TLR activation [46] have been used for a long time to treat SLE [47], our data indicate that autoimmune development and progression could be influenced by estrogen-priming of innate immune responses through up-regulation of endosomal TLR expression.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Endosome-associated TLRs all displayed ligand-induced positive regulation of receptor expression. In contrast, this effect was not observed in TLR4, which is localized to the surface of the cell. Similarly, seminal work in this pathway showed that TLR3 and TLR4 ligands stimulated TLR3 expression, while TLR4 was not inducible by these same stimuli in primary mouse bone marrow cells [48]. Other studies showed results suggestive of this pathway with TLR8 expression in a promonocytic human cell line [25], TLR3 in murine natural killer cells [49] and osteoblastic cells [50], and TLR9 in murine primary mouse bone marrow cells [51]. While this phenomenon may have been previously alluded to, this is the first study to both display this regulation in primary human PBMCs and collectively characterize its presence in endosomal TLRs.

The ligand binding portion of endosomal TLRs is only exposed to the inside of the intracellular compartment, which discourages exposure of potential endogenous self-antigens. This compartmentalized receptor localization is both regulated and controlled in order to prevent unintended signal transduction [52]. Since endosomal TLRs have a much more controlled mechanism of antigen exposure and are not free to sample the environment in an unregulated manner, this may explain why positive agonist-induced regulation of receptor expression may only exist in TLRs localized to endosomes. Endosome-associated TLRs exclusively bind to nucleic acids and SLE patients often secrete high levels of anti-nuclear antibodies (ANA), which results in circulating immune complexes consisting of antibodies to self nucleic acids [53]. Our data suggest that processing of these complexes could cause a ligand-induced up-regulation of receptor expression, which would be more robust in an environment containing estrogen and therefore contribute to autoimmune development. Similarly, viral infection has been shown to be a trigger associated with SLE disease state (reviewed in [54]). Here, estrogen could also lower the threshold of activation and lead to the up-regulation of nucleic acid-binding TLR expression and a heightened innate immune response, which can contribute to autoimmune pathology.

While both female and male cells displayed similar trends of agonist-induced TLR8 expression with E2 enhancing the response, the expression of TLR8 was found to be significantly more robust in females. Hence, heightened expression of TLR8 by agonist (self or non-self) stimulation in the presence of estrogen may contribute to the observation that females are generally more resistant to infection, but may also predispose females to auto-inflammatory responses, as observed in SLE. The distinct responses between male and female PBMCs indicate that these cells may be “hard-wired” to differential responses. This sex-bias likely is achieved through the epigenetic mechanisms, in which genome-wide expression of genes can be altered by influencing chromatin accessibility. In concordance, significantly different methylation patterns have been observed between male and female twins in a genome-wide analysis [55], and women with SLE have been shown to have hypomethylated areas on the X chromosome that corresponded with enhanced gene expression when compared to male counterparts [56]. Since TLR8 and TLR7 are both X-linked, this may explain their basal levels of up-regulation in SLE patients when compared to age and sex-matched healthy females. Collectively, the data suggest a sex-biased, differential expression pattern of genes in SLE that would lead to distinct responses with antigenic challenge and/or estrogen stimulation.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

5. Conclusion

Considering the evidence already linking innate immunity to the pathogenesis of SLE [57], the discovery and further characterization of TLR expression pathways will facilitate the elucidation of their precise role in autoimmune diseases. The results of our work suggest that estrogen can cause an upregulation of endosome-associated TLRs; thus promoting a milieu that is pro-inflammatory and permissive for the development of SLE and potentially other autoimmune diseases that are associated with female predominance. Future work will focus on TLR signaling and estrogen responses to identify additional, viable therapeutic targets in the treatment of SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to extend our appreciation to all the volunteers that participated in this study. Our research would not be possible without the time and effort they put forth. We would also like to thank the Center for Clinical and Translational Science's Research Match program at OSUWMC and the American Red Cross. Both organizations were instrumental in the progress of our research.

