Molecular Basis of Upregulation of IL-17 in Estrogen Model of Inflammation

Deena Khan

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biomedical and Veterinary Sciences

S. Ansar Ahmed Committee Chair

Liwu Li

William R. Huckle

Christopher M. Reilly

June 20th, 2012

Blacksburg, Virginia

Keywords: Interleukin, IL-17, estrogen, immune, induction, response

ABSTRACT

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by

Deena Khan

Interleukin-17 (IL-17) plays a major role in inflammation by regulating the induction of various proinflammatory genes, which aid in the recruitment and activation of neutrophils. Although IL-17 is considered to be protective in infection, overproduction of IL-17 in conditions like autoimmune diseases has been shown to aggravate these diseases and contribute to tissue injury. One of the principal focus of our laboratory is to decipher molecular mechanisms involved in inflammatory cytokine regulation and response in inflammatory disorders. To study this aspect, we employ a murine model of pro-inflammation induced by exposure to a natural immunomodulator, estrogen. In this novel study, we have comprehensively investigated the effect of estrogen on IL-17 induction, an aspect not studied thus far. We are the first to demonstrate that estrogen increases the ability of lymphocytes to secrete IL-17A, and its isoforms IL-17F, IL-17A/F. In addition to the cytokine levels, the percentages of IL-17^{+} cells are also increased by estrogen. Impressively, we found that estrogen fine tunes the balance of multiple transcription factors/signaling pathways. Estrogen upregulates IL-17 by promoting

the activity and expression of positive regulators (RORγt, RORα, NF-κB, JAK-2) and decreases the activity and/or expression of negative regulators (IRF8, ETS-1). In addition, we found that estrogen epigenetically regulates IL-17 induction by miRNAs (miR-326 and miR-223). We also found that majority of IL-17 positive cells are $CD8^+$ suggesting that estrogen-mediated IL-17 induction is predominantly from Tc17 cells. This is possibly due to increased proliferation of $CD8^+$ cells from estrogen-treated mice, as demonstrated by CFSE cell proliferation assay. Furthermore, estrogen also enhances the ability of IL-17-target cells to release proinflammatory molecules when exposed to IL-17. Together, this is the first study to comprehensively show that estrogen calibrates transcription factors and miRNAs to enhance IL-17 induction and promote IL-17 response. This dissertation work will provide a platform to continue further research in estrogen modulation of IL-17 in inflammation and disease conditions.

Dedication

To my Parents

Acknowledgements

I feel truly blessed to be given the opportunity to pursue my graduate studies in state of art Virginia Polytechnic Institute and State University. I express my humble gratitude to all the people I met here in Virginia Tech and United States. I am highly honored to have such a fulfilling learning experience.

Foremost, I would like to express my sincere gratitude to my advisor Dr. S. Ansar Ahmed for the continuous support of my Ph.D study and research, for his patience, motivation, enthusiasm, and immense knowledge. His invaluable advice always helped me whenever I was stuck in research problems and always gave me new ideas to find solutions of any problem I encountered. He helped me think independently and developed confidence in me and showed me how to interpret scientific data in different ways. I could not have imagined having a better advisor and mentor for my Ph.D. study

To my esteemed committee, I express my sincere gratitude to Dr. Liwu Li, Dr. Christopher R. Reilly, and Dr. William R. Huckle for all the intellectual discussions and your inputs and suggestions were very helpful in designing experiments. Special thanks to Dr. Robert Gogal and Dr. Wen Li for their advice and assistance in carrying research. I am highly grateful to Dr. Roger Avery for supporting my graduate research assistantship along with travel grant support and I was able to interact with eminent people in different fields of science and technology. I am also grateful to the wonderful staff in Research and Graduate Studies esp. to Becky Jones and also to Monica Taylor and Patty Goudy in Department of Biomedical Science and Pathobiology for all their help.

I am thankful to all current and previous members of our research group especially Dr. Rujuan Dai, Bettina Heid, and Catharine Cowan. Dr. Dai's guidance and expertise in research helped me tremendously in all stages of my research. You have a special place in my heart and your friendship, generosity and kindness will always be with me for whole life. Special thanks to Bettina and Catharine for their help in all of my animal experiments, collection of tissues and processing of samples. Thank you for running lab so efficiently. I feel obliged to all those people

who were involved in making this project possible including Melissa Makris for all flow analysis and lab animal care staff, lab animal veterinarian and glassware cleaning services personnel and above all the lives of experimental mice that were used in this dissertation.

I express my sincere gratitude to Mrs. Asma Ahmed and Mrs. Nikhat Rizvi for all their prayers and special affection for me. I am very indebted to my friends here in Blacksburg and all those in India for all their good wishes and special moments that we shared which made my stay here pleasant and enjoyable

I dedicate this dissertation to my father Dr. Abdul Qayyum Khan and my mother Mrs. Shehla Khan for their unconditional love, support, encouragement, sacrifice and invaluable prayers throughout my life. It is because of them I am here at this stage of life. I owe my deepest gratitude and also dedicate this dissertation to my maternal grandparents Late Chaudhary Mohammad Arif and Late Mrs. Shahjahan Begum and paternal grandparents Late Zafar Alam Khan and Late Mrs. Maqsoodan Khan for their sacrifices and prayers throughout their life which has a very big role in making this journey possible. Special thanks are reserved to my sister Dr. Yusra Khan, brother-in-law Dr. Aslam Baig and brothers Kunwar Mohammad Hussain Khan, Kunwar Mohammad Taha Khan and niece Amna for their constant love and wishes. I was very fortunate of having my family so nearby which very few international students get this opportunity. I express my sincere gratitude to my sister Dr. Tila Khan, brother-in-law Dr. Mohammed Rabius Sunny for all their untimely help, and support in every aspect of personal and professional life here in Blacksburg. This journey would not have been possible without the support of my husband Syed Makhmoor Mazahir who stood by me through the good and bad times, left his rising career for me and supported me constantly with his unconditional and undemanding love and care and my precious little girl, Sarah whose presence made this entire program a wonderful experience. Lastly, I would like to thank my mother-in-law Mrs. Najma Mazahir Ali for her help and support and for making my life so comfortable, easy and enjoyable.

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Chapter 1 Estrogen and Signaling in the Cells of Immune System

Deena Khan, Catharine Cowan and S. Ansar Ahmed

Advances in Neuroimmune Biology 3 (2012): 73-93.

DOI 10.3233/NIB-2012-012039 IOS Press

Abstract

Hypothalamus-pituitary-gonadal axis regulation of estrogen, which acts on reproductive tissues, is well established. However, it is also evident that estrogens physiologically act on not only reproductive tissues but also on a broad range of tissues such as immune system. It is well documented that estrogen regulates all facets of the immunoregulation thereby affecting the outcome of autoimmune and inflammatory immune responses. Given the broader role of estrogen in immunobiology, it is important to understand how estrogens act on the cells of the immune system. Estrogens act in estrogen receptor dependent and/or independent manner to affect the regulation of cytokines and chemokines. This review focuses on sources and biosynthesis of estrogens, differential expression of estrogen receptors on the cells of immune system, key signaling pathways and its effect on the induction of key pro- and anti-inflammatory cytokines. Since estrogen has contrasting effects in female-predominant autoimmune diseases such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE), we briefly overview the mechanistic role of estrogen on these chronic diseases. Recent evidence suggests that estrogens also alter microRNAs, which regulate a broad range of transcription of genes. The review also addresses this and other newly discovered mechanisms of estrogen-induced immunomodulation in health and disease.

Keywords- estrogen, receptor, immune, autoimmune, cytokine

Sources and Biosynthesis of Estrogens:

There has been a paradigm shift in the understanding of biological effects of estrogen in recent years. Estrogens should no longer be merely considered as reproductive hormones since estrogens act not only on reproductive tissues, but also physiologically act on many non-reproductive tissues. This include: cells of the immune, central nervous, cardiovascular, and skeletal systems, as well as cells from liver, skin and kidneys [1]. Thus, a wide-range of tissues are natural targets for estrogen action. Interestingly, it was originally believed that estrogen exclusively affects females, hence of importance to only women. However, studies in males with either defective estrogen synthesis or signaling have clearly demonstrated that estrogen is also physiologically important in males [2-4]. Estrogens occur in three major natural forms: estrone (E1), 17β-estradiol (E2), and estriol (E3). In non-pregnant female individuals, 17β-estradiol is the predominant form of estrogen in the reproductive stages from puberty to menopause. In postmenopausal women, estrone is more prevalent; whereas during pregnancy, estriol is the primary estrogen. 17β-estradiol is generally believed to be the most potent estrogen synthesized in the human body. Although, estrone and estriol were considered to be inactive in the past, it has now been shown that these compounds have tissue specific roles and are high affinity ligands for estrogen receptors [5-7].

Other sources of exposure to estrogens include: (i) intake of synthetic pharmaceuticals (oral contraceptives and hormone replacement); (ii) unintentional exposure to environmental contaminants (xenoestrogens; e.g. pesticides and herbicides) and certain commercial products (plastics and cosmetics); (iii) consumption of plant-derived estrogens (phytoestrogens), and (iv) exposure to fungus-derived estrogens (mycoestrogens). These structurally diverse compounds act on estrogen receptors to induce estrogenic activity.

17α ethinyl estradiol is a common synthetic estrogen which is prescribed either as oral contraceptive to premenopausal women or as estrogen replacement therapy to postmenopausal women [8]. Some of pharmaceutical estrogens mimic estrogen action in some tissues but behave as antagonist in certain other tissues and are referred to as Selective Estrogen Receptor Modulators (SERMs) [9]. Therefore, these SERMs have been advantageously used clinically to achieve desired effects in certain tissues.

Humans and animals are unintentionally exposed to xenoestrogens. Although these compounds structurally differ with estrogen, yet they are estrogenic and have significant effect on the ecology and human health. Some of the xenoestrogens include: synthetic substances and environmental contaminants found in detergents and surfactants (polycyclic aromatic hydrocarbons; octylphenols), plastics (phthalates, bisphenol A), pesticides (methoxychlor, dichlorodiphenyl-trichloroethane or DDT, hexachlorobenzene, and dieldrin), and industrial chemicals (polychlorinated biphenyls or PCBs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD) [10]. These environmental contaminants are of importance since they interfere with physiological estrogen signaling and thus are commonly termed as endocrine disruptors. In addition, they are also known to alter the synthesis of estrogen and inhibit the inactivation of estrogen by sulphation thus resulting in hormonal imbalance [11]. Even though these compounds are less potent than natural estrogens, they tend to accumulate in body fat and are capable to altering the endocrine function to affect reproductive capacity, immune and other biological functions [12]. Studies in various animals have revealed that endocrine disruptors alter the cytokines, immunoglobulin and T helper cell (Th) profile (reviewed in detail in [13, 14]).

Some plant products also possess estrogenic activity and are termed phytoestrogens [15]. Leguminous plants such as soybeans, whole grain cereals, and some seeds have high amount of phytoestrogens. Soybeans have high levels of isoflavones (e.g. genistein), which are estrogenic and have anti-inflammatory effects that are associated with alteration of chemotactic factors, adhesion molecules, and reactive oxygen radicals. Phytoestrognes are also known to modulate immunoglobulin class, Th1/Th2 balance and IL-4 levels in allergies [16]. Another natural source of estrogens come from fungi and is called mycoestrogens. A common mycoestrogen found in animal feed is zearalenone produced by Fusarium fungi and is recently been shown to modulate immune response in pigs [17]. It is beyond the focus of this review to discuss the biological effects of xenoestrogens, phytoestrogens and mycoestrogens. Therefore, this review is restricted to key findings of natural endogenous estrogens (17β-estradiol) on the cells of the immune system.

