

Published in final edited form as:

Clin Immunol. 2012 July ; 144(1): 1–12. doi:10.1016/j.clim.2012.04.001.

Estrogen receptor alpha modulates toll-like receptor signaling in murine lupus

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Abstract

Systemic lupus erythematosus (SLE) is a disease that disproportionately affects females. Despite significant research effort, the mechanisms underlying the female predominance in this disease are largely unknown. Previously, we showed that estrogen receptor alpha knockout (ER α KO) lupus prone female mice had significantly less pathologic renal disease and proteinuria, and significantly prolonged survival. Since autoantibody levels and number and percentage of B/T cells were not significantly impacted by ER α genotype, we hypothesized that the primary benefit of ER α deficiency in lupus nephritis was via modulation of the innate immune response. Using BMDCs and spleen cells/B cells from female wild-type or ER α KO mice, we found that ER α KO-derived cells have a significantly reduced inflammatory response after stimulation with TLR agonists. Our results indicate that the inflammatory response to TLR ligands is significantly impacted by the presence of ER α despite the absence of estradiol, and may partially explain the protective effect of ER α deficiency in lupus-prone animals.

Keywords

Estrogen receptor alpha; Systemic lupus; erythematosus; Dendritic cells; Toll-like receptors

1. Introduction

Systemic lupus erythematosus and many other autoimmune diseases disproportionately affect females, yet the mechanisms underlying the female predominance in these diseases remain a key unanswered question. The basis of the gender bias is almost certainly multifactorial, likely involving the sex chromosomes themselves, as well as the sex hormones and their receptors. Given that the gender bias is most prominent during the reproductive years, estrogen and its receptors are implicated in disease pathogenesis. Estrogen's primary effects are mediated via estrogen receptors alpha and beta (ER α/β) that are expressed in many tissues, including most immune cells, where they have pleiotropic effects in both the innate and adaptive immune responses [1]. The classical roles of ERs as nuclear hormone receptors are, when ligand-bound, to directly bind estrogen response elements (EREs) in gene promoters to regulate transcription of target genes. ERs can also, however, tether to other transcription factors to regulate transcriptional activity, without

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2012.04.001>.

direct DNA binding [2–4]. Additionally, via “non-classical effects”, cytoplasmic ER and membrane associated ER can impact specific kinase signaling pathways directly to regulate the cellular milieu [5–7], making the potential regulatory impact of ERs even greater. These “non-classical” effects of ERs can occur in the absence of ligand binding, i.e. estradiol-independent.

Previously, we showed that ER α ^{-/-} NZM2410 and ER α ^{-/-}MRL/lpr lupus prone mice had significantly prolonged survival compared to ER α ^{+/+} animals [8]. ER α ^{-/-} mice manifested significantly less pathologic renal disease and proteinuria, despite similar levels of autoantibody production and immune complex deposition as wild-type mice. These findings in ER α deficient mice indicated that autoantibody production/immune complex deposition is necessary, but not sufficient, for full expression of lupus nephritis. Given that the adaptive immune response appears minimally affected by ER α deficiency, we hypothesized that one of the protective effects of ER α deficiency in lupus is by modulating the innate immune response.

Toll-like receptors (TLRs) are critical factors in the innate immune response and TLR activation by autoantigens can potentially amplify autoimmune responses [9]. Multiple lines of evidence suggest that TLRs are involved in the pathogenesis of lupus. For example, the *Yaa* locus, a duplication of the TLR7 gene on the Y chromosome, is responsible for lupus susceptibility in BXSB mice [10,11]. Consistent with this, TLR7- and TLR7/9-deficient lupus prone animals have diminished disease [12]. Interestingly, TLR9 deficiency alone exacerbates disease, however, disease worsening was dependent on the presence of TLR7 [13]. This recent work suggests that TLR9 acts both in parallel and in interdependent pathways with TLR7 to regulate immune responses. TLR3 is also implicated in lupus disease expression [14], but its effects overall are still unclear as renal injury in TLR3-deficient mice was unaffected [15].

TLRs are highly expressed on DCs, potent antigen presenting cells important in the innate immune response that are dysregulated in lupus [16,17]. Estradiol regulates DC differentiation, and is, in fact, required for the development of the Langerhans-like subset of DCs from myeloid progenitors (MP) [18]. A functional ER α is required for this effect. In contrast, estradiol inhibits DC differentiation when MP are supplemented with Flt3 ligand, which predominates in homeostatic DC development [19,20]. This finding suggests a role for estradiol and ERs in differential regulation of DCs depending on the cellular milieu (inflammatory vs. non-inflammatory). Since DCs can stimulate Th1, Th2 and Th17 responses, modulate NK-T cell and plasma cell function, as well as directly impact B cell proliferation, differentiation and isotype switching [21–23], they have far reaching effects on the immune response when inappropriately activated. DCs are dysregulated in SLE; DCs derived from both SLE patients and lupus-prone animals display an activated phenotype characterized by increased CD80/86 and MHCII expression as well as increased inflammatory cytokine production [24–27]. Recently, Seillet et al. showed that 17 β -estradiol treatment of normal post-menopausal women enhanced TLR7/9 pDC production of IFN α , an effect that required ER α [28].

We hypothesized that ER α deficiency impacts lupus disease expression by modulating the innate immune response to TLR signaling. In this study we showed that DC development and TLR-stimulated DC function were affected by ER α absence in murine lupus. The absolute number of DCs isolated from lupus prone ER α KO mice was reduced, and these DCs produced significantly less IL-6 and MCP-1 in response to TLR9 stimulation compared with wild-type littermates. Interestingly, we also find fewer plasmacytoid-type DCs (pDCs) in ER α KO lupus prone mice. After cell-sorting and standardizing numbers, pDCs derived from ER α KO mice also produced less IFN α in the absence of estradiol. We also report for

the first time that ER α deficiency impacts IL-23, IL-23R and IL-17 expression in lupus prone mice. These cytokines were reduced in DCs and ex vivo spleen cells, respectively, when cultured from ER α KO lupus prone mice treated with TLR7/9 agonists in the absence of estradiol. These experiments indicate that ER α may modulate lupus disease expression by influencing DC development and TLR signaling, and may contribute to the development of pro-inflammatory cell types such as pDCs and Th17 cells that are implicated in lupus pathogenesis. This study provides partial mechanistic understanding of how ER α deficiency may protect against development of lupus nephritis in murine lupus.

2. Materials and methods

2.1. Mice

ER α -/- mice on the C57BL/6 background (kind gift of Ken Korach, NIEHS, RTP, NC) were backcrossed for 9 generations to NZM2410 or MRL/lpr mice (Jackson Laboratory, Bar Harbor, ME) and congenic status was verified as previously described [8]. Only female mice were used for these experiments. All mice were maintained at the Ralph H. Johnson VAMC Animal Care Facility (Charleston, SC) using Institutional Animal Care and Use Committee approved protocols #421 and #498 approved August 2008 and 2011 respectively.

2.2. Generation of BMDCs and spleen cells

Bone marrow-derived DCs were generated using a modified version of the protocol originally described by Inaba et al. [29], without lymphocyte depletion. Briefly, BM was flushed from the femurs and tibias of mice and then depleted of red blood cells by incubation with RBC lysis buffer (144 mM NH₄Cl and 17 mM Tris, pH 7.6). Equal numbers of BM cells from WT and knockout mice were suspended in complete RPMI and supplemented with murine GM-CSF (20 ng/ml) and murine IL-4 (20 ng/ml) and cultured in T75 flasks at 1 \times 10⁶ cells/ml (~20 \times 10⁶/flask). On day 3 of culture, fresh complete RPMI medium was added to increase the total volume by 50%. Cytokines were replenished for the final total volume of medium. On day 7, the cells were harvested, washed twice and the phenotype of cDCs (CD11c⁺CD11b⁺) was confirmed by flow cytometry. This method yielded 50–80% conventional myeloid DCs depending on the strain. For spleen cells, mice were sacrificed and spleens were harvested and kept in ice-cold RPMI. Spleens were processed and subjected to red blood cell lysis. Cells were washed twice in cold RPMI before being counted and cultured in 12- or 6-well plates at 1 \times 10⁶ cells/ml (2–4 \times 10⁶/well) for 24 h. For B cell isolation, mouse depletion dynabeads were used (DynaMouse B Cell Negative Isolation Kit, Invitrogen, Carlsbad, CA) with a starting leukocyte working volume of 5 \times 10⁷ cells. An enriched B cell population was validated by FACS analysis and found to be > 92% pure.