References

1. Hoyert DL, Kochanek KD, Murphy SL. Deaths: final data for 1997. Natl Vital Stat Rep. 1999; 47:1–104.
2. Holmes CB, Hausler H, Nunn P. A review of sex differences in the epidemiology of tuberculosis. Int J Tuberc Lung Dis. 1998; 2:96–104. [PubMed: 9562118]
3. Moller AP, Sorci G, Erritzoe J. Sexual dimorphism in immune defense. Am Nat. 1998; 152:605–19. [PubMed: 18811367]
4. Zuk M, McKean KA. Sex differences in parasite infections: patterns and processes. Int J Parasitol. 1996; 26:1009–23. [PubMed: 8982783]
5. Butterworth M, McClellan B, Allansmith M. Influence of sex in immunoglobulin levels. Nature. 1967; 214:1224–5. [PubMed: 4169229]
6. Kotzin BL. Systemic lupus erythematosus. Cell. 1996; 85:303–6. [PubMed: 8616885]
7. Zandman-Goddard G, Peeva E, Shoenfeld Y. Gender and autoimmunity. Autoimmun Rev. 2007; 6:366–72. [PubMed: 17537382]
8. Lockshin MD. Sex differences in autoimmune disease. Lupus. 2006; 15:753–6. [PubMed: 17153846]
9. Tincani A. Cyclophosphamide therapy induces ovarian failure in premenopausal women affected by systemic lupus erythematosus. Clin Exp Rheumatol. 2001; 19:490–1. [PubMed: 11579705]
10. Lahita RG, Bradlow HL, Kunkel HG, Fishman J. Increased 16 alpha-hydroxylation of estradiol in systemic lupus erythematosus. J Clin Endocrinol Metab. 1981; 53:174–8. [PubMed: 7240374]
11. Folomeev M, Dougados M, Beaune J, Kouyoumdjian JC, Nahoul K, Amor B, et al. Plasma sex hormones and aromatase activity in tissues of patients with systemic lupus erythematosus. Lupus. 1992; 1:191–5. [PubMed: 1301981]
12. Whitacre CC, Reingold SC, O'Looney PA. A gender gap in autoimmunity. Science. 1999; 283:1277–8. [PubMed: 10084932]
13. Rider V, Li X, Peterson G, Dawson J, Kimler BF, Abdou NI. Differential expression of estrogen receptors in women with systemic lupus erythematosus. J Rheumatol. 2006; 33:1093–101. [PubMed: 16755656]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

14. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997; 40:1725. [PubMed: 9324032]
15. Young NA, Friedman AK, Kaffenberger B, Rajaram MV, Birmingham DJ, Rovin BH, et al. Novel estrogen target gene ZAS3 is overexpressed in systemic lupus erythematosus. Mol Immunol. 2012; 54:23–31. [PubMed: 23178823]
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–8. [PubMed: 11846609]
17. Schlesinger LS. Macrophage phagocytosis of virulent but not attenuated strains of Mycobacterium tuberculosis is mediated by mannose receptors in addition to complement receptors. J Immunol. 1993; 150:2920–30. [PubMed: 8454864]
18. Rajaram MV, Ni B, Morris JD, Brooks MN, Carlson TK, Bakthavachalu B, et al. Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. Proc Natl Acad Sci U S A. 2011; 108:17408–13. [PubMed: 21969554]
19. Rajaram MV, Brooks MN, Morris JD, Torrelles JB, Azad AK, Schlesinger LS. Mycobacterium tuberculosis activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses. J Immunol. 2010; 185:929–42. [PubMed: 20554962]
20. Burd CJ, Ward JM, Crusselle-Davis VJ, Kissling GE, Phadke D, Shah RR, et al. Analysis of chromatin dynamics during glucocorticoid receptor activation. Mol Cell Biol. 2012; 32:1805–17. [PubMed: 22451486]
21. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell. 2010; 38:576–89. [PubMed: 20513432]
22. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol. 2011; 29:24–6. [PubMed: 21221095]
23. Komatsuda A, Wakui H, Iwamoto K, Ozawa M, Togashi M, Masai R, et al. Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin Exp Immunol. 2008; 152:482–7. [PubMed: 18373699]
24. Krieg AM, Vollmer J. Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. Immunol Rev. 2007; 220:251–69. [PubMed: 17979852]
25. Schlaepfer E, Speck RF. TLR8 Activates HIV from Latently Infected Cells of Myeloid-Monocytic Origin Directly via the MAPK Pathway and from Latently Infected CD4+ T Cells Indirectly via TNF-{alpha}. J Immunol. 2011; 186:4314–24. [PubMed: 21357269]
26. Chang ZL. Important aspects of Toll-like receptors, ligands and their signaling pathways. Inflamm Res. 2010; 59:791–808. [PubMed: 20593217]
27. Hewagama A, Patel D, Yarlagadda S, Strickland FM, Richardson BC. Stronger inflammatory/cytotoxic T-cell response in women identified by microarray analysis. Genes Immun. 2009; 10:509–16. [PubMed: 19279650]
28. Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J Immunol. 2002; 168:554–61. [PubMed: 11777946]
29. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev. 1999; 20:358–417. [PubMed: 10368776]
30. Panchanathan R, Shen H, Zhang X, Ho SM, Choubey D. Mutually positive regulatory feedback loop between interferons and estrogen receptor-alpha in mice: implications for sex bias in autoimmunity. PLoS One. 2010; 5:e10868. [PubMed: 20526365]
31. Borras C, Sastre J, Garcia-Sala D, Lloret A, Pallardo FV, Vina J. Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. Free Radic Biol Med. 2003; 34:546–52. [PubMed: 12614843]
32. Whitacre CC. Sex differences in autoimmune disease. Nat Immunol. 2001; 2:777–80. [PubMed: 11526384]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

