17 β -Estradiol downregulates interferon regulatory factor-1 in murine splenocytes

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Abstract

Interferon regulatory factor-1 (IRF-1) is an important transcription factor that mediates interferon- γ (IFN- γ)-induced cell-signaling events. In this study, we examined whether 17 β -estradiol alters IRF-1 in splenic lymphocytes, in view of the immunomodulatory effects of this natural female sex hormone including its ability to alter IFN- γ levels. We find that IRF-1 expression is markedly downregulated in splenocytes or purified T-cells from estrogen-treated mice at all time points studied when compared with their placebo counterparts. This decrease in IRF-1 in splenocytes from estrogen-treated mice is neither due to upregulation of IRF-1-interfering proteins (nucleophosmin or signal transducer and activator of transcription (STAT)-5) nor due to alternatively spliced IRF-1 mRNA. Given that IFN- γ is a potent inducer of IRF-1, direct addition of recombinant IFN- γ to splenocytes from either wild-type or IFN- γ -knockout mice, or the addition of recombinant IFN- γ to purified T-cells, was expected to stimulate IRF-1 expression. However, robust expression of IRF-1 in cells from estrogen-treated mice was not seen, unlike what was observed in cells from placebo-treated mice. Diminished IFN- γ induction of IRF-1 in cells from estrogen-treated mice was noticed despite comparable phosphorylated STAT-1 activation. These studies are the first to show that estrogen regulates IFN- γ -inducible IRF-1 in lymphoid cells, a finding that may have implications to IFN- γ -regulated immune and vascular diseases.

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Introduction

Interferon- γ (IFN- γ), a major cytokine derived from the immune system, acts on a broad range of lymphoid and non-lymphoid cells. IFN- γ elicits its biological effects on target cells by binding to specific IFN- γ receptors, which results in the activation of receptor associated kinases, such as janus kinase – signal transducer and activator of transcription (JAK-STAT; Bach *et al.* 1997). Specifically, upon the binding of IFN- γ to its receptor, the receptor-linked JAK1 and JAK2 become rapidly phosphorylated. These activated JAKs will then phosphorylate STAT-1. Activation of STAT-1 by IFN- γ is considered to be a primary IFN- γ -mediated downstream signaling event. Activated STAT-1 forms homodimers that translocate into the nucleus and bind to interferon- γ -activated sequence (GAS) elements in the promoters of interferon-responsive genes, such as interferon regulatory factor-1 (IRF-1), to activate their transcription (Remoli *et al.* 2002). The importance of STAT-1 in the induction of IRF-1 is demonstrated by the finding that cells from STAT-1 $^{-/-}$ mice do not upregulate IRF-1 in response to interferons (Ohmori & Hamilton 2001). While IRF-1 mRNA is expressed at low levels in diverse types of cells, these levels increase in response to many biological stimuli, including interferons α , β , and γ , Concanavalin A

(Con-A), interleukin (IL)-2, and tumour necrosis factor α or β (Kroger *et al.* 2002). IRF-1 in turn binds to IRF elements in the promoters of a number of genes including IL-15, which in turn upregulates IFN- γ (Ogasawara *et al.* 1998, Ohteki *et al.* 1998). Thus, IRF-1 may be part of a positive feedback loop for IFN- γ expression. IRF-1 binds to DNA via its amino-terminal end, which has five conserved tryptophan repeats. The carboxyl terminus, which contains many acidic residues and serine/threonine residues, is thought to be the transcriptional activation domain (Harada *et al.* 1989). This domain also includes multiple casein kinase II phosphorylation sites, though it is unclear whether phosphorylation of these sites modifies the transcriptional activity of IRF-1 (Lin & Hiscott 1999).

IRF-1 has also been associated with tumor suppressor activity (Tanaka *et al.* 1994a,b, Yim *et al.* 1997). Overexpression of IRF-1 is associated with growth suppression of human breast cancer cells and decreases the tumorigenicity of those cells when placed in athymic nude mice (Bouker *et al.* 2005). Artificial overexpression of IRF-1 in Ltk $^{-}$ (Certified Cell Line (CCL) 1.3) and mouse fibroblastoid C243 cells led to inhibition of cell growth (Kirchhoff *et al.* 1993). Loss of IRF-1 leads to increased tumorigenicity and IRF-1 has been shown to be absent or altered in some hematopoietic cancers

(Willman *et al.* 1993, Nozawa *et al.* 1999). An analysis of breast tumors showed that the cytosolic form of IRF-1, thought to be inactive, predominates compared with the active, nuclear form (Zhu *et al.* 2006).

