Female and Male Sex Hormones Differentially Regulate Expression of *Ifi202*, an Interferon-Inducible Lupus Susceptibility Gene within the *Nba2* Interval

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Female and Male Sex Hormones Differentially Regulate Expression of *Ifi202*, an Interferon-Inducible Lupus Susceptibility Gene within the *Nba2* Interval

Ravichandran Panchanathan,* Hui Shen,* Melanie Gubbels Bupp,2† Karen A. Gould,‡ and Divaker Choubey3*

Increased expression of IFN-inducible *Ifi202* gene in certain strains of female mice is associated with susceptibility to systemic lupus erythematosus (SLE). Although, the development of SLE is known to have a strong sex bias, the molecular mechanisms remain unknown. Here we report that in vivo treatment of orchietomized (NZB × NZW)*F₁* male mice with the female sex hormone 17β-estradiol significantly increased steady-state levels of *Ifi202* mRNA in splenic cells, whereas treatment with the male hormone dihydrotestosterone decreased the levels. Moreover, increased expression of *Ifi202* in B6.*Nba2* B cells and reduced expression in T cells were associated with increased levels of estrogen receptor-α (ERα) and androgen receptor, respectively. Furthermore, the steady-state levels of *Ifi202* mRNA were higher in splenic cells from C57BL/6, B6.*Nba2*, NZB, and (NZB × NZW)*F₁* female mice as compared with males. 17β-estradiol treatment of B cells and WT276 cells increased *Ifi202* mRNA levels, whereas treatment with dihydrotestosterone decreased the levels. Interestingly, overexpression of ERα in WT276 cells increased the expression of *Ifi202* and stimulated the activity of the 202-luc-reporter through the c-Jun/AP-1 DNA-binding site. Accordingly, ERα preferentially associated with the regulatory region of the *Ifi202* gene in female B6.*Nba2* B cells than in males. Furthermore, *Ifi202* mRNA levels were detectable in splenic cells of wild-type (*Esr1*+/+), but not null (*Esr1−/−), (NZB × NZW)*F₁* female mice. Collectively, our observations demonstrate that the female and male sex hormones differentially regulate the expression of *Ifi202*, thus providing support for the role of *Ifi202* in sex bias in SLE.

Sex hormones, such as estrogen and androgen (2–4). It is well documented that immune reactivity is more enhanced in female SLE patients than in males, and lymphocytes and monocytes from female patients show higher Ag-presenting activity (2, 3). In general, female SLE patients exhibit higher levels of serum IgG than do males and mount more robust humoral immune response. Therefore, it seems likely that enhanced activation of B cells in females contributes to lupus susceptibility. Moreover, female hormone estrogen is known to have immunostimulatory effects, whereas male hormone androgen is known to have immunosuppressive effects (2–4).

As for SLE patients, in a (NZB × NZW)*F₁* spontaneous mouse model of SLE disease, female mice develop the disease earlier and have shorter lifespans than do males (13, 14). Moreover, castrated male (NZB × NZW)*F₁* mice have earlier onset of lupus and shorter lifespan than do their intact littermates (14). Additionally, treatment of these mice with estrogen exacerbates disease activity and causes early mortality (13, 14). In contrast, administration of exogenous testosterone, when begun between at 2 and 6 mo of age, extends the lifespan of ovarietomized (NZB × NZW)*F₁* females (13, 14). These observations suggest that sex hormones, such as estrogen and testosterone, influence the pathogenesis of murine lupus.

Sex hormone estrogen classically functions by activating one of its two nuclear receptors, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) (15–17). Although both estrogen receptors are expressed in most immune cells, ERα is shown to be predominantly expressed (17). Several recent studies involving various mouse models of SLE have suggested a prominent role for ERα in the development of lupus disease (18–20). Interestingly, the ERα deficiency in (NZB × NZW)*F₁* female mice attenuated glomerulonephritis and increased survival of mice (20). Of note, the increased survival of ERα-deficient female mice was associated with reduced development of anti-chromatin and anti-dsDNA Abs as well as reduced serum levels of IFN-γ (20).

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*Abbreviations used in this paper: SLE, systemic lupus erythematosus; AR, androgen receptor; Chip, chromatin immunoprecipitation; DHT, dihydrotestosterone; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen responsive core element.*

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Binding of 17β-estradiol (E2) to ERs results in activation of ERs and transcriptional activation of ER target genes (15–17). Many ER target genes contain a minimal estrogen responsive core element (ERE) sequence (GGTCANNNTGACC) in the 5′ regulatory or promoter region. The ERE sequence functions in an orientation- and distance-independent manner, both of which are characteristics of an enhancer (21). Moreover, ER is also known to bind DNA through half ERE sites (GGTCAN) (15, 21). Because molecular mechanisms of the recruitment of ER to the promoter region of its target genes remain relatively complex, it remains an actively investigated research area. Importantly, proteins that are encoded by the ER target genes mediate many of the biological activities of female sex hormone estrogen (15–17).

Male sex hormone androgen signals via the intracellular androgen receptor (AR), a member of the superfamily of nuclear hormone receptors (22). Androgen-dependent activation and nuclear translocation of the AR is followed by its binding to specific response elements in the promoter regions of target genes to modulate gene expression either positively or negatively (22). Interestingly, expression of AR mRNA has been reported in enriched populations of CD4+ T lymphocytes, CD8+ T lymphocytes, and macrophages (23). However, the enriched populations of B lymphocytes expressed only low levels of AR mRNA (23).