33. Sabry A, Sheashaa H, El-Husseini A, Mahmoud K, Eldahshan KF, George SK, et al. Proinflammatory cytokines (TNF-alpha and IL-6) in Egyptian patients with SLE: its correlation with disease activity. Cytokine. 2006; 35:148–53. [PubMed: 17027280]
34. Vila LM, Molina MJ, Mayor AM, Cruz JJ, Rios-Olivares E, Rios Z. Association of serum MIP-1alpha, MIP-1beta, and RANTES with clinical manifestations, disease activity, and damage accrual in systemic lupus erythematosus. Clin Rheumatol. 2007; 26:718–22. [PubMed: 16924394]
35. Scofield RH. Genetics of systemic lupus erythematosus and Sjogren's syndrome. Curr Opin Rheumatol. 2009; 21:448–53. [PubMed: 19593143]
36. Suarez-Gestal M, Calaza M, Endreffy E, Pullmann R, Ordi-Ros J, Domenico Sebastiani G, et al. Replication of recently identified systemic lupus erythematosus genetic associations: a case-control study. Arthritis Res Ther. 2009; 11:R69. [PubMed: 19442287]
37. Vollmer J, Tluk S, Schmitz C, Hamm S, Jurk M, Forsbach A, et al. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. J Exp Med. 2005; 202:1575–85. [PubMed: 16330816]
38. Li J, McMurray RW. Effects of estrogen receptor subtype-selective agonists on autoimmune disease in lupus-prone NZB/NZW F1 mouse model. Clin Immunol. 2007; 123:219–26. [PubMed: 17336162]
39. Lichtman EI, Helfgott SM, Kriegel MA. Emerging therapies for systemic lupus erythematosus--focus on targeting interferon-alpha. Clin Immunol. 2012; 143:210–21. [PubMed: 22525889]
40. Chauhan SK, Singh VV, Rai R, Rai M, Rai G. Distinct Autoantibody Profiles in Systemic Lupus Erythematosus Patients are Selectively Associated with TLR7 and TLR9 Upregulation. J Clin Immunol. 2013; 33:954–64. [PubMed: 23564191]
41. Wong CK, Wong PT, Tam LS, Li EK, Chen DP, Lam CW. Activation profile of Toll-like receptors of peripheral blood lymphocytes in patients with systemic lupus erythematosus. Clin Exp Immunol. 2010; 159:11–22. [PubMed: 19843090]
42. Li M, Zhou Y, Feng G, Su SB. The critical role of Toll-like receptor signaling pathways in the induction and progression of autoimmune diseases. Curr Mol Med. 2009; 9:365–74. [PubMed: 19355917]
43. Kono DH, Haraldsson MK, Lawson BR, Pollard KM, Koh YT, Du X, et al. Endosomal TLR signaling is required for anti-nucleic acid and rheumatoid factor autoantibodies in lupus. Proc Natl Acad Sci U S A. 2009; 106:12061–6. [PubMed: 19574451]
44. Subramanian S, Tus K, Li QZ, Wang A, Tian XH, Zhou J, et al. A Tlr7 translocation accelerates systemic autoimmunity in murine lupus. Proc Natl Acad Sci U S A. 2006; 103:9970–5. [PubMed: 16777955]
45. Pisitkun P, Deane JA, Difilippantonio MJ, Tarasenko T, Satterthwaite AB, Bolland S. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. Science. 2006; 312:1669–72. [PubMed: 16709748]
46. Macfarlane DE, Manzel L. Antagonism of immunostimulatory CpG-oligodeoxynucleotides by quinacrine, chloroquine, and structurally related compounds. J Immunol. 1998; 160:1122–31. [PubMed: 9570525]
47. Rynes RI. Antimalarial drugs in the treatment of rheumatological diseases. Br J Rheumatol. 1997; 36:799–805. [PubMed: 9255117]
48. Doyle SE, O'Connell R, Vaidya SA, Chow EK, Yee K, Cheng G. Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. J Immunol. 2003; 170:3565–71. [PubMed: 12646618]
49. Duluc D, Tan F, Scotet M, Blanchard S, Fremaux I, Garo E, et al. PolyI:C plus IL-2 or IL-12 induce IFN-gamma production by human NK cells via autocrine IFN-beta. Eur J Immunol. 2009; 39:2877–84. [PubMed: 19728309]
50. Nakamura K, Deyama Y, Yoshimura Y, Suzuki K, Morita M. Toll-like receptor 3 ligand-induced antiviral response in mouse osteoblastic cells. Int J Mol Med. 2007; 19:771–5. [PubMed: 17390082]
51. Schroder K, Spille M, Pilz A, Lattin J, Bode KA, Irvine KM, et al. Differential effects of CpG DNA on IFN-beta induction and STAT1 activation in murine macrophages versus dendritic cells:

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

alternatively activated STAT1 negatively regulates TLR signaling in macrophages. J Immunol. 2007; 179:3495–503. [PubMed: 17785783]

