

Signal Transducer and Activation of Transcription (STAT) 4 β , a Shorter Isoform of Interleukin-12-Induced STAT4, Is Preferentially Activated by Estrogen

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Estrogen, a natural immunomodulatory compound, has been shown to promote the induction of a prototype T helper 1 cytokine, interferon (IFN)- γ , as well as to up-regulate IFN γ -mediated pro-inflammatory molecules (nitric oxide, cyclooxygenase 2, monocyte chemoattractant protein 1). Because IL-12 is a major IFN γ -inducing cytokine, in this study we investigated whether estrogen treatment of wild-type C57BL/6 mice alters IL-12-mediated signaling pathways. A recent study has shown that IL-12 activates two isoforms of signal transducer and activation of transcription (STAT) 4, a normal-sized (full-length STAT4 α) and a truncated form (STAT4 β). Interestingly, we found that estrogen treatment preferentially up-regulates the phosphorylation of STAT4 β in splenic lymphoid cells. Time kinetic data showed the differential activation of STAT4 β in splenic lymphoid cells from estrogen-treated mice, but not in cells from placebo controls. The activation of STAT4 β was mediated by IL-12 and not IFN γ because deliberate addition or neutralization of IL-12, but not IFN γ , affected the activation of STAT4 β . In contrast to IL-12-induced activation of STAT4 β in cells from estrogen-treated mice, STAT4 α was not increased, rather it tended to be decreased. In this context, STAT4 α -induced p27^{kip1} protein was decreased in concanavalin A + IL-12-activated lymphocytes from estrogen-treated mice only. By using the *in vitro* DNA binding assay, we confirmed the ability of pSTAT4 β to bind to the IFN γ -activated sites (IFN γ activation sequences)/STAT4-binding sites in estrogen-treated mice. Our data are the first to show that estrogen apparently has selective effects on IL-12-mediated signaling by preferentially activating STAT4 β . These novel findings are likely to provide new knowledge with regard to estrogen regulation of inflammation. (***Endocrinology* 150: 1310–1320, 2009**)

Estrogen has been involved in immunomodulation at both cellular and molecular levels, and has a role in many inflammatory and autoimmune diseases (1–6). We and others have shown that estrogen up-regulates interferon (IFN)- γ , a T helper 1 (Th1) cytokine (7–11). Estrogen is involved in inflammatory conditions, and our previous studies have demonstrated that *in vivo* estrogen exposure promotes IFN γ -mediated proinflammatory events (9–13). Mechanisms underlying the basis of estrogen-modulated IFN γ are not known, especially with regards to the role of a potent IFN γ -inducing cytokine, IL-12. IL-12 is released from activated antigen presenting cells (APCs) and is

known to act on naive CD4⁺T cells to differentiate these cells into IFN γ -producing Th1 cells (14). Several studies have shown that IL-12 induces IFN γ production (15–18). Thus far, the role of IL-12-mediated signaling in the context of estrogen-induced up-regulation of IFN γ is not known. The present study addressed this aspect.

IL-12 is a heterodimeric cytokine, composed of two subunits, p40 and p35, that forms an active p70 dimer (19). IL-12p70 (IL-12) binds to the IL-12 receptor complex, resulting in the rapid phosphorylation of tyrosine residues on signal transducer and activation of transcription (STAT) 4, a transcription factor

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Abbreviations: APC, Antigen presenting cell; ConA, concanavalin A; DAPA, DNA affinity purification assay; ER, estrogen receptor; GAS, interferon- γ activation sequence; IFN, interferon; NK, natural killer cell; pSTAT4, signal transducer and activation of transcription 4 phosphorylation; rIFN, recombinant interferon- γ ; rIL-12, recombinant IL-12p70; STAT, signal transducer and activation of transcription; Th1, T helper 1.

that is exclusively present in the cells of the immune system (15, 20). The STAT family has three distinct functional domains: a conserved N terminus (necessary for tetramer formation); a DNA-binding domain (allows binding to the promoters of target genes) (21, 22); and a C terminus (acts in transcriptional activation) (23). STAT1, STAT3, and STAT5 have been shown to exist as two isoforms. Full-length proteins are present as α -isoforms and shorter β -isoforms, which are truncated at the C terminus, presumably by either alternative mRNA splicing or proteolytic processing (24–26). With regards to STAT4 isoforms, information is limited. Thus far, only one study, using A139 human $\gamma\delta$ T cell line (expressing IL-12 receptors) (27) or polarized Th1 cells from STAT4-transgenic mice, has demonstrated the existence of both a full-length (STAT4 α) and a natural shorter isoform, STAT4 β , that is missing 44 amino acids at C terminus (28).

A positive feedback regulation between IL-12 and IFN γ is known to exist (29). IFN γ , which is induced by IL-12, in turn positively up-regulates IL-12 secretion. This may be a physiological feedback mechanism that is essential for the local maintenance of Th1-mediated immunity to counter intracellular infections. Overproduction of IL-12 has been observed in several autoimmune diseases such as rheumatoid arthritis (30) and multiple sclerosis (31, 32). In contrast, the absence of IL-12 or a deficiency in IL-12 signaling may result in decreased Th1 differentiation (thereby permitting up-regulation of a Th2 response) and also decreased immunity against Th1-mediated infections such as *Mycobacterium tuberculosis* (33, 34). The significance of IL-12/STAT4 in IFN γ induction is highlighted by the fact that activated lymphocytes from STAT4-deficient mice have remarkably reduced levels of IFN γ (15, 35, 36), impaired Th1 cell development (37, 38), and decreased natural killer (NK) cell cytotoxicity (37).

Given that IL-12 is a potent inducer of IFN γ , in this study we investigated whether estrogen up-regulates IL-12 levels and/or response to IL-12. Our novel findings reported here show that splenocytes from estrogen-treated mice not only have increased phosphorylated STAT4 (pSTAT4) levels, but phosphorylation of STAT4 β was increased when cells were exposed to IL-12, but not IFN γ . To our knowledge this is the first study to report estrogen regulation of IL-12-mediated signaling.

Materials and Methods

Mice

Three to 4-wk-old C57BL/6 wild-type male mice (Charles River Laboratories, Inc., Wilmington, MA) were housed three to five animals per cage, maintained in standard cages in a pathogen-free environment with 12-h light, 12-h dark cycles at the Center for Molecular Medicine and Infectious Diseases Animal Laboratory facility, and fed a diet devoid of synthetic or phytoestrogens (12). All animal-related procedures were in accordance with Virginia Polytechnic Institute and State University Institutional Animal Care guidelines, and were approved by the Institutional Animal Care and Use Committee.

Estrogen treatment

As per our previously reported extensive studies (9, 10, 39, 40), at 4–5 wk of age, mice were anesthetized with ketamine/xylazine, orchi-

ectomized as reported previously (9, 10), and surgically implanted with SILASTIC brand capsules (Dow Corning, Midland, MI) containing powdered 17 β estradiol (Sigma-Aldrich Corp., St. Louis, MO) or empty (placebo) implants as controls. Estrogen-containing implants, 4–5 mm SILASTIC brand medical grade tubing (0.062-in. internal diameter \times 0.125-in. outer diameter), were packed with 3–5 mg 17 β estradiol. Implants are designed to slowly release estrogen over a period of months. Mice were terminated by cervical dislocation after 7 wk treatment. Serum estrogen levels achieved with this method were in the range of estrogen concentrations (41) as reported in our previous studies (42).