The IFI202a gene family includes structurally and functionally related murine (E.G., Ifi202a, Ifi202b, Ifi203, Ifi204, Ifi205, And AminH) and human (e.g., IFIH1, MDA5, AIM2, and IFIX) genes (24–30). The Ifi202a and Ifi202b are highly homologous murine genes that encode p202a and p202b proteins, respectively (26, 27). These two proteins differ only in 7 amino acids (out of 445 amino acids) (27). Because Abs, which have been raised against the p202a protein (31), also detect p202b protein (26, 27), in this study, we have referred both p202a and p202b proteins as p202 protein.

Generation of B6.Nba2 congenic (congenic for NZB-derived Nba2 interval on C57BL/6 genetic background) mice and gene expression analyses identified Ifi202 (probably both Ifi202a and Ifi202b genes) as a lupus susceptibility gene (26, 32). Importantly, consistent with promoter polymorphisms contributing to differential expression of Ifi202a gene between C57BL/6 and NZB mice (26, 32, 33), increased steady-state levels of Ifi202a and Ifi202b mRNAs (as compared with C57BL/6 mice) are detected in splenic cells from NZB and B6.Nba2 mice (33). Moreover, levels of Ifi202a mRNA are relatively higher than the Ifi202b mRNA (33). Interestingly, increased expression of p202 protein (probably both p202a and p202b proteins) in B6.Nba2 splenic B and T cells (more in B cells than in T cells) is associated with defects in apoptosis of B cells and increased susceptibility to develop lupus-like disease (26, 32). Furthermore, the B6.Nba2 congenic female mice produce higher levels of antibacterial autoantibodies than did the age-matched male mice, and (B6.Nba2 × NZWF1)F1 female mice develop severe proteinuria with much higher frequency (34). These observations prompted us to investigate whether sex hormones could regulate expression of the Ifi202 gene. Here, we report that female and male sex hormones differentially regulate the expression of Ifi202.

Materials and Methods

Mice, orchietomy, and sex hormone treatment

Spleens were isolated from wild-type (Esr1+/-) or null (Esr1-/-) (NZB × NZWF1, female mice (20) (age –10 wk) that were housed in animal facilities of University of Nebraska Medical Center (Omaha, NE). Age-matched (6–8 wk old) male and female nonautoimmune (C57BL/6j) and preautoimmune (B6.Nba2, NZB, and (NZB × NZWF1)F1 mice were purchased from The Jackson Laboratory and housed in the animal facilities of the University of Cincinnati. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee at the institution where animals were housed.

Male (NZB × NZWF1)F1 mice were orchietomized at 3 mo of age as described (35). After orchietomy, pellets (Innovative Research of America) releasing E2, dihydrotestosterone (DHT), or placebo for up to 3 wk were inserted s.c. with a 10-gauge needle. Serum was collected on day 7, 8, or 9 and then analyzed by the Endocrine Laboratory at Colorado State University (Fort Collins, CO) for luteinizing hormone, estradiol, and testosterone by radioimmunoassay as described (35). Orchietomized male mice that were treated with E2 pellets exhibited serum E2 levels of 162 pg/ml, while levels in placebo-treated mice were not detectable (data not shown). Intact and orchietomized mice treated with DHT had similarly low luteinizing hormone levels (<1 ng/ml), while orchietomized mice treated with placebo had >20 ng/ml luteinizing hormones (data not shown). Orchietomized male mice treated with placebo demonstrated testosterone levels <2 ng/ml, while intact (NZB × NZWF1)F1 male mice exhibited 12 ng/ml testosterone (35).

Splenocyte isolation, purification of B or T cells, cell culture, and treatments

Total single-cell splenocytes were prepared from age-matched male or female mice as described previously (36). After lysis of RBC, splenocytes were resuspended in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin/glutamatan, and 1% MEM, nonessential amino acids/sodium pyruvate. Unless otherwise indicated, splenic cells from two or more age-matched male or female mice were pooled to purify B or T cells and to prepare total RNA or protein extracts. B or T cells were purified from splenic cells using magnetic beads (purification kit purchased from Miltenyi Biotec), allowing the positive selection of either B or T cells. The purified (>90–95% pure) T and B cells were used immediately for additional experiments.

Estrogen-responsive mouse breast cancer cell line WT276 (37) was provided by Dr. J. Welsh (University of Notre Dame, Notre Dame, IN). Cells were maintained in DMEM medium supplemented with 10% FBS and 1 × antibiotic-antimycotic solution (Invitrogen). For treatment of WT276 cells with E2 or DHT, cells were cultured in phenol red-free RPMI 1640 medium (Invitrogen) and the medium was supplemented with 10% charcoal-stripped FBS (Invitrogen). Cells were treated in vitro with either DHT (23) or E2 (38) at the concentration used previously.

Plasmids and expression vectors

Dr. P. Chambon (Centre National de la Recherche Scientifique, France) provided ERα expression plasmid that allowed expression of ERα (39). The 202-luc-reporter (36) and the mutant 202AP-1mutCS1-luc reporter (40) plasmids have been described.