52. Wagner H. Endogenous TLR ligands and autoimmunity. Adv Immunol. 2006; 91:159–73. [PubMed: 16938540]
53. Ahlin E, Mathsson L, Eloranta ML, Jonsdottir T, Gunnarsson I, Ronnblom L, et al. Autoantibodies associated with RNA are more enriched than anti-dsDNA antibodies in circulating immune complexes in SLE. Lupus. 2012; 21:586–95. [PubMed: 22300829]
54. Zandman-Goddard G, Shoenfeld Y. Infections and SLE. Autoimmunity. 2005; 38:473–85. [PubMed: 16373252]
55. Boks MP, Derks EM, Weisenberger DJ, Strengman E, Janson E, Sommer IE, et al. The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. PLoS One. 2009; 4:e6767. [PubMed: 19774229]
56. Hewagama A, Gorelik G, Patel D, Liyanarachchi P, McCune WJ, Somers E, et al. Overexpression of X-linked genes in T cells from women with lupus. J Autoimmun. 2013; 41:60–71. [PubMed: 23434382]
57. Carroll M. Innate immunity in the etiopathology of autoimmunity. Nat Immunol. 2001; 2:1089–90. [PubMed: 11725293]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Highlights

- Estrogen lowers the threshold of immune system activation
- TLR8 is up-regulated in SLE and estrogen induces TLR8 and all other endosomal TLRs
- Sex-biased TLR8 induction by estrogen and/or ligand is more robust in females
- ERα, but not IFNα, up-regulates TLR8 by binding an ERα response element
- Therefore, TLR8 induction by estrogen and ligand could predispose females to SLE

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

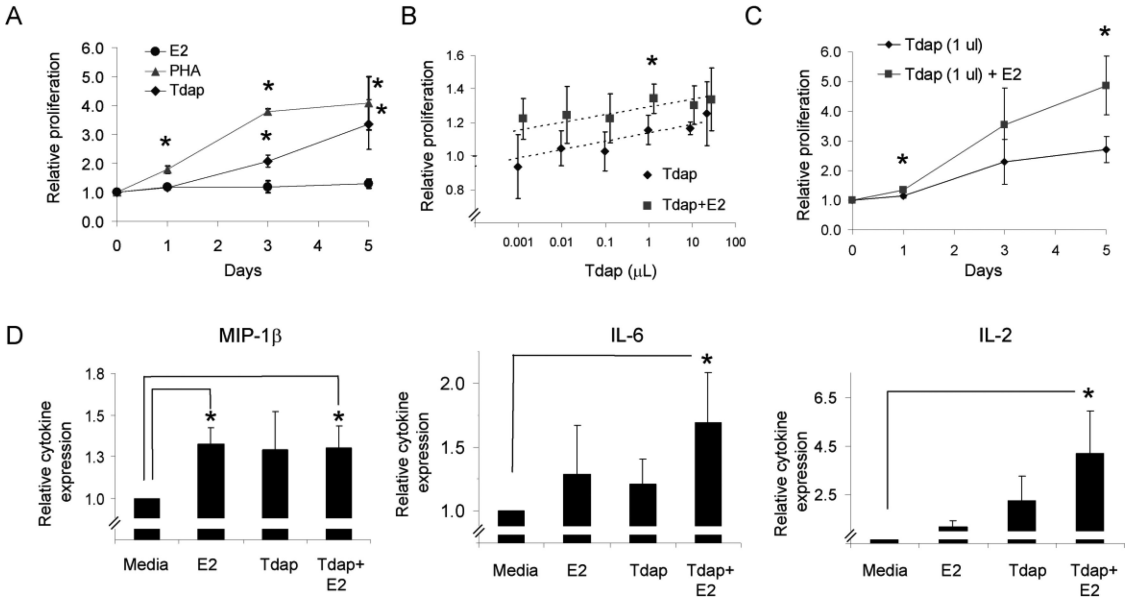

Fig. 1.

Estrogen lowers the threshold of immune cell activation upon acute antigenic challenge. PBMCs were isolated from healthy females (n = 5) and (A) exposed to β-estradiol (E2, 10 nM), 2% phytohemagglutinin (PHA, positive control), or Tdap/BOOSTRIX® (Tdap). The relative fold change in proliferation was determined at the times shown and the statistically significant differences are indicated relative to E2-stimulated levels. (B) Cells were treated with a dilution series of Tdap with and without the addition of 10 nM E2. After 24 hours, relative proliferation was determined. (C) Proliferation was measured with or without 10 nM E2 stimulation and Tdap exposure for the indicated time period. (D) Conditioned media was collected after treatment with 10 nM E2, Tdap, or both for 24 hours. Cytokine levels were measured by multiplex ELISA analysis. Representative experiments are shown. * $p < 0.05$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