Estrogens are primarily synthesized by both gonadal and extragonadal tissues. The synthesis and release of estrogens are centrally regulated by the hypothalamus-pituitary-gonadal axis. Hypothalamus in brain secretes gonadotrophin releasing hormone (GnRH), which then acts on pituitary to release luteinizing hormone (LH) and Follicular stimulating hormone (FSH). These two hormones act on gonads to release estrogen in a cyclic manner during menstrual cycle. Estrogen in turn controls the release of GnRH from hypothalamus in a negative feedback loop. This Hypothalamus-pituitary-gonadal axis regulates reproduction by controlling uterine and ovarian cycles and for proper female development [18]. In premenopausal female, estrogens are produced primarily in the follicles of ovaries, corpus luteum and in the placenta of pregnant women. On the other hand, in postmenopausal women and in men, estrogens are synthesized by extragonadal tissues. These include: cells in the liver and brain, mesenchymal cells of adipose and breast tissue, osteoblasts and chondrocytes of bone, adrenal glands, vascular endothelium, and aortic smooth muscle cells [19]. The estrogen synthesized in these extragonadal sites act locally in a paracrine or intracrine fashion and only occasionally escapes into circulation [20]. The estrogen levels also fluctuate in female mice between 20-35 pg/ml in diestrus; 70-200 pg/ml during estrus, and 5, 000-10,000 pg/ml during pregnancy [21-23]. In women, estrogen levels vary through different physiological stages such as menstrual cycle and pregnancy, and also by age. During menstrual cycle, estrogen levels are highest during ovulation induction 1000 pg/ml; peak (late follicular) around 200-500 pg/ml [24]. In humans, estrogen levels markedly increase to around 16,000-30,000 pg/ml during pregnancy [24, 25]. The levels of estrogen decrease significantly in postmenopausal women to 5-20 pg/ml [19, 24]. Estrogen replacement therapy of post-menopausal women clinically restores estrogen levels to around 100 pg/ml by giving hormone replacement therapy [24]. In men the estrogen level is around 30-35 pg/ml [19, 26].

Cholesterol (C27) acts as a precursor of many steroids found in human/animal body. In theca interna cells of ovary, after hydroxylation and side chain cleavage of cholesterol by delta-5 and delta-4 pathway leads to the generation of a key intermediary androstenedione. Androstenedione then crosses basal membrane into granulosa cells where it is converted to testosterone, which in turn undergoes conversion to estradiol by an enzyme called cytochrome P450 aromatase ($P450_{AROM}$). Alternatively, androstenedione is aromatized to estrone, which is subsequently

converted to estradiol by 17β-hydroxysteroid dehydrogenase. In men, a small amount of estradiol is produced by precursor hormone especially from testosterone in testes. In post menopausal women and in men, aromatase converts androstenedione to estrogen in extragonadal tissue [19].

In the plasma, estrogen and other sex hormone are bound to steroid hormone binding globulin (SHBG). Effectively, SHBG regulates the bioavailability of free estrogen levels in the plasma [27]. SHBG plays an important role in estrogen signal transduction since any change in the levels of SHBG will alter the levels of free estrogen. Free estrogen is now capable of acting on target cells to induce biological activity. Recent studies have shown that SHBG binds to its specific receptor (R_{SHBG}) on surface of various cells [28]. This SHBG- R_{SHBG} complex can be activated by sex hormone to induce cell signaling, thereby providing additional molecular mechanism by which sex hormone can affect cellular functions [29].

Estrogen Receptors in the cells of the Immune System:

Estrogen signaling is an intricate balance between two distinct receptors ERα (NR3A1) and ERβ (NR3A2) and their splice variants from nuclear receptor (NR) family of transcription factors encoded by chromosomes 6 and 14, respectively. ERs are ligand-activated transcription factors that regulate a broad range of estrogen-responsive genes. Nearly six decades ago, an estrogen binding protein was identified by Elwood Jensen, now known as estrogen receptor alpha ERα [30]. Interestingly, ERβ was identified much later (1996) [31]. Generation of ERα knockout and ERβ knockout mice have been invaluable in classifying physiological role of these receptors in various tissues [32]. ERs are evolutionarily conserved and have structural and functional five distinct domains known: the DNA binding domain (DBD; domain C), the ligand binding domain (LBD; E/F domain), the hinge domain (D), and two transcriptional activation function domains AF-1 (in A/B domain) and AF-2 (in F domain) (Fig. 1). The NH2-terminal A/B domain is the only variable domain in both sequence and length. The A/B domain has sites for post-transcriptional modifications by kinases of growth factor pathways, which stimulate AF-1 constitutively in a hormone independent manner. The DNA binding domain or C domain is central and most conserved domain and is involved in recognition and binding to the DNA. The

hinge domain is essential for nuclear localization and for its post-translational modifications by acetylation, lipophilic moieties, and ubiquitination. The C-terminal is essential for ligand binding, ER homo or hetero dimerization, and interaction with co-regulatory proteins through ligand dependent AF-2 region. The AF domains are required for transcriptional activation and aid in the recruitment of co-regulatory proteins for gene expression. Although both ERs have similar affinity for estrogen and bind to the same DNA response element, they have distinct, non-redundant roles and in some tissues entirely opposing roles. Estrogen receptors exert their effects either through genomic or non-genomic signaling pathways depending on the tissue.

Different splice variants or isoform of both ERα and ERβ have been identified in different cell lines [33, 34]. In human, full length ERα is 595 amino acids long [35] and has 2 shorter isoforms (hERα-46 and hERα-36), which lack AF-1 portion of NH2 terminal [33, 34]. ERβ is smaller than ERα and is 530 amino acid long [36] and has 4 other isoforms either because of deletion of 5th exon or due to different COOH-terminal regions [37-40]. Even though ERβ is smaller than ERα, they have similarities in DNA binding domain and ligand binding domain. Similarly, mouse and rats ERs have sequence homology and different isoforms with altered ligand binding and other functionalities [reviewed in detail in [41]]. The binding site of estrogen to its receptor is generous in size, therefore, a wide variety of compounds with diverse structures are capable of binding to the estrogen receptors [42].

The distribution of ERs in various cells and tissues are also critical in determining the overall affect of ligand-dependent signaling. The distribution of ERα and ERβ is overlapping in some tissues and highly specific in some. Analysis and knowledge of tissue distribution of ERs is critical for designing potential ER-targeted therapies. Different molecular mechanisms play important roles in modulating the ER-mediated response. This includes different ligand affinity, signaling events, transactivation, interaction with specific co-factor interaction, homo- or hetero-dimerization, splice variant ER isoforms. Numerous reports have been published regarding differential expression of ERα and ERβ, which is determined by a variety of techniques including polymerase chain reaction, Western blotting or intracellular flow cytometry. ERα is expressed highly in epididymis, bone, breast, uterus, testis (leydig cells), ovary (theca cells),

kidney, adrenal, prostate gland, bladder, liver, and thymus, white adipose tissue and various regions of the brains. ERβ is expressed predominantly in colon, epithelium of prostate gland, testis, granulosa cells of ovary, bone marrow, salivary gland, vascular endothelium, lung, bladder and brain [43-45]. ERβ is predominant in ovarian granulosa cells of follicles, while ERα is mostly present in theca and interstitial cells [31, 46, 47]. The expression of ERs is also dependent on the differentiation and developmental stage of the tissues such as, e.g. in uterus and pituitary gland; ERβ is expressed in immature whereas ERα predominates in fully mature tissue [48, 49].

Since estrogen is known to modulate immune system, extensive studies have been performed to define the role of estrogen-mediated signaling in physiological and pathological conditions. Differential expressions of ERs in cells of the immune system have an impact on the net effect of estrogen on immune responses. To date, ERs have been found in nearly all cells of the immune system including cells of the innate and acquired immunity. This suggests that the immune system is a natural target for estrogen. Hematopoietic progenitors have differentially expressed ERs depending on the developmental stage, with reduced expression during fetus and increased expression after birth [50]. ERα is found in most of the cells of immune system, while ERβ appears to be somewhat restricted. ERα is preferentially expressed in thymus stromal cells, thymocytes, hematopoietic cells, and bone marrow [51-54], and murine splenic DC and peritoneal macrophages [55]. ERβ is expressed in thymus and spleen of human mid-gestational fetus [49], and lymphocytes in human lymph nodes, rat thymocyte and stromal cells [54], and in murine bone marrow and thymus [56-58]. Splenic B cells express both ERα and ERβ [59]. ERα levels are higher than ERβ in $CD4^+$; low levels of both ERα and ERβ are found in $CD8^+$ cells, whereas B cells have more ERβ than ERα [60, 61]. In human blood T lymphocytes, B lymphocytes and NK cells ERα46 isoform is the predominant ER [62]. Overall, the presence of ERs in immune cells indicates that the immune system is a target for estrogen action. Different tissues have varying levels of ERα and ERβ which in turn affects the overall outcome of immune response.

ER-mediated signaling in the cells of the immune system induces a variety of cytokines including IFNγ, IL-17, IL-6 among others. Interestingly, activation of $CD4^+$T cells with

proinflammatory cytokines increases ER expression. For example, IFNγ and IFNα treatment can upregulate ERα expression in mouse splenocytes or cell lines by activating STAT1 pathway [63]. In myasthenia gravis patients, there is upregulation of ERα in thymocytes indicating that there is modulation of ER expression depending on lymphocyte subset, localization and disease condition [61]. The above studies demonstrate cross talk between cytokines and ERs expression.

To define the specific role of ERα and ERβ in immune modulation, mice deficient in either ERα or ERβ and selective agonist or antagonist have been used. ERα is important in thymus and spleen development since, ERα knockout mice have hypoplasia of the thymuses and spleen [58, 64], increase in number of immature $CD4^+CD8^+$ thymocyte and decreased $CD4^+CD8^-$ cells and systemic autoimmune diseases with immune-complex mediated glomerulonephritis, proteinuria, infiltration of B cells in kidney, damage of tubular cells and presence of serum anti-DNA antibodies [58, 65]. Majority of the studies indicate that ERα plays a prominent role in immunmodulation when compared with ERβ [66]. ERβ is required in proliferation of progenitor cells in the bone marrow. Old mice deficient in ERβ have been shown to develop myeloproliferative disease similar to chronic myeloid leukemia with increased number of B cells in bone marrow and spleen; increase in number of Gr-1hi/Mac-1hi-positive granulocytes and B cells in blood and some of the mice even develop severe lymphoproliferative phenotype [67]. ERβ has been shown to be required for estrogen-mediated thymic cortex atrophy and shift of thymocyte phenotype in female mice [58]. ERβ deficient mice have hyperplasia of bone marrow [67]. On the other hand, in ERα and ERβ knockout mice significant reduction of B lymphopoiesis was seen upon estrogen treatment. This reduction in lymphopoiesis is mediated either through ERα or both receptors. Furthermore, estrogen treatment of ERβ knockout mice and wild type littermates resulted in significant increase in immunoglobulin secreting B cells. This indicates that although both ERs are required for complete downregulation of B lymphopoiesis, only ERα is required to up-regulate immunoglobulin production in bone marrow and spleen [68].

It has been shown that ERα and ERβ have opposing role in transcription of estrogen-responsive genes. ERβ either inhibits the action of ERα regulated gene or reduces ERα protein level. For

instance, ERβ protects against ERα-induced hyper proliferation thereby counteracting the biological activity of ERα. ERβ also alters the recruitment of c-Fos and c-Jun to AP-1 regulated promoters antagonizing ERα-mediated regulation of AP-1 dependent transcription. In addition, ERβ also alters the expression of c-Fos and decreases ERα expression by proteolytic degradation [69]. The relative expression of ERs in a cell produces a distinct response to ER selective or partial agonist/antagonists. One possible reason for diversity of responsiveness could be due to differences in activation of AF-1 domain in ERα when compared with ERβ [70].

Estrogen-receptor mediated Cell signaling.

Numerous studies have confirmed that multiple mechanistically distinct molecular pathways are involved in the signaling through ERs. These pathways are broadly classified as ligand dependent or ligand independent (Fig. 2). Binding of either endogenous hormone or synthetic compounds with estrogenic properties to the ligand-binding domain of ERs in the cytosol triggers the ligand dependent pathway. Following this initial step, the ER subtypes undergo different biological processes such as conformational changes, receptor dimerization (either homodimers -ERα: ERα or ERβ: ERβ or heterodimers ERα: ERβ), and then translocate to nuclei and bind to specific estrogen-responsive element (ERE) in the regulatory regions of estrogen responsive genes. This is referred to as the "classical" signaling pathway. The final outcome is dependent on the interaction with effectors such as coregulatory proteins, chromatin remodelers, co-activators, co-repressors and other active signaling pathways in the cells [9, 71-73]. Different ligand binding induces unique ER conformational changes that recruit specific co-regulatory proteins, which interact with AF-1 and AF-2 at N or C-terminal ends of ERs, respectively [74, 75]. Although, the general belief is that ERs bind to the consensus ERE consisting of a 5-base pair palindrome with a 3-base pair spacer: GGTCAnnnTGACC, some reports have suggested deviation in the consensus sequence of ERE [76] or binding to non–estrogen response elements by tethering to other transcription factors [73].