Reporter assays

For reporter assays, subconfluent cultures of WT276 cells (in 6-well plates) were transfected with the 202-luc (2.5 µg) or the 202AP-1mutCS1-luc (with deleted AP-1CS1 site, Ref. 40) reporter plasmid along with pGL3-TK reporter plasmid (0.5 µg), using calcium phosphate transfection kit (Invitrogen), as suggested by the supplier. When indicated, cells were either treated with ethanol (vehicle) or the indicated concentration of E2 or DHT for 16–24 h. Unless otherwise indicated, cells were harvested between 40 and 45 h after transfections. Cells were lysed, and the firefly and Renilla dual luciferase activities were determined as described previously (36). Student’s t test for paired samples was used to determine statistical significance of the reporter activity data. Differences were considered statistically significant at p ≤ 0.05.

Isolation of RNA from splenocytes and RT-PCR

Splenocytes (5–8 × 10^6 cells) were used to isolate total RNA using TRIzol (Invitrogen). Total RNA was digested with DNase I (to remove any contaminating genomic DNA in the preparation), and 0.5–2 µg of RNA was used for RT-PCR reaction using a pair of the ggtcatctaccaactcagaat-3′; reverse, 5′-ctctagagt caacctggtt-3′. For RT-PCR, we used the SuperScript One-Step RT-PCR system from Invitrogen. Primers for Ersl gene (forward, 5′-aatttgacgacgagccagc-3′; reverse, 5′-ctctagagtcaacctggtt-3′). Quantitative real-time TaqMan PCR technology (7300 Real-Time PCR System; Applied Biosystems) and commercially available real-time TaqMan gene expression assays were used to compare expression of genes between male and female mice. The PCR cycling program consisted of denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 s, followed by annealing and elongation at 60°C for 1 min. The TaqMan assays for Ifi202s
statistically significant. These methods were performed using GraphPad

mice contribute to decreased steady-state levels of

lysates containing equal amounts of protein were immunoprecipitated with

Ifi202a/H9252

chased from Santa Cruz Biotechnology. Abs to detect

ectomized (NZB

NZW)F1

control mice. These observations are consistent with the possibility

levels to a measurable extent in most mice as compared with the

mRNA by real-time PCR. As shown in Fig. 1, treatment of male

orchiectomized and reconstituted with slow-releasing pellets re-

were purchased from Cell Signaling Technology.

Total splenocytes, WT276, or NIH 3T3 cells were collected in PBS and

resuspended in a modified radioimmunoprecipitation assay (RIPA) lysis

buffer (50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 1% Nonident P-40, 0.5%

sodium deoxycholate, 0.1% SDS), supplemented with 1× protease

inhibitor (Roche Diagnostics), and incubated at 4°C for 30 min. Cell

lysates were sonicated briefly before centrifugation at 14,000 rpm in a micro-

centrifuge for 10 min at 4°C. The supernatants were collected, and the protein

concentration was measured by Bio-Rad protein assay kit. Equal amounts

of protein were processed for immunoblotting. Antiserum to p202 protein

concentration was measured by Bio-Rad protein assay kit. Equal amounts

of protein were processed for immunoblotting. Antiserum to p202 protein

has been described previously (31). The p202 antisera detects both p202a

and p202b proteins in immunoblotting (27, 31). Abs to detect mouse ERα

(sc-542; MC-20), AR (sc-816), and

β₂-microglobulin (sc-13565) were pur-

chased from Santa Cruz Biotechnology. Abs to detect β-actin (no. 4967)

were purchased from Cell Signaling Technology.

Increased expression of Ifi202 in splenic B cells in female mice is associated with increased levels of ERα and reduced levels of AR

Our earlier studies had revealed that splenic B cells from preautoim

mune (4-mo-old) B6.Nba2 female mice express higher levels of

Ifi202 mRNA as compared with T cells (32). Therefore, our

above observations (Fig. 1) that in vivo treatment of orchiecto-

mized (NZB × NZW)F1, male mice with estrogen increased

Ifi202 mRNA levels, whereas treatment with DHT reduced the

mRNA levels, prompted us to compare steady-state levels of Ifi202

mRNA in splenic B and T cells from male and female preautoim-

mune (age ~10 wk) B6.Nba2 mice (32). We noted that steady-

state levels of Ifi202 mRNA were significantly (~7-fold) higher in

splenic B cells than in T cells in the female mice (Fig. 2A). Inter-

estingly, we found that levels of Ifi202 mRNA were consistently

higher in B and T cells from female mice than the age-matched

male mice (Fig. 2A). Because sex hormones, such as E2 and DHT,

regulate gene expression through binding to their respective rece-

ptors (15, 22), we also compared expression levels of ERα, AR,

and p202 proteins in B and T cells from male and female B6.Nba2

mice. As shown in Fig. 2B, increased levels of p202 protein in

splenic B cells from B6.Nba2 female mice as compared with age-

matched male mice (compare lane 4 with lane 2) were associated

with increased levels of ERα protein and reduced levels of AR

protein. In contrast, reduced levels of p202 protein in T cells from

male mice as compared with age-matched female mice (compare

lane 1 with lane 2) were associated with increased levels of AR

and reduced levels of ERα protein. Furthermore, consistent with

our above observations (Fig. 2, A and B), basal steady-state levels

of Ifi202 mRNA were relatively high in B cells from C57BL/6 or

(NZB × NZW)F1, female mice than from the age-matched male

mice, and treatment of B cells with estrogen (10 nM for 24 h)

resulted in further increases in the steady-state levels of the mRNA

(Fig. 2C). Taken together, these observations revealed that in-

creased expression of the Ifi202 in splenic B cells from B6.Nba2 female mice is associated with increased levels of ERα and re-

duced levels of AR. Additionally, our observations revealed that the basal steady-state levels of the Ifi202 mRNA are relatively

higher in B cells from nonautoimmune (C57BL/6) and preautoim-

mune (B6.Nba2 and (NZB × NZW)F1) female mice than from age-

matched males, and the treatment with E2 can increase the

mRNA levels further.