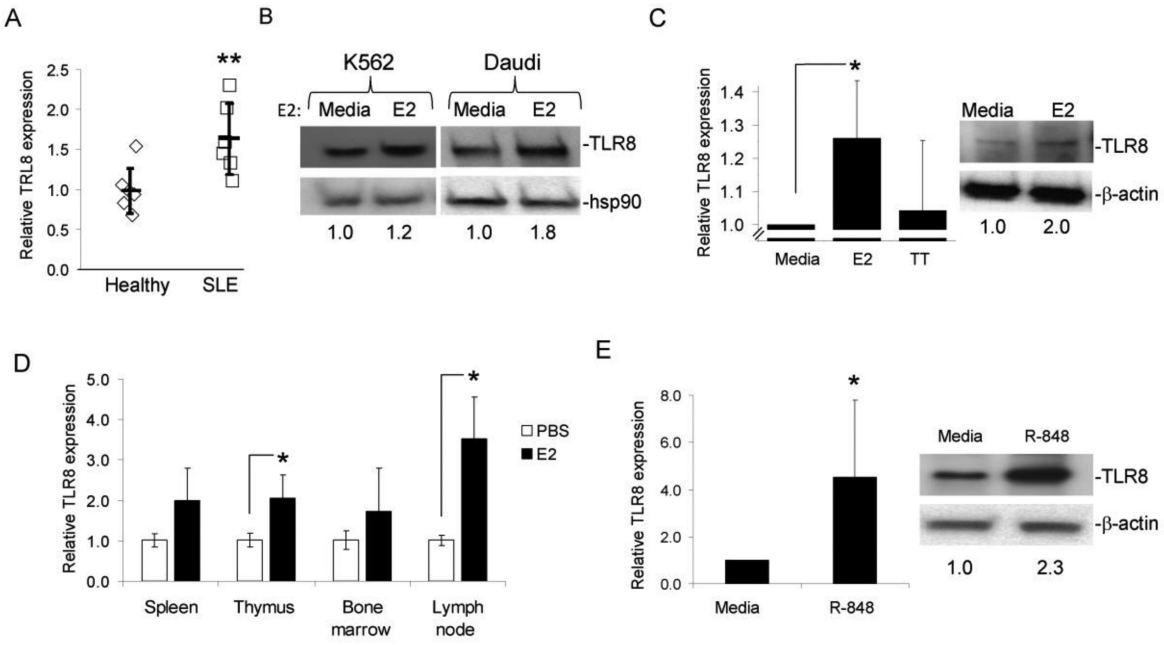

Fig. 2.

TLR8 expression is higher in PBMCs from SLE patients compared to healthy controls and is induced by both estrogen and TLR8 agonist. (A) Real time-RT-PCR of RNA isolated from whole blood of SLE patients (n = 6) compared to age and sex-matched healthy controls (n = 6). (B) Human hematopoietic cell lines K562 and Daudi were treated with 10 nM E2 for 24 hours. Total cell lysates were collected for Western blotting using antibodies against TLR8 and hsp90 (loading control). (C) Healthy male and female PBMCs (n = 15) were treated with 10 nM E2 and testosterone (TT, 100nM) for 48 hours. (left) RNA was extracted and analyzed by real time-RT-PCR. (right) Western blot analysis was performed with total cell lysates to measure TLR8 and β-actin (loading control) expression. (D) E2 (0.5 μg/day) was injected subcutaneously into wild-type mice (n = 10).

Lymphoid tissue was collected after six days for real time-RT-PCR analysis. TLR8 expression is shown relative to PBS-injected control mice (n = 10). (E) Healthy male and female PBMCs (n = 8) were treated with TLR8 agonist (R-848, 0.3 μg/mL). After 48 hours, total cell lysates were collected for (left) real time RT-PCR and (right) Western blotting to measure TLR8 expression. All relative protein expression levels were determined using semi-quantitative analysis and are indicated below the respective Western blot lanes. Representative experiments are shown. * $p < 0.05$, ** $p < 0.005$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