2.3. Treatment of DCs with TLR agonists

BMDCs were harvested on day 7 from BM cultures as described above, washed twice with PBS and then counted with a hemocytometer. Cells were seeded at 1 \times 10⁶ cells/ml into 6- or 12-well plates in estrogen free phenol red-free RPMI with 10% charcoal-dextran-stripped FCS and treated with vehicle or TLR agonist: loxoribine (TLR7/8 agonist, 50–200 μ mol), polyI:polyC (TLR3 agonist 10–100 μ mol), LPS (TLR4 agonist; 1 μ g/ml) or mouse CpG DNA (TLR9 agonist; 1 μ g/ml) for 24 h.

2.4. Antibodies and reagents

Antibodies including anti-mouse CD16/CD32 (Fc receptor block), and fluorescent conjugated anti-CD11b, anti-CD11c, anti-CD317 (PDCA1), anti-CD3e and anti-IL-17A were purchased from eBiosciences, Inc. (San Diego, CA). Anti-human/mouse ROR γ -APC was purchased from R&D Systems (Minneapolis, MN). PE conjugated-120G8.04 mAb to

pDC was purchased from Imgenex/Dendritics (San Diego, CA). Murine recombinant GM-CSF and IL-4 cytokines were purchased from R&D systems (Minneapolis, MN) and reconstituted in PBS before use. TLR agonists: polyI:polyC, loxoribine, and lipopolysaccharide were all purchased from Sigma–Aldrich, (St. Louis, MO). The TLR9 agonist CpG was purchased from Hycult Biotech (Canton, MA).

2.5. Flow cytometry

Fresh single-cell suspensions were prepared and 1×10^6 cells were treated with anti-CD16/CD32 for 5 min on ice. Cells were then stained with the indicated conjugated mAbs, and incubated for 30 min on ice. These cells were washed twice and re-suspended in 0.3 ml of 0.5% BSA, 0.02% sodium azide in PBS solution. To determine IL-17 and ROR γ t expression, spleen cells were cultured in complete RPMI±GolgiStop before intracellular staining was performed. Cells were fixed and permeabilized using an intracellular staining kit from BD Biosciences (San Jose, CA), and stained for IL-17 and ROR γ t. FACS acquisition was done on BD FACS Calibur machine. Cells were analyzed by flow cytometry using the FlowJo software package (Tree Star, Inc., Ashland, OR).

2.6. RNA preparation

Following 24 h culture, media was removed and RNA was prepared from cells using a hybrid Trizol/RNeasy protocol (adapted from Alvydas Mikulskis and the Qiagen RNeasy protocol). Briefly, samples were suspended in an appropriate volume of trizol and left at room temperature for 30 min. Chloroform was then added at a volume of 1:5. After a 15 min centrifugation at $12,000 \times g$, the aqueous phase was removed and 70% EtOH added. Samples were loaded into RNeasy columns and washed per protocol before elution in TE buffer.

2.7. Analysis of gene expression in DCs and spleen cells by quantitative RT-PCR

Primers (IL1 β , IL23, IL23R, GAPDH, 18 S) were purchased from SABiosciences (Frederick, MD); L19 was synthesized by Integrated DNA Technologies (Coralville, Iowa). All primers were used at 5 mM. First Strand Kit (SABiosciences) was used to generate cDNA. RT-PCR was performed as follows: for each real-time qPCR, 1 μ g cDNA was added to a master mix containing primers, IQ SYBR-green (Bio-Rad) and nuclease-free water. PCR conditions were 10 m at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at the appropriate annealing temperature (generally 60 °C), 60 s at 95 °C, and 60 s at 55 °C. Subsequent melt curve analysis commenced at 55 °C for 10 s and increased by 0.5 °C every 10 s for 80 cycles. A melt curve for each qPCR, with a single peak at the correct melting temperature, was indicative of a reliable and desired PCR product. All samples and controls were analyzed in triplicate, and a no-cDNA control was included. The relative gene expression of the genes of interest was calculated after normalization to at least 1 housekeeping gene (GAPDH, 18 s, or L19).

2.8. Cytokine release assay

Cytokine release by DCs and spleen cells was determined by culturing 1×10^6 /ml cells with either vehicle or TLR agonist. After 24 h, culture supernatants were harvested and cytokine (IL-6, MCP-1 or IL-23) concentrations were measured by sandwich ELISA per the manufacturer's protocol (eBioscience, Inc. San Diego, CA) using a microplate luminometer (Thermo Scientific Multiskan Ascent).

2.9. WISH cell assay

WISH cells, purchased from ATCC Cat #CCL-25 (Manassus, VA), were plated in 12-well plates at 3×10^5 , and cultured in either control media (RPMI +10% FCS), control media plus Universal Type I Interferon 100 units (PBL Interferon Source, Piscataway, NJ) or

conditioned media from PDCA1+ sorted pDCs (ER α ^{+/+} or ER α ^{-/-}) post-treatment with CpG for 18 h. RNA was extracted from WISH cells after 6 h incubation and qRT-PCR was performed as above with 3 primer pairs (PKR, IFIT, MX1) to IFN target genes as described by Hua et al., 2006. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA): *MX1 (forw)* 5' - TAC CAG GAC TAC GAG ATT G -3'; *MX-1 (rev)* 5' - TGC CAG GAA GGT CTA TTA G -3'; *PKR (forw)* 5' - CTT CCA TCT GAC TCA GGT TT -3'; *PKR (rev)* 5' - TGC TTC TGA CGG TAT GTA TTA -3'; *IFIT (forw)* 5' - CTC CTT GGG TTC GTC TAT AAA TTG -3'; *IFIT (rev)* 5' - AGT CAG CAG CCA GTC TCA G 3'.

2.10. Statistics

Numerical data obtained from each experiment were expressed as mean \pm SEM and the statistical differences between experimental and control groups were assessed using GraphPad Prism software. *P* values less than 0.05 were considered statistically significant. For experiments that included both mouse strain and treatment variables, a grouped analysis was done by performing a two-way ANOVA with subsequent Bonferroni's post-test correction. A sign test was performed for paired animals (WT versus KO).

3. Results

3.1. TLR-induced IL-6 production is significantly reduced in spleen cells and isolated B cells

To test the hypothesis that TLR-induced inflammation is downregulated in immune cells from ER α deficient lupus prone mice, we examined production of IL-6 by spleen cells and isolated B cells following treatment with TLR agonists under estrogen-free conditions. Animals were sacrificed prior to onset of disease at 8–12 weeks of age and littermates were utilized when possible. Spleens were harvested and cells were isolated for overnight culture. A subset of spleen cells was used for isolation of B cells by negative selection (>92% pure). Treatment for 24 h with LPS 1 μ g/ml (TLR4), polyI:C 10 or 100 μ g/ml (TLR3), or loxoribine 200 nmol (TLR7) resulted in significantly decreased levels of IL-6 production by spleen cells (Fig. 1A) and isolated B cells (Fig. 1B) from lupus prone ER α KO animals compared to wildtype littermates, as measured by ELISA. This result suggested that TLR-induced inflammatory cytokine production is blunted in the absence of ER α .