Increased expression of Ifi202 in splenic B cells from female mice is associated with increased levels of ERα and reduced levels of AR

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Sex-dependent regulation of *Ifi202* expression

Our earlier studies had revealed that increased expression of *Ifi202* in preautoimmune B6.Nba2 female mice (as compared with nonautoimmune C57BL/6 mice) is associated with increased lupus susceptibility (26, 32). Because steady-state levels of *Ifi202* mRNA are very low (as compared with age-matched B6.Nba2 mice), but detectable in C57BL/6 mice (33), we decided to compare the steady-state levels of *Ifi202* mRNA between male and female C57BL/6 mice. We noted that basal levels of *Ifi202* mRNA were detectable in splenic cells from C57BL/6 male and female mice (age ~10 wk), and IFN treatment of cells increased the levels further (Fig. 3A). Interestingly, the basal levels of *Ifi202* mRNA were ~5-fold higher in C57BL/6 females than age-matched males. This observation indicated that steady-state levels of *Ifi202* mRNA are regulated in sex-dependent manner in C57BL/6 splenic cells.

Encouraged by the above observations, we also compared the steady-state levels of *Ifi202* mRNA between young male and female lupus-prone mice (B6.Nba2, NZB, and [NZB×NZWF1]), as shown in Fig. 3B. Basal steady-state levels of the *Ifi202* mRNA were consistently higher in splenic cells from the females than from the age-matched males. Taken together, these observations suggested that the steady-state levels of *Ifi202* mRNA in splenic cells are regulated in a sex-dependent manner.

*FIGURE 3.* Sex-dependent regulation of *Ifi202* expression. A, Splenic cells were prepared from age-matched C57BL/6J male or female mice (age ~10 wk; cells pooled from two age-matched male or female mice) and cells were either left untreated or treated with IFN-γ (1000 U/ml, for 14 h). Total RNA was isolated from control and IFN-treated cells, and steady-state levels of *Ifi202* mRNA were analyzed by quantitative TaqMan real-time PCR. The ratio of *Ifi202* mRNA to β2-microglobulin mRNA was calculated in units, with 1 unit being the ratio of *Ifi202* mRNA to β2-microglobulin mRNA in C57BL/6J splenocytes. The relative levels of *Ifi202* mRNA in the male mice are indicated as Fig. 1. A representative experiment is shown. Results are mean values of triplicate experiments, and error bars represent SD (*, p < 0.005; **, p < 0.0005), B, Splenic cells were prepared from age-matched nonautoimmune (C57BL/6J) or preautoimmune (B6Nba2, NZB, and [NZB×NZWF1]) male or female mice (age ~10 wk; cells pooled from two age-matched male or female mice). Total RNA was isolated and steady-state levels of the *Ifi202* mRNA were analyzed by quantitative TaqMan real-time PCR. The ratio of *Ifi202* mRNA to β2-microglobulin mRNA was calculated in units, with 1 unit being the ratio of *Ifi202* mRNA to β2-microglobulin mRNA in C57BL/6J. A representative experiment is shown. Results are mean values of triplicate experiments, and error bars represent SD (*, p < 0.005; **, p < 0.0005).

Treatment of WT276 cells with female or male sex hormone regulates *Ifi202* expression

The mouse mammary tumor cell line WT276 has been reported to be estrogen-responsive (37). Therefore, to identify molecular mechanisms by which E2 treatment up-regulates expression of *Ifi202* gene, we explored whether treatment of WT276 cells with sex hormones could regulate the expression of *Ifi202* gene. For this purpose, we treated cells with increasing concentrations (1, 5, or 10 nM) of female sex hormone E2 (these concentrations were chosen based on an earlier studies; see Ref. 38) for 16 h. As shown in Fig. 4A, the treatment with E2 resulted in an increase in steady-state levels of *Ifi202* mRNA as determined by semiquantitative RT-PCR. Moreover, consistent with E2-mediated up-regulation of *Ifi202* expression by EREα, treatment of cells with tamoxifen (100 nM), a selective estrogen receptor modulator (42), which resulted in increases in EREα mRNA and protein levels (data not shown), abrogated the E2-mediated increases in *Ifi202* mRNA levels (data not shown). Consistent with the above observations, we also noted increases in p202 protein levels after treatment of cells with increasing concentrations of E2 (Fig. 4B). Interestingly,