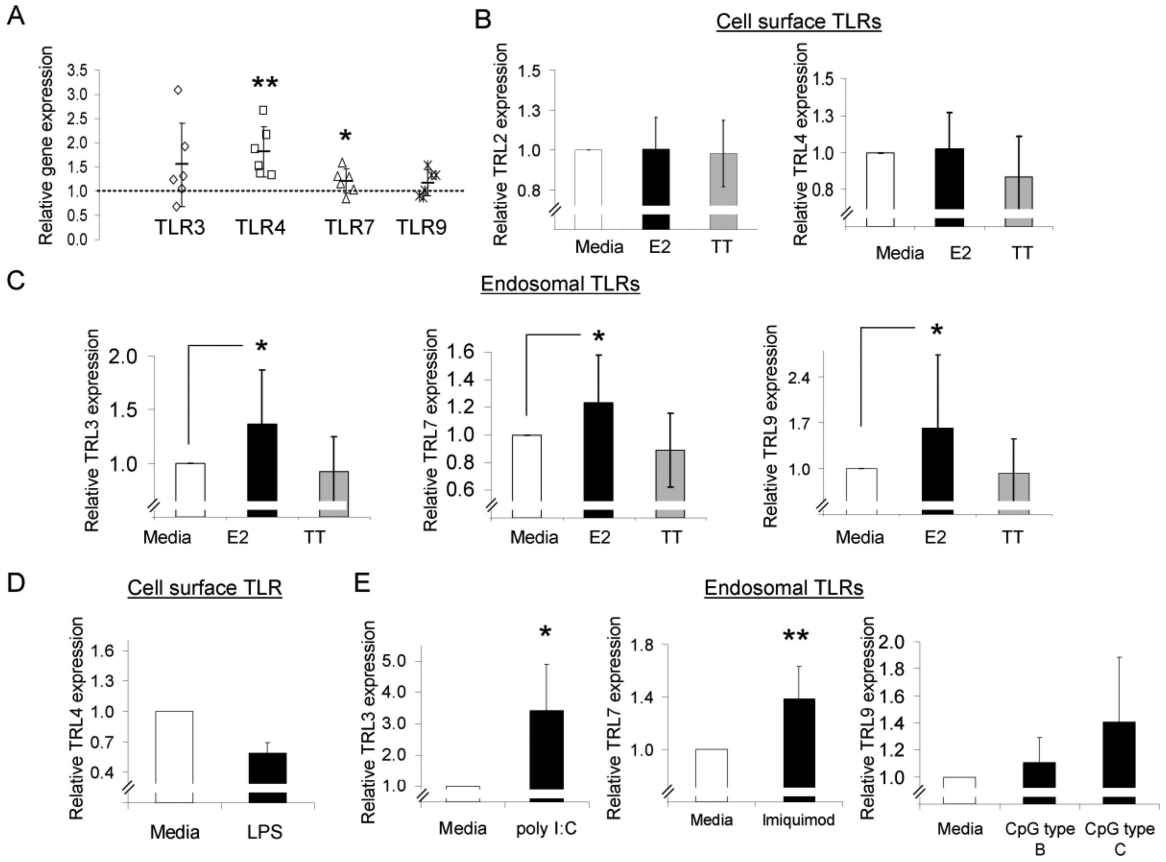

Fig. 3.

Endosome-associated TLRs are induced selectively by estrogen and agonist stimulation. (A) Real time-RT-PCR analysis of RNA isolated from whole blood samples obtained from SLE (n = 6) and healthy subjects (n = 6) to measure TLR3, TLR4, TLR7, and TLR9 transcript levels. The dashed line (--) indicates the average relative expression level of each TLR in healthy samples. (B-E) Real time-RT-PCR of PBMCs from healthy male and female donors after 48 hours of treatment. (B-C) E2 (10 nM) and testosterone (TT, 100 nM). (B) Expression of the cell surface TLRs (left) TLR2 (n = 5) and (right) TLR4 (n = 12). (C) Endosomal-TLR expression was measured for (left) TLR3 (n = 11), (middle) TLR7 (n = 14), and (right) TLR9 (n = 20). (D) Cells were stimulated with LPS to measure TLR4 expression. (E) Endosomal TLR agonist treatment of cells: (left) Polyinosine-polycytidylic acid (poly I:C) treatment for TLR3, (middle) Imiquimod for TLR7, or (right) CpG DNA for TLR9. Representative experiments are shown. * $p < 0.05$, ** $p < 0.005$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

