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IFN- γ -Inducing Transcription Factor, T-Bet is Upregulated by Estrogen in Murine Splenocytes: Role of IL-27 but not IL-12[#]

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Abstract

Estrogen is believed to be involved in regulation of the differentiation, survival, or function of diverse immune cells as well as in many autoimmune and inflammatory diseases. However, the mechanisms behind the immunomodulatory effects of estrogen are poorly understood. Previously, we have shown that natural estrogen can upregulate IFN- γ and IFN- γ -mediated-inflammatory events (iNOS, nitric oxide, COX-2). Since IFN- γ is regulated by T-bet, in this study, we investigated whether estrogen induces T-bet expression in primary murine splenocytes. We found that *in vivo* estrogen treatment primes splenocytes for early upregulation of T-bet upon activation by T cell stimulants, Concanavalin-A (Con-A) or anti-CD3 antibodies. The expression of T-bet protein was not altered by IL-12 while IFN- γ had partial effects on T-bet in splenocytes from estrogen-treated mice. Notably, T-bet expression increased the most in Con-A-activated splenocytes from estrogen-treated mice in the presence of IL-27. Together, our studies show that *in vivo* estrogen exposure primes lymphocytes towards Th1 type development by promoting/upregulating T-bet expression, which is upregulated in part by IFN- γ and IL-27. Given that T-bet is a potent inducer of IFN- γ , these studies may lead to new lines of investigation in relation to many female-predominant autoimmune diseases and inflammatory disorders.

Keywords

T-bet; IFN- γ ; IL-27; IL-12p70; Estrogen; T cells; Splenocytes

Introduction

The immune system exquisitely and effectively responds to external challenges by calibrating the most effective type of immune response. For example, an effective immune response to intracellular pathogens involves the induction and promotion of interferon-gamma (IFN- γ), which is secreted by T helper 1 (Th1) subsets of cells as well as other cell types such as natural killer (NK), invariant natural killer T (iNKT), B, and dendritic cells. A

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notable advance in the molecular understanding of Th1 generation is the identification of the Th1 specific transcription factor T-bet, which is crucial for the commitment and differentiation of naïve CD4⁺ T cells to CD4⁺ Th1 cells (Lugo-Villarino et al., 2003; Szabo et al., 2000; Szabo et al., 2002). T-bet is also involved in IFN- γ induction in NK (Szabo et al., 2002), dendritic cells (Lugo-Villarino et al., 2003), CD8a⁺ and CD8a⁻ murine dendritic cells, but not in CD8⁺ T cells. T-bet expression is believed to be restricted to the immune system. In unstimulated naïve CD4⁺ T cells, T-bet is expressed at very low levels. However, upon activation its expression is upregulated (Szabo et al., 2000). The absence of T-bet in CD4⁺ T cells from T-bet deficient mice results in decreased IFN- γ production, a decrease in the number of IFN- γ producing cells, as well as an increase in Th2 type cytokines (Szabo et al., 2002). The strong role of T-bet in IFN- γ induction is demonstrated by the fact that T-bet transfection of Th2 type murine cells results in decreased IL-4 and IL-5 expression redirecting them to a Th1 profile (Szabo et al., 2000).

Recent data show that T-bet expression induces IL-12R β 2 expression on lymphocytes, especially on T cells, making them more responsive to IL-12/STAT-4 pathway and IL-12-induced IFN- γ (Mullen et al., 2001). IFN- γ itself can upregulate T-bet. This positive feedback loop via IFN- γ presumably enhances the expression of T-bet to stabilize the Th1 response (Lighvani et al., 2001). It bears mentioning that the data on whether T-bet is induced by the IL-12/STAT-4 pathway in either T cells or antigen presenting cells are not conclusive (Afkarian et al., 2002; Mullen et al., 2001; Szabo et al., 2000). Recently, a new member of the heterodimeric family of cytokines, Interleukin-27 (IL-27), which is composed of a p40 protein chain [Epstein-Barr virus (EBV)-induced gene 3 (EIB3)] and a p28 chain, has also been reported to regulate T-bet levels. In one putative model, IL-27 is secreted by activated antigen presenting cells prior to IL-12 to promote early Th1 development and T-bet expression (Hibbert et al., 2003; Lucas et al., 2003; Takeda et al., 2003). Overall, data suggest that T-bet can be upregulated by IFN- γ , IL-27, and possibly IL-12.