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**FIGURE 2.** Increased steady-state levels of *Ifi202* mRNA and protein in splenic B cells from B6.Nba2 female mice are associated with increased levels of ERα and reduced levels of AR. Total splenocytes isolated from preautoimmune (age ~9 wk) male or female mice (spleen cells pooled from three age-matched male or female mice) were subjected to purification of B or T cells using a kit (from Miltenyi Biotec) that allowed positive selection of either B or T cells. Total RNA and proteins were prepared from the purified (cells ~90% pure) T or B cells. A, Steady-state levels of *Ifi202* mRNA were analyzed by quantitative TaqMan real-time PCR. The ratio of *Ifi202* mRNA to β2-microglobulin mRNA was calculated in units (one unit being the ratio of *Ifi202* mRNA to β2-microglobulin mRNA in B6.Nba2 splenocytes). The relative levels of *Ifi202* mRNA in B cells from C57BL/6 male mice are indicated as in Fig. 1. A representative experiment is shown. Results are mean values of triplicate experiments and error bars represent SD (**, p < 0.005; ***, p < 0.0005). B, Total cell extracts prepared from T (lanes 1 and 2) or B (lanes 3 and 4) cells were analyzed by immunoblotting using Abs specific to the indicated proteins. A representative experiment is shown. M, male; F, female. The numbers below the figure indicate relative fold change (FC) in the p202 protein levels as compared with levels detected in T cells (indicated as 1.0) from male mice. C, Steady-state levels of *Ifi202* mRNA were analyzed in splenic B cells (isolated from male or female mice) without any or after E2 (10 nM, 24 h) treatment by quantitative TaqMan real-time PCR. The ratio of *Ifi202* mRNA to β2-microglobulin mRNA was calculated in units, with 1 unit being the ratio of *Ifi202* mRNA to β2-microglobulin mRNA. The relative levels of *Ifi202* mRNA in B cells from C57BL/6 male mice are indicated as in Fig. 1. A representative experiment is shown. Results are mean values of triplicate experiments and error bars represent SD (**, p < 0.03; ***, p < 0.005).
Ifi202 cells was analyzed by semiquantitative PCR for steady-state levels of 1, 5, or 10 nM) for 16 h in phenol red-free medium. Total RNA isolated from cells was analyzed by semiquantitative PCR for steady-state levels of 1, 5, or 10 nM) for 16 h in phenol red-free medium. Total RNA isolated from treated with ethanol (vehicle) or increasing concentration of estrogen (1, 5, or 10 nM). Forty to 45 h after transfections, cells were either treated with ethanol (vehicle) or E2 (5 or 10 nM). Forty to 45 h after transfections, cells were processed for dual luciferase activity. B. Subconfluent cultures of WT276 cells in a 6-well plate were transfected with 202-luc-reporter plasmid (2.5 µg), pRL-TK (0.5 µg) plasmid along with either empty vector, or a plasmid encoding ERα using calcium phosphate precipitation method. Twenty-four hours after transfections, cells were either treated with ethanol (vehicle) or E2 (5 or 10 nM). Forty to 45 h after transfections, cells were processed for dual luciferase activity. C. Subconfluent cultures of NIH 3T3 cells in a 60-mm plate were transfected either empty vector or a plasmid encoding ERα using calcium phosphate precipitation method. Twenty-four hours after transfections, cells were processed for immunoblotting using Abs specific to the indicated proteins. We also included a positive control for p202 protein in our experiment (lane 1). The numbers below the figure indicate the fold difference (FD) in p202 protein levels as compared with control (lane 2).

FIGURE 4. Treatment of WT276 cells with sex hormones regulates the Ifi202 expression. A. Subconfluent cultures of WT276 cells were either treated with ethanol (vehicle) or increasing concentration of estrogen (1, 5, or 10 nM) for 16 h in phenol red-free medium. Total RNA isolated from cells was analyzed by semiquantitative PCR for steady-state levels of Ifi202 or actin mRNA levels. B. Subconfluent cultures of WT276 cells were treated with ethanol (vehicle; lane 2), 5 (lane 3), or 10 nM (lane 4) concentration of E2, or 5 (lane 5) or 10 nM (lane 6) concentration of DHT for 16 h in phenol red-free medium. Total cell lysates from treated and control cells were analyzed by immunoblotting using Abs specific to the indicated proteins. We also included a positive control for p202 protein in our experiment (lane 1). The numbers below the figure indicate the fold difference (FD) in p202 protein levels as compared with control (lane 2).

treatment of cells with 5 nM concentration of E2 resulted in increases in p202 protein levels (compare lane 3 with lane 2). However, treatment of cells with 10 nM E2 resulted in moderate decreases in p202 protein levels (compare lane 4 with lane 3). Furthermore, treatment of WT276 cells with the male sex hormone DHT decreased basal levels of p202 protein in a dose-dependent manner. Taken together, the above observations demonstrated that in vitro treatment of WT276 cells with female sex hormone E2 or male sex hormone DHT differentially regulated the levels of the p202 protein.

Extrogen through ERα up-regulates expression of the Ifi202 gene

To further investigate whether estrogen treatment of cells activates transcription of the Ifi202 gene through ERα, we first transfected estrogen-responsive WT276 cells with 202-luc-reporter plasmid and treated cells with increasing concentrations of E2. As shown in Fig. 5A, treatment of cells with increasing concentrations of E2 stimulated the activity of the 202-luc-reporter in a concentration-dependent manner. Next, we transfected WT276 cells with 202-luc-reporter along with either an empty vector or a plasmid encoding the ERα receptor. After transfections, cells were either treated with ethanol alone (vehicle) or 10 nM E2. As shown in Fig. 5B, transfection of cells with the plasmid encoding ERα protein stimulated the activity of the 202-luc-reporter. Interestingly, transfection of cells with the plasmid encoding the ERα protein and subsequent treatment of the transfected cells with E2 strongly stimulated the activity of the reporter. To further test whether ERα regulates expression of the Ifi202 gene, we transfected NIH 3T3 mouse fibroblasts (we chose these cells because basal levels of p202 protein are detectable and these cells are not known to express ERα) with either an empty vector or the plasmid encoding ERα (cells treated with 5 nM E2 in phenol red-free medium) and analyzed the expression of p202 protein. As shown in Fig. 5C, ectopic expression of ERα protein in NIH 3T3 cells resulted in increases in p202 protein levels. Taken together, these observations suggested that activation of ERα by E2 in WT276 and NIH 3T3 cells up-regulates the expression of the Ifi202 gene.