The process whereby ERs interact with other transcription factors is referred as transcription factor cross talk or the "tethered" pathway. It is noteworthy that ERs can affect ER responsive genes even in the absence of binding to ERE. This is accomplished by binding of ERs to

different transcription factors and thus not interacting with ERE directly. There are studies that demonstrate ligand activated ERs interact with various transcription factor complexes such as Fos/Jun AP-1 (activating protein-1) [77], SP-1 (stimulating protein-1; GC-rich motifs), CREB, Runx1, NF-κB, STAT5 and p53 [78-81]. While most of these associations have been demonstrated in epithelial cancer cells, many of these transcription factors play well-characterized roles in immune cell responses as well. For example, estrogen is known to inhibit NF-κB-driven transcription by preventing p65 translocation to the nucleus [82]. It has been demonstrated that in vitro estrogen (1nm) treatment of Raw 264.7 cells decreases LPS induced DNA binding and transcriptional activity of p65 by blocking nuclear translocation and also activates phosphatidylinositol 3-kinase without activating or modifying Ikappa-Balpha degradation or MAPK.This activity is mediated through ERα and not ERβ [82].

Studies from our laboratory have added new thoughts to estrogen-mediated NF-κB signaling pathway that promotes inflammation. *In vivo* estrogen treatment of mice, inhibited nuclear localization of NF-κB p65, c-Rel, and RelB, but not p50. However, estrogen treatment increased the activity of NF-κB as measured by luciferase reporter assay increased binding of NF-κB to DNA, and the levels of cytokines regulated by NF-κB (IL-1 alpha, IL-1 beta, IL-10, and IFN-gamma) [83]. Interestingly, we have recently demonstrated that estrogen promotes truncation of p65 by serine protease [84] . While estrogen inhibited full-length p65 translocation, shorter form of p65 was found in nuclei of estrogen-treated cells, when detected in Western blotting by using antibody specific for N-terminal p65. Inhibition of serine protease activity restored the full-sized length of p65 in nuclear extracts of cells from estrogen-treated mice comparable to placebo-treated mice [84]. In lymphoid cells, NF-κB regulates TNF secretion, and estrogen has also been demonstrated to inhibit TNF secretion in T cells, macrophages, and dendritic cells [85]. However an ER-mediated mechanism for this inhibition has not been demonstrated.

Third ligand dependent pathway is also known as “non-genomic pathway” termed due to its rapid physiological effect within seconds to minutes without involving gene regulation. In this process, there is ligand interaction with ERs localized to the plasma membrane via palmitoylation on cysteine447 [86]. Recently, a genetically and structurally unrelated membrane

receptor, GPR30 (an orphan G-protein coupled receptor 30) has been demonstrated to bind to estrogen and mediate downstream non-genomic signaling. However, it has been shown that estrogen binding to GRP30 induces expression of the ERα variant ERα36, which then mediates downstream non-genomic signaling; demonstrating that the GPR30 pathway still relies on ER expression and function [87]. The role of GPR30 in immune cells remains unclear, as knockout mouse models demonstrate thymic atrophy; however GPR30 expression could not be detected in thymocytes [88]. This implies that GRP30 may have indirect effects on the thymus.

In non-genomic pathway, after activation of ERs, cytoplasmic signaling pathways are stimulated, including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), phosphoinositide-3 kinase (PI3K), as well as increased release of intracellular Ca and activation of calcium-calmodulin-dependent kinases. Activation of non-genomic pathway likely depends on expression levels of the different ERs, splice variants and post-translational modifications [73].

Some studies on immune cells have demonstrated the existence of the non-genomic actions of estrogen and ERs interactions. Membrane bound ERα has been detected in peripheral blood mononuclear cells [89]. Estrogen has been shown to rapidly induce ERK (MAPK) phosphorylation in glial cells, indicating at least this non-genomic pathway is conserved between epithelial and immune cells, and implying others likely are as well [90, 91]. In another example, estrogen decreases the activity of the small GTPase Rac1 in monocytes, without altering Rac1 expression. Rac1 regulates actin polymerization in the cytoplasm and modulates monocyte adhesion [92]. However it is unclear whether this effect is due to direct ER interaction or via downstream proteins.

The fourth ER signaling pathway, is known as the ligand-independent pathway. In this pathway, ERs are phosphorylated by activated signaling cascades [93]. Ligand-bound growth factor receptors activate downstream kinases, which then phosphorylate ERs and stimulate both direct ERE-dependent genomic actions and transcription factor associations. For example, HER2 signaling activates the MAP-kinase ERK, which can phosphorylate ER on serine 118, and lead to increased ER-AF-1 activity [94]. The role of this pathway in immune cells is perhaps the least

characterized. Whether activation of kinases such as ERK by immunologic stimuli leads to similar ER phosphorylation events in immune cells, although likely, needs to be determined.

The signaling mechanism via ERs is dependent on number of variables including but not limited to ER type, expression and its post-translational modification; type and concentration of ligand availability; tissue type and coregulators present in the cell. Post-translational modifications of ERs modulate their signaling ability. While phosphorylation triggers downstream events, glycosylation is involved in directing ERs to their final intracellular localization. Acetylation has been shown to enhance ER-DNA binding activity, hormone sensitivity and transcriptional activity. Ligand mediated sumoylation facilitates ERalpha-dependent transcription, nitrosylation impairs their genomic action, DNA binding ability and ubiquitination tags ERs to undergo degradation. In addition, myristoylation and palmitoylation affect interaction of ERs with membrane proteins, trafficking, as well as signal transduction [95].

Estrogen-mediated regulation of pro-inflammatory cytokines

Estrogen treatment is known to markedly regulate cytokine synthesis from a number of different cell types. Estrogen-regulated cytokine secretion is variable, depending on the cell type, ER expression profile, concentration of ligand, type of ligand and length of exposure and *in vitro* versus *in vivo* treatment [85, 89]. In many cases, low doses of estrogen (roughly diestrus levels) may stimulate secretion while high doses (approximately pregnancy levels) are inhibitory [85]. The converse can also be true. These variability likely results from the multiple signaling pathways and cross-talk that can occur upon ligand binding to ERs, as well as variability in expression of ER subtypes and splice variants among immune cells. Following is a brief review of selected key pro- and anti- inflammatory cytokines secreted from cellular players of innate and adaptive immune system that are responsive to estrogen treatment, a subject reviewed in detail earlier [85, 89, 96].

Cytokines from Innate Immune cells: The cells of innate immune cells predominantly macrophages and dendritic cells (DCs) produce cytokines which influence the direction of adaptive immune response. As mentioned earlier, the influence of estrogen on these cytokines

depends on number of factors including but not limited to, dose of estrogen, method (*in vitro* or *in vivo*) and duration of treatment, species and cell type studied and the kind of activation/stimuli used [85]. For example, on exposure to estrogen, PMA- activated human monoblastic U937 cells have increased TNFα production [97], while LPS-activated bone marrow derived macrophages have decreased TNFα production [98]. 17β-estradiol at 0.01-0.001 ng/ml increases TNFα secretion from LPS-activated rat peritoneal macrophages while both low (<0.1 pg/ml) and high dose (> 0.1 ng/ml) doses of estradiol decreased TNFα secretion from the LPS-activated rat peritoneal macrophages [99]. *In vivo* estrogen or estriol treatment also increases TNF expression from peritoneal macrophages, Kupffer cells and in sera of animals challenged with LPS [100, 101] Treatment of $CD11c^+$ murine spleen DCs with estrogen, upregulated intracellular IL-6 and IL-10, but did not affect TNFα and IL-12 expression [102]. Another recent report has shown that *in vivo* estrogen or ERα select agonist exposure of wildtype and ERβ knockout mice downregulated TNFα and IL-6 secretion from splenocytes and macrophages following trauma-hemorrhage [103]. In ovariectomized mice, there is decreased TNFα expression in brain following intracerebral injection of LPS. However, addition of estrogen upregulated TNFα levels [104]. These findings suggest that both ERα and ERβ are essential for mediating immunoprotective effect by decreasing the production of proinflammatory cytokines [103] and the affect of estradiol on cytokine induction is dependent on the dose and route of treatment.

Similarly, IL-1α and IL-1β, are proinflammatory cytokines that are secreted mainly from macrophage or monocyte, and have been shown to be critical in not only fighting off infections but also is a key player in diverse autoinflammatory diseases [105]. IL-1β signal through IL-1R of Toll like receptor (TLR) family activates NF-κB translocation and expression of multiple pro-inflammatory genes. There are reports that have shown that estrogen upregulate IL-1β induction, possibly by direct binding to IL-1β promoter. *In vitro* constructs demonstrated ER binding directly to IL-1β promoter in murine macrophages at moderate physiological estrogen concentrations [106]. Likewise, in peritoneal macrophages from male and female rats, moderate to low estrogen stimulated secretion of IL-1β [107]. Exposure of estrogen to LPS-stimulated HL-60 promyelocytic leukemia cells also upregulated IL-1β induction [108].

Increased IL-1α mRNA has been demonstrated in 17β-estradiol-treated rheumatoid fibroblast-like synovial cells and from primary synovial cells from RA patients in ERα dependent manner. It has been demonstrated that estrogen–mediated dissociation of corepressor HDAC2 from ERα, results in physical interaction of ER with Sp1 transcription factor and activation of Sp1 through the GC-rich region within the IL-1α gene promoter [109]. Administration of estrogen in ovariectomized rats with acute endoluminal arterial injury decreases IL-1α induction when compared with untreated ovariectomized rats [110].

Activation of estrogen-treated splenic lymphoid cells with LPS increased both IL-1α and IL-1β via NF-κB mediated signaling [83]. Increased IL-1α, IL-1β and IL-6 and increased astroglial responses have been observed in male rats *in vivo* treated with 17β -estradiol following spinal cord injury [111]. Conversely, short term *in vitro* estrogen exposure downregulates IL-1 secretion from macrophage by activating Akt pathway while chronic administration enhances IL-1 levels from LPS-activated peritoneal macrophages [112]. However, LPS-activated PBMCs from postmenopausal women given estrogen for 6 months had decreased IL-1β but not IL-1Ra [113]. LPS activation of microglia and astrocytes exposed to estrogen or ER agonist decreases IL-1β and TNFα induction [114]. Interestingly, IL-6, TNFα, IL-1Ra, IL-1β, and ratio of IL-1β /IL-1Ra were decreased in whole blood cell cultures exposed to varying dose of estrogen (10^{-12}-10^{-6} mol/l) [115]. These studies demonstrate that the duration and type of estrogen exposure plays a crucial role in modulating IL-1α and IL-1β levels and therefore influence the overall immune response.

Another important pro-inflammatory cytokine secreted by antigen presenting cells is IL-6, which together with TGFβ is involved in priming naïve $CD4^+$ T cells to commit to IL-17 secreting Th17 cell type. Increase in estrogen-mediated IL-6 has been shown in human peripheral monocytes with and without LPS treatment [116]. Various reports have demonstrated that IL-6 is variably inhibited by estrogen treatment in whole blood, PBMCs and whole bone marrow, but increased from macrophages [115, 117]. Even in macrophages, it is chronic *in vivo* estrogen exposure that leads to stimulation, while short-term in vitro exposure causes suppression [112]. However, others have shown that short-term estrogen exposure of immature DCs has been

demonstrated to increase IL-6, IL-8, and MCP-1 secretion. In addition, estrogen provides signal migration of mature DCs towards lymph-node derived CCL19/MIP3beta in migration assay, indicating that estrogen regulates DC-mediated T and B cell responses [118].

Cytokines from cells of the adaptive immune system: The key effector cells of adaptive immunity comprise of (i) IFNγ-secreting Th1 cells, which are critical for immunity against intracellular pathogens; (ii) IL-4-secreting Th2 cells essential for immunity against helminth parasite and key player in allergic response; (iii) IL-17-secreting cells which regulate neutrophilic inflammation; and (iv) suppressor T regulatory cells (Treg), which downregulate immune responses by secreting TGFβ and IL-10.