Understanding T-bet control of IFN- γ levels is important not only for discernment of immune regulation but is also of significance in many female-predominant organ-specific autoimmune diseases, where abnormal levels of IFN- γ have been reported (Ahmed and Karpuzoglu-Sahin, 2005; Ansar Ahmed et al., 1999). The importance of T-bet regulation of autoimmune diseases is evidenced by findings that T-bet knockout mice are resistant to the induction of Experimental Autoimmune Encephalomyelitis (EAE) (Bettelli et al., 2004). It is noteworthy that estrogen has been shown to alter the course of various autoimmune diseases (Ansar Ahmed et al., 1999; Lahita, 1999; Olsen and Kovacs, 1996). We (Karpuzoglu et al., 2006; Karpuzoglu-Sahin et al., 2001a; Karpuzoglu-Sahin et al., 2001b) and others (Fox et al., 1991; Maret et al., 2003) have shown that estrogen treatment promotes IFN- γ . We also have shown that estrogen promoted IFN- γ -mediated pro-inflammatory events such as induction of iNOS, nitric oxide, and COX-2 (Karpuzoglu et al., 2006). Therefore, it was crucial to determine whether *in vivo* estrogen treatment upregulates T-bet in primary splenic lymphocytes. Since, thus far, no studies have addressed this important issue. To date, this is the first study to demonstrate that *in vivo* estrogen treatment alters the expression of the transcription factor T-bet in splenic lymphocytes. Further, we have demonstrated that T-bet appears to be regulated in part by IL-27, to a lesser extent by IFN- γ , but not by IL-12p70.

Materials and Methods

Mice and Estrogen Treatment

Three-to-four week old C57BL/6 male mice were obtained from Charles River Laboratories and housed 3-5 animals per cage. All mice were maintained at the Center for Molecular Medicine and Infectious Diseases (CMMID) Animal Laboratory facility. Mice were fed on a

diet (7013 NIH-31 Modified 6% Mouse/Rat sterilizable diet, Teklad, Madison, WI) that is devoid of synthetic or phytoestrogens and maintained on a 12/12 light/dark cycle. Mice were housed in standard cages and terminated by cervical dislocation in accordance with the Virginia Polytechnic Institute and State University Institutional Animal Care guidelines. After one week of acclimatization, male mice were orchietomized and given silicone implants prepared as either a placebo (empty implant as a control) or estrogen implants containing 17- β estradiol (Sigma-Aldrich Inc., St. Louis, MO) by standard procedures that have been extensively reported previously (Karpuzoglu-Sahin et al., 2001a; Karpuzoglu-Sahin et al., 2001b). Mice were terminated after 6 to 7 weeks of treatment.