Several recent studies in non-lymphoid tissues have suggested that IRF-1 can be regulated hormonally. Exposure of human mammary epithelial cells (HMEC-E6) with DNA damage to tamoxifen induced IRF-1 mRNA (Bowie *et al.* 2004). Prolactin has been shown to induce IRF-1 in Nb2 T lymphoma cells (Yu-Lee *et al.* 1990). Recent studies have also suggested that IRF-1 is one of the proteins that affects endocrine responsiveness (Zhu *et al.* 2006). It is therefore plausible that estrogen may also regulate IRF-1 in lymphoid tissues. To date, no studies have addressed this aspect. These studies are particularly relevant considering estrogen is a potent immunomodulator (Olsen & Kovacs 1996, 2002, Ahmed *et al.* 1999, Ahmed 2000) and has been shown to upregulate IFN- γ (Sarvetnick & Fox 1990, Maret *et al.* 2003). The present studies address this critical gap in the literature and report novel observations that estrogen downregulates IRF-1 in splenocytes.

Materials and methods

Mice and splenic lymphocytes

Male, C57BL/6, wild-type mice from Charles river laboratories (Wilmington, MA, USA), were used except where it is noted that male C57BL/6 IFN γ ^{-/-} (bred at Virginia–Maryland Regional College of Veterinary medicine) knockout mice were used. At 4- to 5-weeks of age, mice were orchietomized and surgically implanted with silastic capsules containing 17 β estradiol or empty (placebo) silastic implants, by standard procedures that have been extensively described previously (Ahmed & Verthelyi 1993, Karpuzoglu-Sahin *et al.* 2001). Briefly, 4–5 mm of silastic medical grade tubing (0.062 in. internal diameter $\times$ 0.125 in. outer diameter) was packed with estrogen and the flanking ends were plugged with sterile wooden plugs and sealed with silicone. The animals were anesthetized with ketamine/xylazine and orchietomized as reported previously. Implants (estrogen or placebo) were surgically placed through an incision on the lower back and the implants were pushed to the neck region. The surgical wounds were closed with wound clips and in our experience the animals recover within a few hours. Our Institutional Animal Care Committee approved all procedures on mice. Mice were kept in the Center for Molecular Medicine and Infectious Diseases animal facility, fed a commercial pellet diet devoid of estrogenic hormones (7013 National Institute of Health (NIH)-31 modified 6% mouse- or rat-sterilizable diet), given water *ad libitum*, and housed three to five animals per cage. The mice were euthanized by cervical dislocation at 5–7 weeks

after treatment. Splenic lymphocytes were isolated with ammonium chloride potassium (ACK)–Tris–NH₄Cl lysis buffer per our previous studies, and cultured in Roswell Park Memorial Institute (RPMI)-1640 media devoid of estrogenic phenol red (CellGro, Mediatech, Herndon, VA, USA), and supplemented with steroid-free 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA), 2 mM L-glutamine (ICN, Costa Mesa, CA, USA), 50 IU/ml penicillin (Mediatech), 50 μ g/ml streptomycin (Mediatech), and non-essential amino acids (Fisher, Pittsburgh, PA, USA). Five hundred microliters of splenic lymphocytes (5×10^6 cells/ml) from estrogen- and placebo-treated mice were stimulated for various time points from 45 min to 48 h with 500 μ l purified anti-CD3 antibodies (10 μ l/ml, (14-0032-86, eBioscience, San Diego, CA, USA)), Con-A (10 μ l/ml, (C0412, Sigma)), recombinant mouse IFN- γ (100, 1000, or 10 000 pg/ml as designated) (554587 BD Pharmingen, San Jose, CA, USA), or left unstimulated in media alone. Cells were then harvested and used for the following described experiments.

Isolation and purification of splenic T-lymphocytes

The purification of T-lymphocytes from fresh whole splenocyte samples from estrogen- and placebo-treated mice was performed as per the manufacturer's instructions (EasySep, Mouse T Cell Enrichment Kit; #19751; StemCell Technologies) using the RoboSep Cell automated magnetic cell separator (StemCell Technologies, Seattle, WA, USA). The purity of the isolated T-cells was confirmed by staining cells with the following monoclonal antibodies: fluorescein isothiocyanate- or phycoerythrin-(PE) conjugated anti-Thy1.2 (CD90.2, clone 53-21), and anti-CD45RB (B220; clone RA3-6B2; eBioscience Inc.) and conducting flow cytometry. For the detection of cell-surface markers on purified T-lymphocytes, 100 μ l of each fluorochrome-conjugated monoclonal antibody in PBS were added to each well followed by incubation at 4 °C in the dark for 30 min. Samples were washed with PBS followed by analysis on an EPICS XL-MXL flow cytometer (Coulter, Hialeah, FL, USA). Results from flow cytometry showed that the negative isolation of T-cells resulted in 97% purity in T-cells.

