Regulation by Estrogen and Progesterone of Interferon Alpha Signaling in Human Leukocytes

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Abstract

**Purpose of Study:** 90% of systemic lupus erythematosus (SLE) patients are female, and the incidence peaks during reproductive years when estrogen (Es) and progesterone (Pg) levels are at their highest. Studies show that Es increases the risk of SLE in both humans and animal models, while Pg may be protective. Interferon alpha (IFN-α) is a central pathogenic cytokine in SLE and can directly activate multiple immune cell types to favor autoimmunity. Though recent studies suggest a link between Es and IFN-α signaling in immune cells, the relationship is not well understood; and very little is known about the effects of Pg. To investigate the relationship between female reproductive hormones and lupus autoimmunity, we tested the hypothesis that, in human leukocytes, Es enhances IFN-α signaling while Pg suppresses it.

**Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors. PBMCs were cultured in medium alone or medium supplemented with vehicle, physiologic concentrations of β-estradiol (E2), Pg, or a combination of both hormones, with or without IFN-α. Total RNA was isolated, and expression of known IFN-α response genes (IRGs: CXCL10, MX1, PKR, IFIT1, ISG20) was measured by quantitative PCR and normalized to housekeeping gene 18sRNA.

**Results:** Consistent with our hypothesis, we observed that E2 can increase IFN-α induced expression of IRGs; however, we did not observe significant effects of Pg on IRG expression. E2 treatment significantly increased IFN-α induced expression of CXCL10 ($p=0.0273$, two-tailed paired T-test). Importantly, E2 alone failed to increase expression of any IRGs, including CXCL10, consistent with the idea that E2 was acting
on IFN-α signaling pathways to enhance CXCL10 expression. Finally, we observed that E2 effects on IFN-α induced CXCL10 expression were bi-modal, in that PBMCs from some donors consistently showed E2 sensitivity while others did not.

**Conclusions:** Our research shows that E2 can enhance IFN-α induced gene expression in human leukocytes from healthy donors, possibly by regulating IFN-α signaling pathways. This suggests a novel mechanism by which Es increases a woman’s risk of developing SLE. Additional experiments will allow us to determine whether Es regulates all or a subset of IFN-α response genes and what factors determine Es sensitivity, such as Es receptor (ER) expression. We plan to extend these findings to PBMCs of SLE patients to ask whether abnormal sensitivity or resistance to Es or Pg may contribute to disease.
Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the production of anti-nuclear autoantibodies and the formation of immune complexes (ICs). The deposition of ICs can cause tissue damage in a variety of organs, including kidneys, brain, joints, and skin, etc. 90% of all SLE patients are female, and disease incidence closely shadows the rise of systemic levels of estrogen (Es) and progesterone (Pg) with menarche, and their subsequent fall with menopause (18). Epidemiologic studies show that the use of Es-containing oral contraceptives by women is associated with significantly increased dose-dependent risk of developing SLE (2, 3). Limited evidence suggests that, in the same setting, Pg is protective against human lupus (19). In addition, women with SLE demonstrate normal to high serum levels of Es and deficient levels of Pg (5, 19). These observations are corroborated in animal models of SLE, where Es treatment accelerated development and progression of autoimmunity, while Pg treatment protected against the production of autoantibodies against nuclear antigens and the development of lupus nephritis (9, 10).

Interferon alpha (IFN-α) is the central pathogenic cytokine in SLE and is linked to disease development and progression in both human lupus and animal models of lupus autoimmunity. IFN-α, through its direct effects on leukocytes, plays an important role in immune activation, maturation and survival of dendritic cells (DCs), B cells, and T cells (20). In active SLE, ICs induce circulating plasmacytoid DCs to produce IFN-α. While the importance of IFN-α in disease activity is confirmed by recent successes with
specific IFN-α blockade in SLE patients, recent genetic studies in human and experimental lupus highlight the importance of IFN-α in disease development as well (8). Importantly, Es can enhance IFN-α production by pDCs (16), while Pg can inhibit it (11), together suggesting a common mechanism by which Es and Pg differentially regulate lupus autoimmunity. Moreover, whole-genome transcriptional analysis in T cells from SLE patients identified IFN-α signaling as one of several pathways selectively up-regulated by Es (22), suggesting that Es, in addition to increasing IFN-α production, also can enhance the effects of IFN-α on immune cells. Moreover, IFN-α induced the expression of estrogen receptors (ERs) in mouse cells, and Es enhanced IFN-α responses, suggesting the possibility of mutual enhancement between Es and IFN-α signaling (17). However, the specific effects of Es and Pg on IFN-α signaling in healthy human leukocytes have to our knowledge not been reported. Addressing this question will provide novel insight into potential mechanisms linking female sex, hormonal abnormalities and SLE pathogenesis. We hypothesize that, consistent with their effects on SLE disease development, Es will enhance IFN-α signaling, while Pg will suppress it, in human leukocytes.