Isolation and Culture of Splenic Lymphocytes

Spleens were collected under sterile conditions and lymphocytes were isolated as described in previous studies (Karpuzoglu-Sahin et al., 2001a; Karpuzoglu-Sahin et al., 2001b). Briefly, 1.5 ml of cells at 5×10^6 cells/ml, were added to 24-well round flat-bottom plates containing complete phenol red free RPMI-1640 with or without an optimal concentration of the T cell stimulants, Concanavalin-A (Con-A, 10 μ g/ml; Sigma-Aldrich Inc., St. Louis, MO) or anti-CD3 antibodies (10 μ g/ml, eBioscience Inc., San Diego, CA). Splenic lymphocytes were also cultured with one of the following reagents: recombinant IL-12p70 (rIL-12, 20 ng/ml), anti-IL-12 antibodies (3 μ g/ml), recombinant IL-27 (rIL-27; 10 ng/ml) (R&D Systems Inc., Minneapolis, MN), recombinant IFN- γ (rIFN- γ , 100, 1,000, 10,000 pg/ml, BDPharmingen San Diego, CA). Cells were cultured for 3, 6, 18, or 24 hrs at 37°C with 5% CO₂. At the end of the culture period, the cells and supernatants were frozen at -80°C until use. In selected cultures, splenic T lymphocytes were purified from estrogen and placebo-treated mice per the manufacturer's instructions (EasySep, Mouse T Cell Enrichment Kit; #19751; StemCell Technologies, Seattle, WA). Briefly, 80×10^6 splenic lymphocytes were suspended in $1 \times$ PBS (phosphate buffered saline) and 2% Fetal Bovine Serum (FBS) containing 5% normal rat serum provided in the Easy Sep kit. To these cells, EasySep Negative Selection Mouse T cell Enrichment Antibody Cocktail[®] was added and incubated at 4°C for 15 min followed by 15 min incubation with Easy Sep Biotin Selection Cocktail[®]. The cell suspension was combined with EasySep Magnetic Nanoparticles[®] and incubated at 4°C for 15 min and placed into the magnet base of RoboSep Cell automated magnetic cell separator (Stem Cell Technologies). T cells were isolated by initiating a T cell separation program. The purity of the isolated T cells was confirmed with flow cytometry analysis on an EPICS XL-MXL flow cytometer (Coulter, Hialeah, FL) using the following monoclonal antibodies: fluorescein isothiocyanate- (FITC) conjugated anti-Thy1.2 (CD90.2) or phycoerythrin- (PE) conjugated anti-CD45RB (B220) (eBioscience Inc. San Diego, CA). The negative isolation of T cells resulted in T cell purity of 97%.

Nuclear/Cytoplasmic Extracts and Western Blot Analysis

Twenty micrograms of the nuclear or cytoplasmic extracts from splenic lymphocytes from estrogen or placebo-treated mice were utilized for subsequent Western immunoblotting to detect T-bet and IFN- γ protein expression according to the manufacturer's instructions (NE-PER kit, Pierce Biotechnology Inc., Rockford, IL) and as per our previously published studies (Karpuzoglu et al., 2006). As a loading control for the extracts, β -actin protein was detected by Western immunoblotting using a rabbit anti- β -actin polyclonal antibody (Abcam Inc., Cambridge, MA). The β -actin protein was observed to be loaded equally in all Western blots (data not shown). Samples were electrophoresed on a 7.5% or 18% SDS-PAGE gel and were transferred to PDVF transfer membranes (Amersham Biosciences, Piscataway, NJ). Blots were incubated with primary antibody: T-bet (1:500; BioLegend, San Diego, CA; or Santa Cruz Biotechnology Inc., Santa Cruz, CA), or IFN- γ (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The secondary HRP-conjugated antibody was applied, the bands were visualized using the ECL protocol (Amersham Biosciences, Piscataway, NJ).

and quantitated as relative densitometry values using a Kodak Image Station (Perkin Elmer Life Sciences Inc., Boston, MA).

Determination of IFN- γ Protein Levels

IFN- γ protein levels in the supernatants of splenic lymphocytes were detected using an IFN- γ specific ELISA as per in our previous studies (Karpuzoglu et al., 2006). The intracellular detection of IFN- γ protein in freshly isolated splenocytes was also performed using flow cytometry per manufacturer's instructions (BDPharmingen San Diego, CA).

Statistics

Statistical significance of the differences observed between experimental groups was determined by one-way ANOVA using INSTAT software (GraphPad, San Diego, CA). Post-hoc comparisons between means of treatment groups were made with the Bonferroni test for multiple comparisons. Two-tailed t-test was also used to assess statistical differences. *P*-values less than 0.05 were considered to be significant.