3.2. TLR9-induced IL-6 and MCP-1 production by dendritic cells is reduced in both B6 and lupus prone ER α KO mice

Dendritic cells (DCs) express high levels of TLRs, are key mediators of the innate immune response, and are implicated in the pathogenesis of lupus. We isolated bone marrow hematopoietic cells from WT or ER α KO mice (B6, MRL/lpr and NZM2410) and derived DCs with selective/supplemented media. Following harvest on d7, a subset of DCs was analyzed by FACS (see Supplemental Fig. 1) and the remaining DCs were divided into 6- or 12-well plates. As DC development requires estrogen, the cells were derived in usual complete media (which contains the equivalent of micromolar concentrations of estradiol). Post derivation, stimulation of the DCs was performed under estrogen-free conditions. Following treatment with vehicle (PBS), loxoribine (50 nmol), or CpG DNA (1 μ g/ml) for 24 h, media was removed and the pro-inflammatory cytokines, IL-6 and MCP-1, were measured. In DC cultures derived from wild-type B6 mice, the TLR9 agonist CpG stimulated robust IL-6 production, however, ER α KO DC IL-6 production was significantly decreased. CpG stimulation increased IL-6 levels more than 20-fold in media from B6 WT DCs, with the stimulation index reduced by ~50% in ER α KO animals (Fig. 2A). MCP-1 levels increased 3-fold in response to CpG, and again there was a significantly reduced response by ER α KO DCs. Cytokine levels were in the range of 120 pg/ml–47 ng/ml,

indicating a robust response. These data indicate that ER α significantly modulates TLR9 responses by DCs.

Consistent with our findings in B6 ER α KO mice, DCs from ER α -/- lupus prone MRL/lpr and NZM2410 mice also exhibited a significantly blunted response to TLR agonists. In NZM2410 derived DCs, CpG treatment resulted in 60-fold increase in IL-6, with a significant reduction in IL-6 production in the absence of ER α (Fig. 2B). Similarly, MCP-1 production was significantly increased in CpG-treated DCs isolated from NZM mice, and this effect was reduced by ~50% in the setting of ER α deficiency, again indicating a role for ER α in modulating the effect of the TLR stimulated inflammatory response independent of ligand. Similar trends were observed in MRL/lpr WT and ER α KO animals (data not shown). TLR7 stimulation with loxoribine (50 nmol) also variably increased IL-6 and MCP-1 levels, with a trend towards decreased levels in ER α -deficient mice, however, the effects were not significant due to a low stimulatory index in both WT and ER α KO mice.

3.3. IL-1 β and IL-23 expression is induced by a TLR9 agonist in wild-type but not ER α KO mice

CD4⁺ T-cells differentiate to become Th1, Th2, Tregs, or Th17 cells (among other subsets), based on intercellular interactions with APCs and the cytokine environment. IL-6 is an essential cytokine for driving the development of Th17 cells, which are implicated in the pathogenesis of SLE. Because TLR-induced levels of IL-6 were modulated by the presence or absence of ER α , we investigated the expression of IL-1 β and IL-23, two TLR-induced cytokines that are produced by APCs and also promote the expansion and survival of Th17 cells.

In DCs isolated from B6 WT mice, IL-1 β was induced >90-fold by CpG treatment. This effect was reduced by 80% in the ER α -/- animal (Fig. 3A). Loxoribine induced IL-1 β mRNA expression by 10-fold and 3-fold in B6 WT and ER α KO mice respectively. CpG also stimulated IL-23 mRNA expression by B6 WT DCs, but did not induce IL-23 in ER α KO animals, suggesting that TLR9 stimulation of IL-23 requires ER α . IL-23 expression was also determined at the protein level by ELISA. Low concentrations of IL-23 were detected in media removed from cultured B6 WT DCs, but only in CpG-treated cultures. This effect was abrogated in B6 ER α -/- DC cultures (Fig. 3B). Similar results were found in NZM2410 mice, with the exception that higher levels of IL-23 were produced. In summary, expression of the pro-inflammatory cytokines IL-1 β and IL-23 by B6 or NZM2410 DCs, following TLR9 stimulation, was significantly reduced in the setting of ER α deficiency, further implying an important role for ER α in modulating TLR9 signaling independent of estradiol.

3.4. TLR9 stimulation induces IL-23R expression in wild-type but not ER α KO mice

To further define ER α effects on immune responses, we evaluated IL-23R expression. In addition to IL-23R upregulation on Th17 cells, a subset of dendritic cells (and macrophages) expressing IL-23R can be directly activated by IL-23, resulting in the production of inflammatory mediators [30,31]. We assessed the level of IL-23R mRNA expression in both WT and lupus prone animals to determine if its expression was affected by the presence or absence of ER α . As with cytokine levels of IL-23, IL23 receptor levels were also significantly reduced in the setting of ER α deficiency. In B6 WT DCs, IL-23R mRNA expression was stimulated 2–3-fold by both TLR7 and 9 ligands, however, TLRs did not induce IL-23R expression in DCs isolated from ER α KO mice (Fig. 4). TLR9 stimulation of DCs and spleen cells from MRL/lpr mice resulted in a robust increase in IL-23R expression, which was decreased by >50% in ER α KO mice (data not shown). A similar trend was noted in NZM2410 mice, but did not reach statistical significance. Combined, these data suggest

that ER α plays a role in modulating TLR stimulation of the IL-23/IL-17 pathway that contributes to the pro-inflammatory phenotype in these animals.

3.5. Percent of IL-17+/ROR γ t+spleen cells are reduced in lupus prone ER α KO mice

ROR γ t is a key transcription factor for Th17 differentiation and expansion, including upregulation of IL-23R to stabilize the Th17 phenotype. Evaluation of IL-17+/ROR γ t+spleen cells by flow cytometry was performed to determine whether the presence or absence of ER α in lupus prone animals impacted the development of IL-17 producing cells. Spleen cells were harvested and cultured for 24 h prior to being fixed and permeabilized for intracellular staining. In each experiment, spleen cells isolated from NZM ER α -/- animals had significantly fewer IL-17+/ROR γ t+ cells than NZM WT animals (Fig. 5), again supporting the hypothesis that ER α is necessary for a robust inflammatory response to TLR stimulation, with one endpoint being production of IL-17-secreting cells.

3.6. BMDCs from ER α KO mice display a decreased plasmacytoid phenotype

In addition to finding fewer IL-17 producing cells in the absence of ER α , we tested the hypothesis that IFN α production is also impacted by ER α . The plasmacytoid subset of DCs is the major percentages were decreased significantly compared to their wild-type littermates, indicating that ER α impacts the development of pDCs under these culture conditions. producer of IFN α and expresses high levels of TLR7 and TLR9. Of note, in this report, DCs were derived from BM cells cultured for 7 d in GM-CSF and IL-4 supplemented media, an established culture method for deriving conventional, rather than plasmacytoid DCs (pDCs), while selecting against macrophages. With this method, approximately 50–80% of cells cultured expressed both CD11c and CD11b (Supplemental Fig. 1). Only a few cells (2–10%) were stained with the pan-macrophage marker F4-80 (data not shown) suggesting very little macrophage contamination. Thus we hypothesized that the remaining cells were pDCs. Mouse pDCs express low levels of CD11c or lose CD11c expression, but have other specific markers. To determine whether pDCs were present in our DC cultures, we used 2 different murine pDC markers, PDCA1 and 120 G8. By both markers, pDC percentage and number were significantly increased in NZM2410 (ER α +/+ approximately 42%) compared to B6 mice (5–10%) (Fig. 6). Most notably, we also found that in ER α KO strains, pDC numbers and

3.7. IFN α and IL-6 production by plasmacytoid dendritic cells derived from ER α KO lupus-prone mice is significantly reduced

pDCs produce both IFN α and IL-6. Since we previously found decreased levels of IL-6 production by BMDCs, we first wanted to determine whether the overall reduced number of pDCs in the ER α KO animals was responsible for the cytokine decrease rather than being a functional difference. BMDCs were harvested on d7 and pDCs were stained with PDCA1. PDCA1+ cells were then sorted by MoFlo to separate pDCs from cDCs. Similar to the prior experiment, approximately 40–50% of NZM2410 ER α WT-derived DCs were PDCA1+ whereas only 20–30% of the DCs derived from ER α KO mice were PDCA1+. Once sorted, the pDC numbers were standardized to 1×10^6 cells/well and CpG added for 18 h. Media were removed and IL-6 levels were measured by ELISA. Consistent with our whole BMDC cultures, IL-6 production by ER α KO pDCs was significantly reduced compared with NZM2410 WT (Fig. 7A).