In our series of published studies, we have shown that estrogen has similar effects in both males and females (10, 43). In female mice, ovaries secrete not only estrogen, but also progesterone and other hormones that can affect the immune cells. Moreover, extragonadal sites [mesenchymal cells from adipose tissue and skin (44), osteoblasts (45), aortic smooth muscle (46), and vascular endothelial cells (47)] can also secrete estrogen. To explore the effects of estrogen on immune parameters, we preferred the use of male orchiectomized mice because these male mice have not only low levels of male hormones (which could confound the results) but also have very low levels of endogenous estrogens. Moreover, unlike in males, ovarian-intact females also secrete significant levels of progesterone, FSH and LH, which will complicate the results because these hormones are immunomodulatory. Further low levels of estrogen are also produced by extragonadal tissues in ovariectomized female mice (48, 49). Finally, orchiectomy is a simpler surgical procedure compared with ovariectomy, which is abdominally invasive.

Isolation and culture of splenocytes

Spleens were collected under sterile conditions, and lymphocytes were isolated as described in previous studies (9, 10, 40). One and a half milliliters of cells (5×10^6 cells per ml) were cultured with 1.5 ml per well of complete phenol-red free RPMI 1640 with or without T-cell mitogen, concanavalin A (ConA) (10 μ g/ml; Sigma-Aldrich). Splenocytes were also cultured with ConA and/or recombinant IL-12p70 (rIL-12) (20 ng/ml; R&D Systems, Inc., Minneapolis, MN), recombinant IFN γ (rIFN γ) (10 ng/ml; BD PharMingen, San Diego, CA), anti-IL-12, or anti-IFN γ antibodies (3 μ g/ml; R&D Systems) for 3, 18, or 24 h.

RT-PCR

Total RNA was isolated using RNeasy Mini kits (QIAGEN, Inc., Valencia, CA) and reverse transcribed to cDNA (Invitrogen Corp., Carlsbad CA) as in our previous studies (12). To detect STAT4 α and STAT4 β mRNA expressions, Primer1 (5'-ACGGGGAGACAAGGGTTACG-3') was paired either with Primer2 (5'-TTGGAGACATGGGGAGAAGGT-3') or Primer3 (5'-AGGGCAGCTTTATCATTCCAGT-3'), respectively. The genomic accession number for murine STAT4 is NW_001030680. Primer1 [binds to exon22 (394028–394094)] and Primer2 [binds to exon23 (394490–394598)] product: STAT4 α (107 bp), Primer1 [binds to exon22 (394028–394094)] and Primer3 [binds to intron22 (394094–394490)] product: STAT4 β (278 bp). β -Actin (110 bp), the housekeeping gene, was used as a control with sense 5'-CCCTAAGGCCAACCCTGAAAAG-3' and antisense 5'-TGGCGTGAGGGAGAGCATAGC-3' primers. Conditions for cDNA amplification were: 94 C (4 min), 35 cycles of 94 C (15 sec), 55 C (30 sec), and 72 C (1 min).

IL-12p70 and IFN γ ELISA

Protein levels of IL-12p70 in culture supernatants were determined with IL-12p70 Quantikine ELISA kits per slightly modified (overnight incubation at 4 C) manufacturer's instructions (R&D Systems). Plates were read at 450 nm with a Molecular Devices maximum velocity (Vmax) microplate reader (Molecular Devices, Sunnyvale, CA). IL-12p70 levels were calculated using SoftMaxPro software (Molecular Devices). IFN γ levels were determined using an IFN γ ELISA as reported in our previous studies (9, 10, 13).

Nuclear extracts and Western blot analysis

Nuclear extracts from 7.5×10^6 whole splenocytes were prepared according to the manufacturer's instructions (NE-PER kit; Pierce, Rockford, IL) and as described in our previous studies (12). Equal amounts of nuclear extracts (25 μ g protein per well) were resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Amersham Biosciences Inc., Piscataway, NJ) as described elsewhere (12). Total and phosphorylated STAT4 and p27^{Kip1} were detected with pSTAT4tyrosine (1:1000, Tyr693; Invitrogen), STAT4 (1:1000, sc-485; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p27^{Kip1} (1:500; eBioscience, San Diego, CA), and with secondary antibodies (1:3000; Santa Cruz Biotechnology). The pSTAT4 antibody was specially concentrated at 1 mg/ml by Invitrogen. Specific binding sites of pSTAT4 and STAT4 antibodies are explained in *Results* section. As a loading control, anti- β -actin antibody was used (Abcam Inc., Cambridge, MA). Detection was determined by using an enhanced chemiluminescence protocol (Amersham Pharmacia Biotech, Uppsala, Sweden). Densitometric analysis of the data was completed using a Kodak Image Station (Carestream Health, Inc., Rochester, NY), which determines the relative densitometry values of the bands.

In vitro DNA-protein binding assay

For the qualitative detection of pSTAT4 DNA-binding activity, a DNA affinity purification assay (DAPA) was performed, in which DNA-binding proteins were precipitated from nuclear extracts using agarose-conjugated consensus oligonucleotides and then analyzed with Western blotting. DAPA was done as described before (50), with minor modifications. In brief, nuclear extracts were prepared cultured splenocytes. The 5' biotin-labeled DNA binding probes containing IFN- γ activation sequence (GAS) (forward, GATCGTGATTTCCTCCGAAATGACG; and reverse complementary, CGTCATTTCCGGGAAATCACGATC) were synthesized (Integrated DNA Technologies, Coralville, IA) and annealed. For the binding assay, 150 μ g nuclear protein was incubated with 50 pmol biotin-labeled double-stranded DNA probe in 500 μ l 1 \times binding buffer [12 mM HEPES (pH7.5) 80 mM NaCl, 5 mM dithiothreitol, 5 mM MgCl₂, 0.5 mM EDTA, and 5% glycerol] containing 25 μ g polydIdC at 4 C with rotation overnight. After incubation, 40 μ l streptavidin-agarose beads were added and incubated for an additional 2 h. Agarose beads were pelleted and washed three times with 1 \times binding buffer (without polydIdC). Proteins were eluted with sodium dodecyl sulfate sample buffer, separated on a 4–15% SDS-PAGE gel, transferred on polyvinylidene fluoride membranes, and subjected to Western blot analysis using antibody pSTAT4tyrosine.

Statistics

Statistical significance of differences between experimental groups was determined by one-way ANOVA using InStat software (GraphPad Software Inc., San Diego, CA). *Post hoc* comparisons between treatment group means were made with Bonferroni for multiple comparisons. The significance of differences between placebo and estrogen-treated samples was also assessed with two-tailed *t* tests. Significant differences are indicated with brackets and an asterisk denoting *P* values less than 0.05. Data are presented as means with SE bars.