Results

Estrogen treatment promotes early induction of IFN- γ by splenocytes

The intracellular IFN- γ protein levels were significantly increased in freshly-isolated lymphocytes from estrogen-treated mice when compared to placebo-treated samples (Fig. 1A, $*p<0.05$). Representative histograms of intracellular staining for IFN- γ in fresh splenocytes are shown in Fig. 1B. The extracellular IFN- γ level was significantly upregulated in supernatants from Con-A (pan T cell stimulant)-stimulated splenocytes from estrogen-treated mice when compared to placebo-treated mice after 3 hrs of culture (Fig. 1C). There was no detectable IFN- γ protein in the supernatants from unstimulated (media only) splenocytes from either estrogen or placebo-treated mice.

Estrogen induces early T-bet protein expression in activated splenic lymphocytes

As there is strong evidence that IFN- γ is regulated by T-bet, we next investigated whether estrogen also induces an increase in T-bet levels in activated splenocytes in a time kinetic fashion. The expression of T-bet protein was significantly upregulated in nuclear extracts from Con-A-activated splenocytes that were cultured for 3 hrs from estrogen-treated mice, compared to controls (Fig. 2A, $*p<0.05$). At 6 and 18 hrs of culture, T-bet protein levels from Con-A-activated splenocytes from estrogen-treated mice had an increasing trend compared to placebo-treated mice (Fig. 2A). By 24 hrs of culture, T-bet levels from splenocytes from estrogen-treated mice tended to be similar to those observed at 18 hrs of culture. In the case of placebo-treated mice, T-bet protein was upregulated after 24 hrs of culture (Fig. 2A). The supernatants of these cultures were analyzed for IFN- γ . Analogous to increased T-bet levels from estrogen-treated mice at 3 hrs, IFN- γ levels in the supernatants were significantly increased compared to placebo-treated mice (Fig. 2B, $*p<0.05$). With time, IFN- γ levels increased in estrogen-treated mice. By 24 hrs of culture, IFN- γ protein from Con-A-stimulated splenocytes from estrogen-treated mice was significantly increased compared to placebo-treated mice (Fig. 2B, $*p<0.05$).

Estrogen upregulates T-bet expression in anti-CD3-activated lymphocytes

Since Con-A-activated splenocytes from estrogen-treated mice demonstrated increased T-bet expression after 3 hrs of culture, we next determined whether activation of splenocytes with another T cell stimulant, anti-CD3 antibodies would also result in increased T-bet protein in cells from estrogen-treated mice at this time point. As shown in Fig. 2C, the expression of T-bet was significantly upregulated in anti-CD3-stimulated splenocytes

cultured for 3 hrs from estrogen-treated mice when compared to that from placebo-treated mice (Fig. 2C, $*p<0.05$). The increase in T-bet expression upon anti-CD3 stimulation was similar to the increase observed in Con-A-activated lymphocytes from estrogen-treated mice.

IL-12 does not alter T-bet in Con-A-activated splenocytes from estrogen-treated mice

To determine the potential role of IL-12p70 in upregulation of T-bet protein expression in activated splenocytes from estrogen-treated mice, Con-A-activated cells were cultured for 3 hrs either in the presence or absence of rIL-12 or with or without anti-IL-12 antibodies. Consistent with data shown in Figure 2, estrogen treatment compared to placebo treatment significantly induced the expression of T-bet protein in cells activated by Con-A alone (Fig. 3A-B). However, in the estrogen-treated groups further addition of rIL-12 to Con-A-activated cells did not alter T-bet levels compared to cultures exposed only to Con-A (Fig. 3A). In addition, the neutralization of IL-12 did not affect the protein expression of T-bet in nuclear extracts from estrogen or placebo-treated mice when compared to T-bet expression in Con-A alone activated samples (Fig. 3B).