We next determined IFN α activity via the WISH cell assay. Again, pDCs (PDCA1+ cells) were sorted and numbers standardized as above. Cells were treated with CpG for 18 h and media were removed for use in the WISH assay. WISH cells were incubated in either control or conditioned media (from pDCs treated with CpG) for 6 h prior to RNA isolation and qRT-PCR. A 2–3 fold induction of IFN α activity was observed in cells incubated with

media from NZM2410-WT pDCs which was reduced to near baseline when media from NZM2410 ER α KO pDCs were tested (Fig. 7B).

4. Discussion

We previously reported that ER α KO NZM2410 and ER α KO MRL/lpr lupus prone mice had significantly reduced renal disease and significantly prolonged survival [8]. The mechanism of the renal protection afforded to ER α KO animals is currently unknown, which is the major focus of the current report. In this study, we demonstrated that ER α modulates TLR signaling. In the absence of ER α , the inflammatory response to TLR9 stimulation was significantly blunted. We also demonstrated, for the first time, that ER α is required for TLR-induced stimulation of IL-23R expression, which may have paracrine and autocrine effects on T cells and DCs involved in the IL-23/IL-17 inflammatory pathway. This may ultimately impact the numbers of Th17 cells that are expanded/stabilized, as evidenced by data in this report that fewer IL-17/ROR γ t-expressing spleen cells were found in ER α KO mice.

Previously published studies revealed a critical role for both estradiol and ER α in murine DC differentiation in normal animals [18–20,32,33]. Since dysregulated DC maturation and function are implicated in the pathophysiology of SLE (Reviewed in [34,35]), we investigated the role of ER α in two lupus-prone mouse strains. Our results demonstrate that ER α is an important factor in the regulation of DC development in lupus-prone mice (Supplemental Fig. 1), and that there are strain differences in the differentiation of conventional DCs, which are reduced in NZM2410 compared with MRL/lpr and B6 mice. It is possible that this difference is a reflection of the fact that BMDCs derived from NZM2410 mice have a propensity to develop into plasmacytoid DCs, despite culture conditions that favor cDC development. This is consistent with reports that a large subset of active SLE patients has increased numbers of pDCs and elevated IFN α levels [36,37]. Data by Pascual and others led to the discovery of the now well-known interferon α -signature in lupus patients and the hypothesis that SLE may be driven by unabated DC activation. Interestingly, we show in this study that pDC numbers are diminished in the absence of ER α in both B6 and NZM2410 animals, suggesting that ER α partly mediates the development of this subset of interferon-producing DCs. ER α also appears to have a functional impact on these cells; purified pDCs from ER α KO mice produced less IL-6 and IFN α than equivalent numbers of pDCs from ER α +/+ animals. Further work is necessary to characterize these cells and determine the effect on Type I interferon production and other functional parameters affected by the presence or absence of ER α .

As noted above, changes in DC maturation, as well as migration, are observed in both human and murine lupus [38–41]. DCs derived from both SLE patients and lupus-prone mice have an altered costimulatory profile, with an increase in the expression of maturation markers [25,42]. For example, in mice carrying *Sle3*, a lupus susceptibility locus in the NZM2410 strain, DCs are not only significantly more mature/activated, they are also less apoptotic, more proinflammatory and better at costimulating T cells in vitro [25]. DCs also express abundant TLRs and multiple lines of evidence have implicated these receptors in the development of SLE, particularly the subgroup of nucleic-acid specific TLRs.

Since ER α deficiency is renal protective in lupus-prone animals, we investigated the mechanism of ER α protection by assessing TLR-induced inflammation in the presence or absence of ER α . Perhaps not surprisingly, TLR9 stimulation caused a significant increase in pro-inflammatory mediator production by lupus-derived DCs vs. B6-derived, consistent with the notion that DCs have a more activated and/or pro-inflammatory phenotype in lupus. Importantly, we found a significant decrease in TLR-induced inflammation by multiple

endpoints in ER α KO mice, indicating that ER α modulates TLR signaling. Our laboratory has generated similar data in ER α KO splenocytes and B cells in this report, as well as kidney mesangial cells (Svenson, Gilkeson, Cunningham, unpublished data) with multiple TLR agonists (TLRs 3,4,7,9); thus the effect does not appear to be TLR specific. We concentrated efforts in studying TLR7 and 9 due to their clear role in lupus pathogenesis. We further focused on TLR9 because we found the greatest stimulatory effect with CpG DNA and this was markedly different in ER α KO cells. This is the first report of which we are aware, to demonstrate that ER α is required, independent of estrogen, for robust TLR signaling. The mechanism of this effect, however, is still unclear, and our next task will be to investigate the role of ER α in TLR signaling on a molecular level.

On a more systemic level, our results indicate a role for ER α in the TLR induced IL-23/IL-17 inflammatory pathway. TLR7 and TLR9 agonists induced significant increases in IL-1 β and IL-23 expression in ER α +/+, but not ER α KO mice. Furthermore, TLR7 and TLR9-induced upregulation of IL-23R, which is important for the stabilization of the Th17 cell phenotype, could not be demonstrated in the absence of ER α . These results led us to hypothesize that the capacity to expand Th17 cells is partially regulated by ER α (Fig. 8). In this report we provide evidence to support this hypothesis; ex vivo spleen cells taken from NZM ER α KO mice expressed significantly less IL-17 and ROR γ t. A reduction in IL-17 producing cells may partially explain the decrease in inflammatory renal damage and increased survival in these animals. Since numerous autoimmune diseases have a female predominance, and Th17 cells are implicated in the pathogenesis of many of these diseases, it is tempting to theorize that ER α modulation of Th17 cells is a common feature among affected females. Although more studies are required to fully understand and integrate the effects that ER α has on the immune system in SLE, these findings are an important step forward in determining more specifically the role of ER α in inflammation.

There is growing evidence that the molecular mechanisms by which ER α exerts its effects are more complicated than the classic pathway of ligand-activated transcriptional activation via ERE binding. ER α also acts via multiple non-classical signaling pathways to regulate cellular responses. This study provides additional evidence for ligand-independent actions of ER α since the experiments reported herein were done under estradiol-free conditions. The full stimulatory effect of CpG and loxoribine on DC cytokine production, for example, required the presence of ER α but did not require the presence of estradiol. In addition to utilizing estradiol-free conditions, we also performed a set of experiments in the presence of ICI 182780, an estradiol antagonist, to ensure that there was no effect of any residual estradiol present in our media (phenol red-free, charcoal-dextran stripped). ICI had no effect (data not shown). Other studies have also demonstrated ligand-independent effects of ER α , and that DNA binding is not necessary for all ER α actions. For example, ER α may bind to other transcription factors such as AP-1, Sp1, and NF κ B to regulate transcription [2–4]. It is also possible that ER α exerts some of its effects by recruiting co-activators or co-repressors, such as p300 [43,44], to the transcriptional complex to affect gene expression depending on the cellular milieu. This study provides evidence for a ligand-independent effect of ER α on TLR induced gene expression in dendritic cells, but does not identify definitively the molecular mechanism(s) for ER α effects on TLR signaling. Further work is needed to better understand the specific binding partners of ER α , whether DNA binding is necessary, and whether and which sites require ER α phosphorylation for these effects.