Results

Estrogen-induced early increase in IL-12 levels

In our previous studies, we have demonstrated that estrogen induces IFN γ and IFN γ -mediated proinflammatory molecules from ConA-activated splenocytes (9–12, 42). To demonstrate whether *in vivo* estrogen induces a key IFN γ -inducing cytokine, IL-12p70, splenocytes from estrogen- and placebo-treated mice were cultured with ConA for 3, 18, and 24 h, and IL-12p70 levels were determined in culture supernatants. IL-12p70 levels were

significantly increased in supernatants of ConA-activated splenocytes from estrogen-treated mice at 3 h compared with placebo controls (*P* < 0.05; Fig. 1). IL-12p70 levels increased at 18 h, and were comparable in samples from estrogen- and placebo-treated mice. IL-12p70 levels tended to decline by 24 h in both estrogen- and placebo-treated mice. IL-12p70 protein was not detectable in supernatants from unstimulated (media only) cells (data not shown).

Estrogen up-regulates tyrosine phosphorylation of the STAT4 β isoform

Because estrogen promoted a significant increase in IL-12p70 levels in the supernatants of ConA-activated cells, especially after 3 h culture, we investigated whether estrogen also augments IL-12-mediated signaling, as assessed by STAT4 phosphorylation (pSTAT4) at tyrosine residues. We used two different STAT4 antibodies. One STAT4 antibody was specific for the N-terminal region and detected total (phosphorylated and unphosphorylated) STAT4, including both the α and β -isoforms of STAT4. The second antibody detected phosphorylated STAT4 by binding to a phosphorylated tyrosine residue (amino acid 693) on both phosphorylated STAT4 α and phosphorylated STAT4 β (denoted as pSTAT4 α or β in this paper).

We next determined the time kinetic profile of pSTAT4 α and β in nuclear extracts of splenocytes from estrogen- and placebo-treated mice that were cultured in the presence of ConA for 3, 18, and 24 h culture. Surprisingly, at 3 h culture, samples from estrogen-treated mice showed two distinct bands; these were determined to represent the full-length (pSTAT4 α) and the shorter (pSTAT4 β) isoforms (Fig. 2A). Only pSTAT4 α was observed in samples from control mice. Levels of pSTAT4 β were high in samples from estrogen-treated mice at 3 h. By 18 h, pSTAT4 β protein was still evident in samples from estrogen-treated mice, albeit at lower levels than observed at 3 h. By 24 h, pSTAT4 β was diminished in samples from estrogen-treated mice, and only the full-length pSTAT4 α protein was expressed (Fig. 2A). These findings revealed that there is a temporal, early, and selective

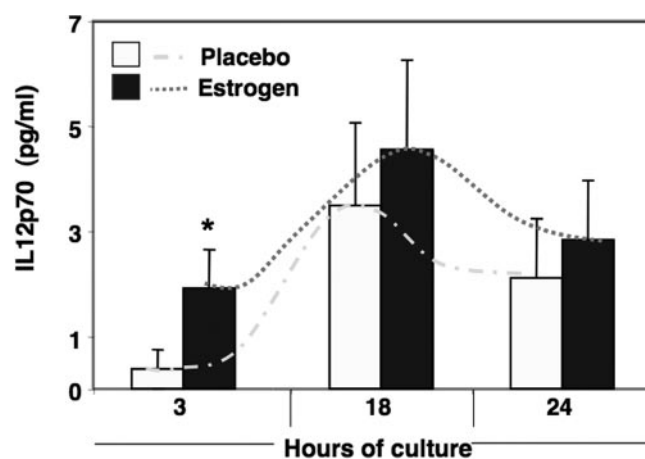


FIG. 1. Estrogen up-regulates IL-12p70 protein at 3 h. IL-12p70 protein levels were determined in the supernatants of ConA (10 μ g/ml)-stimulated splenocytes cultured for 3, 18, and 24 h from estrogen- or placebo-treated mice. IL-12p70 levels were markedly up-regulated at 3 h (*, *P* < 0.05; *n* = 4 mice per placebo or estrogen).

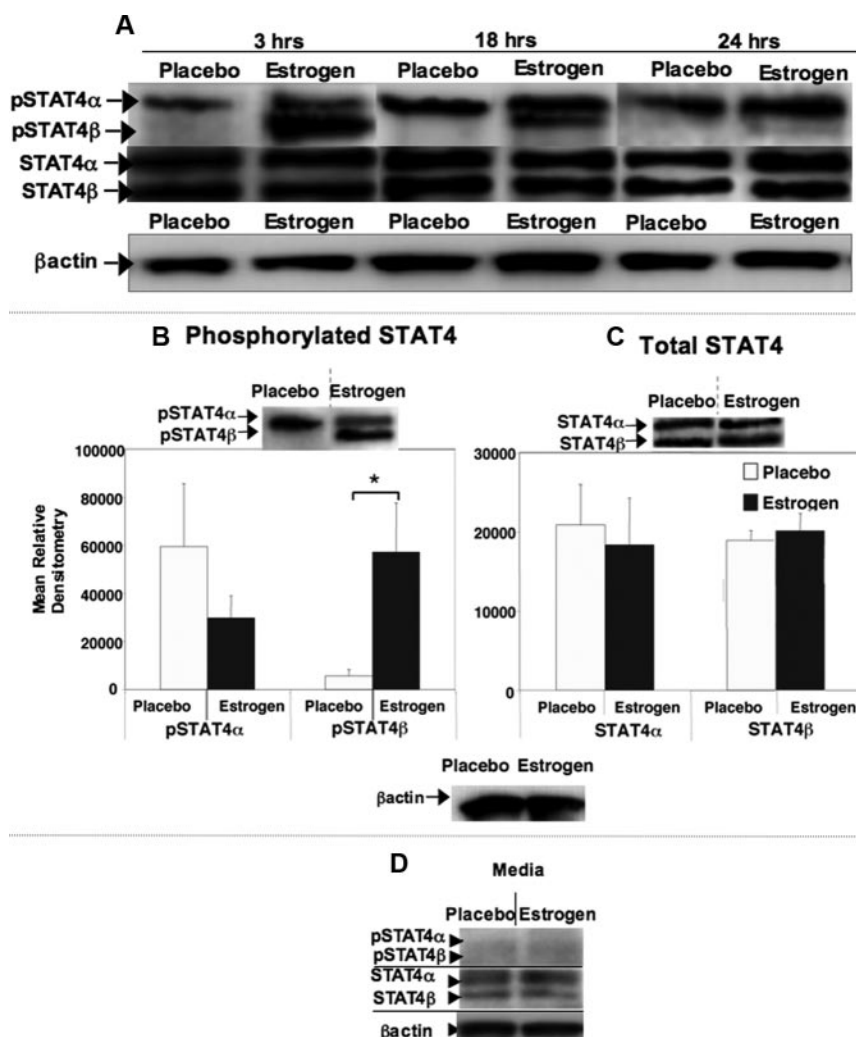


FIG. 2. Estrogen up-regulation of the tyrosine phosphorylated STAT4 β isoform occurs at early culture time points. Nuclear extracts from ConA-activated splenocytes from estrogen- or placebo-treated mice were analyzed for tyrosine phosphorylated STAT4 (pSTAT4) or total STAT4 with Western blot assays. All blots were stripped and reprobed with an antibody specific for β -actin, showing equal protein loading in each lane. One representative individual experiment is shown per panel (placebo: $n = 4$ mice; estrogen: $n = 4$ mice; *, $P < 0.05$). A, Time kinetic profile of pSTAT4 α or pSTAT4 β isoforms in nuclear extracts from splenocytes from estrogen- or placebo-treated mice cultured for 3, 18, or 24 h are depicted in parentheses and brackets, respectively. B and C, A representative blot and the densitometry data of pSTAT4 (B) and total STAT4 (C) protein expression in nuclear extracts from ConA-activated splenocytes cultured for 3 h are shown. D, A representative blot for pSTAT4 and total STAT4 protein expression in nuclear extracts from unstimulated (media only) splenocytes cultured for 3 h are demonstrated.