Transcriptional activation of Ifi202 by ERα

The 5′-regulatory region (~800 bp) of Ifi202 gene contains at least two potential ERE half-sites (Fig. 6A). Moreover, one of the potential ERE half-sites is located next to an AP-1 DNA-binding site (AP-1CS1) that can bind to c-Jun/AP-1 in gel-mobility shift assays (40). Molecular mechanisms through which the ERα regulates the transcription of its target genes through the half ERE sites are relatively complex (15, 16) and known to involve collaborations with other transcription factors, such as c-Jun/AP-1 (15, 16). Therefore, to investigate the role of ERα in the regulation of Ifi202 expression, we compared the activity of the wild-type (202-luc) and the mutant (202-AP-1CS1-luc, in which the AP-1CS1 site is mutated) reporters without or after E2 treatment of WT276 cells. As shown in Fig. 6B, the activity of the wild-type reporter was stimulated ~2.5-fold by the treatment of cells with E2. However, the mutation in the AP-1CS1 site in the 5′ regulatory region of the Ifi202 gene abrogated the stimulation of the activity of the reporter after E2 treatment. This suggested that E2 treatment of WT276 cells stimulates transcription of the Ifi202 gene through the AP-1CS1 site. To further examine the role of ERα in the transcriptional activation of the Ifi202 gene by E2, we also compared in vivo association of ERα with the 5′ regulatory region of the Ifi202 gene in splenic B6.Nbal B cells between female and age-matched males by ChIP assays. As shown in Fig. 6C, some binding of ERα to Ifi202 regulatory region was detected in male B cells (lane 5). Interestingly, relatively more ERα bound to the Ifi202 regulatory region in female B cells (compare lane 6 with lane 5). Moreover, a quantitative real-time pPCR revealed (Fig. 6D) that there was ~4-fold more binding of ERα to the Ifi202 regulatory region in the female B cells than in males. These observations indicated that relatively higher levels of ERα associated with the regulatory region of the Ifi202 gene in female B6.Nbal B cells than in males.

FIGURE 5. Treatment of estrogen-responsive WT276 cells with E2 or overexpression of ERα stimulated the activity of 202-luc-reporter. A. Subconfluent cultures of WT276 cells in a 6-well plate were transfected with 202-luc-reporter plasmid (2.5 µg) along with pRL-TK (0.5 µg) plasmid using calcium phosphate precipitation method. Twenty-four hours after transfections, cells were either treated with ethanol (vehicle) or E2 (5 or 10 nM). Forty to 45 h after transfections, cells were processed for dual luciferase activity. B. Subconfluent cultures of WT276 cells in a 6-well plate were transfected with 202-luc-reporter plasmid (2.5 µg), pRL-TK (0.5 µg) plasmid along with either empty vector, or a plasmid encoding ERα using calcium phosphate precipitation method. Twenty-four hours after transfections, cells were either treated with ethanol (vehicle) or E2 (5 or 10 nM). Forty to 45 h after transfections, cells were processed for dual luciferase activity. C. Subconfluent cultures of NIH 3T3 cells in a 60-mm plate were transfected either empty vector or a plasmid encoding ERα using calcium phosphate precipitation method. Forty to 45 h after transfections, cells were processed for immunoblotting using Abs specific to the indicated proteins.
FIGURE 6. ERα associates with the potential DNA-binding site in the 5′ regulatory region of Ifi202 gene in ChIP assays. A, Schematic representation of the 5′ regulatory region of the Ifi202 gene containing two potential ERα half DNA-binding sites, which are next to an AP-1 DNA-binding site. Relative locations of PCR primers that were used to amplify the immunoprecipitated chromatin are shown. B, Subconfluent cultures of WT276 cells were either transfected with 202-luc reporter or 202AP1CS1-luc reporter plasmid along with pRL-TK plasmid. Twenty-four hours after transfections, cells were left untreated or treated with E2 (10 nM). Forty hours after transfections, cells were processed for dual luciferase activity as described in Materials and Methods. Normalized reporter activity is indicated. C, Soluble chromatin was prepared from B6.Nba2 male (lanes 1, 3, and 5) or female (lanes 2, 4, and 6) B cells. Chromatin was incubated with Abs to ERα (lanes 5 and 6) or, as a negative control, with isotype IgG1 Abs (lanes 3 and 4). DNA was extracted from immunoprecipitates and PCR amplified (30 cycles) using a pair of primers that covered ERα DNA-binding site in the 5′ regulatory region of the Ifi202 gene. As a positive control for PCR, we also amplified the input chromatin DNA from male (lane 1) and female (lane 2) B cells. D, Soluble DNA precipitated in B was also subjected to quantitative real-time PCR using PCR primers flanking the 5′ regulatory region of the Ifi202 gene as described in Materials and Methods.