IFN- γ has a partial role in regulating T-bet expression in estrogen-treated mice

We next investigated whether deliberate addition of recombinant IFN- γ (rIFN- γ) to splenocytes from estrogen or placebo-treated mice would alter T-bet expression. The expression of T-bet protein was markedly increased in nuclear extracts from Con-A-activated splenocytes after 3 hrs of culture from estrogen-treated mice when compared to that from placebo-treated mice (Fig. 4, $*p<0.05$). The addition of 1000 pg/ml, but not at a lower dose at 100 pg/ml, of rIFN- γ to Con-A stimulated cells significantly increased T-bet expression when compared to samples stimulated with Con-A alone. At 1000 pg/ml dose of rIFN- γ , T-bet levels also increased in samples from placebo-treated mice. However, this increase of T-bet from placebo samples was much less than the increase observed for estrogen samples. T-bet protein levels were not further enhanced in 10,000 pg/ml rIFN- γ and Con-A-stimulated lymphocytes compared to samples stimulated with 1,000 pg/ml rIFN- γ and Con-A (Fig. 4A).

We then investigated whether estrogen treatment would alter T-bet protein levels in freshly isolated and unstimulated cells left in media for 3 hrs of incubation. There was no detectable T-bet expression in freshly-isolated cells from either estrogen or placebo-treated mice. However, T-bet expression was observed in nuclear extracts of unstimulated cells from estrogen, but not placebo-treated mice, that were cultured for 3 hrs (data not shown). Addition of neither rIFN- γ nor rIL-12 markedly altered T-bet protein expression in samples from estrogen-treated mice (data not shown).

Estrogen upregulates T-bet protein expression in purified T cells

Next, purified T cells from estrogen and placebo-treated mice were stimulated with Con-A for 3 hrs in the presence or absence of rIFN- γ (10,000 pg/ml) and the expression of T-bet was determined in nuclear extracts. As observed in Fig. 4B, there was a marked upregulation of T-bet protein levels in Con-A-activated T cells from estrogen-treated mice when compared to their placebo counterparts ($**p<0.01$). The addition of rIFN- γ tended to increase T-bet expression in Con-A-activated samples from estrogen-treated mice compared to samples stimulated with Con-A alone (Fig. 4B, $*p<0.05$). Since the level of extracellular IFN- γ protein in the supernatants from Con-A-stimulated T cells from estrogen-treated mice was low (Fig. 4C), we determined the expression of intracellular IFN- γ protein in cytoplasmic extracts from Con-A-activated T cells (Fig. 4D). IFN- γ protein levels were noticeably increased in cytoplasmic extracts from Con-A-activated T cells from estrogen-treated mice compared to placebo-treated mice (Fig. 4D, $*p<0.05$). The deliberate addition

of rIFN- γ to Con-A-activated T cell cultures did not further alter the expression of IFN- γ in cytoplasmic samples from splenocytes of estrogen-treated mice, but increased the expression in placebo-treated mice (Fig. 4D, $*p<0.05$). This suggests that estrogen-treated samples may have reached saturating levels of intracellular IFN- γ with Con-A activation alone.

Deliberate addition of IL-27 markedly upregulated T-bet in samples from estrogen-treated mice

Recent studies have shown that IL-27 is critical for the induction of T-bet expression in Th1 cells (Owaki et al., 2005; Takeda et al., 2003). We therefore explored whether deliberate addition of IL-27 altered T-bet protein in nuclear extracts from splenocytes stimulated with Con-A for 3 hrs of culture. Exposure of Con-A-activated splenocytes to rIL-27 significantly increased T-bet protein expression in estrogen-treated mice when compared to samples activated with Con-A alone indicating the importance of IL-27 in the induction of T-bet protein in primary splenic lymphocyte cultures from estrogen-treated mice (Fig. 5, $*p<0.05$). Unlike cells from estrogen-treated mice, addition of rIL-27 to comparable cultures from placebo-treated mice did not markedly induce upregulation of T-bet (Fig. 5)