Materials and Methods

Peripheral blood mononuclear cells (PBMC) isolation and cell culture

Heparinized peripheral blood was collected from both healthy male and female donors and diluted 1:1 with sterile PBS. PBMCs were isolated by density gradient centrifugation. Viable cells were counted by trypan blue exclusion under a light
microscope and cultured in human cell culture medium (RPMI supplemented with 1% Penicillin/Streptomycin, amino acids, sodium pyruvate, and 10% charcoal-stripped fetal bovine serum to minimize effects of bovine steroid hormones). Cells were cultured at 2x10^6 cells/ml in 48-well plates. To test the effects of hormones on IFN-α signaling in PBMCs, cells were co-cultured in medium alone or physiologic concentrations of β-estradiol (E2, 10^{-7} – 10^{-8} M), progesterone (Pg, 10^{-7} M), a combination of both hormones, or vehicle (ethanol), with or without IFN-α (50 - 100 IU/ml). Stock solutions of 10^{-3} M E2 and Pg, dissolved in ethanol, were diluted to desired concentrations with cell culture medium prior to use. An equal volume of ethanol diluted in medium was used as the vehicle control. IFN-α stock solution of 10,000 IU/ml was prepared from dissolving IFN-α in PBS with 0.1% BSA and was also diluted to final concentrations with medium. Cells were incubated at 37°C with 5% CO_2 for 20 hours.

RNA isolation and cDNA synthesis

Cells were collected by pipetting, transferred to 1.5ml centrifuge tubes, and pelleted by centrifugation for 10 minutes. RNA was isolated with Qiagen RNeasy Mini kit per manufacture’s protocol, and RNA samples were treated with DNase to minimize genomic DNA contamination. RNA concentrations were determined by optical density (260 nm) by mass spectroscopy. Total RNA was used as template for cDNA synthesis using OligoDT primers, according to Invitrogen Superscript RT III First-Strand Synthesis System protocol.
Quantitative real-time PCR

Expression of selected known interferon response genes (IRGs) was measured by qRT-PCR. Gene specific primers for CXCL10, MX1, PKR, IFIT1, ISG20, and housekeeping 18sRNA were used, and their sequences and final concentrations used are listed below. Relative expressions were measured using ABI SYBR Green system and normalized to constitutively expressed housekeeping gene, 18sRNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Final Concentration</th>
</tr>
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<tbody>
<tr>
<td>CXCL10</td>
<td>ATTTGCTGCCTTTATCTTTCTG</td>
<td>TCTCACCCCTTTTTTTCAACG</td>
<td>500nM</td>
</tr>
<tr>
<td>MX1</td>
<td>AGCCACTGGACTGACGACCTT</td>
<td>ACCACGGCTAACGGATAAG</td>
<td>500nM</td>
</tr>
<tr>
<td>PKR</td>
<td>CTTCCATCTGACTCAGGTTT</td>
<td>TGCTTCTGACGGTGATGATTA</td>
<td>1000nM</td>
</tr>
<tr>
<td>IFIT1</td>
<td>CTCCTTGGGTTGGATACAAATTG</td>
<td>AGTCACGACCCAGTCCTAG</td>
<td>1000nM</td>
</tr>
<tr>
<td>ISG20</td>
<td>GCGGCTACACAAATCTACGAC</td>
<td>AGGCTGTTTCTGGGATGCTCTT</td>
<td>400nM</td>
</tr>
<tr>
<td>18sRNA</td>
<td>CATTAATCAGTTATGCTTTTTTG</td>
<td>CCCGTGCGCATGTATTAGCT</td>
<td>100nM</td>
</tr>
</tbody>
</table>

Data analysis and Statistics

Relative expression of each IRG was calculated by normalizing its expression to that of 18sRNA, using the following formula: relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{18sRNA}$. Then, fold change in relative expression compared to medium control was calculated for each treatment group in each experiment. Means for fold change were compared with paired two-tailed T-tests using GraphPad Prism 5.0 software. A p-value of less than 0.05 was selected as a cutoff for statistical significance.
Results

IFN-α up-regulated the expression of selected IRGs in PBMCs from healthy donors in a dose dependent manner.