Nuclear extraction

Nuclear extracts from mixed splenocytes were prepared by lysing 5×10^6 cells in 400 μ l buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and mammalian protease inhibitor cocktail) for 15 min on ice, after which 25 μ l of 10% Nonidet P-40 were added. The lysates were vortexed briefly, nuclei pelleted by centrifugation, and the supernatants discarded. Pelleted nuclei were

resuspended in buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and mammalian protease inhibitor cocktail), vortexed, and incubated on ice for 30 min. Lysates were then centrifuged and the supernatants (nuclear extracts) were collected and stored at -80°C for use in western blotting. Nuclear and cytoplasmic extracts from purified T-cells were prepared from 2.5×10^6 T-cells cultured with Con-A (10 $\mu\text{g}/\text{ml}$) with or without recombinant IFN- γ (10 ng/ml) in a 24-well plate incubated for 3 h at 37°C according to the manufacturer's instructions (NE-PER kit, Pierce Biotechnology, Inc., Rockford, IL, USA). Twenty micrograms of the nuclear extracts from cultured cells were used for western immunoblotting.

Western blot

Nuclear extracts or whole cell lysates were mixed with an equal volume of $2 \times$ Laemmli sample buffer and boiled for 5–10 min. The samples from purified T-cells were electrophoresed on a SDS-7.5% PAGE at 25 mA constant current until the dye ran off the bottom. The proteins were transferred to polyvinylidene fluoride (PDVF) transfer membranes (Amersham Biosciences) by blotting for 1 h and 30 min at 240 mA constant current. After transfer, the membranes were blocked in 3% BSA in Tris buffered saline-tween (TBST) for 1 h at room temperature. All other samples were electrophoresed on a SDS-10% PAGE and transferred to a PVDF membrane (Bio-Rad). These membranes were blocked with Tris buffered saline (TBS) with 0.1% Tween-20 and 5% dried non-fat milk for 1 h at room temperature on a shaking platform. The membranes were then incubated with optimal concentrations of polyclonal antibodies specific to IRF-1 (1:2000), STAT-1 (1:2000), STAT-5 α/β (1:2000), nucleophosmin (1:1000), or β -actin (1:8000) diluted in TBS with 0.1% Tween-20 and 5% dried non-fat milk overnight at 4°C on a shaking platform. Antibodies reactive against IRF-1, STAT-1, STAT-5, and nucleophosmin were purchased from Santa Cruz (Santa Cruz, CA, USA); the antibody against β -actin was purchased from AbCam (Cambridge, MA, USA). The membranes were washed for 10 min, three times in TBS with 0.1% Tween-20, incubated with the appropriate secondary antibody diluted in TBS with 0.1% Tween-20 and 5% dried non-fat milk for 1 h at room temperature on a shaking platform. They were then washed for 10 min five times in TBS with 0.1% Tween-20 and developed using ECL-Plus Reagents (Amersham). Bands were visualized and quantitated using a Kodak Image Station 440F. HeLa cell extracts (Cell Signaling Technology, Danvers, MA, USA) were used as a positive control for STAT-1.

RNA isolation

RNA was isolated from murine splenocytes using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Briefly, $5\text{--}10 \times 10^6$ cells were pelleted by centrifugation and lysed in 1 ml of TRI Reagent. After incubating for 5 min at room temperature, 0.2 ml chloroform was added to each sample and shaken vigorously for 15 s. The samples were incubated at room temperature for 10 min and centrifuged at $12\,000g$ for 15 min at 4°C . The aqueous phase was transferred to a new tube, mixed with 0.5 ml isopropanol, and incubated at room temperature for 15 min. RNA was then pelleted by centrifugation at $12\,000g$ and 4°C for 8 min. The supernatant was decanted and the RNA pellet was washed once in 1 ml 75% ethanol. The samples were centrifuged briefly at $12\,000g$ at 4°C , the ethanol wash decanted, and the pellets air-dried for 5 min. RNA pellets were resuspended in RNase-free water and quantitated using a RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions.

Real-time PCR

In each reaction, 500 ng total RNA were used. Reverse transcription and PCRs were performed using the QuantiTect SYBR Green RT PCR kit (Qiagen) according to the manufacturer's instructions. Real-time PCR was carried out on an iCycler machine (Bio-Rad) programmed for 40 cycles of 95°C for 20 s, 58°C for 30 s, and 72°C for 45 s. The primers used were as follows: IRF-1: 5' TCT GAG TGG CAT ATG CAG ATG GAC; 3' GGT CAG AGA CCC AAA CTA TGG TGC; IRF-1 (spanning exons 2 and 3): 5' TCG GGC ATC TTT CGC TTC GT; 3' GAT GTC TGG CAG GGA GTT CA; 18s rRNA: 5' CGA CGA CCC ATT CGA ACG TCT; 3' GCT ATT GGA GCT GGA ATT ACC G; β -actin: 5' TGG AAT CCT GTG GCA TCC ATG AAA C; 3' TAA AAC GCA GCT CAG TAA CAG TCC G.