Discussion

We and others (Fox et al., 1991; Maret et al., 2003) have shown that estrogen regulates IFN- γ and IFN- γ dependent molecules (Karpuzoglu et al., 2006; Karpuzoglu-Sahin et al., 2001a; Karpuzoglu-Sahin et al., 2001b). In this study, we investigated whether *in vivo* estrogen treatment upregulates T-bet in activated splenocytes and the contribution of IFN- γ , IL-12, and IL-27 on T-bet induction. We found that *in vivo* estrogen treatment upregulated intracellular IFN- γ protein expression in fresh splenic lymphocytes. Interestingly, even though IFN- γ was detectable in freshly-isolated splenocytes, T-bet protein expression was not detectable in these cells. This may be because of technical limitations of detection, due to a transient appearance of T-bet that was not covered in our kinetic study, or simply because T-bet is not expressed at that time. Nevertheless, an important novel finding of this study is the early, selective, and significant induction of T-bet protein expression in splenocytes from estrogen-treated mice after 3 hrs of culture. Supernatants of these cultures from estrogen-treated mice also demonstrated enhanced levels of IFN- γ protein. Our data clearly suggest that *in vivo* estrogen treatment can prime the cellular environment for a Th1 type profile and splenic lymphocytes to express T-bet protein upon activation.

The IL-12, IFN- γ , and IL-27 are three known cytokines that have been shown to regulate T-bet. The role of IL-12 in upregulating T-bet, however, is unclear. It has been shown that even though IL-12 is an important IFN- γ -inducing cytokine, T-bet expression does not seem to be directly altered by the IL-12/STAT-4 pathway in Th1 cells (Afkarian et al., 2002; Mullen et al., 2001; Szabo et al., 2000). On the other hand, it is possible that the IL-12/STAT-4 pathway can indirectly upregulate T-bet expression. T-bet can increase IL-12R β 2 expression, which in turn upregulates the IL-12/STAT-4 signaling pathway resulting in augmented IFN- γ , subsequently altering T-bet activity (Afkarian et al., 2002). IFN- γ -induced T-bet expression and subsequent activation of IL-12R β 2, is believed to be an important player in Th1 development of naïve T cells (Afkarian et al., 2002; Lighvani et al., 2001). In our study, the presence or absence of IL-12p70 protein did not directly alter T-bet expression from Con-A-activated primary splenic lymphocytes from estrogen-treated mice when compared to Con-A only stimulated samples. Interestingly, in separate recent studies we have shown that estrogen upregulated IL-12-mediated STAT-4 signaling and IFN- γ induction (manuscript submitted). Overall, our data suggest that IL-12 may not be critical in the direct regulation of T-bet expression but instead may be more important in the induction of IFN- γ via the IL-12/STAT-4 pathway.

A positive bidirectional feedback loop interactions between T-bet and IFN- γ are known to exist. T-bet instigates chromatin remodeling of the IFN- γ gene (Mullen et al., 2001) and therefore promote IFN- γ gene activation (Szabo et al., 2000) as well as increase the production of IFN- γ protein. The upregulation of IFN- γ protein due to increased T-bet expression can lead to a positive feedback loop, which further primes the cells for Th1 differentiation (Lighvani et al., 2001; Szabo et al., 2000). In our studies, we have shown that deliberate addition of rIFN- γ partially increased T-bet protein expression in nuclear extracts from Con-A-activated splenocytes or purified T cells from estrogen-treated mice. This suggests that in our model, IFN- γ itself in part upregulates T-bet.

Recent studies have shown that IL-27 binds to an orphan receptor called TCCR (WSX-1) on naïve T cells to induce the expression of IL-12R β 2 and T-bet in naïve T and NK cells, regardless of IFN- γ (Chen et al., 2000; Hibbert et al., 2003). Thus, IL-27 prepares these cells to be differentiated into Th1 lymphocytes (Lucas et al., 2003; Takeda et al., 2003). The upregulation of IL-27-induced IL-12R β 2 expression on these naïve T cells suggests that IL-27 acts earlier than IL-12 inducing an early commitment to Th1 type lineage. Our data show that IL-27 significantly upregulates T-bet protein expression in splenic lymphocytes from estrogen-treated mice when compared to only Con-A-activated samples from estrogen-treated mice. The induction of T-bet via IL-27 in splenocytes from estrogen-treated mice is analogous to the increased T-bet expression observed in IL-27 exposed-CD4⁺ Th1 cells (Hibbert et al., 2003; Kamiya et al., 2004; Lucas et al., 2003). Significant difference of our study is that these cells (Hibbert et al., 2003; Kamiya et al., 2004; Lucas et al., 2003) were stimulated between 24 to 72 hrs of culture whereas our data demonstrated increased T-bet protein expression in estrogen-treated mice after 3 hrs of culture. Interestingly, unlike cells from estrogen-treated mice, deliberate addition of IL-27 to Con-A activated splenocytes from placebo-treated mice did not markedly alter T-bet expression. Our data suggest that estrogen and IL-27 may induce commitment of splenocytes to Th1 profile.