4.1. Conclusions

In summary, ER α plays an important role in TLR-induced inflammation, and appears to support the generation of both plasmacytoid dendritic cells and IL-17 producing cells, both of which are implicated in SLE (and other female predominant autoimmune diseases). Despite significant research effort and abundant evidence that sex hormones and their

receptors influence disease expression, it has been difficult to disentangle the timing and specifics of their seemingly ubiquitous immune effects. Isolating the role of ER α , which clearly impacts both DC development and inflammation, suggests that modulation of lupus disease activity may be possible by targeting ER α .

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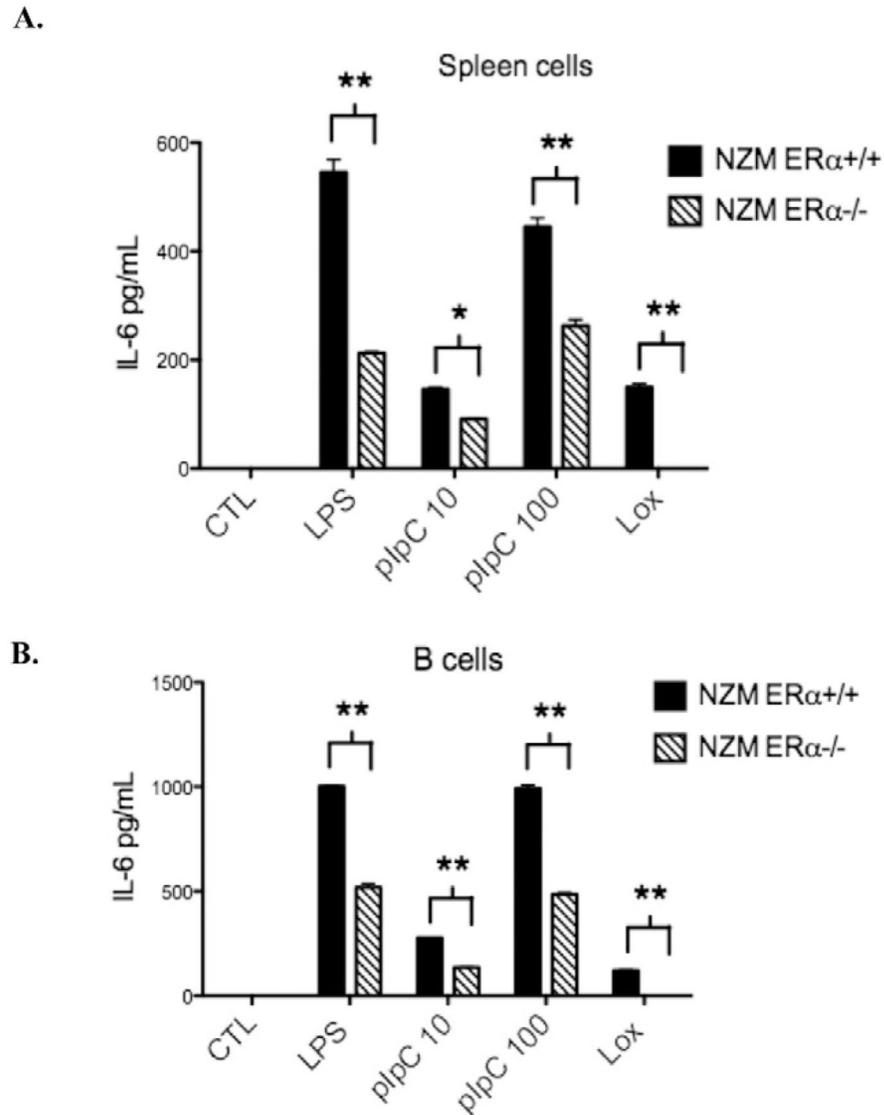


Figure 1. TLR-induced IL-6 production is significantly reduced in isolated spleen cells (A) and B cells (B) from ER α ^{-/-} mice. Treatment with a TLR4 agonist (LPS 1 μ g/ml), TLR3 agonist (polyI:C 10 or 100 μ g/ml) or a TLR7 agonist (loxoribine 200 mmol) for 24 h resulted in IL-6 induction that was reduced by at least 50% in spleen cells or B cells from ER α ^{-/-} animals. Representative of 3 independent experiments with a total of 10 animals per group minimum. *= p <0.05, **= p <0.001.

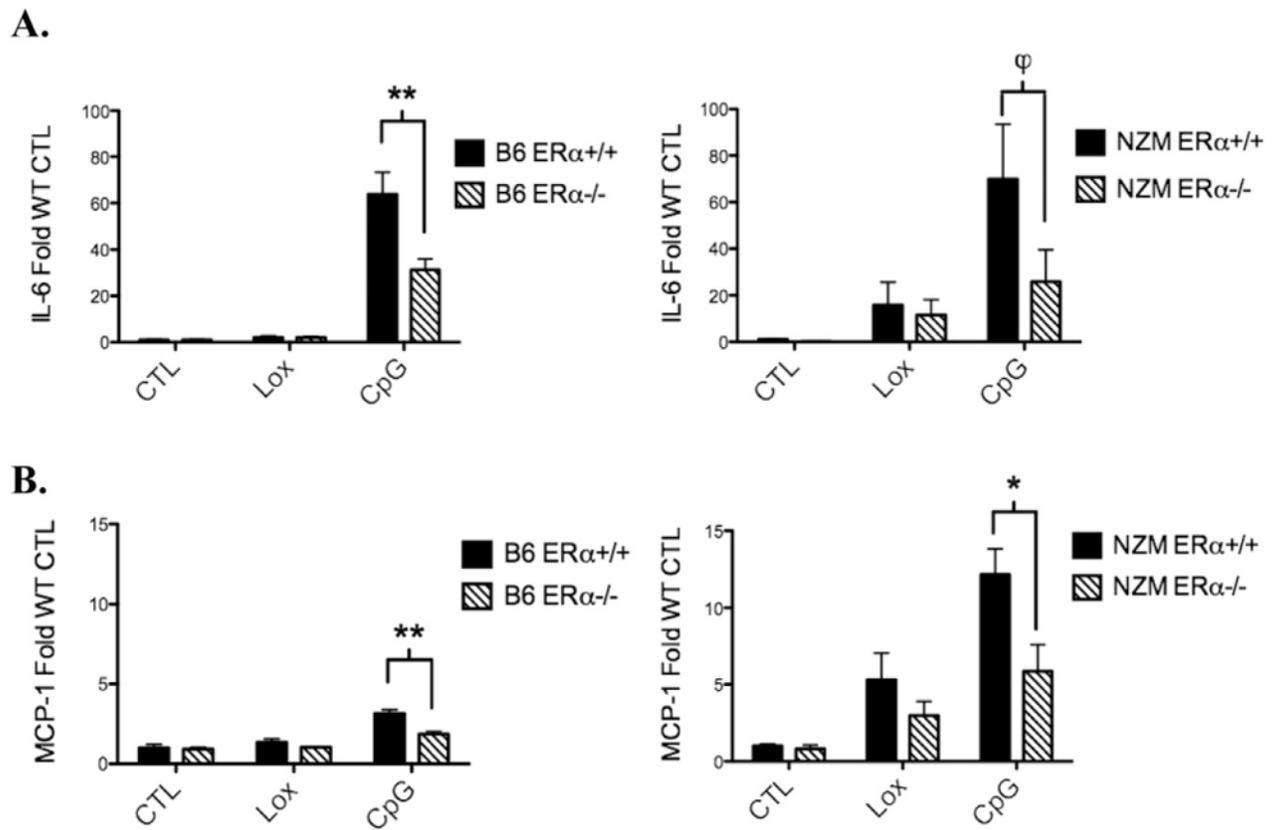


Figure 2.