Cytometric bead assay

Whole cell lysates were prepared according to the manufacturer's instructions (BD Pharmingen). The lysates were incubated with fluorescent beads coated with a capture antibody specific for STAT-1 phosphorylated on tyrosine 701 and with a PE-conjugated detection antibody. The beads were then washed and analyzed on a flow cytometer. Serial dilutions of a phosphorylated STAT-1 standard were also analyzed to generate a standard curve in order to quantitatively measure levels of phosphorylated STAT-1 (phosphorylated at tyrosine residue 701) in denatured cell lysate samples, which were analyzed using a Coulter Epics XL

flow cytometer and BD CBA software (BD Biosciences, San Diego, CA, USA).

Statistical analysis

One-way ANOVA with Tukey–Kramer multiple comparisons post test was performed using GraphPad InStat version 3.0a for Macintosh, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

Results

IRF-1 is downregulated in splenocytes from estrogen-treated mice, at all time points studied

Splenocytes from estrogen- or placebo-treated mice were cultured for 24 h in the absence of any stimulation (media only) or in the presence of T-cell stimulants, anti-CD3 antibodies, or Con-A. Both Con-A and anti-CD3 activation of splenocytes induced strong expression of IRF-1 in placebo-treated mice. Interestingly, mitogen-activated splenocytes from estrogen-treated mice consistently displayed suppressed levels of IRF-1 compared with placebo-treated controls (Fig. 1A and B). In Fig. 1A and B, representative blots (IRF-1 and β -actin) are shown above graphs displaying densitometry values (mean $\pm$ s.e.m.) for IRF-1. Stimulation with anti-CD3 resulted in significantly increased (* denotes $P < 0.001$) expression of IRF-1 in placebo-treated mice compared with estrogen-treated mice (Fig. 1A; $n = 9$ placebo; $n = 7$ estrogen, media only; $n = 10$ estrogen, anti-CD3). Likewise, Con-A stimulation caused significant induction ($P < 0.001$) of IRF-1 in placebo, but not estrogen-treated mice (Fig. 1B; $n = 18$ placebo; $n = 16$ estrogen, media; $n = 17$ estrogen Con-A). Since similar results were obtained whether splenocytes were stimulated with Con-A or anti-CD3 antibodies, we chose to use Con-A for subsequent experiments. Activation of splenocytes was necessary to induce IRF-1, since in unstimulated cultures, IRF-1 was not detectable in cells from estrogen-treated mice and was only weakly expressed in cells from placebo-treated mice.

Since IRF-1 has a very short half-life (about 30 min), it was important to examine the kinetics of its expression

in freshly isolated splenocytes and at various time points in order to avoid missing a possible transient increase in IRF-1 expression, followed by a decrease. Therefore, splenocytes were examined immediately after isolation and at various time points after culture. In freshly isolated splenocytes (i.e. prior to culture and hence unstimulated), IRF-1 was detectable in placebo-treated mice, but not in splenocytes from estrogen-treated mice (Fig. 1C). Furthermore, in the aliquots of splenocyte cultures, either left unstimulated (media only) or stimulated with Con-A, at every culture time point examined (45 min, 1 h 30 min, 2 h 15 min, 3 h, 6 h, 8 h, and 24 h of culture), the expression of IRF-1 was decreased in Con-A-activated splenocytes from estrogen-treated mice when compared with similar cultures from placebo-treated mice (Fig. 1D).

At 48 h of culture, Con-A-activated splenocytes from estrogen-treated mice had only weak expression of IRF-1 compared with strong expression of this transcription factor in activated splenocytes from placebo-treated mice (Fig. 1E). Additionally, in separate studies, Con-A-activated splenocytes cultured for 18 h also demonstrated markedly decreased expression of IRF-1 in cells derived from estrogen-treated mice when compared with placebo control cultures (data not shown). Overall, splenocytes from estrogen-treated mice, when examined immediately after isolation up to 48 h of culture, consistently demonstrated markedly decreased ability to induce IRF-1 compared with placebo-treated mice.

IFN- γ upregulates IRF-1 in splenocytes from placebo-, but not estrogen-treated mice

Since IRF-1 is inducible by IFN- γ , splenocytes from estrogen- or placebo-treated mice were cultured with or without the addition of recombinant IFN- γ (100 or 1000 pg/ml) for 6 h, after which whole cell lysates were prepared to determine IRF-1 protein levels. Con-A was not added to the cultures of wild-type cells since it is known to induce IFN- γ (especially in estrogen-treated mice) and we wished to examine the effects of added recombinant IFN- γ . Figure 2A shows that IRF-1 is only induced by IFN- γ in splenocytes from placebo-treated mice. In contrast, even the deliberate addition of IFN- γ