increase in pSTAT4 β in activated splenocytes from estrogen-treated mice. In freshly isolated splenocytes (*i.e.* not activated by ConA) or after 30 min activation with ConA, neither pSTAT4 β nor pSTAT4 α was evident in estrogen- or placebo-treated mice (data not shown), implying that stimulation is required for pSTAT4.

Because the levels of pSTAT4 β protein were markedly increased after 3 h stimulation, we focused on early expression of pSTAT4 α and β in splenocytes in this study. Although the levels of pSTAT4 β were noticeably enhanced in ConA-activated splenocytes from estrogen-treated mice, but not in control mice, after 3 h incubation with ConA (Fig. 2B), nearly equivalent protein expression of total STAT4 (α and β) was detected in nuclear extracts from estrogen- and placebo-treated mice (Fig. 2C).

Phosphorylated (activated) STAT4 α and β was not detectable in nuclear extracts from unstimulated (media only) cells from estrogen- or placebo-treated mice (Fig. 2D). The expression of total STAT4 in nuclei from unstimulated (media only) cells was not changed between estrogen- and placebo-treated mice.

STAT4 α and β mRNA are both expressed in splenocytes from estrogen- and placebo-treated mice

As indicated previously, *in vivo* estrogen treatment selectively up-regulated the phosphorylation of STAT4 β protein. We next determined whether estrogen alters the presence of alternatively spliced STAT4 β at the mRNA level. Previous studies have demonstrated alternative splicing of STAT4 in human peripheral blood lymphocytes (where a 369 bp sequence, intron22, is included leading to an early stop codon) (28). Furthermore, formation of both STAT4 α and STAT4 β isoforms by alternative mRNA splicing has been detected in studies with an A139 human γ DT cell line [expressing IL-12 receptors (27)] and primary Th1 cells from STAT4-transgenic mice (28). To investigate the expression of alternatively spliced STAT4 isoforms in placebo- and estrogen-treated splenocytes, we designed two sets of primers to amplify STAT4 α and STAT4 β , respectively. The first set of primers (Primer1 and Primer2) was designed to span exons22 and 23 of the mouse STAT4 gene, and was expected to detect STAT4 α mRNA, which is 107 bp (Fig. 3). The second set of primers (Primer1 and Primer3) was designed to span exon22 and intron22 to amplify specifically the STAT4 β isoform (278 bp). RT-PCR assays demonstrated the expression of both STAT4 α and STAT4 β in unstimulated and ConA-activated splenocytes from estrogen and placebo-treated mice (Fig. 3).

Estrogen-induced increase in pSTAT4 β is dependent upon IL-12 but not IFN γ

To address cytokine-induced activation of STAT4 β , ConA-activated splenocytes from estrogen or placebo-treated mice were exposed to either rIL-12 or rIFN γ for 3 h, and pSTAT4 β levels were determined. As expected, pSTAT4 β levels were increased in nuclear extracts from ConA-activated splenocytes from estrogen-treated mice, but not in extracts from their placebo counterparts (Fig. 4A). In placebo-treated mice, addition of rIL-12 to ConA-activated splenocytes markedly induced pSTAT4 α but had minimal effects on pSTAT4 β compared

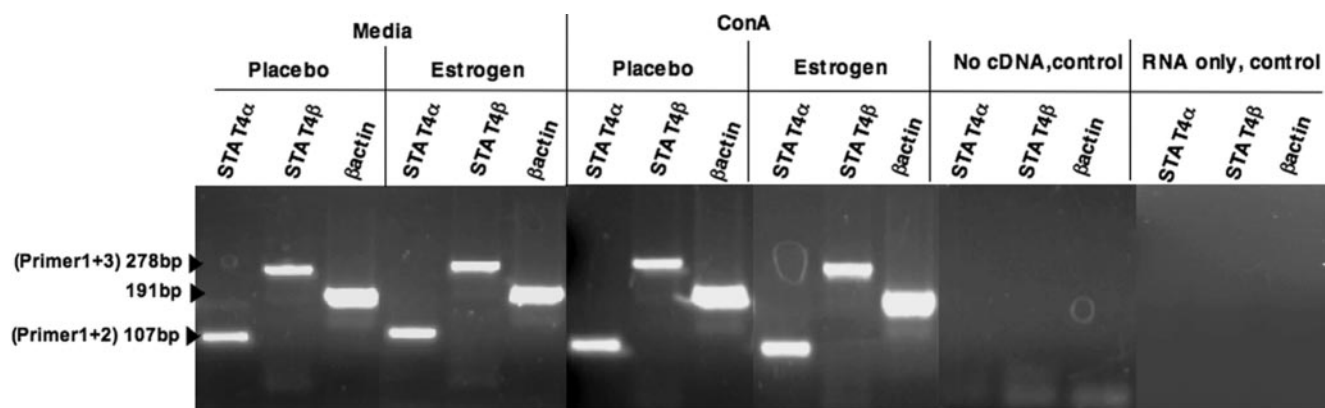


FIG. 3. mRNA expression of STAT4 α and β -isoforms. One representative experiment is shown for RT-PCR performed to detect STAT4 α (107 bp) and β (278 bp) and β -actin (housekeeping gene, 110 bp) in unstimulated (media only) and ConA-activated splenocytes from estrogen- and placebo-treated mice cultured for 3 h. No template and RNA only controls are shown. Experiments were performed at least four times.

with cultures activated with ConA alone. In sharp contrast, the addition of rIL-12 to ConA-activated splenocytes from estrogen-treated mice resulted in a notable increase in pSTAT4 β and pSTAT4 α expression compared with estrogen-treated cells activated with ConA alone (Fig. 4A). Activation of STAT4 α was significantly higher in samples from placebo-treated mice when compared with controls stimulated with ConA plus rIL-12, whereas pSTAT4 β was less in samples from placebo-treated mice.