TLR9-induced IL-6 and MCP-1 production by bone-marrow derived dendritic cells cultured from ERα^{-/-} mice is reduced in both B6 and NZM2410 strains. BMDCs from ERα^{+/+} and ERα^{-/-} mice were cultured for 24 h with vehicle (CTL), loxoribine (Lox) 50 nmol, or CpG 1 μg/ml under estradiol-free conditions. Media was removed and IL-6 (A) or MCP-1 (B) was measured by ELISA. Cytokine levels were in the range of 120 pg/ml–47 ng/ml. Pooled data from n=4 separate experiments with total number of 12 animals per group minimum. φ=p<.05, *=p<.01, **=p<0.0001.

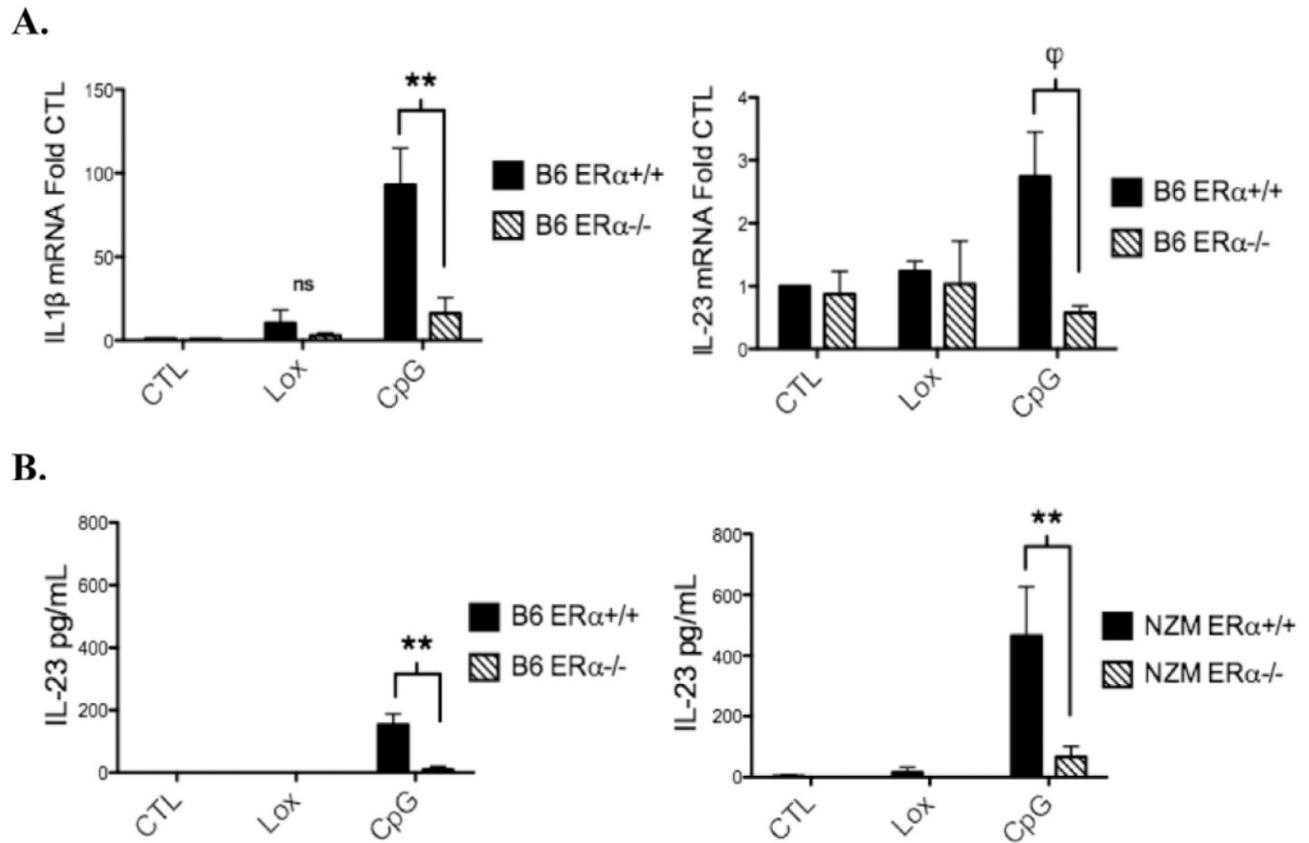


Figure 3.

TLR9-induced mRNA expression of the inflammatory cytokines IL-1 β and IL-23 by dendritic cells is reduced in B6 ER α ^{-/-} mice. BMDCs from ER α ^{+/+} and ER α ^{-/-} mice were cultured for 24 h with vehicle (CTL), loxoribine (Lox) 50 nmol, or CpG 1 μ g/ml under estradiol-free conditions. *A*, RNA was isolated and IL-1 β and IL-23 mRNA were measured by qRT-PCR. *B*, IL-23 protein levels were measured by ELISA (p19-specific capture antibody and a p40 specific detection antibody). Cytokine levels were in the range of 60 pg/ml–700 pg/ml. CpG-induced IL-23 production was reduced in ER α ^{-/-} animals in both B6 and NZM strains, n=3 or 4 separate experiments, **=p<0.001, ϕ =p<0.05.

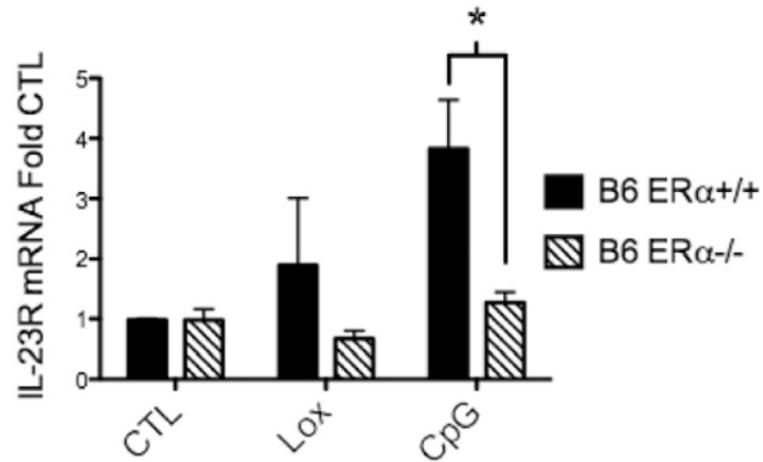


Figure 4.

TLR9-induced mRNA expression of IL-23R by dendritic cells was reduced in B6 ERα^{-/-} mice. BMDCs from ERα^{+/+} and ERα^{-/-} mice were cultured for 24 h with vehicle (CTL), loxoribine (Lox) 50 nmol, or CpG 1ug/ml under estradiol-free conditions. RNA was isolated from DCs and IL-23R was measured by qRT-PCR. A similar trend was observed in NZM and MRL/lpr mice that reached statistical significance in MRL DCs and spleen cells (data not shown), n=3 separate experiments, min. 5 animals per group, *=p<0.01.