Basal steady-state levels of Ifi202 mRNA are not detectable in ERα-deficient mice

Because our above observations demonstrated that activation of ERα by E2 up-regulates the expression of the Ifi202 gene in WT276 cells, we compared steady-state levels of Ifi202 mRNA between ERα-expressing wild-type (Esr1+/+ and ERα-deficient (Esr1−/−) age-matched (NZB × NZW)F1 female mice (20). As shown in Fig. 7, we found that basal levels of Ifi202 mRNA were detectable in splenic cells from the wild-type mice. However, Ifi202 mRNA levels were not detectable in splenic cells from the Esr1-null mice. These observations indicated that the basal steady-state levels of the Ifi202 mRNA in (NZB × NZW)F1 splenic cells are regulated by expression levels of ERα.

FIGURE 7. Levels of Ifi202 mRNA in splenic cells depend on the ERα status. Total RNA isolated from spleens of wild-type (Esr1+/+; lanes 1–3) or ERα-deficient (Esr1−/−; lanes 4–6) age-matched (NZB × NZW)F1 female mice was analyzed by semiquantitative RT-PCR for expression levels of Esr1, Ifi202, and actin mRNA.

Discussion

Increased levels of estrogen in certain lupus-prone strains of female mice are known to activate and increase survival of autoreactive cells in a naive repertoire (3, 4, 43–45). However, molecular mechanisms that contribute to increased cell survival in female mice remain to be elucidated. Therefore, identification of lupus-susceptibility genes whose expression is regulated by genetic factors and sex hormones, as well as elucidation of the role of encoded proteins in cell survival, is expected to provide insights into the molecular basis of sex bias in lupus susceptibility. Interestingly, estrogen treatment of female BALB/c transgenic (transgenic for R4A-γ2b H chain) mice results in up-regulation of Bcl-2 expression in splenic B cells (43). Furthermore, a recent study has identified a number of genes whose expression is regulated by sex hormones in splenic cells of (NZB × NZW)F1 lupus-prone mice (35). The study also identified the Trp53 gene (encoding the p53 protein), whose expression is up-regulated in male mice (as compared with female mice). p53 represses transcription of the Ifi202 gene (46).

Therefore, our observation that expression of the Ifi202 gene is down-regulated by male sex hormone (DHT) in orchietomized (NZB × NZW)F1 male mice (Fig. 1) makes it likely that increased levels of male sex hormone negatively regulate Ifi202 expression, in part by up-regulating the p53 expression. Moreover, the study by Xin et al. (35) also identified other genes that are known to encode proteins with immunomodulatory functions. However, none of the identified estrogen-responsive genes mapped within the NZB-derived Nba2 lupus susceptibility interval, which is syntenic to the human lupus susceptibility locus (26, 32).

Promoter polymorphisms-dependent increased expression of Ifi202 gene in female mice of certain strains is associated with defects in apoptosis of B cells and the development of lupus-like disease (26, 32, 33). Because the development of lupus-like disease in B6.Nba2 mice has sex bias (34), we investigated whether sex hormones could regulate the expression of the Ifi202, an IFN-inducible lupus susceptibility gene within the Nba2 interval. Our experiments revealed that: (1) in vivo treatment of orchietomized (NZB × NZW)F1 male mice with female sex hormone E2 increased steady-state levels of Ifi202 mRNA, whereas treatment with male sex hormone DHT decreased the mRNA levels (Fig. 1); (2) increased steady-state levels of Ifi202 mRNA and protein in splenic B cells from B6.Nba2 male and female mice were associated with increased levels of ERα and reduced levels of AR (Fig. 2); (3) steady-state levels of Ifi202 mRNA were relatively higher in C57BL/6, B6.Nba2, NZB, and (NZB × NZW)F1 female mice than in age-matched male mice (Fig. 3); (4) treatment of E2-responsive WT276 cells with increasing concentrations of E2 increased the steady-state levels of Ifi202 mRNA and protein, whereas treatment of cells with DHT decreased the p202 protein levels (Fig. 4); (5) treatment of WT276 cells with E2 or overexpression of ERα stimulated the activity of 202-luc-reporter plasmid (Fig. 5, A and B), and overexpression of ERα in NIH 3T3 cells up-regulated the p202 protein levels (Fig. 5C); (6) E2 treatment of WT276 cells activated transcription from 202-luc-reporter through the AP-1CS1 site and increased levels of ERα associated with the 5′ regulatory region of the Ifi202 gene in B6.NBa2 female B cells than in age-matched males (Fig. 6); and (7) steady-state levels of Ifi202 mRNA were detectable in (NZB × NZW)F1 splenic cells of wild-type (Esr1+/+), but not ERα-deficient (Esr1−/−), age-matched female mice (Fig. 7). Taken together, these observations demonstrated that female sex hormone and male sex...
hormone differentially regulate the expression of the *Ifi202* gene in immune cells.

Of note, our observations revealed that basal and IFN-induced steady-state levels of *Ifi202* mRNA were significantly higher in nonautoimmune C57BL/6 females than in age-matched males (Fig. 3A). Moreover, steady-state levels of *Ifi202* mRNA were also significantly higher in preautoimmune B6.Nba2, NZB, and (NZB × NZW)F₁ female splenic cells than in age-matched males (Fig. 3B). Similarly, steady-state levels of *Ifi202* mRNA and protein were significantly higher in preautoimmune B6.Nba2 female splenic B or T cells than age-matched males (Fig. 2). Taken together, these observations are consistent with sex hormone-dependent (and disease-independent) regulation of *Ifi202* expression.