Figure 1 IRF-1 is downregulated in splenocytes from estrogen-treated mice at all time points studied as determined by western blots. (A) and (B) Culture of splenocytes from placebo- (P) or estrogen- (E) treated mice with anti-CD3 antibodies (A) or Con-A (B) for 24 h resulted in significant (* denotes $P < 0.001$) induction of IRF-1 in placebo-treated mice only (A: $n = 9$ placebo; $n = 7$ estrogen, media; $n = 10$ estrogen, anti-CD3) (B: $n = 18$ placebo; $n = 16$ estrogen, media; $n = 17$ estrogen, Con-A). Data are presented as mean relative densitometry values with standard error bars accompanied by a representative blot. (C) IRF-1 is decreased in freshly isolated, unstimulated splenocytes from estrogen-treated mice compared with placebo controls (representative of $n = 4$). (D) A kinetic analysis of IRF-1 was performed and at all time points studied, estrogen-treated mice had lower levels of IRF-1 than their placebo-treated controls (representative blots of $n = 4$ each). (E) Splenocytes from placebo- or estrogen-treated mice were cultured with or without Con-A for 48 h (representative of $n = 8$ each).

A IRF-1 24 hrs Stimulated with Anti-CD3

B IRF-1 24 hrs Stimulated with Con-A

C IRF-1 in Freshly isolated, unstimulated splenocytes

D IRF-1 Time kinetics

E IRF-1 48 hrs Stimulated with Con-A

did not result in upregulation of IRF-1 in splenocytes from estrogen-treated mice.

We next employed IFN- γ knockout mice (IFN- γ ^{-/-}) to examine the potential role of IFN- γ in the induction of IRF-1 and exclude the possibility that estrogen-induced IFN- γ *in vivo* in the wild-type mice may have triggered a negative feedback loop, dampening the expression of IRF-1. Using western blot analysis of whole cell lysates, IRF-1 expression was examined in splenocytes from these mice cultured for 24 h with media alone (unstimulated cells), Con-A, or Con-A plus various doses

of recombinant IFN- γ . Figure 2B shows the mean densitometry results for these experiments as well as a representative blot. As expected, the IRF-1 levels in media or Con-A-activated splenocytes from placebo-treated IFN- γ -knockout mice were markedly decreased compared with placebo-treated wild-type mice (as shown in Fig. 1), demonstrating an important role of IFN- γ in the induction of IRF-1. This was further substantiated by the observation that intentional addition of recombinant IFN- γ to Con-A-activated splenocytes from placebo-treated IFN- γ -knockout mice exhibited a pronounced increase in IRF-1 protein expression. Importantly, IRF-1 expression failed to be comparably upregulated in splenocytes from estrogen-treated mice. Even when the cells were cultured with recombinant IFN- γ (100 pg or 1000 pg/ml), IRF-1 expression was significantly higher ($P<0.001$) in placebo-treated mice compared with estrogen treated mice (Fig. 2B; $n=2$ for placebo; $n=3$ for estrogen, except media $n=1$ and Con-A+100 pg IFN- γ $n=2$). Similar results were obtained after 18 h of culture (data not shown). These results reveal that estrogen treatment alters the expected response of splenocytes to IFN- γ .

Figure 2 IRF-1 expression after deliberate addition of IFN- γ to splenocyte cultures from wild-type and IFN- γ -knockout mice. (A) Culture of splenocytes from wild-type placebo- (P) or estrogen- (E) treated mice ($n=6$ each) with recombinant IFN- γ for 6 h resulted in upregulation of IRF-1 only in cells from placebo-treated mice. (B) Culture of splenocytes from placebo- (P) or estrogen- (E) treated IFN- γ -knockout mice ($n=3$ each) with Con-A and recombinant IFN- γ (100 or 1000 pg/ml) for 24 h caused significantly (* denotes $P<0.001$) increased expression of IRF-1 only in placebo-treated mice. Data are presented as mean relative densitometry values with standard error bars accompanied by a representative blot.

IFN- γ upregulates IRF-1 in T-cells from placebo-, but not estrogen-treated mice

Next, T-cells from placebo- or estrogen-treated mice were purified from splenocytes and cultured in the presence of either Con-A alone or Con-A plus IFN- γ and the expression of IRF-1 was determined. Unstimulated cultures (media only) served as controls (Fig. 3A). As shown in Fig. 3B, where representative blots for IRF-1 and β -actin as well as mean densitometry values $\pm$ S.E.M. for IRF-1 ($n=6$ placebo and estrogen) are shown, IRF-1 was rapidly and significantly ($P<0.01$) induced in Con-A-stimulated purified T-cells from placebo-treated mice but not in cultures from estrogen-treated mice. Addition of recombinant IFN- γ (10 000 pg/ml) to aliquots of these cultures significantly ($P<0.001$) increased IRF-1 expression in T-cells from placebo-treated mice compared with estrogen-treated mice. Importantly, even the deliberate addition of recombinant IFN- γ to purified T-cell cultures from estrogen-treated mice did not robustly induce IRF-1 unlike those from placebo cultures (Fig. 3B).