IFNγ is a prototypic cytokine released by Th1 cells, but a number of other cell types such as CD8 T cells, NK cells, NKT cells, are also know to secrete IFNγ. Numerous reports have indicated that there is gender bias in IFNγ production. There is increased IFNγ secretion in virus or *Listeria mexicana* or *mycobacteria*-stimulated spleen or lymph node cultures from female BALB/c, C57BL/6 [119], NZBxNZW- F1[120], DBA/2 mice [121] when compared to their male counterparts etc. Elevated levels of IFNγ have been demonstrated in Concanavalin A or antiCD3 antibody activated splenic lymphoid cell cultures and purified T cells from *in vivo* estrogen treated mice [122]. A putative ERE has been identified in the promoter 5'-prime flanking region of *IFNγ g*ene [123]. In presence of IL-27, estrogen induces T-bet, which primes $CD4^+$ cells to differentiate into Th1 cell type and IFNγ-mediated downstream proinflammatory events [124]. Studies have also demonstrated that 17β-estradiol, estrone and estriol treatment stimulates secretion of IFNγ from neuroantigen specific $CD4^+$ T cells from patients with MS [125, 126]. There is increase in $IFN\gamma^+$-secreting cells in lymph nodes of estrogen-treated female $ER\alpha^{+/+}$ mice and not in ERα deficient mice demonstrating that ERα, but not ERβ, is critical for the enhanced E2-driven Th1 cell responsiveness [66]. *In vitro* and *in vivo* exposure to estrogen increased IFNγ synthesis from alpha-GalCer activated iNKT via ERα receptor [127]. On the contrary, estrogen has been shown to inhibit secretion in T cells from postmenopausal women and in dendritic cells [89, 128, 129]. Physiological (preovulatory) levels of estrogen increase IFNγ secretion from PHA and LPS-stimulated PBMCs, whereas exposure to pregnancy levels of

estrogen decreases IFNγ production [130]. In the third trimester of pregnancy, there is decreased IFNγ expression in RA patient compared to healthy women.

Although estrogen has been shown to increase the secretion of Th-1 defining cytokine, IFNγ, there are other reports, which have demonstrated that estrogen primes CD4 T cells to entirely opposite pathway of IL-4 and IL-10-secreting Th2 cell subset. In vivo ovalbumin treatment of ovarietomized rats enhances estrogen mediated IL-4 secretion from bone marrow cells when compared to control rats [131]. High physiological levels of estrogen in T cells increase IL-4 secretion and GATA-3 expression, a transcription factor that can bind the IL-4 promoter [132]. GATA-3 over-expression is associated with ER over-expression in hormonally responsive breast cancer, however estrogen treatment in that model did not increase GATA-3 expression [133]. Exposure to high levels of estrogen either during pregnancy or given experimentally, shifts the cytokine balance to Th2 type [130]. Increased IL-4 and IL-10 accompanied with decreased IFNγ have been reported in PHA-stimulated PBMCs from pregnant women when compared to cells from non-pregnant controls suggesting the role of higher estrogen levels in skewing the response from Th1 to Th2 [134, 135].

Th17 cells, Tc17 cells, NKT and γδ T cells secrete various proinflammatory cytokine but the most potent and important of them all is IL-17 [136]. IL-6 along TGFβ is required for commitment of naïve $CD4^+$ T cells to Th17 cells. IL-17 aids in recruitment of neutrophil to the site of tissue damage and has been shown to either protect and enhance immunity against different pathogens and vaccine or enhance their clearance e.g. *Mycoplasma pulmonis, Shigella flexneri, Listeria monocytogenes, Francisella tularensis, Yersinia pestis, Helicobacter pylori,* influenza A [137-143]. However, exaggerated or dysregulated IL-17 production results has been observed in various chronic and autoimmune diseases such as MS and SLE. A recent report from our laboratory has shown that *in vivo* estrogen treatment primes splenic lymphocytes to secrete copious amounts of IL-17 when activated with IL-6+TGFβ and antiCD3 antibodies [144].

Cytokines from Tregs cells- T-regulatory cells (Tregs) exist as natural regulatory cells in the thymus and induced Tregs cells in the peripheral lymphoid organs, which are typically CD4+ CD25+FoxP3+ [145]. These cells exert profound effects to dampen the immune responses

especially after the antigens are cleared. Tregs can downregulate all types of T cells including pro-inflammatory Th-1 and Th-17 cells. Tregs use a variety of mechanisms to downregulate these cells including through secretion of "suppressive" cytokines, IL-10 and TGFβ. Disturbances in Treg cells can promote autoimmunity and are discussed in the following section.

Estrogen has been shown to have opposing effects in autoimmune diseases by exaggerating the pathogenesis in systemic lupus erythematosus (SLE) and attenuating diseases like multiple sclerosis (MS). Anti-inflammatory action of estrogen is mediated by decreasing the production of pro-inflammatory cytokines or by increasing the secretion of anti-inflammatory cytokine such as IL-10 and TGFβ secreted by Treg cells. These cytokines are known to dampen Th1, Th2 and Th17-mediated immune response by blocking of IFNγ, IL-4 and IL-17, respectively. During pregnancy, when there are high levels of estrogens, there is increase in anti-inflammatory IL-10 levels, decreased maturation of DCs and IL-12p70 [146]. TGFβ is conventionally regarded as an anti-inflammatory cytokine, transcribed by SP-1, a known estrogen-binding partner via the tethered pathway [73, 147]. TGFβ secretion is stimulated by estrogen treatment in astrocytes and fibroblasts [148, 149]. Estrogen at high physiological levels stimulated production of IL-10 [125] in T cells from human patients. IL-10 transcription can be induced by CREB [150], a transcription factor known to associate with ER in breast cancer models [81, 151], and likewise a putative ERE has been identified in the IL-10 promoter region [151]. It has been demonstrated that Treg suppresses osteoclast differentiation from human embryonic bone marrow cells by upregulating TGFβ and IL-10 induction. Addition of estrogen at concentrations between 10^{-7} and 10^{-9} mol/l further suppressed osteoclast differentiation by increasing IL-10 and TGFβ expression in Tregs cells. This indicates the stimulatory effect of estrogen on Tregs [152]. Estrogen treatment also increases FoxP3 expression in Tregs [153]. Additionally, programmed death -1 (PD-1), a negative co-stimulatory molecule that is expressed intracellularly by Tregs is also upregulated by estrogen treatment. In ER knockout mice, there is suppressed PD-1 expression and Tregs activity [154]. It has been demonstrated that the protective effects of estrogen on EAE, by increasing FoxP3 expression and suppressive activity of Tregs are mediated through ERα but not ERβ [155]. Furthermore, physiological levels of estrogen *in vitro* directly converted activated $CD4^{+}CD25^{-}$ T cells into $CD4^{+}CD25^{+}$ Treg cells which correlated with expression of ERα in

these cells [156]. From published reports, it is evident that estrogen plays an important role in regulating the number and activity of Treg cells in various autoimmune diseases such as MS and RA. In the following section we will compare the opposing roles of estrogen on two female predominant autoimmune diseases.

Contrasting Influence of Estrogen on MS and SLE

Clearly estrogen can mediate both inflammatory and anti-inflammatory effects, depending on the cellular context, specific ligand as well as receptor repertoire. Likewise varying effects of estrogen on immune diseases has been observed. Estrogens can both promote autoimmune disease, as in the case of systemic lupus erythematosus (SLE), and reduce or even protect against autoimmune disease, as in the case of multiple sclerosis (MS). Experimental autoimmune encephalitis (EAE) is a Th-1 cell mediated autoimmune disease and serves as a model for MS, an inflammatory demyelinating disease of the central nervous system.

MS and EAE are characterized by activation of auto-reactive $CD4^+$ T cells that target myelin antigens such as myelin oligodendrocyte glycoprotein (MOG) [157, 158]. This results in infiltration of $CD4^+$ T cells (predominantly Th-1) and other pro-inflammatory cells into the central nervous system (CNS) and de-myelination of axons by macrophages, ultimately leading to axonal death and decreased CNS function [158]. Clinically, MS often presents as a relapsing-remitting disease, with variable rates of relapse between individuals.

Estrogen treatment has been shown to decrease clinical and histological signs of EAE in mice. It is thought that estrogen accomplishes this by inhibiting the autoantigen specific proinflammatory cytokine production, and inhibiting inflammation and demyelination. For example, estrogen has been shown to inhibit production of inflammatory molecules (such as IFNγ, tumor necrosis factor-alpha (TNFα), iNOS, and MCP-1) in microglia cells from EAE mice [157]. Estrogen treatment is neuroprotective as it helps in preserving the integrity of neurons and axon with decreased activation of microglial and monocytes in the central nervous system [159]. The anti-inflammatory and neuroprotective effects of estrogen on EAE are mediated through ERα but not ERβ [157, 159, 160]. Immuno-modulatory effects of estrogen are also demonstrated in a study

where splenic dendritic cells (DC) exposed to estrogen, *in vitro*, had therapeutic effects on acute EAE with inhibition of $CD4^+$ T cells expansion, increased proportions of Tregs and $CD4^+CD8^-$ suppressor T cells [129], increased T cell apoptosis [161], and increased Th2 cytokines such as IL-10, IL-4 accompanied with decreased Th1 cytokines such as TNFα and IFNγ [162]. Interestingly, estrogen profoundly altered expression pattern of 315 genes in spinal cord tissue of mice protected from EAE, of which 302 genes were down-regulated and only 13 genes up-regulated [163].

In pregnant mice with EAE, there is reduced CNS pathology and less TNFα and IL-17 production along with reduced T cells activation when compared to non-pregnant controls [164]. In addition, myeloid-derived cells especially DCs have shown to mediate protective effects of estrogen. *In vivo* treatment of mice with pregnancy specific estrogen, estriol, generated tolerogenic DCs [165]. These DCs had upregulated activation markers (CD80 and CD86) and inhibitory costimulatory markers (PD-L1, PD-L2, B7-H3, and B7-H4). Furthermore, DCs from estriol treated mice had increased IL-10 and TGFβ but decreased proinflammatory IL-12, IL-23 and IL-6 mRNA expression. Transfer of DCs from estriol treated mice protected the recipient mice from active induction of EAE [165]. With use of conditional ERα deficient mice and bone marrow chimeras, it has been demonstrated that ERα in hematopoietic cells but not in endothelial cells is essential for estrogen-mediated inhibition of Th1 and Th17 cell differentiation and protection from EAE [166]. ERα signaling in T cells is indispensable and sufficient for estrogen-mediated protection against Th17-cells driven CNS inflammation in EAE mice [166] and $CD4^+$ T cells homing in the CNS [167].

The anti-inflammatory role of estrogen was implicated when it was observed that rates of relapse in women were significantly reduced during pregnancy when estrogen levels are high, however rates rose substantially post-partum when estrogen drops precipitously [168, 169]. A small clinical trial of women with MS given E3 at pregnancy levels showed a decrease in the number and size of brain lesions measured by MRI [170]. Experiments in mouse EAE models have demonstrated E2 and to a greater extent E3 treatment is protective against development of EAE and reduces the severity of disease after onset [157] . There is a decrease in inflammation upon

estrogen treatment by a number of mechanisms, including reduced secretion of TNFα by autoreactive T cells and macrophages, reduced recruitment of inflammatory cells to the CNS, as well as induction of $CD4^+CD25^+$ Tregs. It also down-regulates cellular adhesion molecules such as VCAM and ICAM in endothelial cells and modulates the GTPase Rac1 in macrophages, inhibiting adhesion and migration [171]. The anti-inflammatory actions of estrogens thus inhibit autoimmunity in the context of a Th-1 T cell-mediated disease such as MS.

In the case of SLE, estrogen is associated with increased severity and increased flares of the disease, both in humans and in animal models [85, 89]. The female: male ratio of 9:1 to 20:1 has been reported for SLE susceptibility [172, 173]. Lupus disease manifests as multiple symptoms that vary by patient. Lupus involves nephritis, skin rash, arrhythmias and neuronal effects such as numbness, tingling, and psychiatric changes [174]. Dysregulation of the immune system at multiple levels, including loss of tolerance, altered T cell signaling, and T cell, B cell, and monocyte hyperactivity, lead to activation of autoreactive B cells and secretion of pathogenic antibodies including anti-ds DNA and anti-phoshpholipid antibodies [175-178]. The accumulation of immune complexes which lodge in small capillaries, triggering further localized inflammatory cascades, have been shown to cause SLE nephritis, skin rash and are likely responsible for many other symptoms. There is a shift in T cell populations from Th-1 to Th-2, associated with an increase in serum concentrations of Th-2 associated cytokines IL-4, IL-6 and IL-10 and reduction in the Th-1 cytokine IL-2 and IFNγ [89]. IFNγ still plays a role in glomerulonephritis implying different effects of estrogen in various tissue pathology. Gender differences in SLE predilection and pathogenesis are well documented. Clinically, females have more frequent relapses and Raynaud's phenomenon, arthritis and leucopenia, while males have more skin manifestations, serositis and renal disease. Female patients have more psychiatric symptoms and headaches whereas males have more seizures and peripheral neuropathy [179] .