To verify induction of IRGs in our system, PBMCs from a single donor were stimulated with graded doses of IFN-α ranging from 50 IU/ml to 1000 IU/ml for 20 hours, and the relative expression of CXCL10, MX1, and PKR were measured by qRT-PCR. As expected, IFN-α up-regulated the expression of CXCL10, MX1, and PKR in a dose dependent manner (Fig. 1). PKR demonstrated a lower level of relative expression when compared to CXCL10 and MX1 at any given dose of IFN-α, and it reached maximum induction at a lower dose of IFN-α. The heterogeneity in dose-responses to IFN-α among IRGs presented a challenge in selecting an optimal dose of IFN-α to best assess the modulatory effects of hormone treatments, and subsequent experiments were performed with IFN-α dose in the steepest part of the CXCL10 response curve, at 50 IU/ml and 100 IU/ml.

E2 treatment significantly increased IFN-α-induced expression of CXCL10.

PBMCs from seven healthy donors were cultured with IFN-α at 50 IU/ml or 100 IU/ml in the presence of medium, vehicle, E2 (10^{-7} or 10^{-8} M), Pg (10^{-7} M), or combination of both hormones. Again, IFN-α treatment markedly increased the expression of IRGs at both 50 IU/ml and 100 IU/ml (data not shown). When compared to vehicle control treatment, E2-treated PBMCs showed significantly (p = 0.0273) higher IFN-α induced CXCL10 expression (Fig. 2). A similar trend in CXCL10 expression was seen in
PBMCs treated with both E2 and Pg, but this was not statistically significant. We did not observe marked hormone effects on IFN-α induced expression of the four other IRGs tested: MX1, IFIT1, PKR and ISG20. Importantly, hormone treatments alone, in the absence of IFN-α, did not significantly alter the expression of IRGs (data not shown).

**Donor-dependent enhancement of IFN-α induced CXCL10 expression by E2.**

There was considerable heterogeneity among donors in terms of E2 effects on IFN-α-induced CXCL10 expression; however, this did not appear to be random, but bi-modal, where some donors showed E2 sensitivity, while others did not (Fig. 3). The differences in E2 responses appeared to be independent of donor sex or the concentration of IFN-α and E2 used.

**Discussion**

Systemic lupus erythematosus (SLE) has a female predominance, and disease incidence increases with rise in estrogen (Es) and progesterone (Pg) during women’s reproductive years or with the use of Es containing oral contraceptives (2, 3, 18). Animal studies also suggest a link between Es and Pg and SLE development and progression (9,10). In addition, recent studies demonstrated a link between Es and IFN-α signaling in immune cells—a central pathogenic mechanism in SLE; however, the relationship is poorly understood, and very little is known about the effects of Pg. The connection between female reproductive hormones and lupus autoimmunity, while striking, remains poorly explained. Here, we sought to test the idea that Es and Pg,
female reproductive hormones with opposing effects on lupus development in humans and mice, have correspondingly opposite effects on IFN-α signaling in human leukocytes. Specifically, we tested the hypothesis that E2 will enhance IFN-α signaling in healthy human leukocytes, using IRG induction as surrogate measurements, while Pg will suppress it.

