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Short communication

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 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

1. 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 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, 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-12Rβ2 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

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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 (Ansar 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,b) 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. 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.

2. Materials and methods

2.1. 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 1 week of acclimatization, male mice were orchiectomized 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,b). Mice were terminated after 6–7 weeks of treatment.

2.2. 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,b). 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 μg/ml; Sigma–Aldrich Inc., St. Louis, MO) or anti-CD3 antibodies (10 μg/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 μg/ml), recombinant IL-27 (rIL-27; 10 ng/ml) (R&D Systems Inc., Minneapolis, MN), recombinant IFN-γ (rIFN-γ, 100, 1000, 10,000 pg/ml, BDPharmingen, San Diego, CA). Cells were cultured for 3, 6, 18, or 24 h at 37 °C with 5% CO_2. 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× 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 a 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 the 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 isothyocynate- (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%.

2.3. Nuclear/cytoplasmic extracts and Western blot analysis

Twenty micrograms of 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