Estrogen can promote SLE progression via a number of mechanisms including induction of cytokine and autoantibodies and autoantigens [180, 181]. First, by induction of Th-2 type cytokines including IL-4, IL-6 and IL-10, as discussed in the review of cytokines, estrogen promotes skewing of T cell populations towards Th-2 , the canonical B cell "helper" which then

activates auto-reactive B cells. This imbalance in cytokine is accompanied with flares of SLE and thrombosis and has been confirmed in pregnant females, where there is high amount of estrogen in the circulation, and also in females given exogenous estrogen [182]. However, from randomized trials and large observational studies it is now demonstrated that SLE patients on HRT have only marginal increase in the risk of mild/moderate flare and thrombosis and no risk of major flare [183, 184]. Estrogen also stimulated secretion of IL-10 in monocytes along with increased anti-double-stranded DNA antibody and immunoglobulin G production by peripheral blood mononuclear cells from patients with SLE [185, 186].

Estrogen has been shown to regulate B cell maturation and selection, in a mouse model of lupus [187]. It has been demonstrated that high levels of estrogen in serum promotes maturation of a pathogenic naive autoreactive B cell population and decreases the maturation of a potentially protective autoreactive B cell repertoire [188]. In addition, estrogen treatment increased IgG and IgM secretion from PBMCs as well as serum levels of IgG and IgA in rodents and humans [186, 189-191]. Furthermore, in mouse model for induced lupus, exposure to estrogen increases the titer of antiDNA antibodies and also leads to systemic inflammation with increased B cell-activating factor and IFN levels and induction of an IFN signature [192]. Estrogen exposure increases calcineurin expression from T cells from SLE patients when compared with healthy females [193]. Not only this, estrogen also upregulates the expression of endogenous autoantigens such as human endogenous retroviruses [194], and reactivity to exogenous antigens [195]. HERV is found to be increased in SLE wherein HERV act as autoantigens by molecularly mimicking RNP antigens in the patient's body [196].

In mice model for SLE, it has been reported that there is differential expression of ERs in MRL/MP-lpr/lpr and NZB/W mice when compared with BALB/c mice [197]. One reason for hyper-responsiveness of estrogen in SLE patients could be due to altered quantitative and/or qualitative expression of ERs. For example, in SLE patients, there is high expression of ERα on $CD4^+$ T cells while there is decreased ERβ on PBMCs, which inversely correlates with the SLE disease activity index (SLEDAI) or prednisolone dose [198]. In addition, estrogen treatment modulated expression of ERs in immune cells of autoimmune-prone $SNF_{(1)}$ and non-autoimmune $DBF_{(1)}$ mice. Estrogen exposure increased ERα expressing $CD4^+$ and $CD8^+$ T cells and percent

$ER\alpha^{+}$ DCs and macrophages in $SNF_{(1)}$ mice but not in $DBF_{(1)}$ [199]. Studies have shown that ERα -mediated signaling is important in estrogen-induced development of lupus phenotype. Estrogen treatment of wildtype mice had accelerated lupus development, increased autoantibody and IL-5, IL-6, IL-10, IFNγ and TNFα production with increased kidney damage when compared with estrogen-treated ERα deficient mice [199]. In lupus-prone (NZB x NZW)$F_{(1)}$ mice, ERα deficiency attenuated glomerulonephritis, decreased anti-histone/DNA antibodies and increased survival [200]. Furthermore, with ER selective agonists, importance of ERα in lupus development has been demonstrated [201]. Together, these studies indicate that ERα is essential in estrogen-mediated exaggeration of lupus disease.

Polymorphism in ERα gene expression has also been reported in SLE patients [202, 203]. Furthermore, out of 13 genes identified as significantly altered during menstrual cycle in females but not in males, six were statistically different in SLE patients when compared with normal controls [204]. Tumor necrosis factor receptor superfamily member (TNFRSF14) also called Herpes virus entry mediator (HVEM) is one such gene, which is quantitatively altered in both females and SLE patients. It is a ligand for B and T lymphocyte attenuator (BTLA). Interaction of TNFRSF14 with BTLA downregulates lymphocyte activation and homeostasis [205]. In menstrual cycle, estrogen increases expression of TNFRSF14 mRNA in PBMCs, however in SLE patients TNFRSF14 mRNA is lowered which results in partial activity of BTLA thus leading to net immune enhancement [205, 206].

In addition, SLE patients have other risk alleles susceptibility loci such as interferon regulatory factor (IRF5)[207]. It is suggested that immune complex of nuclear antigens activate intracellular TLRs such as TLR7 and TLR9- mediated IRF5 pathway, which upregulates IFNα secretion in SLE patients and MRL/lpr mice, [208, 209]. Increased levels of IRF5 have been reported in female NZB and NZB/W F(1) mice when compared to male NZB and NZB/W F(1) or C57BL/6 aged matched controls [210]. Furthermore, the mRNA level of IRF5 in splenic cells was found to be decreased in $ER\alpha^{-/-}$ mice when compared with female $ER\alpha^{+/+}$ mice and treatment of splenic cells with 17β–estradiol upregulated IRF5 mRNA levels. Impressively, IRF5 mRNA was high in nuclei of splenic B cells from female mice when compared to male mice [210]. Interestingly,

IFNγ and IFNα can upregulate ERα, which demonstrates the feedback interactions of cytokines and ERs. Overall, it is clear that estrogen effects vary based on the autoimmune disease setting.

miRNA and Estrogen

From recent reports it is now evident that estrogens play an important in immune response by epigenetic microRNA regulation [211, 212]. MicroRNAs (miRNA) are endogenous non-coding RNAs of an average of 21-22 nt in length [213]. The role of miRNA in post-transcriptional gene regulation has recently been defined. MiRNA bind to the 3' untranslated region (UTR) of target mRNA by partial homology resulting in either degradation of mRNA or inhibition of translation. miRNAs also participate in the regulation of autoimmunity: deficiency in Dicer or Drosha leads to autoimmunity in mice [214]. Aberrant miRNA expression has been demonstrated in several autoimmune diseases including multiple sclerosis (MS), and SLE [215]. MicroRNAs have recently been implicated in the pathogenesis of MS by multiple studies, however there is great variability between these studies and the precise disease-associated miRNAs [216-221]. This variation is likely due to differences in tissue and patient populations studied as well as techniques employed to identify miRNAs. The possible contribution of hormonal regulation of these disease-associated miRNAs has not been addressed directly.

Of the MS-associated miRNAs identified, only a few have been shown to be regulated by estrogen [212]. These include miR-145, miR-200b, and miR-486. Keller et al demonstrated upregulation of miR-145 in whole blood from relapsing-remitting MS patients compared to healthy controls [219]. While the target of miR-145 in immune cells is unknown, it has been show to be critically involved with multiple stem cell differentiation pathways, including Sox9 and Oct4 [222, 223]. *In vivo* estrogen treatment downregulated expression of miR-145 in murine splenic lymphocytes [212]. Both miR-200b and miR-486 were found to be upregulated in $CD4^+$ cells from relapsing-remitting MS suffers compared to healthy controls. Estrogen downregulated miR-200b, which has a broad range of target mRNAs in different cell types, including the transcription factors ZEB1/2, RND3 and Ets-1 [224-226]. It is possible that estrogen downregulation of miR-145 and miR-200b could be one of the reasons for estrogen-regulated alleviation of MS since both of them are found to be upregulated in MS patients. Unlike the

previous miRNAs, estrogen treatment upregulated expression of miR-486 by nearly 7 fold, in splenic lymphoid cells [212]. The antiapoptotic glycoprotein OLFM4 is a putative target of miR-486 in gastric cancers, however its expression and role in lymphocytes remains to be determined [227]. Together, these miRNAs can be used as potential targets to treat MS.

In pediatric SLE patients, miR-181a is significantly downregulated [228]. It is noteworthy that, miR-181a is critical modulator of B and T cell differentiation, maturation and function. In another report, 11 miRNA were significantly altered in $CD4^+$ T cells of SLE patients of which miR-126 was increased which targeted DNA methyltransferase 1 (Dnmt1). The decrease in Dnmt1 resulted in demethylation and increased expression of CD11a and CD70 resulting in hyperactivity of T and B cells [229]. MiR-146a and miR-125a are decreased in human SLE patients [230, 231]. Decrease in expression of miR-146a contributes to alteration in type 1 IFN signaling with increased IRF-5, STAT-1 and IFN scores [232]. Decreased miR-125a in SLE patient results in upregulated miR-125a target RANTES via KLF13 expression [231]. miR-21 is upregulated in SLE patients and positively correlates with disease activity. miR-21 has been shown to target a protein translation inhibitor, PDCD4, which results in aberrant T cell activity [233].

In our recent study, common set of dysregulated miRNAs, in splenic lymphocytes, purified T and B cells, were identified in three genetically different murine lupus models, MRL-lpr, B6-lpr and NZB/W(F_1) [234]. By miRNA microarray assays and Real-time RT-PCR analysis, miR-182-96-183 cluster, miR-31, and miR-155 were found to be decreased in splenic lymphocytes of all three strains with active disease when compared with their age-matched controls. However, miR-146a, miR-101a, and miR-17-92 were markedly upregulated only in splenic T, and not in B cells from MRL-lpr mice. There was difference in level of expression across different strains e.g, miR-127 and miR-379 were greatly upregulated in splenocytes from lpr mice, but were only moderately increased in diseased NZB/W mice [234].

Microarray analysis data revealed regulation of 25 miRNA by estrogen in splenic lymphocytes. While miR-223, miR18a and miR-708 were increased, miR-146a, miR-125a, miR-125b, miR-143, miR-145, let-7e, miR-126 were found to be decreased in freshly-isolated splenic

lymphocytes from estrogen-treated mice when compared to placebo controls. Increased activity of miR-146a significantly lowered LPS-induced IFNγ and iNOS expression, however decreased activity of miR-223 decreased LPS-induced IFNγ in cells from estrogen-treated mice suggesting important role of miRNA in estrogen-mediated immune regulation [212]. Since some of the miRNA dysregulated in SLE and modulated by estrogen are common such as miR-146a, miR-125a etc, it is plausible that estrogen-mediated exaggeration of SLE may be due to dysregulated miRNA expression. Recent studies add new dimension to our current understanding of immune regulation by estrogen.

Conclusion

Sex hormone regulation of the immune system is now very well recognized. Estrogens act on the immune system by estrogen receptor or non-receptor dependent mechanisms. While it is clear that estrogen regulates the immune system, the outcome of estrogen-induced immune response is variable. It is therefore difficult to generalize the effect of estrogen since the overall outcome i.e. either immuno-suppression or immune enhancement depends on multiple factors such as type of tissue; health of host; presence or absence of estrogens; dose and type of estrogen; type of receptor expression; qualitative or quantitative expression of receptor; and signaling pathways, presence of adaptor molecules, coactivators and corepressors. It is now recognized that estrogen exists in multiple forms natural, synthetic, plant-derived or fungal form. It is possible that there may be a subset of individuals who could be very sensitive to estrogen. It is noteworthy that a majority of autoimmune diseases are female predominant [235]. The precise reasons for female gender predisposition of autoimmune disease remain unclear. The evident gender bias has been inconclusively associated with different hypothesis such as X-chromosomal inactivation, fetal microchimerism, X chromosomal abnormalities, epigenetic miRNA and histone deacetylation. It is well established that sex hormones play a critical role in the disease pathogenesis since the fluctuations in the levels of estrogen with age and estrus cycle modulate the immune parameters and disease severity. Although studies are being conducted to define the role of estrogen in these chronic lifelong debilitating diseases, their precise affects needs to be answered. The complexity of genetic factors, age, gender and other signaling pathways together with environmental

exposure to new chemicals makes it hard to conclusively predict the outcome of sex hormone in clinical conditions. It is unwise to generalize the estrogen-mediated immune cell signaling and thus each disease state must be examined independently. Therefore, future mechanistic studies are warranted to understand the specific role of estrogen in immune regulation in a particular clinical condition and disease.