This study is the first to demonstrate that *in vivo* estrogen treatment upregulates T-bet protein expression in activated primary splenocytes as early as 3 hrs of culture. The induction of T-bet protein was significantly upregulated by IL-27, partially modulated by IFN- γ , but not altered by IL-12 in splenic lymphocytes from estrogen-treated mice. Although in a non-lymphoid system (i.e. reproductive tract epithelial human cell lines) *in vitro* estrogen exposure has been shown to induce T-bet (Kawana et al., 2005), our data demonstrates that *in vivo* estrogen treatment primes splenic lymphocytes for Th1 differentiation in the context of increased T-bet expression. In summary, our work demonstrates that estrogen is a natural regulator of T-bet. These findings are significant since T-bet influences the generation of Th1 dependent immunity, thus affecting key downstream IFN- γ -mediated events including effective defense against intracellular pathogens or promoting inflammation. In view of the fact that there are marked physiological sex differences in immune capabilities and autoimmune inflammatory diseases, as well as increased pharmaceutical use of estrogen, our studies provide new molecular information regarding estrogen regulation of IFN- γ . Therefore, these data have the potential to be useful in molecular design of new therapeutic strategies for IFN- γ induction/suppression.

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Figure 1. Estrogen upregulates early production of IFN- γ protein

(A) Intracellular IFN- γ protein was detected in freshly isolated lymphocytes from estrogen and placebo-treated mice. The cells were stained with anti-IFN- γ antibodies and evaluated by flow cytometry. The relative expression of IFN- γ -stained cells was presented as means $\pm$ SEM (standard errors of mean) (placebo: n=5 mice, estrogen: n=5 mice; * p <0.05). (B) Representative histograms from placebo and estrogen-treated mice are overlaid on top of each other. The faint unidentified histogram represents background control. (C) The protein levels of IFN- γ were determined in the supernatants from Concanavalin-A (Con-A; 10 μ g/ml)-stimulated or unstimulated (media only) splenic lymphocytes from estrogen or placebo-treated mice. Extracellular protein levels of IFN- γ were upregulated as early as 3 hrs (placebo: n=4 mice, estrogen: n=4 mice; * p <0.05). Data are presented as means with standard errors of the mean. There was no detectable IFN- γ in supernatants from unstimulated cultures (media only).

Figure 2. Estrogen upregulates T-bet protein expression in activated splenic lymphocytes in a time-dependent fashion

(A) Nuclear extracts from splenic lymphocytes from estrogen or placebo-treated mice which were Con-A-stimulated for 3, 6, 18, or 24 hrs of culture were analyzed for T-bet protein expression with a Western blot assay. One representative experiment is shown above the graph. The mean relative densitometry data of T-bet protein expression from nuclear extracts from Con-A stimulated splenic lymphocytes from estrogen or placebo-treated mice are shown (placebo (P): $n=4$ mice, estrogen (E2): $n=4$ mice; * $p < 0.05$). (B) IFN- γ protein levels in supernatants from Con-A-activated splenocytes cultured for 3, 6, 18, or 24 hrs from estrogen and placebo treated mice were analyzed with an ELISA (placebo: $n=4$ mice,