Although the level of pSTAT4 β protein was increased in nuclear extracts from both ConA and ConA plus rIL-12 stimulated splenocytes from estrogen-treated mice (Fig. 4A), the respective levels of total STAT4 β expression were not markedly changed in samples cultured with rIL-12 and ConA (Fig. 4B). To investigate further the role of IL-12 in up-regulation of pSTAT4 β , splenocytes were cultured with ConA alone or ConA plus anti-IL-12 antibodies (to block IL-12 induced by ConA activation) (Fig. 4C). There was no significant change in the expression of total STAT4 between types of stimulation or estrogen/placebo treatments. Blocking IL-12 protein in ConA-activated splenocytes with anti-IL-12 antibodies significantly decreased pSTAT4 β in samples from estrogen-treated mice (Fig. 4C). Neutralization of IL-12 protein was confirmed by the absence of detectable levels of IL-12 (data not shown). No marked changes in the expression of total STAT4 in ConA and ConA plus anti-IL-12 stimulated splenocytes from estrogen and placebo treatments were observed (Fig. 4D). There was no detectable activation of STAT4 α and STAT4 β , and no difference in the expression of total STAT4 in unstimulated splenocytes from estrogen- and placebo-treated mice.

The potential role of IFN γ in induction of the pSTAT4 β was next investigated by coculturing ConA-activated splenocytes with rIFN γ . Splenocytes from placebo- and estrogen-treated mice were cultured for 3 h with ConA alone, ConA plus rIFN γ , ConA plus anti-IFN γ antibodies, or cultured with ConA plus anti-IFN γ antibodies for 2 h (to block IFN γ). They were subsequently exposed to rIL-12 for 1 h to determine the effects of IL-12 in the absence of IFN γ . Addition of rIFN γ with ConA or blockade of IFN γ (by anti-IFN γ antibodies) in the presence of ConA had no marked effects on the induction of pSTAT4 β in cultures

from estrogen-treated mice when compared with cells activated with ConA alone (Fig. 5A). Impressively, the addition of rIL-12, while also blocking IFN γ , significantly increased the induction of pSTAT4 β expression in nuclear extracts from ConA-activated splenocytes from estrogen-treated mice (Fig. 5B). The level of activation of pSTAT4 β by rIL-12 was higher in estrogen-treated mice compared with placebo-treated mice. In placebo-treated mice, brief exposure to rIL-12 (in the absence of IFN γ) mostly induced full-length pSTAT4 α (Fig. 5B). Supernatants from anti-IFN γ -exposed cells had no detectable levels of IFN γ protein, confirming the blockade of IFN γ (data not shown). There was no marked difference in total STAT4 α / β protein expression from any of the groups of stimulated splenocytes from estrogen- and placebo-treated mice (Fig. 5C). No detectable activation of STAT4 α or β and no difference in the expression of total STAT4 in unstimulated (media only) splenocytes from estrogen- and placebo-treated mice were observed (data not shown). At 18 h culture, similar results were acquired, suggesting a potential role of IL-12 (and lack of an IFN γ effect) in selectively inducing the pSTAT4 β isoform in ConA-activated splenocytes from estrogen-treated mice (data not shown).

Selective and functional DNA binding of phosphorylated STAT4 β protein in samples from estrogen-treated mice

We next tested the functional ability of phosphorylated STAT4 α / β proteins in nuclear extracts from ConA-activated splenocytes. We determined the binding activity of phosphorylated STAT4 to the STAT response element (a GAS binding site) using a DAPA. Protein-GAS DNA binding complexes were precipitated, eluted, and separated on a SDS-PAGE gel. GAS binding-pSTAT4 α and pSTAT4 β proteins were detected by Western blot assays (Fig. 6). In cells from placebo-treated mice, we observed the binding of both pSTAT4 α and pSTAT4 β to GAS-DNA probe. However, in cells from estrogen-treated mice, only the binding of pSTAT4 β to the GAS-DNA probe was evident (Fig. 6). Results from Western blot (Figs. 2, 4, and 5) and DNA binding assays (Fig. 6) revealed interesting observations. In cells from placebo-treated mice,

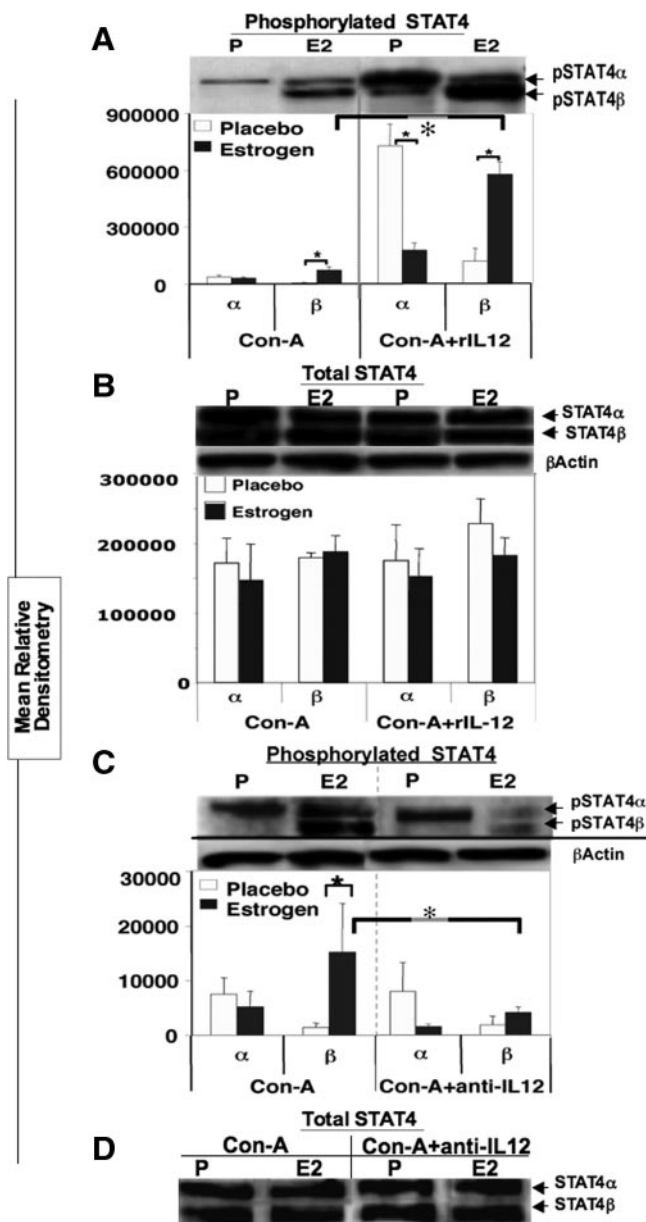


FIG. 4. Increased expression of pSTAT4 β is due to IL-12 in estrogen-treated mice. Splenocytes from estrogen (E2)- or placebo (P)-treated mice were stimulated with ConA in the presence or absence of recombinant IL-12 or blocking antibodies for 3 h (placebo: $n = 4$ mice; estrogen: $n = 4$ mice; *, $P < 0.05$). The top portions of all panels show representative blots above densitometry graphs as detected with Western blot assays. All blots were reprobed with an anti- β -actin antibody showing equal protein loading in each lane. A and B show densitometry data for pSTAT4 α/β and total STAT4 α/β , respectively, in nuclear extracts from ConA or ConA plus rIL-12 (20 ng/ml) stimulated cells from estrogen- or placebo-treated mice. C and D show pSTAT4 α/β and total STAT4 α/β , respectively, in nuclear extracts from ConA or ConA plus anti-IL-12 antibodies (3 μ g/ml) stimulated cells from estrogen- or placebo-treated mice.