Figure 1

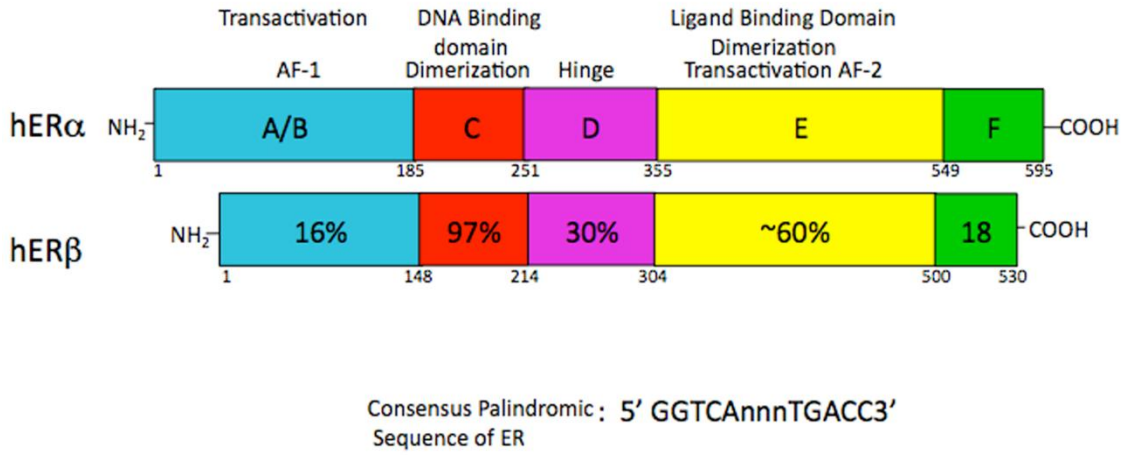

Figure 1: Structural description of domain structure and percent sequence homology of human ERα and ERβ

Full length human ERα comprise of 595 amino acids and has 2 shorter isoforms, while ERβ has 530 amino acid and 4 other splice variants. ERs usually bind to Estrogen Response Element (ERE), which is a 5 base pair palindrome (GGTCA) with a 3 base pair spacer (nnn).

Figure:2

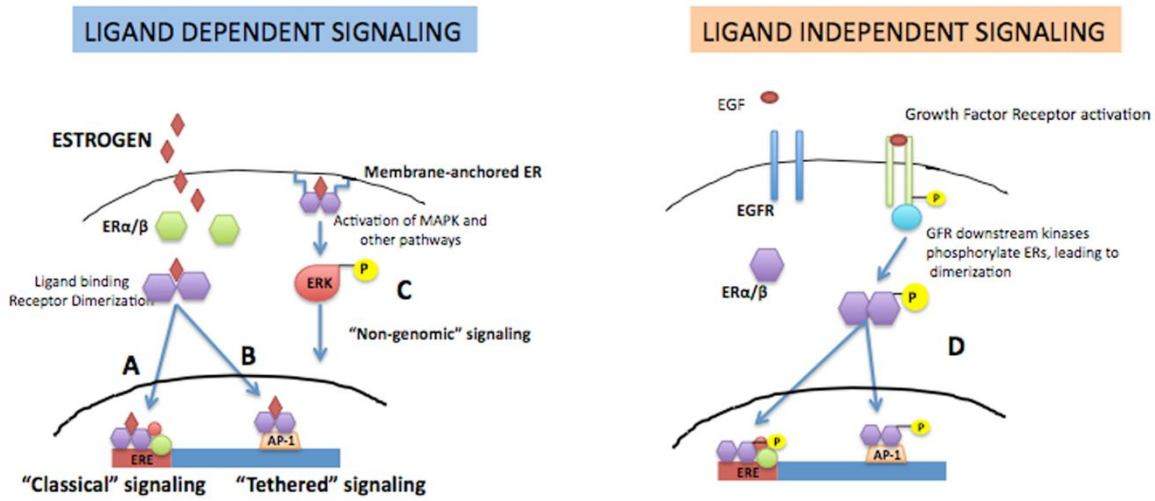

Figure 2: Pictorial illustration of key estrogen signaling pathways.

Estrogen signals via membrane or intracellular ERα/β in either ligand dependent or ligand independent pathway. *A*) In the ligand dependent pathway, estrogen binds to its receptor, which then dimerizes and translocates to nucleus and binds to estrogen response element (ERE) and regulates estrogen responsive genes in "classical" pathway. *B*) In "tethered" pathway, activated ERs interact with other signaling molecules and transcription factors and bind to non-ERE sites and regulate gene expression. *C*) In another ligand dependent pathway, upon ligand receptor interaction, there is rapid physiological "non-genomic" signaling by activation of MAPK or other cytoplasmic signaling pathways. *D*) In the ligand independent pathway, ERs are activated downstream of other activated signaling cascades such as growth factor receptor (GFR) and modulate gene expression in absence of estrogen in both ERE dependent genomic fashion or by interacting with other transcription factors.

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Chapter 2: IL-17: Biological and Pathological Role

Introduction

Interleukin 17 (IL-17) was initially termed in 1993 as cytotoxic T lymphocyte-associated antigen-8 (CTLA-8) when Rouvier et al. [1] first cloned it from a rodent cDNA sequence. Subsequently, IL-17 was also identified in humans [2]. It is now known that the IL-17 cytokine family includes six cytokines, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. The IL-17 isoforms are glycoproteins of 155 amino acids and range from 20 to 30 kDa in size. These IL-17 isoforms have overlapping, but not identical biological activities. They share 4-conserved cysteine residues at C-terminal region. So far, major focus has been on IL-17A (a founding member of IL-17, hence referred as IL-17) and IL-17F because of their important functional and biological properties. IL-17A is a powerful proinflammatory cytokine and is now known to be secreted by many cell types including: $CD4^+$ cells (Th17), $CD8^+$ cells (Tc17), γδ T cells, natural killer cells and mast cells, neutrophils, among others [3-6]. IL-17F largely has similar action as IL-17A and is produced by $CD4^+$ cells, $CD8^+$ cells, monocytes, basophils, mast cells, γδ T cells, NKT cells, etc [7-10]. IL-17A and IL-17F are 50% homologous and map to the same chromosomal loci. They exist as either homodimers of IL-17A or IL-17 or as IL-17A/F heterodimers [11]. Compared to IL-17A or IL-17F, little is known about IL-17B or IL-17C and their cellular source. IL-17D is secreted by resting $CD4^+$ cells and B cells, whereas, IL-17E, also termed IL-25, is known to favor Th2-mediated and allergic immune responses [12], and is mainly secreted by Th2 cells, mast cells [13].

It is now well documented that IL-17 and IL-17-mediated proinflammatory events are upregulated in different infections and autoimmune diseases. Although IL-17 is known to have protective effects in infection, over production of IL-17 has been shown to aggravate disease conditions and contribute to tissue injury as observed in different autoimmune diseases. IL-17A and IL-17F have potential to mobilize, recruit, and activate neutrophils, thus linking adaptive and innate immunity [14]. Cytokines and chemokines released in response to IL-17 promotes granulopoiesis and neutrophils accumulation; protection of mucosal membrane by mucin

secretion and tight junction formation [15, 16]. IL-17A has been shown to induce iNOS and NO production, which has relevance in autoimmune and inflammatory disorders.

IL-17 and Infections

Upregulation of IL-17 has been reported in a variety of infections including *Klebsiella pneumoniae* [17], *Porphyromonas gingivalis* [18], *Helicobacter pylori* [19], *Borrelia burgdorferi* [20], *Aspergillus fumigatus*, and *Nippostrongylus brasiliensis* [21]. Protective effects of IL-17 in infection is well-documented. For example, deficiency of IL-17R resulted in higher susceptibility to *Klebsiella pneumoniae* and *Candida albicans* in mice [22, 23]. Production of IL-17A by cells such as T cells aids in the clearance of *pathogens*, *Bacteroides fragilis* [24], *Borrelia burgdorferi*, *Mycobacterium tuberculosis* [20], and the fungal species [25]. Null mutation in *IL12B* and *IL12RB1* genes results in impaired IL-17-producing T cell development in patients with autosomal-recessive susceptibility to myobacterial diseases [26]. IL-17R knockout mice have decreased infiltration of neutrophils in alveolar space with more *Klebsiella pneumonia* dissemination and 100% mortality when compared with control mice with only 40% mortality. This was associated with decreased IL-17-induced G-CSF and MIP-2 [22].

IL-17 and Autoimmune Diseases

IL-17-secreting cells are well documented to be involved in the pathogenesis of chronic autoimmune diseases [27-29]. A growing list of reports has associated autoimmune disorders with overproduction of IL-17. Interference in IL-17 production or action by using IL-17R antagonist [30] and IL-17A-blocking antibodies [31, 32] has been shown to attenuate autoimmune diseases. Although, in healthy homeostatic conditions the levels of IL-17A in human sera are undetectable, the levels in serum and tissue are markedly increased in inflammatory bowel disease MS and RA [33-35]. Interaction of IL-23-producing APCs and Th17 cells has been shown to have a role in many autoimmune diseases such as psoriasis, MS, inflammatory bowel disease and SLE. Animal models of MS, EAE was long thought to be IL-12 driven Th1-mediated disease. This view is changing with the recent finding that IL-17-producing $CD4^+$ T cells, driven by IL-23, also play a pivotal role in the pathogenesis of EAE. Further, the

involvement of IL-17 producing $CD4^+$ T cells in EAE disease severity has been well established by recent reports where IL-17 deficient mice had significantly suppressed disease development [36]. IL-27, a member of the IL-12/IL-23 family, potently inhibits Th17 development. In microglia cells from brain, and macrophages from spleen and lymph nodes of mice with EAE, there was increased expression of both IL-27 and its receptor, IL-27Rα [37]. Evidence is now emerging that IL-27 may aid in downregulation of EAE by suppressing proinflammatory IL-17 [38]. Moreover, treatment of effector cells with IL-27 suppressed encephalitogenic Th17 responses [39]. These studies further show IL-17 is important in EAE pathogenesis. Since IL-17 is a potent proinflammatory cytokine and its exaggerated response results in tissue damage, the regulation of IL-17 expression is considered to have valuable potential for clinical applications in the diagnosis or treatment of complicated immune disorders [40, 41].

Molecular Aspects of IL-17 Induction

Role of Cytokines:

Although IL-17 was discovered nearly 20 years ago, it was not until 2005, this cytokine gained attention of immunologists when two groups independently identified a new IL-17 secreting $CD4^+$-Th cell sub-population named Th17 cell (Figure 1) [42, 43]. Th17 cells not only secrete IL-17A, but also other cytokines such as IL-21, IL-17F, IL-22 etc. (Figure 2). It is now well established that exposure of naïve T cells to IL-6 and TGFβ1 drives the cells towards the development and differentiation of Th17 subpopulation. In human Th17 development, TGFβ1 is absolutely needed [44]. Although initial reports showed that TGFβ1 inhibits IL-17A production in a dose-dependent manner [45, 46], recent studies have shown that low concentrations of TGFβ1 in combination with either IL-21 [47], or IL-1β and IL-23 [48] or IL-1β, IL-23, and IL-6 [49] are necessary to promote differentiation of human naïve $CD4^+$ T cells into Th17 cells. However, high levels of TGFβ1 and the absence of inflammatory cytokines skew T cell differentiation towards regulatory T cell development by increasing transcription factor, FoxP3 [48, 50], (Figure 1 and 2). Low levels of TGFβ1 synergize with cytokines such as IL-1β, IL-6, IL-21, and IL-23 [47, 48] to promote expression of heterodimer IL-23R (which facilitates Th17 cell proliferation). Initially IL-23 was considered to be important for commitment of naïve cells

to Th17 cells. However, later it was found that IL-23 amplifies and stabilizes the proliferation of IL-17-producing cells but is not essential for differentiation of Th17 cells [26, 51]. Th17 cells also express CC chemokines receptor (CCR6), which is a receptor for chemokine CCL20 (also known as macrophage inflammatory protein-3alpha; MIP-3alpha) suggesting Th17 cells also respond to this chemokine [52]. The cytokine microenvironment in tissue plays an important role in deciding the divergent route of $CD4^+$ T cell differentiation and commitment. Exposure of naïve $CD4^+$ T cells to cytokine such as IL-12 or IL-4 will preferentially differentiate into Th1 and Th2, respectively (Figure 1). Interestingly, it has been demonstrated that Th17 cells demonstrate plasticity, i.e. these cells do not have a fixed phenotype. A low dose of TGFβ appears to be essential in maintaining Th17-phenotype. In absence of TGFβ, but presence of IL-23 or IL-12 alone, Th17 cells switch to Th1-like phenotype with increased IFNγ and decreased IL-17A or IL-17F secretion in STAT4 and T-bet dependent fashion [53]. The potential plasticity of Th17 to Th1 cells by IFNγ and IL-12 has been observed in both mice and human [54, 55]. Th17/Th1 cells express both RORγt and T-bet [55] and IL-23R, CCR6 [54]. Similarly, other studies have also shown that IL-17A and IL-17F expression may be transient and is not a terminal/end-stage Th cell differentiation [56, 57].