Estrogen does not alter STAT-1 phosphorylation in splenocytes at 24 h

Since IRF-1 was not robustly upregulated in IFN- γ -exposed splenocytes from estrogen-treated mice, it implied that estrogen impairs IFN- γ downstream signaling events. To investigate this issue, we next examined whether splenocytes from estrogen-treated

A IRF-1 in Purified T cells
No stimulation

B IRF-1 in Purified T cells
Stimulated with Con-A and recombinant IFN γ

Figure 3 IFN- γ upregulates IRF-1 in T-cells from placebo-, but not estrogen-treated mice. (A) Unstimulated, purified T-cells from placebo-treated mice express increased IRF-1 compared with estrogen-treated mice. (B) After 3 h of culture with Con-A or Con-A and recombinant IFN- γ (10 000 pg/ml), the protein expression of IRF-1 was significantly (* denotes $P < 0.01$ for Con-A, † denotes $P < 0.001$ for Con-A and recombinant IFN- γ) increased in nuclear extracts of purified T-cells from placebo-treated mice compared with estrogen-treated mice ($n = 4$ each). Data are presented as mean relative densitometry values with standard error bars accompanied by a representative blot.

mice have diminished ability to activate STAT-1 (a transcription factor involved in downstream signaling in response to IFN- γ). Splenocytes from estrogen- or placebo-treated mice were cultured in the presence or

absence of Con-A for 24 h and nuclear extracts were used to access the levels of activated STAT-1 protein by western blot analysis. As demonstrated by a representative blot and densitometry in Fig. 4A ($n = 3$ placebo and estrogen), we found that activated STAT-1 levels were not markedly altered in splenocytes from estrogen-treated mice at 24 h ($P > 0.05$ for (placebo-Con-A versus estrogen-Con-A) for STAT-1 α and STAT-1 β). Similar results were obtained when western blots were performed using whole cell lysates probed with an antibody specific for tyrosine-phosphorylated STAT-1 (data not shown) and serine-phosphorylated STAT-1 (Fig. 4B). To confirm these results, we also analyzed the level of phosphorylated (and therefore activated) STAT-1 using a flow cytometric bead assay. Our data from these experiments show that lysates from estrogen- and placebo-treated mice do not differ significantly in phosphorylated STAT-1 levels (Fig. 4C, $n = 11$ placebo and estrogen) thus suggesting that decreased IRF-1 in estrogen-treated mice is not due to impaired STAT-1 activation at 24 h of culture.

Decreased IRF-1 is not due to upregulation of IRF-1 interfering factors

To determine a possible mechanism by which estrogen treatment might result in decreased IRF-1 protein levels, we checked to see whether estrogen upregulates the IRF-1 interfering factors nucleophosmin (B23) and STAT-5. Estrogen has been shown in vascular smooth muscle cells (Koike *et al.* 1996) and MCF-7 breast cancer cells to induce the expression of nucleophosmin, a non-ribosomal nuclear phosphoprotein, known to bind to proteins containing nuclear localization sequences, including IRF-1 thereby suppressing IRF-1 activity (Kondo *et al.* 1997, Skaar *et al.* 1998, Hsu & Yung 2000, Gu *et al.* 2002). Our data show that nucleophosmin was not detectable in nuclear extracts of splenocytes from either placebo- or estrogen-treated mice, regardless of the expression levels of IRF-1 in these cells. (Fig. 5A).

Studies have shown that both STAT-5 α and STAT-5 β bind to the IRF-1 GAS element, inhibit the IRF-1 promoter, and suppress transcription of IRF-1 in some cell types (Luo & Yu-Lee 1997, Yu-Lee 2002). To address the possibility that this mechanism was responsible for the decreased levels of IRF-1 in estrogen-treated mice, western blots were performed on nuclear extracts from Con-A-activated and unstimulated splenocytes from placebo- and estrogen-treated mice. We found that the levels of STAT-5 in nuclear extracts are significantly decreased (* denotes $P < 0.05$) in splenocytes from estrogen-treated mice cultured for 24 h with Con-A (Fig. 5B; $n = 6$ placebo; $n = 4$ estrogen, media; $n = 5$ estrogen, Con-A), similar to the observed pattern for IRF-1. Comparable results were also obtained when western blots were performed on whole cell lysates

Figure 4 Estrogen does not alter STAT-1 at 24 h. (A) Activated STAT-1 is present in similar amounts in splenocytes from both estrogen-treated mice and placebo-treated controls ($n=3$ each) at 24 h of culture. Data are presented as mean relative densitometry values with standard error bars accompanied by a representative blot. (B) Protein expression of both serine-phosphorylated STAT-1 (top) and total STAT-1 (bottom) is unchanged in whole cell lysates of splenocytes from placebo- versus estrogen-treated mice. (C) Cytometric bead array analysis also demonstrates that levels of phosphorylated STAT-1 in splenocyte extracts from placebo- or estrogen-treated mice are not different. Error bars represent standard error of the mean ($n=11$ each).