Our data show that E2 can enhance IFN-α-induced gene expression in human leukocytes from healthy donors, possibly by regulating IFN-α signaling pathways. This identifies a potential mechanism linking female reproduction and lupus autoimmunity. It is noteworthy that IFN-α-induced expression CXCL10, but not other ISGs tested, was enhanced by E2. CXCL10 is a chemokine that plays an important role in leukocyte activation and recruitment to sites of inflammation by binding to CXCR3. It is found at elevated levels in SLE, and high serum level of CXCL10 is strongly correlated with SLE disease activity (15). Thus, E2 may target IFN-α-induced CXCL10 to increase risk of SLE. However, it is possible that the induction of other IRGs may have been saturated at the doses of IFN-α used (Fig. 1), thus limiting the modulatory effects of hormones. It will be important in future experiments to test the effects of both E2 and Pg on IRG induction by a wide range of IFN-α concentrations. Similarly, inter-donor variability and small sample size may have precluded detecting small effects of either E2 or Pg on IRG induction. This could be addressed by using cell lines and PBMCs from more donors. Temporal response may also be important and should be explored with RNA collection at different time points.
E2 failed to induce \textit{CXCL10} expression (or that of any IRGs) in the absence of IFN-\(\alpha\), suggesting E2 is modulating IFN-\(\alpha\) signaling in leukocytes. In all nucleated cells, IFN-\(\alpha\) signals by binding IFN-\(\alpha\) receptors (IFNARs), triggering the phosphorylation and formation of STAT1/2 heterodimers, which complex with interferon regulatory factor 9 (IRF9), translocate to the nucleus and induce the transcription of IRGs (7). In reproductive tissues, E2 is known to enhance STAT1 activity by increasing tyrosine phosphorylation and DNA binding activity (14), while Pg can interfere with STAT1’s transcriptional activity (24). Whether this occurs in immune cells is not known but could be an important mode mediating hormonal influence on the development of lupus autoimmunity in susceptible women.

Though we observed considerable inter-donor heterogeneity in terms of E2 effects on \textit{CXCL10} induction, there appeared to be a bi-modal distribution, where some donors consistently showed E2 sensitivity, while others did not. E2 sensitivity in these donors appeared to be independent of donor sex, E2 or IFN-\(\alpha\) dose used, but may be related to the differential expression of estrogen receptors (ERs). There are two known forms of ERs expressed in human leukocytes, ER-\(\alpha\) and ER-\(\beta\), encoded by \textit{ESR1} and \textit{ESR2}, respectively (4). Ligand-bound ERs function as transcriptional factors but also can directly activate cytoplasmic signaling pathway via secondary messengers (4). It has been demonstrated that expression of an ER gene (\textit{ESR1}) is significantly increased in PBMCs from SLE patients compared to healthy controls (12). It is reasonable to speculate that high ER expression might underly increased sensitivity of IFN-\(\alpha\) signaling
to E2 (as suggested in lupus T cells), providing a molecular mechanism linking female reproduction and lupus autoimmunity.

In summary, our data show that E2 increases IFN-α-induced expression of *CXCL10*, but not other IRGs, in leukocytes from healthy individuals, perhaps through modulation of IFN-α signaling downstream of IFNAR. Thus, Es may increase a woman’s risk of SLE by enhancing IFN-α effects on immune cell functions. Interestingly, sensitivity to E2 effects did not appear to be universal, but was restricted to a subset of healthy donors that included both men and women. Causes for this, such as differential expression of ERs, and how this relates to abnormal E2-sensitivity in SLE T cells, remains to be explored.
References


Figure 1. PBMCs were isolated from a single healthy donor and stimulated with graded doses of IFN-α from 50 to 1000 IU/ml. IFN-α stimulation of PBMCs up-regulated the expression of CXCL10, MX1, and PKR in a dose dependent manner. PKR showed a lower level of relative expression compared to CXCL10 and MX1 at any given dose of IFN-α, and it reached a maximal response at a lower dose of IFN-α.
Figure 2. PBMCs were isolated from seven healthy donors, and cells were stimulated with medium, vehicle, E2, Pg, or both hormones, and with or without IFN-α for 20 hours. Data were pooled from multiple experiments using two doses of E2 (10^{-8} and 10^{-7} M) and IFN-α (50 and 100 IU/ml). E2 treatment significantly increased IFN-α-induced expression of CXCL10 (p=0.0273, two-tailed paired T-test). Hormone treatments alone did not significantly alter the expression of IFN-α response genes (data not shown).
Figure 3. Data from Figure 2 plotted with each line represent a single donor. E2 sensitivity of IFN-α induced CXCL10 expression was restricted to only a subset of donors, and the sensitivity appeared to be independent of sex or E2 and IFN-α concentration used.