Studies have also shown that activation of dendritic cells (DCs), in a MyD88 dependent fashion by different Toll-like receptors (TLR) ligands viz., CpG, LPS, polyI:C, are crucial for CD4 differentiation into IL-17-producing cells [58]. The importance of IL-1β in the induction of IL-17 is evidenced by the finding that in IL-1 receptor type-1 deficient (IL-1RI $^{-/-}$) mice, there is lowered induction of autoantigen-specific Th17 cells but normal Th1 and Th2 cells. Importantly, IL-1RI $^{-/-}$ mice had decreased EAE presumably due to decreased pathogenic Th17 cells [59]. Cross-linking of CD3 on T cells has also been shown to release IL-17 [60]. Under *in vitro* conditions, ICOS, IL-15, IL-23 and nonspecific stimuli ionomycin, and phorbol 12-myristate 13-acetate (PMA) stimulate IL-17 production [61-63]. Microbial lipopeptide such as from *Borrelia burgdorferi* have also been reported to have stimulatory effects on IL-17 production from human and murine T cells [20].

Although Th17 are undoubtedly important in IL-17 induction and secretion, cells from innate immune system can also secrete IL-17 even when $CD4^+$ cells have not been activated. Sentinel innate cells reside in the host-environment interface such as in lung, mucosal lining of gut and in skin. These cells are γδ T cells, Lti cells, Paneth cells, iNKT cells, neutrophils ($GR1^+CD11b^+$ cell) etc, which are in pre-active state and do not require antigen processing to start secreting IL-17 [64] (*reviewed extensively by Cua and Tato 2010*). In Rag-/- mice (which lack B and T cells), there was increased IL-23-mediated IL-17 production thereby indicating that the innate immune cells also play an important in early immune responses [65]. These cells constitutively express transcriptional regulators for IL-17 induction (discussed in the next section), therefore, upon activation by IL-23 or IL-1β alone or in combination with activated toll-like receptors (TLR) or T cell receptors (TCR), these cells produce IL-17 within hours of stimuli [64, 66]. Overall, the above studies indicate that IL-17 induction is highly dependent on the kind of stimuli received from non-immune and innate immune cells.

Role of IL-23 in IL-17 maintenance

IL-23, a heterodimeric cytokine of IL-6/IL-12 family that is primarily secreted by antigen presenting cells (APCs), T cells, B cells, and endotheial cells. IL-23 is composed of two subunits- IL-12p40 (common with IL-12) and IL-23p19 [67-69]. IL-23 receptors (IL-23R) are expressed on different cells including $CD4^+$ T cells, DCs etc. Contrary to the initial understanding that IL-23 is critical of Th17 differentiation; it is now evident that IL-23 is required for expansion, and/or survival and stabilization of Th17 cells by activating STAT3 and partially STAT4 [31, 42, 70-73]. IL-23 alone is not able to drive Th17 differentiation from naïve T cells, which lack IL-23R (Figure 3). However, once committed Th17 cells have enhanced expression of IL-23R and become responsive to IL-23. For initial differentiation of naïve $CD4^+$ cells into Th17 cells activation by IL-6 together with TGFβ1 is critical but in the later stage of differentiation, IL-23 appears to play a key role in Th17 commitment [51, 74, 75]. Similar to IL-6-mediated STAT3 activation, activation of IL-23R by IL-23 results in autophosphorylation and transphosphorylation of receptor-associated JAK-family proteins, Tyk and JAK2 and tyrosine moieties on the receptor. These phosphorylated tyrosine act as the recruiting/docking site for

STAT3 molecules, which in turn get phosphorylated. Phosphorylated STAT3 proteins then homodimerize and migrate to the nucleus and activate RORγt and IL-17 gene [76]. It has also been shown that expression of IL-23R is dependent on positive feedback by IL-23-mediated activation of JAK-2, STAT1 and STAT3 activation in human CD4 cells [77].

IL-23 also plays an important role in a range of autoimmune and inflammatory disorders [78]. Impressively, IL-23p19 and IL-12p40 deficient mice are resistant to EAE, and collagen-induced arthritis (CIA) [79, 80]. IL-23-activated pathogenic T cells have been shown to produce IL-17A, IL-17F, IL-6, TNFα but not IFNγ or IL-4. Experimentally, it has been shown that treatment of mice with active EAE with anti-IL-23p19-specific antibodies, decreased serum IL-17 levels and IFNγ, IP-10, IL-17, IL-6, and TNFα mRNA in CNS [81]. Additionally, IL-23-driven T cell autoreactivity has been shown to be IL-17 dependent but IL-12- and IFNγ- independent [31]. IL-23p19 levels have been found to be increased in patients suffering from other autoimmune diseases including: Crohn's disease (CD) [82], rheumatoid arthritis (RA) [83], and MS [84]. Recent genetic studies have identified that a non-synonymous nucleotide substitution in exon 9 of the IL-23R gene, which results in an exchange of arginine to glutamine (Arg381Gln) in the cytoplasmic domain of the receptor has protective effect on psoriasis [85, 86], Crohn's disease, ulcerative colitis [87], ankylosing spondlytis [88], graft-versus-host disease [89] and partially in celiac disease and MS [90]. These findings indicate that IL-23-IL-23R activation plays an important role in pathogenesis of autoimmune diseases potentially by modulating Th17 differentiation. Therefore, targeting IL-23 pathway has therapeutic potential for treatment of these chronic diseases. For psoriasis, monoclonal antibodies targeting p40 subunit of IL-23 (shared with IL-12; termed ustekinumab) is under clinical trial since it simultaneously inhibits both IL-23- and IL-12-mediated inflammatory events [91].

Transcriptional Regulation of IL-17 induction

It is well established that the differentiation of naïve $CD4^+$ cells into *bona fide* distinct T-cell lineages, Th1, Th2 and Th17, is regulated by specific transcription factors. T-bet has been shown to be important for Th1, while GATA-3 is critical for Th2 differentiation (Figure 1). Since the

role of these transcription factors is not germane to the Th17-induction, this literature review will not focus on these transcriptional regulators. Therefore, this review will focus on multiple transcription factors involved in the positive and negative regulation of IL-17 (Figure 4 and 5).

Positive Regulators

RORγt and RUNX1: *In vitro* and *in vivo* studies have shown that the differentiation of Th17 cells require TGFβ1 and IL-6-induced upregulation of unique lineage-specific transcription factor, RORγt, an retinoic acid related-orphan nuclear receptor that is encoded by *RORc* gene [92-94] (Figure 4). Alternate promoter usage and exon splicing of *RORc* results in related isoforms: RORγt (also known as RORγ2), and RORγ (RORγ1), which differs with RORγt, at the amino terminal [95]. RORγ is also expressed in Th17 cells and specifies Th17 phenotype [96]. For optimal transcription of *Il17*, a 2 kilobase promoter and atleast one conserved non-coding (enhancer) sequence, CNS2, is required [97]. These cis-regulatory elements have RORγt and RUNX1 (Runt-related transcription factor 1) binding regions. The transcription factor RUNX1 regulates Th17 differentiation by upregulating RORγt expression. Further, RUNX1 binds to RORγt directly to induce IL-17 induction [97]. Deficiency of RORγt in the T helper precursor (Thp) cells led to markedly reduced Th17 differentiation, on the other hand expression of RORγt in Thp cells resulted in increased IL-17 and IL-23R expression [98, 99]. Additionally, other transcription factors such as RORα [94] and aryl hydrocarbon receptor [100, 101] have also been shown to be important for IL-17 induction.

STAT3: Activation of IL-6R (ligand binding IL-6Rα and signal transducing gp130) by IL-6 results in autophosphorylation and transphosphorylation of receptor-associated JAK-family proteins, Tyk and JAK2 and tyrosine moieties on the receptor gp130 of IL-6R. These phosphorylated tyrosine act as the recruiting/docking site for STAT3 molecules, which in turn get phosphorylated. Phosphorylated STAT3 proteins then homodimerize and migrate to the nucleus and activate different proinflammatory genes [76]. It has been shown that STAT3 binds to the promoter of IL-17A and IL-17F [102]. In addition, a report has shown that depletion of either STAT3 or gp130 in $CD4^+$ T cells results in decreased RORγt expression and Th17 differentiation, suggesting that IL-6-gp130-STAT3 regulate IL-17 induction in at least in part by

regulating RORγt levels [103]. Similarly, RORα is also induced in a STAT3-dependent manner [104].

NF-κB: Interestingly, it has recently been shown that promoters of RORγ and RORγt bind to RelA (p65) and c-Rel, members of NF-κB family, respectively [96]. The positive role of NF-κB in IL-17 induction was further substantiated by the findings that activation of NF-κB increases secretion of IL-17 [105, 106]. c-Rel deficient mice have decreased EAE due to impaired activation of *RORc* gene and subsequently decreased Th17 development [107]. Peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) patients have increased IL-17 induction by activation of PI3K/Akt, which increases the DNA binding activity of NF-κB [108]. RelB silenced DCs have decreased IL-12p70, IL-23 and IL-6 as compared to control DCs. In addition, co-culturing $CD4^+$ T cells and RelB-silenced DCs decreased IL-17 and IFNγ but increased IL-4 levels. Further, *in vivo* transfer of RelB-silenced cells in experimental autoimmune myasthenia gravis (EAMG) mice showed decreased Th1 and Th17 phenotype but increased Th2 and Tregs population, indicating RelB is essential for IL-17 and IFNγ induction [109]. Additionally, the potent role of IκB/NF-κB proteins in the induction of IL-17 has been demonstrated even in the absence of known IL-17 inducers, IL-6 and TGFβ. Ectopic expression of transcription factor IkappaBzeta (encoded by NF-κBiz gene), which binds to *Il17a* gene, along with RORα and RORγt is sufficient to induce IL-17 in CD4+ T cells even in the absence of IL-6 and TGFβ [110]. Furthermore, mice deficient in NF-κBiz were resistant to develop EAE due to a defect in Th17 development [110]. Cyclosporine A inhibition of phosphorylation of Akt and IkappaB consequently decreased the binding of NF-κB to the IL-17 promoter and decreased IL-17 induction [111]. In addition, inhibition of NF-κB by the specific inhibitor, BAY 11-7082, or a PI3K inhibitor, LY294002, decreased IL-17 induction. Interestingly, inhibitors of the MAP kinase ERK (UO126) and p38 MAPK (SB203580) did not inhibit IL-17 induction [59], thereby further suggesting the positive role of NF-κB in the induction of IL-17.

IRF4: Increasing evidence suggests that interferon regulatory factor 4 (IRF4) is also critical for IL-17 and IL-21 induction. It has been shown that IRF4-deficient mice have decreased RORα and RORγt expression but increased FoxP3 levels [112, 113]. IRF4-deficient mice have a defect

in IL-17 induction and IL-21 autocrine loop [113]. TGFβ-mediated activation of Rho-ROCK pathway, promotes phosphorylation of IRF4 by ROCK kinase. Once phosphorylated IRF4 translocates to nuclei and binds to IL-17 and IL-21 promoters [114]. Impressively, in autoimmune models such as MRL/lpr, there is enhanced ROCK2 activation concomitant with increased IRF4 function and IL-17 levels [114]. Furthermore, it has been demonstrated that IRF4 is critical for mucosal Th17 cell differentiation by direct binding to the IL-17 promoter. In an IRF4-deficient experimental colitis model, there is impaired RORγt and IL-17 expression [115].

Other Stimulatory Transcription Factors: Another transcription factor, Kruppel-like factor (KLF) 4, has been shown to regulate Th17 development by binding to the IL-17 promoter directly without altering RORγt expression [116, 117]. Sphingosine 1-phosphate, secreted by innate immune cells and RBCs, signal through type 1 S1P receptors ($S1P_1$s), which are expressed on T cells to augment Th17 cell development and IL-17 production via S1P-$S1P_1$s axis [118]. Administration of a modulator and an agonist of S1P receptor 1 have been shown to sequester lymphocyte and macrophage migration from secondary lymphoid organs and also decreased local IL-17 levels in autoimmune neuritis model and decreased Th17 cells in the blood of MS patients [119-121].