ERα-deficient mice are reported to have elevated levels of estrogen and testosterone (47). Therefore, our observations that in vivo treatment of orchietomized (NZB × NZW)F₁ male mice with DHT reduced the mRNA levels (Fig. 1) and the lack of detection of *Ifi202* mRNA in *Esr1*-null mice (Fig. 7) make it likely that increased levels of testosterone in ERα-deficient female mice through AR down-regulate the expression of *Ifi202* gene. Further work will be needed to test this possibility.

A study (48) has revealed that treatment of BALB/c mice with ER subtype-selective agonists that results in activation of ERα, but not ERβ, plays a major role in estrogen-induced thyromatosis and thymic atrophy and thymic T cell and splenic B cell phenotype alterations. Moreover, the study also revealed that ERα, but not ERβ, mediates the estrogen-induced up-regulation of IFN-γ. Consistent with a role for ERα in IFN-γ production, our study (20) involving generation of ERα knockout (NZB × NZW)F₁ mice and their characterization revealed that estrogen through ERα promotes lupus disease, at least in part, by inducing IFN-γ production. Moreover, estrogen is known to enhance IFN-γ production by CD11c⁺ cells (49). Taken together, these observations raise the possibility that activation of ERα by E2 in immune cells of certain strains of female mice up-regulates *Ifi202* expression in part by increasing IFN-γ production. Therefore, further work will be needed to test this hypothesis.

Previous studies (43, 44, 50) have suggested that estrogen treatment of R4A-γ2b BALB/c mice (transgenic for the H chain of an anti-DNA Ab) with E2 leads to the survival and activation of autoreactive cells in a naïve repertoire. Moreover, studies (43) also revealed that estrogen treatment of B cells also up-regulates the expression of several genes, such as *cd22*, *shp-1*, and *bcl-2*, which are involved in B cell activation and survival. Interestingly, treatment of mice with tamoxifen, a selective ER modulator (42), blocked estrogen-induced B cell maturation but not survival (50). Because increased expression of p202 protein in splenic B cells from B6.Nba2 congenic mice is associated with defects of apoptosis of cells (26, 32, 33) and down-regulation of expression of p53 and E2F-responsive proapoptotic genes (36, 41), we compared basal transcriptional activity of NF-κB between B6.Nba2 female B cells and age-matched males. These experiments indicated that increased levels of p202 in female B cells (as compared with males) are associated with increased transcriptional activity of NF-κB (data not shown). Collectively, our observations support the idea that female sex hormone-dependent increased levels of p202 protein in B cells by increasing cell survival contribute to sex bias in lupus susceptibility (Fig. 8).

The 5' regulatory region (~800 bp) of *Ifi202* gene contains at least two ERE half-sites (Fig. 6A). Interestingly, one of the ERE half-sites is located next to an AP-1 consensus DNA-binding site (the AP-1CS1), which can bind to AP-1 and can stimulate the transcription of the *Ifi202* gene (40). Because the ERα can regulate transcription of its target genes through the c-Jun/AP-1 DNA-binding sites (15, 16), we tested whether ERα regulates transcription of the *Ifi202* gene through the AP-1CS1 site-dependent manner. Our experiments demonstrated that E2-mediated stimulation of the activity of 202-luc-reporter was abrogated due to a mutation in the AP-1CS1 site. Further work will be needed to investigate how ERα and c-Jun/AP-1 collaborate with each other to up-regulate the expression of the *Ifi202* gene in B cells.

A search for an AR-responsive element (ARE) in the 5'-regulatory region of the *Ifi202* gene did not result in identification of an ARE. Therefore, further work will be needed to determine whether 5' regulatory region of the *Ifi202* gene, which is upstream to an ~800-bp region, contains an ARE. In this regard, note that bone marrow stromal cells mediate androgenic suppression of B lymphocytes development through up-regulation of TGF-β expression in response to DHT treatment (51). Consistent with these observations, we have noted that TGF-β treatment of splenic cells reduced steady-state levels of *Ifi202* mRNA (data not shown). Therefore, further work will be needed to determine how androgens negatively regulate the expression of *Ifi202* in T cells.

Interestingly, a recent study (29) has provided evidence that the p202 protein can recognize double-stranded DNA in cytoplasm (29). Moreover, the study proposed that increased levels of p202 protein in immune cells could inhibit the ability of the AIM2 protein, a pyrin domain containing member of the p200-family (28, 30), which can also sense DNA in cytoplasm and can form a caspase-1-activating inflammasome (30). In light of these observations it will be important to investigate whether the expression of the murine *Aim2* is also differentially regulated by the female and male sex hormones in immune cells.

In summary, our observations provide support for our model (Fig. 8). The model predicts that increased levels of male hormone androgen through activation of AR down-regulate the expression of the *Ifi202* gene. In contrast, increased levels of female hormone estrogen through activation of ERα up-regulate the expression of *Ifi202*. Consequently, increased levels of p202 protein in immune cells of certain strains of female mice contribute to increased survival of autoreactive cells, resulting in increased susceptibility to lupus disease. Our observations will serve as a molecular basis to identify signaling pathways and molecules that contribute to sex bias in the development of SLE in human patients.
ROLE OF If202 in SEX BIAS IN LUPUS SUSCEPTIBILITY