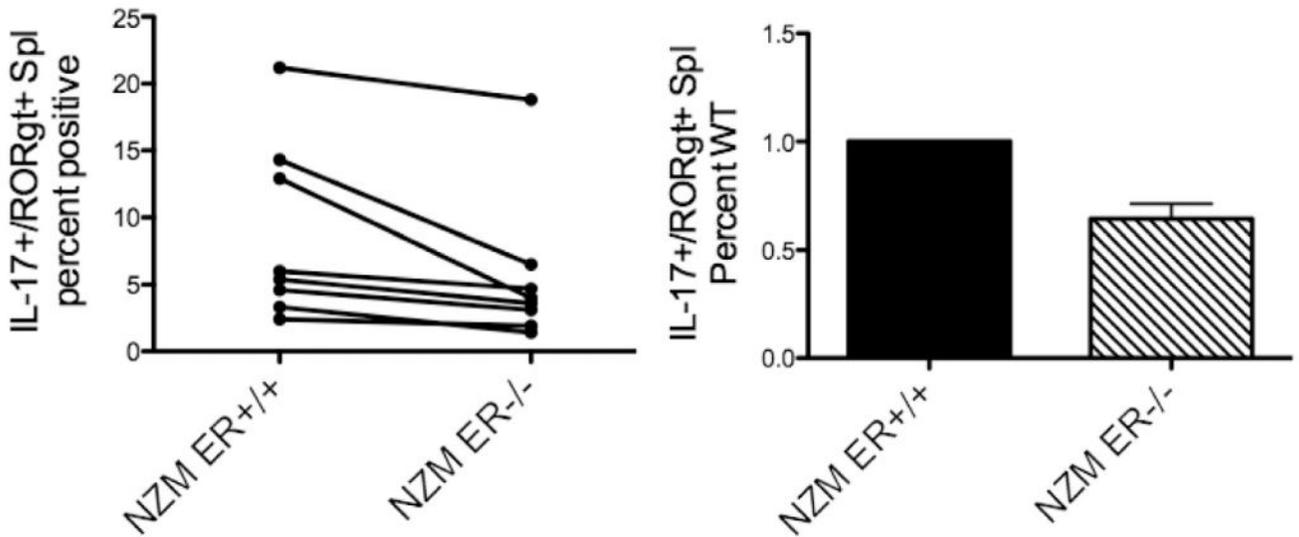
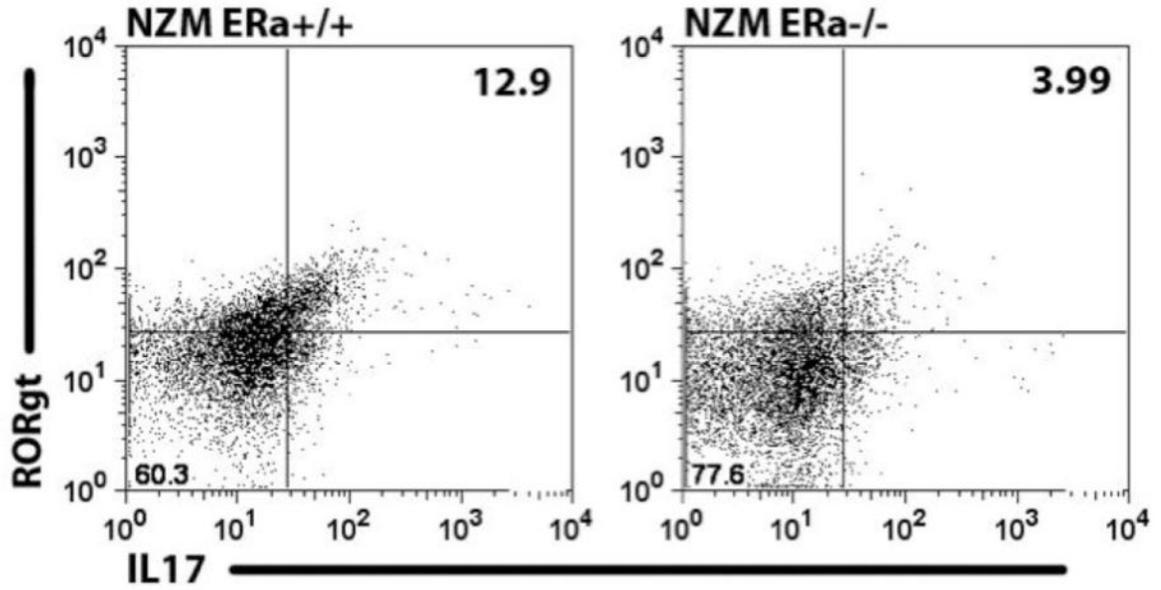


Figure 5. Unstimulated spleen cells derived from NZM ER $\alpha^{-/-}$ animals displayed reduced numbers of IL-17 $^{+}$ /ROR γ t $^{+}$ cells. Spleen cells were isolated from NZM ER $\alpha^{+/+}$ and NZM ER $\alpha^{-/-}$ mice and cultured for 24 h +/- GolgiStop. Cells were fixed, permeabilized and stained with IL-17-PE and ROR γ t-APC. Isotype controls: rat IgG2a for IL-17 and anti-human/anti-mouse IgG mix for ROR γ t were used to set gates. Results are compiled from 8 sets of animals, n=5 separate experiments. For the difference between WT and ER α KO, both a paired *T* test and a sign test indicate a significantly larger percent of IL17 $^{+}$ /ROR γ t $^{+}$ spleen cells in WT relative to KO animals (p=0.025 paired *t*-test, p=0.008 sign test).

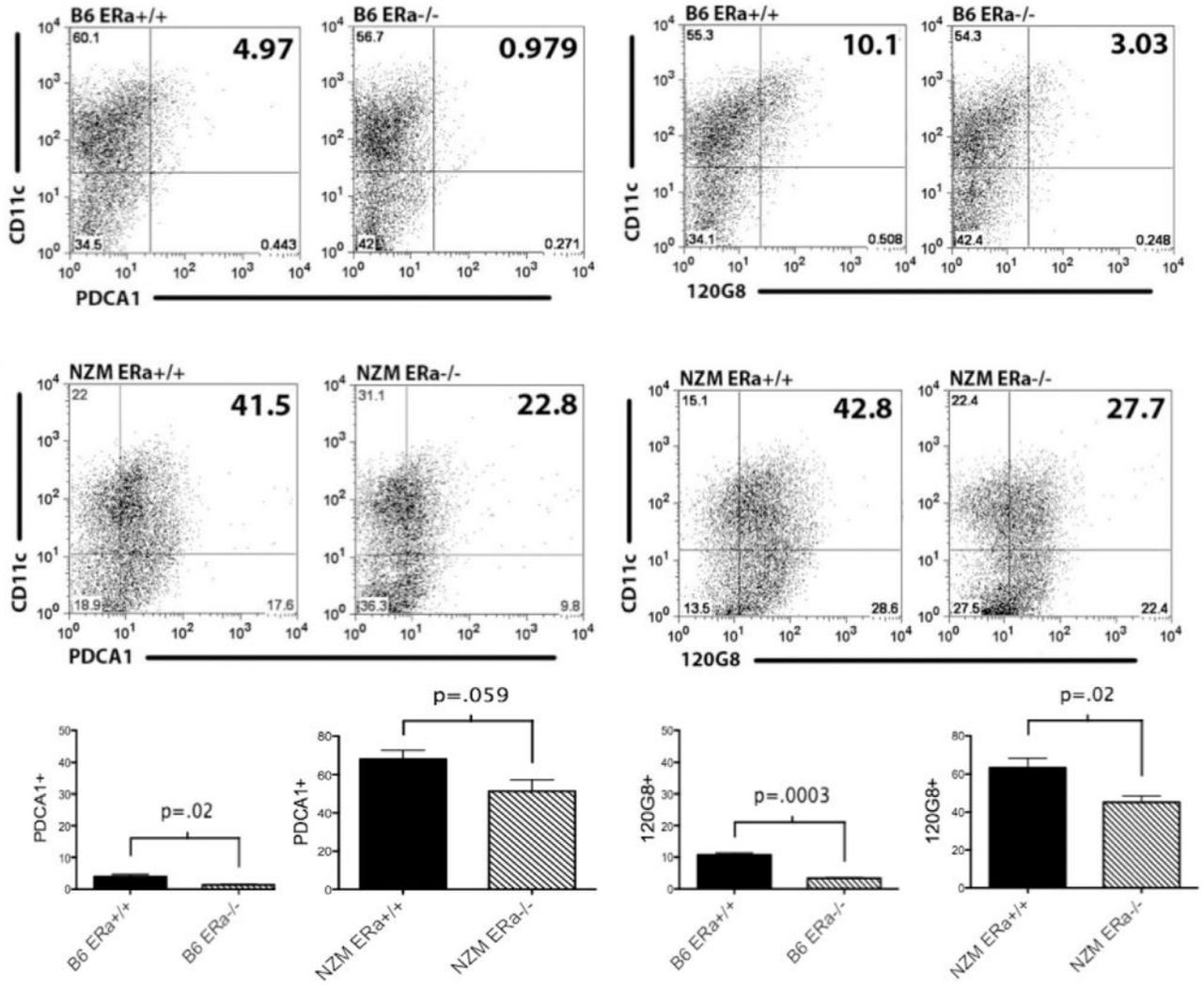
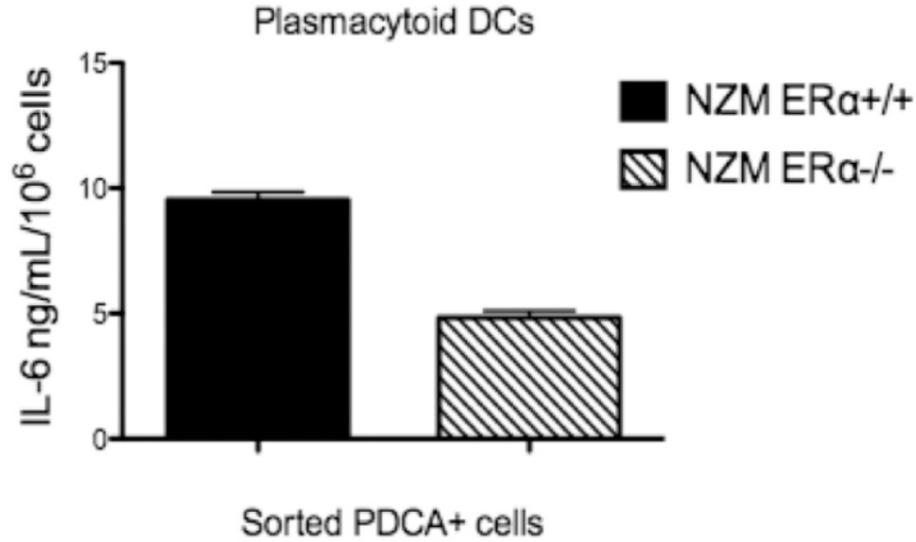