Western blot detected only pSTAT4 α , but not pSTAT4 β , whereas GAS binding assay revealed both isoforms. This possibly could be due to inherent differences in these two methods, which may relate to a significant difference in the amount of nuclear extract proteins used in these two assays (150 μ g for the DNA binding assay *vs.* 25 μ g for the Western blot assay). It is plausible that in cells from placebo-treated mice, the level

of pSTAT4 β may be too low and beyond the detection limit of the Western blot assay. An additional possibility is that STAT4 β may have higher affinity for DNA binding than STAT4 α based on recent studies, which have demonstrated that tyrosine phosphorylated STAT3 β can bind to DNA with 20- to 50-fold more avidity *in vitro* (51) and 10–20 times more avidity *in vivo* (52) than STAT3 α . Interestingly, in our present studies, we also noticed that in cells from estrogen-treated mice, whereas both pSTAT4 α and pSTAT4 β isoforms were evident by the Western blot analysis, only pSTAT4 β was found to bind to the GAS-DNA probe. Together, the data suggest that estrogen treatment (at the DNA-binding level) preferentially allowed the activated pSTAT4 β isoform but tended to inhibit the activation and binding of the pSTAT4 α isoform in splenocytes.

Addition of rIL-12 induced IFN γ in ConA-activated splenocytes from estrogen-treated mice

Because the pSTAT4 β isoform was significantly up-regulated by rIL-12 in splenocytes from estrogen-treated mice, we next investigated whether the increased presence of pSTAT4 β would alter IL-12-dependent-downstream events, *i.e.* induction of IFN γ . ConA-activated splenocytes from estrogen- and placebo-treated mice were cultured in the presence or absence of rIL-12 for 3 h, and IFN γ levels were determined as an indication of IL-12p70-induced pSTAT4 β downstream events (Fig. 7A). Exposure of splenocytes from estrogen-treated mice to rIL-12 induced a significantly higher level of IFN γ compared with placebo-treated mice (Fig. 7A), indicating that pSTAT4 β does not appear to act in an inhibitory manner as reported for other STAT β isoforms (26, 53).

Decreased STAT4 α -inducible p27^{Kip1} protein in cells exposed to rIL-12 from estrogen-treated mice

Recent studies from STAT4 α or β -transgenic mice and T-cell lines demonstrated that expression of p27^{Kip1} (a cell cycle inhibitor) is induced by STAT4 α , but not STAT4 β (28). We next explored whether STAT4 α -mediated downstream events are altered in estrogen-treated mice by checking for changes in the expression of p27^{Kip1} protein from splenocytes stimulated with ConA, ConA plus rIL-12, or ConA plus anti-IL-12 for 3 h culture. Although there was no difference in the expression of p27^{Kip1} protein in ConA-activated samples from estrogen- or placebo-treated mice, addition of rIL-12 to ConA-activated cultures down-regulated p27^{Kip1} protein expression in samples from estrogen-treated mice (Fig. 7B).

Discussion

Estrogens are believed to play a role in various aspects of immune function and modulate female-predominant autoimmune diseases (6, 54). For example, estrogen worsens experimental autoimmune lupus (55, 56). Interestingly, recent evidence also suggests that estrogen exacerbates experimental autoimmune myasthenia gravis by promoting Th1 polarized immune responses (57). It is not surprising that increased levels of IFN γ have been noted in many female

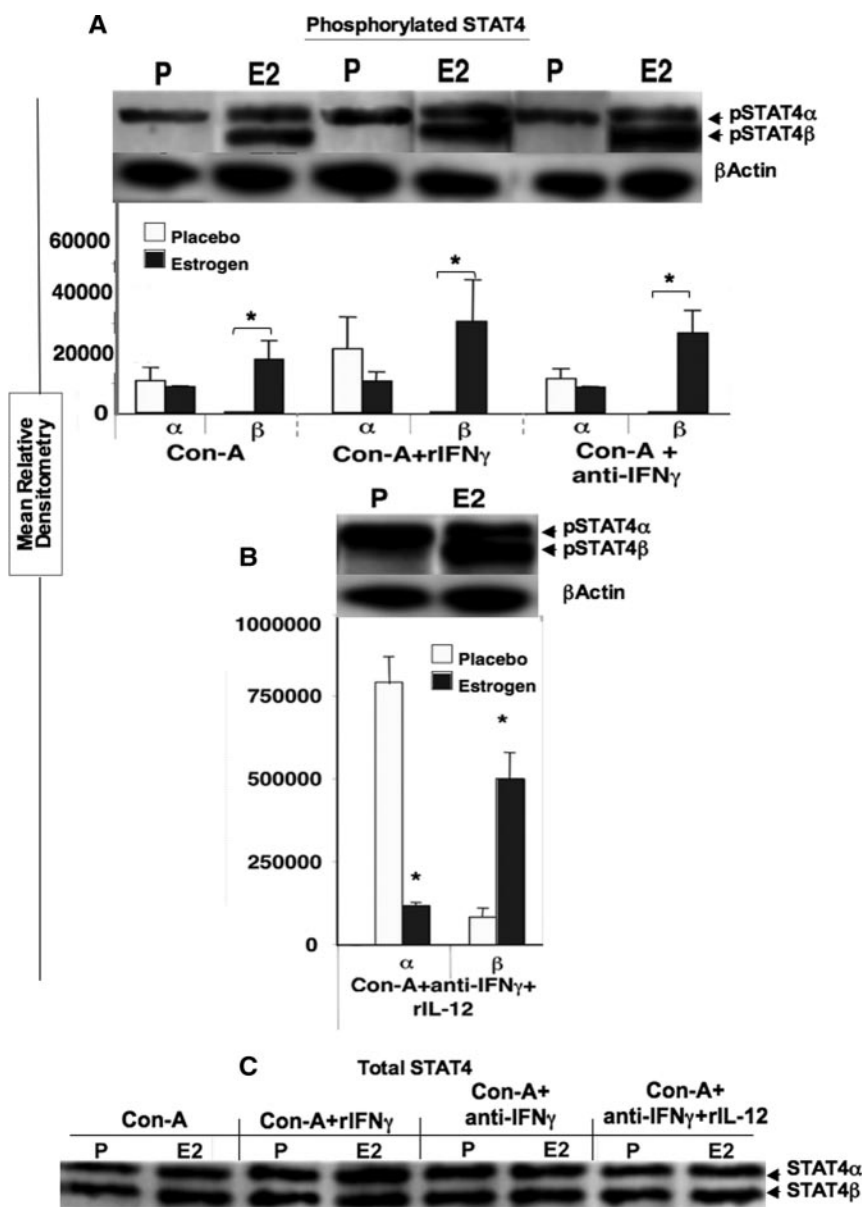


FIG. 5. Activation of STAT4 β is not mediated by IFN γ but by IL-12p70 in estrogen (E2)-treated mice. A, pSTAT4 α/β in nuclear extracts from samples cultured with ConA, ConA plus rIFN γ (10 ng/ml), and ConA plus anti-IFN γ antibodies (3 μ g/ml). B Shows pSTAT4 α/β in samples stimulated with rIL-12 (20 ng/ml) for an additional 1 h after 2 h culture with ConA and anti-IFN γ antibodies (3 μ g/ml) for a total of 3 h of culture [placebo (P) $n = 4$, estrogen (E2) $n = 4$; *, $P, 0.05$]. C, A representative blot picture for pSTAT4 α/β (above densitometry graphs) and total STAT4 expression (below) are shown. β -Actin for all representative Western blot pictures are shown in A and B.