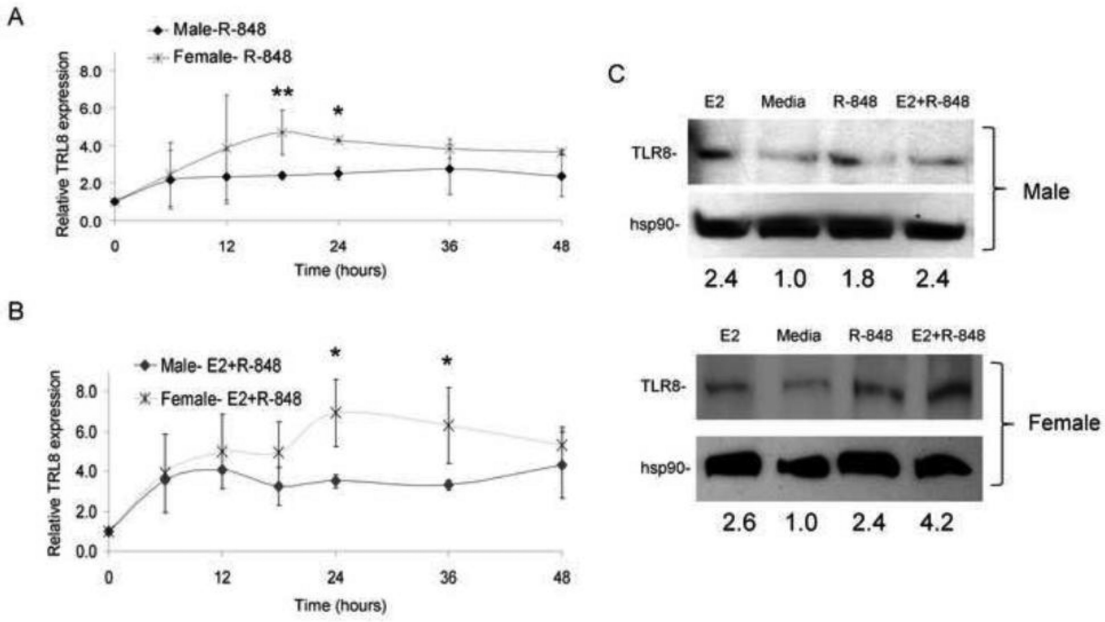

Fig. 4.

Female sex-biased TLR8 induction is observed in PBMCs when stimulated with estrogen and/or TLR8 agonist. Healthy male and female PBMCs ($n = 6$) were treated with 10 nM E2, R-848 (0.3 μg/mL), or both for the indicated time period. RNA was analyzed by real time-RT-PCR for TLR8 expression. (A) Time course analysis of TLR8 expression after R-848 treatment and (B) R-848 with E2. Expression levels were normalized for each sample and expressed relative to time zero. (C) Western blot analysis for TLR8 and hsp90 (loading control) expression after 24 hours. Semi-quantitative analysis of TLR8 protein expression levels from Western blots are indicated below the respective Western blot lanes. Representative experiments are shown. * $p < 0.05$, ** $p < 0.01$.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

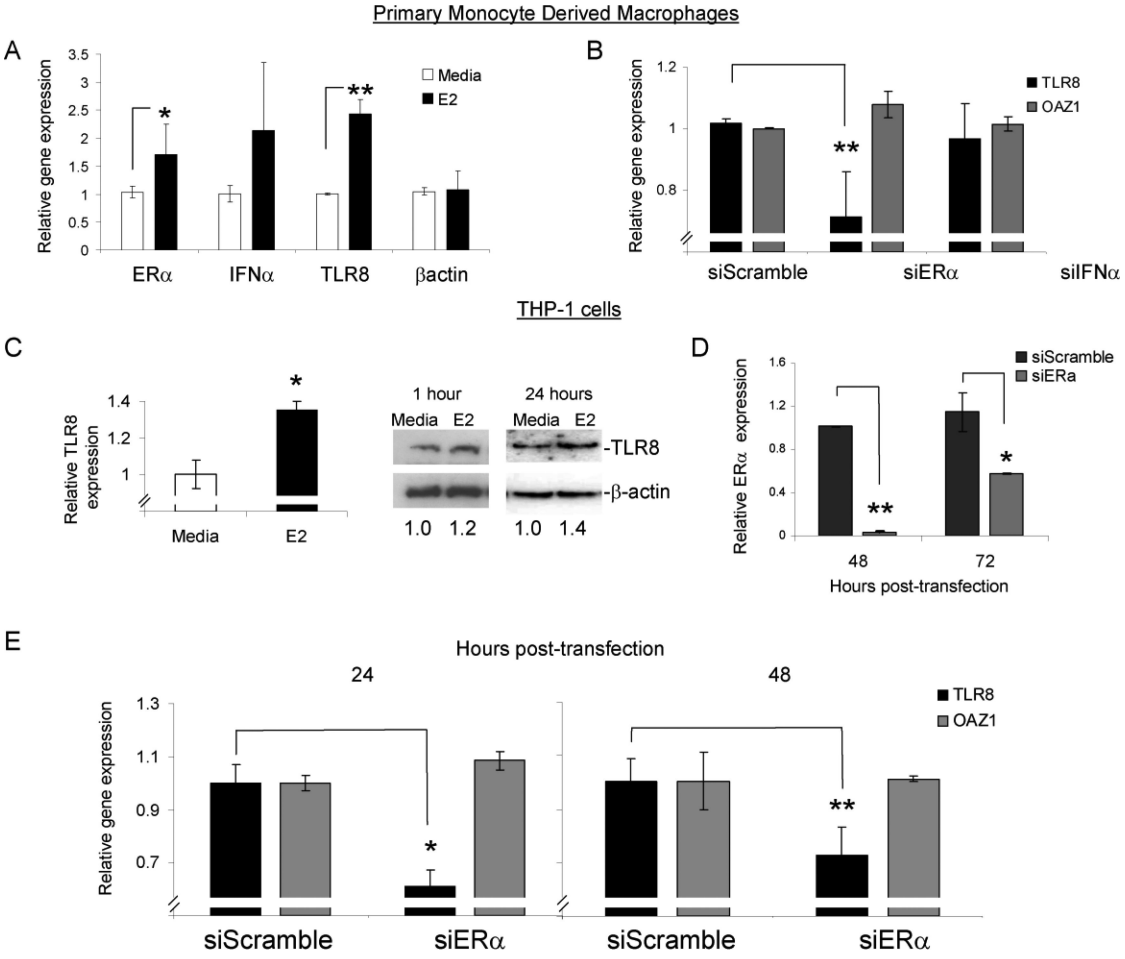

Fig. 5.