estrogen: n=4 mice; * $p<0.05$). (C) T-bet expression is increased in anti-CD3 antibody-stimulated splenocytes from estrogen-treated mice. Nuclear extracts from anti-CD3 antibody (10 $\mu\text{g/ml}$) stimulated splenic lymphocytes or unstimulated (media only) cells from placebo or estrogen-treated mice incubated for 3 hrs were assayed to determine T-bet protein expression. The top portion of the figure shows one representative experiment (n=4 mice per placebo (P) or estrogen (E2)-treatment; * $p<0.05$). The bottom portion of the figure depicts the mean relative densitometry data for T-bet protein expression as detected with Western blot analysis. Data are presented as means with standard error bars.

Figure 3. IL-12 does not directly alter T-bet protein expression in activated splenocytes from estrogen-treated mice

Splenocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 $\mu\text{g}/\text{ml}$), Con-A and rIL-12 (20 ng/ml), or Con-A and anti-IL-12 antibodies (3 $\mu\text{g}/\text{ml}$) for 3 hrs and the expression of T-bet protein in the nuclear extracts was determined. (A) One representative experiment of T-bet protein expression in cells stimulated with Con-A or Con-A and rIL-12 is shown in the top portion of the figure. The bottom portion shows the mean relative densitometry data of T-bet protein expression (placebo (P): $n=6$ mice, estrogen (E2): $n=6$ mice; $**p<0.01$). (B) One representative experiment of T-bet protein expression is shown in the top portion of the figure. The bottom portion shows the mean relative densitometry data of T-bet protein expression in nuclear extracts from Con-A or Con-A and anti-IL-12-antibodies-activated splenocytes from estrogen or placebo-treated mice (placebo: $n=10$ mice, estrogen: $n=10$ mice; $**p<0.01$). Data are presented as means with standard error bars.

Figure 4. T-bet expression is partially regulated by IFN- γ in activated splenocytes and purified T cells from estrogen-treated mice

(A) Splenic lymphocytes from placebo or estrogen-treated mice were stimulated with Con-A (10 μ g/ml) or Con-A plus various doses of rIFN- γ (100, 1,000, 10,000 pg/ml) for 3 hrs of culture and T-bet expression was explored in nuclear extracts of splenocytes. The top portion shows one representative experiment for T-bet expression. The figure demonstrates the mean relative densitometry data for T-bet protein expression (placebo: n=4 mice, estrogen: n=4 mice; * p <0.05). (B) Purified splenic T cells from estrogen or placebo-treated mice were cultured with Con-A (10 μ g/ml) or Con-A and rIFN- γ (10,000 pg/ml) for 3 hrs and T-bet protein expression was explored in nuclear extracts from T cells (placebo: n=4

mice, estrogen: n=4 mice; $**p<0.01$). One representative experiment is shown at the top of Panel A. (C) IFN- γ protein levels in the supernatants from Con-A-activated T cells from estrogen and placebo-treated mice are shown (placebo: n=4 mice, estrogen: n=4 mice). (D) Intracellular IFN- γ protein in the cytoplasmic extracts from Con-A or Con-A and rIFN- γ -activated T cells was detected with Western blot assay. The mean relative densitometry data and one representative Western blot experiment are shown (placebo: n=4 mice, estrogen: n=4 mice; $*p<0.05$). Data are presented as means with standard error bars.

Figure 5. The early expression of T-bet is increased in response to deliberate addition of IL-27 to Con-A-activated splenocyte cultures from estrogen-treated mice

Nuclear extracts from Con-A (10 μ g/ml), Con-A and rIL-27 (10 ng/ml) cultured splenic lymphocytes from placebo or estrogen-treated mice that were incubated for 3 hrs were assayed to determine T-bet expression. The top portion of the figure shows all 4 pairs of experiments (n=4 mice per placebo or estrogen-treatment). The bottom portion of the figure depicts the mean relative densitometry data for T-bet protein expression as detected with Western blot analysis (n=4 mice per placebo or estrogen-treatment; * p <0.05). Data are presented as means with standard error bars.