predominant autoimmune diseases (58–60). IL-12 is a major cytokine in the development of Th1 cells and induction of IFN γ through activation of the STAT4 pathway. In this study we inves-

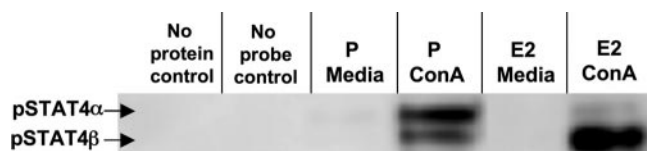


FIG. 6. Selective DNA binding affinity of pSTAT4 β in splenocytes from estrogen (E2)-treated mice. Nuclear extracts from ConA-activated splenocytes cultured for 3 h were subjected to a qualitative DAPA. No probe and no protein controls were included to demonstrate the specificity of the pSTAT4 α/β -DNA binding complex. Data shown represent at least three independent experiments. P, Placebo.

tigated whether *in vivo* estrogen modulates IFN γ -inducing cytokine, IL-12, and activation of IL-12-dependent STAT4 signaling.

A novel observation in this study is the selective phosphorylation of STAT4 β (pSTAT4 β), the short isoform of STAT4 α , in estrogen-treated but not from placebo-treated mice. The presence of STAT4 β in this work is in agreement with a recent study, which showed that STAT4 can occur in two forms, a full-length STAT4 α and a shorter STAT4 β , in primary murine Th1 and A139 human $\gamma\delta$ T cells, as well as human peripheral blood leukocytes (28). Our data are the first to show that *in vivo* estrogen appears to sensitize splenocytes to up-regulate preferably the activation of STAT4 β (pSTAT4 β).

Precisely how estrogen up-regulates pSTAT4 β is not readily apparent because it was not the objective of this study. One conjunctural possibility is that estrogen may selectively enhance the activity of kinase(s) that can phosphorylate STAT4 β . Interestingly, cross talk between estrogen receptors (ERs) and STATs has been reported in several studies (61–63). It is not known whether ER has a role in preferential activation of STAT4 β . Future studies need to be designed to address this aspect. Other recent studies also demonstrate that estrogen can rapidly induce phosphorylation/activation of STAT3 and STAT5 (64, 65). Non-genomic and indirect activation of STAT3 and 5 is regulated by estrogen-induced activation of MAPK, Src-kinase, and phosphatidylinositol 3-kinase signaling pathways (65–70). The precise mechanism(s) of how STATs cooperate with other transcription factors and signaling cascades to activate transcription of target genes is not known. Our data demonstrate that only in cells from estrogen-treated mice was there a distinct and rapid activation of STAT4 β by 3 h, which suggests that activation of the

β -isoform occurs at early time points in response to increased IL-12 levels. Thus, in light of previous studies, it can be speculated that molecular mechanisms of estrogen-modulated rapid and specific activation of STAT4 β can occur through non-genomic and/or genomic action of ERs and transcriptional cross talk between STAT4-estrogen/ERs-protein kinase signaling pathways. However, ER activation of STAT4 in splenocytes remains to be elucidated.

The occurrence of pSTAT4 β in nuclear extracts from splenocytes from estrogen-treated mice appears to be time dependent. Increased activation of pSTAT4 β was observed until 18 h, showed a tendency to decrease after 18 h, and entirely disap-

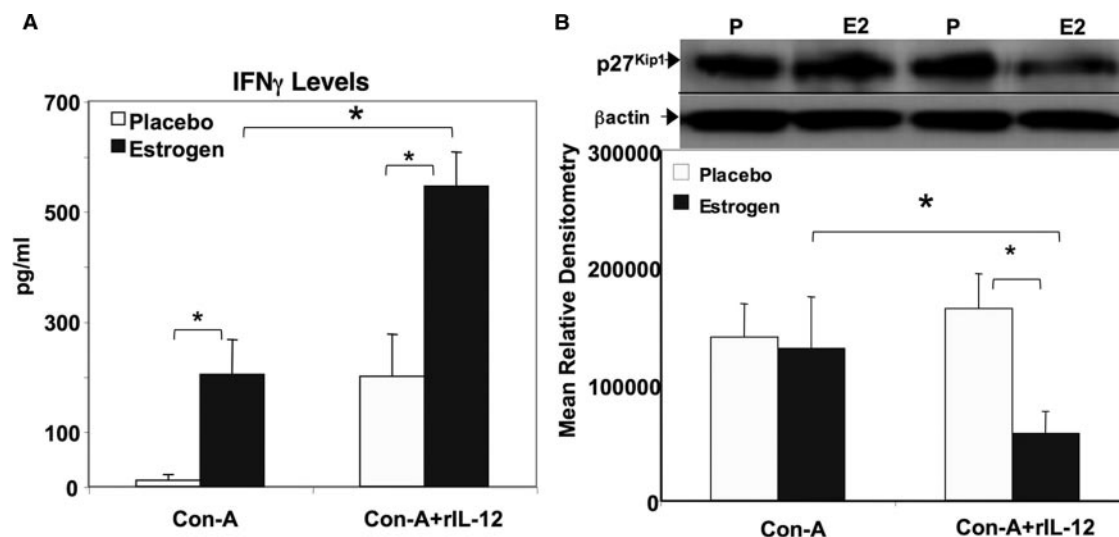


FIG. 7. Estrogen alters IL-12-mediated downstream events. **A**, Deliberate addition of IL-12 further up-regulates IFN γ from estrogen-treated mice. Splenocytes from estrogen (E2)- or placebo (P)-treated mice were stimulated with ConA or ConA plus rIL-12 (20 ng/ml) for 3 h. **A**, IFN γ protein levels in supernatants determined with an ELISA ($n = 6$ mice per placebo or estrogen; *, $P < 0.05$). IFN γ protein was not detectable in supernatants from unstimulated cells (media only; data not shown). **B**, STAT4 α -inducible p27^{Kip1} protein expression was diminished in response to addition of rIL-12. The expression of p27^{Kip1} was determined with Western blotting in cytoplasmic extracts. The top portion shows one representative experiment for p27^{Kip1} and β -actin. The bottom portion shows the densitometry data for p27^{Kip1} protein (placebo: $n = 4$ mice; estrogen: $n = 4$ mice; *, $P < 0.05$).

peared after 24 h. It is possible that IL-12-mediated responses may be blunted later due to decreased activation of STAT4 β (*i.e.* 24 h). In support of this view, other studies have illustrated that repeated and prolonged administration of IL-12 to human T cells results in decreased pSTAT4 and lower IFN γ levels (71).