Figure 5 Neither nucleophosmin nor STAT-5 is detectable in nuclear extracts from splenocytes. (A) Nucleophosmin (B23) protein is not detectable at 24 h of culture (representative of $n=11$ each), regardless of the expression level of IRF-1 (representative of $n=16$ each). HeLa cell nuclear extracts were included as a positive control for nucleophosmin. (B) STAT-5 is significantly (* denotes $P<0.05$) decreased in splenocytes from estrogen-treated mice when compared with placebo-treated controls ($n=6$ each). Data are presented as mean relative densitometry values with standard error bars accompanied by a representative blot.

using an antibody specific for phosphorylated STAT-5 (data not shown). Therefore, it is unlikely that transcriptional inhibition by STAT-5 is the mechanism responsible for the decrease in IRF-1 protein levels in splenocytes from estrogen-treated mice.

Estrogen regulation of IRF-1 transcription

We examined whether exon skipping could be a mechanism for the downregulation of IRF-1 protein and mRNA expression seen in our experiments. This rationale is based on the observation that humans expressing high levels of aberrantly spliced IRF-1 mRNAs were also found to express reduced levels of full-length IRF-1 transcript (Harada *et al.* 1994, Green *et al.* 1999, Tzoanopoulos *et al.* 2002). Primers were chosen that span exons 2 and 3 of the *IRF-1* gene in order to detect the possible presence of alternate transcripts lacking these exons and real-time PCR was conducted on RNA from freshly isolated, unstimulated

splenocytes harvested from estrogen- or placebo-treated mice. A full-length transcript amplified by this set of primers would generate a PCR product of 373 bp. A transcript missing exon 2 would result in a PCR product of 281 bp, while a transcript missing both exons 2 and 3 would result in a PCR product of 181 bp. To detect the presence of alternately spliced IRF-1 transcripts, the SYBR green method of real-time PCR was performed using this primer set and a melt curve was performed. We used a sensitive melt-curve technique to check for alternative transcripts because this method has been widely cited as sensitive and reliable (Ririe *et al.* 1997, Bernard & Wittwer 2000, Rodriguez *et al.* 2002, Dufresne *et al.* 2006, Schneider *et al.* 2006). Primers for 18s rRNA and β -actin were both employed as endogenous controls (Fig. 6A). The presence of a single melt-curve peak indicates a single product from PCR (Fig. 6B). Together, these data show that exon skipping is not the mechanism responsible for the decrease in IRF-1 levels in estrogen-treated mice. As reported earlier in the mouse *IRF-1* gene

(Harada *et al.* 1994), exon skipping was also not observed in this study.

Discussion

In this study, we show that IRF-1 protein levels are markedly decreased in splenocytes from estrogen-treated mice compared with placebo-treated controls after *in vitro* stimulation with T-cell stimulants. This decrease in IRF-1 in splenocytes from estrogen-treated mice was seen at all culture time points examined, including freshly isolated, unstimulated splenocytes. Further, purified T-cells from estrogen-treated mice also demonstrated diminished ability to induce IRF-1 after Con-A stimulation. To our knowledge, this is the first study to show that estrogen downregulates IRF-1 in lymphoid cells. These findings are in agreement with the recently reported studies in non-lymphoid MCF-7 cells, in which exposure to estradiol significantly

Figure 6 Transcriptional regulation of IRF-1 does not involve exon skipping but does appear to be downregulated. (A) Real-time PCR was performed, on samples of RNA from fresh, unstimulated splenocytes, using primers for β -actin or IRF-1, as indicated. (B) Real-time PCR was performed using primers spanning exons 2 and 3. Melt curves are presented to show the absence of alternately spliced transcripts. ($n=4$ placebo; $n=5$ estrogen for both A and B).

downregulated IRF-1 mRNA expression in MCF-7 cells (Bouker *et al.* 2004). These studies, using co-cultures of estradiol and ICI 182 780, a high-affinity estrogen receptor antagonist, implied that estradiol affected IRF-1 cells via the estrogen receptor. Other studies have also illustrated a correlation between cytosolic (likely inactive) IRF-1 and estrogen receptor- α expression (Zhu *et al.* 2006). This implies that estrogen may be acting via the α -form of the estrogen receptor to downregulate IRF-1.