Estrogen-induced TLR8 expression requires ERα but not IFNα. Primary human monocyte derived macrophages (MDMs) were isolated from healthy female PBMCs ($n = 5$) and analyzed by real time RT-PCR. (A) MDMs treated with 10 nM E2 for 48 hours to measure the relative expression of ERα, IFNα, TLR8, and β-actin. (B) MDMs transfected with siRNA targeting ERα, IFNα, or scramble (control) in the presence of 10 nM E2 for 48 hours. TLR8 or OAZ1 (control) expression was measured relative to scrambled siRNA levels for each gene. (C) THP-1 cells were treated with 10 nM E2 and TLR8 expression was measured by (left) real time-RT-PCR and (right) Western blotting. (D) Transfection of THP-1 cells with siRNA directed against ERα. Expression of ERα is shown relative to siScramble levels following real time-RT-PCR analysis. (E) Relative TLR8 or OAZ1 expression in THP-1 cells after 24 or 48 hours transfection with siRNA targeting ERα. Representative experiments are shown. * $p < 0.05$, ** $p < 0.01$.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

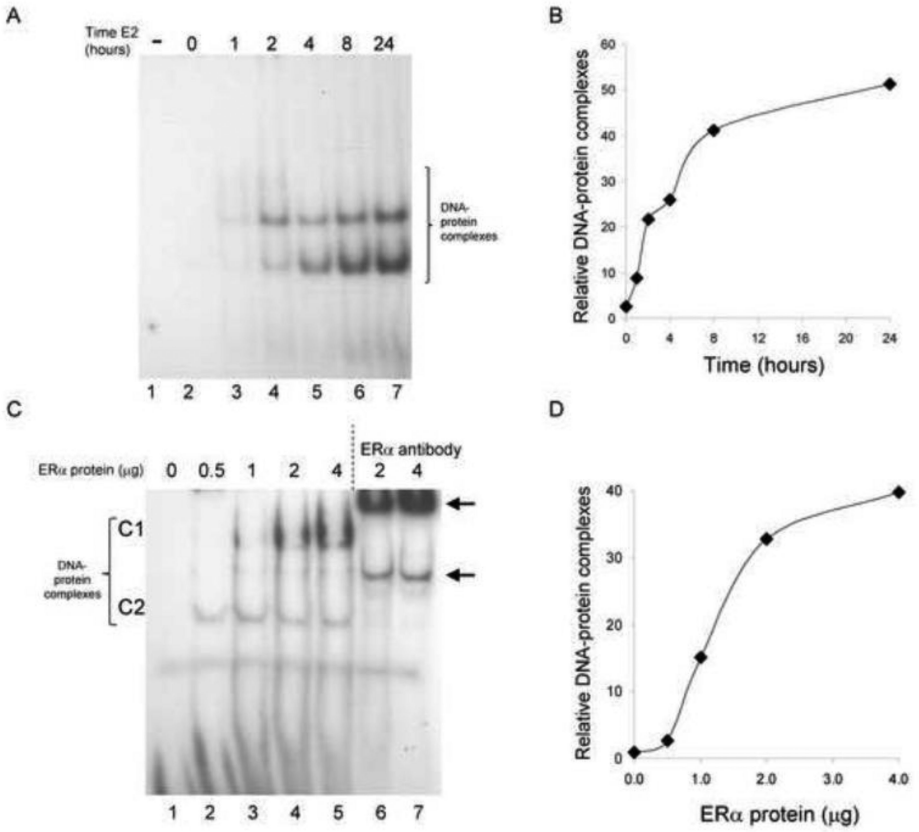

Fig. 6.

ERα binds directly to a putative estrogen responsive element near the TLR8 locus. (A) EMSA of nuclear extracts prepared from THP-1 cells stimulated with E2 (10 nM) for the time indicated and incubated with ^{32}P-labeled ERα probes (lanes 2 to 7). (-) no nuclear extracts (lane 1). (B) Semi-quantitative analysis of the amounts of DNA-protein complex formed over time with E2 incubation. (C) EMSA and antibody gel supershift assays. Radio-labeled ERα probes were incubated with increasing amounts of recombinant human ERα protein (lanes 2 to 5), and supplemented with anti-ERα antibodies (lanes 6 and 7). DNA-protein complexes are labeled (C1 and C2) and antibody gel supershifted complexes are indicated with arrows. (D) Semi-quantitative analysis of the amounts of DNA-protein complex formed (C1 + C2) with increasing amounts of rhERα protein. Representative experiments are shown.