The expression of STATs can be regulated at many levels, including alternative splicing at the posttranscriptional level. An alternatively spliced transcript for STAT4 β is expressed in human T-cell lines and primary murine T cells as a result of an insertion in the STAT4 α intron sequence, which creates a premature stop codon and generates the shorter STAT4 β transcript (28). The mRNA expression of both STAT4 α and STAT4 β in splenocytes from estrogen- and placebo-treated mice implies that estrogen does not affect expression of STAT4 at mRNA level but, rather, it alters the activation of STAT4 β protein.

Intriguingly, in contrast to the β -isoforms of STAT1 and STAT5, which are generally considered to be dominant negative regulators that inhibit STAT-related downstream responses (26, 53), both pSTAT4 α and β can positively regulate transcription of IL-12-induced target genes in Th1 lymphocytes (28). It is believed that pSTAT4 α modulates the levels of IL-12-induced IFN γ production in Th1 cells, whereas pSTAT4 β has a role in IL-12-stimulated proliferative and cellular responses, as well as activation of surface proteins that may be involved in cellular adhesion (28). On the other hand, a recent study has suggested that STAT4 β -transgenic mice, when induced to develop experimental autoimmune encephalomyelitis, expressed higher levels of IFN γ , IL-12, and IL-27 from splenocytes when compared with STAT4 α -transgenic mice (72). These observations (72) and our current data suggest that the activation and presence of STAT4 β is more critical than STAT4 α in the induction of IFN γ -inducing cytokine IL-12, IFN γ , and Th1 responses.

It is well known that activated STATs form dimers, translocate into the nuclei to bind to the target genes through IFN- γ -like

sequences (GAS), and induce their transcription (28, 73, 74). In addition, the β -isoform of STAT proteins is shown to have sustained activation for longer periods of time compared with its α -isoform after tyrosine phosphorylation (24, 28, 75). For instance, STAT3 β isoform had increased DNA-binding activity, delayed dephosphorylation (75), and increased transcription of target genes (76). Recent reports showed that STAT4 β from STAT4 β -transgenic mice remained activated for a longer time compared with STAT4 α observed from STAT4 α -transgenic mice (28). Interestingly, our current study demonstrates that in cells from placebo-treated mice, both pSTAT4 α and pSTAT4 β bound to the GAS elements, whereas estrogen preferentially induced the binding of pSTAT4 β to the GAS site. This implies that the estrogen-modulated IL-12-induced Th1 profile can be regulated through differential and sustained activation of STAT4 isoforms.

Intriguingly, several recent studies have suggested that different isoforms of STATs can have unique roles in the modulation of certain genes. For example, even though STAT5A and B are highly homologous in structure (*i.e.* TAT4 α and β), these isoforms have been shown to have differential functions. STAT5B-deficient mice had diminished NK cytolytic activity (77) and decreased numbers of CD4⁺CD25^{high}T cells (78). This was not evident in STAT5A-knockout mice. STAT4 β -transgenic experimental autoimmune encephalomyelitis mice (but not STAT4 α -transgenic mice) demonstrated specific induction of IFN γ -inducing cytokines and IFN γ (72), which can be attributed to the specific induction of target genes through the binding of activated STAT4 β dimers to GAS sites. Estrogen-induced specific DNA-binding of pSTAT4 β , but not pSTAT4 α , can very likely regulate the activation of genes with GAS binding sites and up-regulation of IFN γ protein expression. Although the precise molecular mechanism(s) is at present unclear, the possibility of estrogen-modulated predominant activation of pSTAT4 β may

be important for the determination of Th1/Th2 balance and the expression of proinflammatory biomolecules. This aspect will be the subject of future in-depth studies. Our findings in wild-type mice may have relevance to female-predominant autoimmune diseases.

IL-12-induced STAT4 α and β can stimulate a number of overlapping (28) as well as distinctive genes. One of the IL-12-responsive-STAT4 α -induced genes is the cell cycle inhibitor, p27^{Kip1} (28). The absence of p27^{Kip1} results in increased proliferation of STAT4-deficient T cells in response to IL-12 and up-regulation of IFN γ levels (79). *In vitro* estrogen treatment of mouse embryonic stem cells (80) or the breast cancer cell line, MCF-7 (81), resulted in down-regulation of p27^{Kip1}. We have shown that p27^{Kip1} protein was decreased in the presence of rIL-12 in ConA-activated splenocytes from estrogen-treated mice. It is possible that the decreased presence of pSTAT4 α , as consistently observed in ConA plus rIL-12-stimulated samples from estrogen-treated, but not control mice, may result in decreased expression of p27^{Kip1}. Although expression of p27^{Kip1} was lower in rIL-12-treated splenocytes from estrogen-treated mice compared with controls, the proliferation of splenocytes was not altered after 3 h culture as detected with propidium iodide analysis (data not shown), which may be attributed to the short incubation period. Because p27^{Kip1} is an inhibitor of cell growth, a promoter of apoptosis, as well as a modulator of tumor development in some lymphoid and endocrine cells, the estrogen-induced and IL-12-mediated down-regulation of p27^{Kip1} may lead to differential development and/or response of lymphocytes, and, thus, homeostasis of the immune system.

Our data demonstrating the immunomodulatory effect of estrogen on Th1-type proinflammatory molecules is consistent with that of Bao *et al.* (59), in which they demonstrated that estrogen-mediated IL-12-induced IFN γ and phosphorylation of STAT4. However, our study differs in several aspects compared with that of Bao *et al.* (59), in which purified CD4⁺T cells were incubated with estrogen (25 nM) *in vitro* for 20 h. Cells were rested and then treated with rIL-12 for 48 h to detect IFN γ or for 20 min to determine pSTAT4. IFN γ was up-regulated from CD4⁺T cells after IL-12 and *in vitro* estrogen treatment. In the study by Bao *et al.* (59), the antibody that was used to immunoprecipitate STAT4 binds to the C terminus of STAT4, and, therefore, only one band was evident (catalog no:07–913; Upstate Biotechnology, Waltham, MA) and would be able to detect only the α -isoform of STAT4 (28). In contrast, our data demonstrate the differential activation of pSTAT4 α/β with increasing incubation time.

In this study we chose to use splenic lymphoid cells rather than isolated purified cells to simulate *in vivo* splenic cellular interactions. It is important to recognize that in natural conditions in the spleen, various cellular subsets (APCs, T cells, B cells, NKs, and DCs) interact and cross-regulate each other for a collective immune response. Studying only purified subsets introduces an element of artificiality by removing cross-regulatory signals. The importance of using splenocytes rather than isolated purified subsets was supported in our previous findings in which we showed that the release of inflammatory molecules (nitric oxide and IFN γ) from splenocytes of estrogen-treated mice was mark-

edly diminished when only purified T cells were used or when interactions between T and APCs were molecularly blocked (12). Using purified subsets is beyond the scope of the present studies and will be a subject of future separate detailed studies.

Considering that IL-12 and IL-12-induced IFN γ have significant effects on the immune system, including defense against microbial agents, cellular differentiation, proliferation (14, 82), as well as in the pathogenesis of female-predominant autoimmune diseases (1, 2, 83), our studies add new knowledge in relation to IL-12-mediated signaling. These studies may have relevance to estrogen regulation of inflammatory and autoimmune conditions.

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