The decreased IRF-1 in splenocytes from estrogen-treated mice, which were activated with T-cell stimulants, was surprising considering that we and others have shown that estrogen promotes IFN- γ , a cytokine that induces IRF-1 (Fox *et al.* 1991, Karpuzoglu-Sahin *et al.* 2001, Maret *et al.* 2003, Gourdy *et al.* 2005, Nakaya *et al.* 2006). The importance of IFN- γ in IRF-1 induction is evidenced by the findings that IRF-1 was markedly decreased in Con-A-activated splenocytes from IFN- γ ^{-/-} mice. While the overall levels of IRF-1 were reduced in these mice compared with wild-type mice, a further decrease of IRF-1 was seen in splenocytes from estrogen-treated IFN- γ -knockout mice when compared with those from placebo-treated IFN- γ knockout mice. Importantly, the addition of recombinant IFN- γ to splenocytes resulted in a dramatic increase in IRF-1 protein levels in placebo-treated IFN- γ knockout mice, but not in estrogen-treated knockout mice. Comparable results were also evident in wild-type mice. Similar results were also evident when recombinant IFN- γ was added with Con-A to purified T-cells from estrogen-treated mice. It is noteworthy that the decreased response of lymphocytes from estrogen-treated mice to IFN- γ was not due to diminished activation of STAT-1, since the levels of STAT-1 phosphorylation, as measured by the western blots of nuclear extracts and by the flow cytometry assay, were comparable between cells from estrogen- and placebo-treated mice. These results suggest that estrogen inhibition of IRF-1 may be independent of STAT-1. The diminished response (observed here as a lack of IRF-1 induction) of splenic lymphocytes to IFN- γ from estrogen-treated mice appears to be selective, since we have recently shown that the direct addition of recombinant IFN- γ to splenocytes from either estrogen-treated wild-type or IFN- γ knockout mice markedly increased iNOS protein or nitric oxide when compared with placebo-treated mice (Karpuzoglu *et al.* 2006). It is also noteworthy that in recent studies we find that splenic T-cells from estrogen-treated mice when exposed to IFN- γ upregulate T-bet expression compared with similar cultures from placebo-treated mice (unpublished observation). Together, these results demonstrate that estrogen selectively alters the response of splenic lymphocytes to IFN- γ .

The observed estrogen-induced decrease in IRF-1 in the present study does not appear to be due to upregulation of the IRF-1 binding factor nucleophosmin or of IRF-1 interfering STAT-5. In our experiments, we found that nucleophosmin was undetectable and therefore seems an unlikely explanation for the estrogen-mediated decrease in IRF-1 levels. In addition, we found that STAT-5 levels are decreased in the nuclear extracts of splenocytes from estrogen-treated mice; hence, STAT-5-mediated transcriptional inhibition seems to be an unlikely mechanism for the decreased IRF-1 levels as well. The precise reasons underlying this estrogen-induced decrease in STAT-5 are not known and may be independent events. In other recent studies involving non-lymphoid systems, ER α and ER β were found to physically interact with STAT-5 and potentially repress PRL-induced STAT-5 activation using a β -casein promoter construct (Faulds *et al.* 2001). These findings support and provide a potential mechanism for our observation of decreased STAT-5 in the nuclear extracts of splenocytes from estrogen-treated mice. It is likely that IRF-1 in splenocytes from estrogen-treated mice may interfere with other transcription factors that were not studied.

Increased frequency of alternate IRF-1 transcripts, in which exon 2, or exons 2 and 3, have been reported in humans with myelodysplastic syndrome or chronic myeloid leukemia (Harada *et al.* 1994, Green *et al.* 1999, Tzoanopoulos *et al.* 2002). These alternate transcripts are missing the normal translational start site, which is found in exon 2. Patients expressing high levels of aberrantly spliced IRF-1 mRNAs were also found to express reduced levels of full-length IRF-1 transcript compared with healthy individuals, suggesting that exon skipping may be an important mechanism of tumor-suppressor gene inactivation in hematopoietic malignancies. Likewise, exon skipping could be a mechanism for downregulation of IRF-1 protein and mRNA expression. Our studies show that estrogen does not promote accelerated exon skipping of the IRF-1 gene in estrogen-treated mice. A previous study, involving RT-PCR analysis of IRF-1 using mouse primers in R27-3 (a mouse NIH3T3-derived cell line) and BAF/B03 (a mouse hematopoietic cell line), suggested that exon skipping may be unique to the human *IRF-1* gene (Harada *et al.* 1994). Accelerated exon skipping was not observed in the mouse cell lines. It was only observed in human samples. Harada *et al.* only observed the full-length IRF-1 transcript in the mouse cell lines when using mouse-specific primers. However, the exon skipped forms were observed in the R27-3 cell line, when it was transfected with the human IRF-1 gene and human primers were used. In our study, we wished to check for the presence of alternate transcripts of IRF-1 in splenocytes from C57BL/6 mice. We found no differences in RNA end products between placebo-versus estrogen-treated mice.

Together, our studies are the first to show that estrogen downregulates the ability of IFN- γ to induce IRF-1 in splenic cells. Since both estrogen and IFN- γ act on diverse lymphoid tissues and vascular tissues, these studies have implications to immune and vascular diseases since they suggest that transcription factors other than IRF-1 may act to mediate events downstream of IFN- γ .

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