Figure 6. BMDCs from lupus-prone mice (middle panel) display increased plasmacytoid DC markers compared with wild-type B6 mice (top panel), despite culturing under traditional cDC conditions, using 2 different pDC-specific markers (PDCA or 120 G8). There is a clear trend toward reduced numbers of PDCA⁺ or 120 G8⁺ cells in ERα^{-/-} BMDCs which is statistically significant in B6 animals and in NZM mice staining with 120 G8. Flow cytometry plots represent 3 separate experiments, n=6 B6 and n=15 NZM animals (compiled in bottom panel).

A.



B.

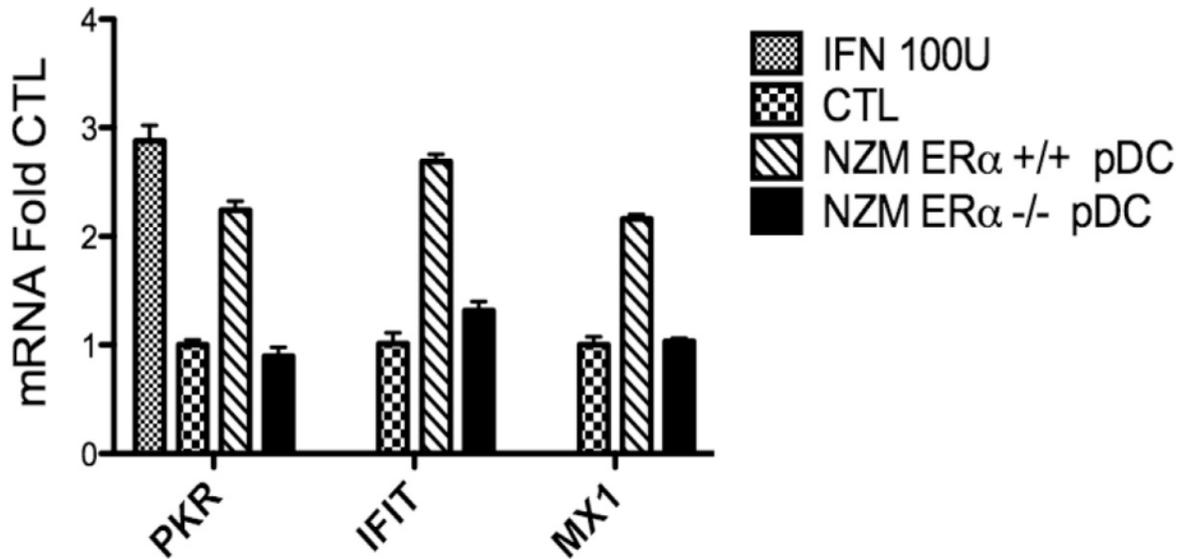
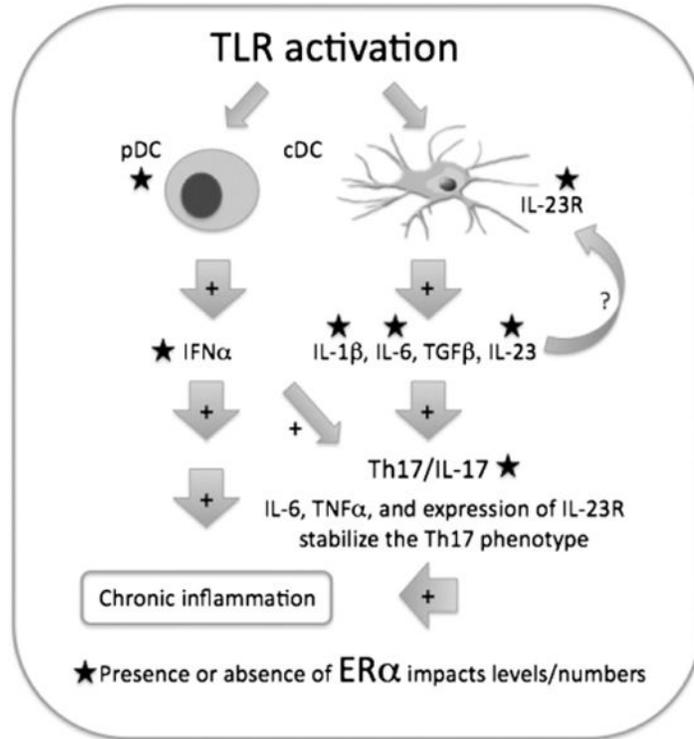


Figure 7.

IL-6 (A) and IFN α (B) production by plasmacytoid dendritic cells derived from ER α ^{-/-} lupus-prone mice is significantly reduced. The number of PDCA⁺ cells after cell-sorting was standardized to 1.0 \times 10⁶ cells/ml and cells were treated for 24 h with CpG 1 μ g/ml. A, IL-6 production was measured by ELISA and was significantly diminished in ER α ^{-/-} pDCs taken from NzM2410 mice, $p < .001$, representative of 2 independent experiments. B, IFN α activity was measured via WISH assay using conditioned media taken from CpG-treated pDCs. WISH cells were incubated with control media +/- Type I Interferon 100 units or with conditioned media from ER α ^{+/+} or ER α ^{-/-} pDCs for 6 h. qRT-PCR was done using IFN α -responsive gene primer sets. ER α ^{+/+} vs. ER α ^{-/-} are significantly different, $p = .003$, representative of 2 independent experiments.

**Figure 8.**

Working model for how ER α may modulate signaling by TLR-activated DCs. Activated DCs produce pro-inflammatory cytokines (ex. IL-23, IL-1 β , IFN α) that promote ongoing inflammation via multiple mechanisms. As demonstrated, the expansion and stabilization of the Th17 subset of T cells via IL-6 and IL-23 and the upregulation of IL23R are all impacted by the presence of ER α . In the absence of ER α , TLR stimulation cannot fully induce IL-6, IL-1 β , IL-23 or IL-23R upregulation on spleen cells (or DCs), leading to fewer IL-17-producing cells. Additionally, lupus-prone ER α ^{-/-} mice have decreased numbers of plasmacytoid DCs that feed into this pathway via production of IFN α .