Another identified positive regulator of IL-17 is basic leucine zipper transcription factor, ATF-like (BATF), which is highly expressed in hematopoietic cells and activated Th1, Th2, Th17 cells [122-124]. It is a basic leucine zipper (bZIP) transcription factor that dimerizes with Jun class factors of the AP-1 family [125]. It synergizes with RORγt to induce IL-17 by direct interaction with conserved intergenic elements of *Il17A/ Il17F* loci. In addition, BATF also binds to *Il17*, *Il21*, *Il22* promoters. BATF-deficient mice have decreased IL-17 but increased Treg cells resulting in decreased EAE development [124]. It is still unclear whether BATF modulates STAT3 activation or RORγt and IRF4-DNA binding.

Overall, these above studies indicate IL-17 induction is tightly regulated by multiple transcription factors and is dependent on various signaling pathways.

Negative Regulators of Th17 (T-bet; SOCS3; FoxP3; Ets-1; IRF8):

Both IFNγ (a defining cytokine for Th1) and IL-4 (a signature cytokine of Th2) markedly inhibit Th17 differentiation (Figure 5) [42, 43]. More recently, a member of the IL-17 family, IL-25 that is involved in Th2 response [126], has been found to negatively regulate Th17 cells by inhibiting the expression of IL-1β and IL-23 by DC [127]. Similarly, IL-27, which drives the initial Th1 cell responses, also acts as an IL-17 inhibitor [38, 128, 129]. Suppressor of cytokine signaling (SOCS3) also negatively regulates IL-6-gp160 signal transduction resulting in decreased IL-17 [130].

Given that TGFβ induces transcription factor, FoxP3, the presence of IL-6 or IL-21 is critical to decrease FoxP3, and to enhance the activation of RORγt for the induction of IL-17 [74, 114, 131]. FoxP3 interacts directly with RORγt through the exon 2 region and forkhead domain of Foxp3 and suppresses the activation of the IL-17 promoter [132]. Interestingly, it has also been shown that the transcription factor Runx1 interacts with FoxP3 and negatively regulates Th17 differentiation [97]. T-bet, a Th1 lineage specific transcription factor, also suppresses Th17 development by binding to the transcription factor Runx1 via tyrosine 304 of T-bet. This T-bet-Runx1 binding has been shown to block the transactivation of *RORc* gene and therefore IL-17 induction [133]. These studies indicate that Runx1 differentially associates with RORγt to upregulate, or with either T-bet or FoxP3 to suppress Th17 differentiation. T-bet also regulates IL-23R gene expression, and inhibition of T-bet by siRNA decreased both IFNγ and IL-17 induction and improved EAE. Since IL-23 is required for optimal IL-17 induction, any change in the levels of IL-23R by T-bet will affect IL-17 levels [134].

As mentioned earlier, IRF4 is also essential for IL-17 and IL-21 induction, any pathway inhibiting IRF4 activation can inhibit IL-17 induction [113]. Recently, an IRF-4 binding protein Def6, has been identified which prevents the phosphorylation of IRF4 and subsequent IL-17 and IL-21 induction. Further, deficiency of Def6 results in unrestrained ability of IRF4 to induce IL-17 and IL-21, which eventually led to the development of lupus-like autoimmune diseases [114]. A recent report suggests that Def6 deficiency results in the activation of the Rho-ROCK pathway (also activated by TGFβ). Activation of ROCK2 in Th17 skewing conditions results in

phosphorylation of IRF4 and therefore results in the production of IL-17 and IL-21 in wild type mice. Impressively, in autoimmune models such as MRL/lpr there is enhanced ROCK2 activation concomitant with increased IRF4 function and IL-17 levels [114]. It has been shown that deliberate inhibition of ROCK2 activity by treatment of Def6 deficient DO11.10-arthritis murine model and MRL/lpr autoimmune mice with Fasudil, decreased IRF4 phosphorylation and its binding to IL-17 and IL-21 promoters, and consequently reduced RORγt, IL-17 and IL-21 levels in $CD4^+$ cells of these mice [114]. This suggests that inhibition of ROCK2 activity is important for regulating Th17-related genes [114]. Another T-bet interacting transcription factor, v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1), has been shown to inhibit Th17 differentiation. ETS-1-deficient mice have increased IL-17 levels (without affecting RORγt), suggesting that ETS-1 is a negative regulator of IL-17. So far it has not been defined whether or not there is direct interaction between ETS-1 and *IL-17* gene [135]. Recently, transcription factor of interferon regulatory factor (IRF) family, IRF8, also known as ICSBP, has been shown to regulate IL-17 by silencing Th17 differentiation and downregulating Th17-associated genes. IRF8 is expressed by B cells, macrophages, DCs and activated T cells [136]. IRF8 is important for myeloid cell differentiation, DC development and an important regulator for immune cell growth and survival [137, 138]. IRF8 physically interacts with RORγt and inhibits *IL-17* transcription by binding to its promoter. However IRF8 does not regulate IL-23 mediated expansion and maintenance of Th17 cells [139].

A nuclear receptor like RORγt, peroxisome proliferator-activated receptor γ (PPARγ) also acts as an intrinsic suppressor of Th17. It prevents the removal of repressor complexes from RORγt promoter, resulting in decreased RORγt expression and RORγt-induced Th17 differentiation [140]. There are compounds which act as inhibitors of IL-17 such as cardiac glycoside digoxin. Digoxin and its non-toxic derivatives inhibit IL-17 induction by inhibiting RORγt activity but not RORα, thereby delaying the onset and reducing the severity of autoimmune disease in mice [141, 142]. There are other transcription factors and other signaling proteins (e.g. STAT1, STAT4, STAT5, STAT6, NFAT, SOCS1, epidermal fatty acid binding protein (E-FABP)) which also affect Th17 differentiation and IL-17 induction, in this review; however, I have focused on main ones reported in the literature.

Concluding comments: Together, these reports confirm that there are multiple transcription factors, which are involved in the induction and suppression of IL-17. It appears that induction or suppression of IL-17 is dependent on whether positive or negative transcription factor regulators predominate. Since most of the studies conducted utilize either specific knockout mice or the affect of gene ablation in a particular cell type, there is a need for more mechanistic studies to better understand the interaction of these multiple transcription factors at the gene level.

IL-17 and miRNA

Cytokines such as IL-6 are also known to regulate microRNAs (miRNA) which are now shown to be the novel regulators of genes at the post-transcription level. miRNAs are endogenous non-coding RNAs of an average of 21-22 nt in length [143]. miRNA binds to the 3' untranslated region (UTR) of target mRNA by partial homology resulting in either degradation of mRNA or inhibition of translation. Functionally, miRNAs are believed to target multiple functionally related proteins or a key protein target. From recent reports on miRNA, it is evident that miRNA regulate important functions in the development of hematopoietic lineages, differentiation and activation of the immune system. Specific deletion of dicer, an enzyme critical in miRNA biogenesis pathway in T cells [144-146], regulatory T cells [147-149], or B cells [150] results in aberrant development and function of these cells [151-154]. miRNAs also participate in the regulation of autoimmunity: deficiency in Dicer or Drosha leads to autoimmune disease in mice [149].

The distinct role of different miRNAs in the development and function of immune cells is now becoming evident. For example, miR-150 and miR-155 are critical in lymphocyte differentiation and function [155, 156]. miR-150 is rapidly downregulated under Th1 or Th2 conditions, while miR146 is selectively upregulated in Th1 lymphocytes [157]. miR-150 regulates maturation of pro-B to pre-B cell by fine-tuning expression of c-myb, an essential transcription factor for normal lymphocyte development [156]. It has been reported that miR-155 is essential for normal immune function, since miR-155- deficient mice have defective germinal center responses and humoral, B cell immunity decreased Tregs [155, 158, 159]. There are numerous other miRNA

and their target proteins which regulate innate and acquired immune cell development and differentiation have been identified *reviewed in detail in Dai and Ansar Ahmed, 2011* [160].

Given that miRNAs regulate key immuno-regulatory functions, dysregulated and aberrant expression of miRNAs have been reported in several human autoimmune diseases such as SLE, RA, psoriasis, MS, in neurodegenerative diseases such as Parkinson's and Alzheimer's disease, metabolic diseases, viral infections, chronic inflammatory skin disease, human cancers, including leukemia and cancer of lung, brain, liver and colon [154, 161-167]. Dysregulated miRNA expression profiles have the potential to serve as good diagnostic markers [168], prognostic markers [169] or therapeutic targets [170]. In this section, I will focus on the miRNAs which epigenetically either regulate IL-17 levels or are regulated by IL-17 in different disease conditions.

For example, ten miRNA including miR-19a, miR-21, miR-31, miR-101, miR-223, miR-326, miR-142-3p, miR-142-5p, miR-146a, and miR-155, were found to be increased with colonic inflammation in IL-10- knockout mice. Of these miRNAs, miR-19a, miR-21, miR-31, miR-101, miR-223, and miR-155 were decreased with *in vitro* IL-10 treatment of colonic intraepithelial lymphocyte. In addition, there was decrease in IL-17 but increase in Roquin expression in IL-10-treated intraepithelial lymphocytes. It was also confirmed that miR-223 targeted Roquin which resulted in increased IL-17 expression [171]. These findings suggest that IL-10-mediated decreased miR-223 is an important molecular pathway by which IL-10 inhibits IL-17A-mediated inflammation. A study has shown that miR-135b mediates nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-driven oncogenicity and suppresses Th-2 regulators STAT6 and GATA3 expression, which results in the upregulation of Th17 phenotype of anaplastic large cell lymphoma. This suggests that inhibition of miR-135b could be the potential therapy to limit IL-17 induction and tumor angiogenesis and growth [172].

miR-155 expression is required for proper Th1 and Th17 differentiation to control *Helicobacter pylori* infection and immunopathology [173]. In *Helicobacter pylori* infection, there is upregulation of miR-155 in gastric mucosa of experimental mice. In miR-155 deficient mice, there is impaired T cell responses (Th1 and Th17) which result in less severe infection induced

inflammation. Another study has demonstrated that miR-155 was co-expressed with IL-17A in PBMCs of acute coronary syndrome patients [174]. During EAE, there is increased miR-155 expression in $CD4^+$ T cells and decrease in severity of EAE in miR-$155^{-/-}$ mice is associated with decreased Th1 and Th17 responses in CNS and peripheral lymphoid cells [175]. miR-155 is also required for optimal IL-6, IL-23 release from DCs for Th17 differentiation [176]. In experimental model of rheumatoid arthritis (RA), collagen induced arthritis (CIA), there are increased IL-17 levels which correlate with disease severity. In miR-$155^{-/-}$ mice, there was impaired Th17 polarization and decreased levels of IL-17 and IL-22 and the mice did not develop CIA [177]. The above studies indicate that there is a strong correlation between miR-155 expression and Th17 differentiation.

In the skin and sera of systemic sclerosis (SSc) patients, there is significant increase in IL-17A expression. However, there is decreased IL-17RA expression due to intrinsic activation of TGFβ1 in fibroblasts of SSc patients. This decreased IL-17RA is important to inhibit IL-17A-mediated upregulation of miR-129-5p, which targets α1(I) collagen. This study shows that TGFβ regulates IL-17-mediated collagen accumulation and fibrosis in SSc patients by inhibiting IL-17RA expression [178]. There is increased IL-17 in skin lesions and sera of psoriasis vulgaris patients. Interestingly, levels of miR-1266, a putative regulator of IL-17A, were increased in the sera of psoriasis patients. This suggests that miR-1266 may be involved in pathogenesis of psoriasis by targeting other proteins. However, increased in miR-1266 is not regulating IL-17A expression directly [179].

miR-146 is also positively correlated with IL-17A levels in RA disease severity, and is co-expressed with IL-17A in the PBMC and synovium in RA patients [180]. MicroRNA *Let-7f*, inhibits IL-23R expression in human $CD4^+$ memory T cells which results in downregulation of IL-17 induction [181]. A study has shown that there is positive correlation in expression of miR-133b and miR-206 and IL-17 in both αβ and γδ T cells in human and inbred mouse strains. It was found that these miRNAs were clustered nearly 45 kb upstream of *Il17a/f* locus [182].

Importantly, Du et al. reported that miR-326 expression correlated with MS disease severity in human patients. Further in EAE mice, miR-326 played an important role in pathogenesis by

regulating Th-17 cell differentiation through translational inhibition of Ets-1, a negative regulator of Th17 differentiation [183]. It was found that miR-326 expression correlated with IL-17 expression in MS patients. By FISH assay, it was confirmed that IL-17A and miR-326 are present particularly in $CCR6^{+}CD4^{+}$ T cells from the peripheral blood of patients with relapsing MS and in *in vitro* differentiation model [182].

Concluding Comments: Together, these studies indicate that miRNA profile can be used novel biomarkers for Th17-type immune reactions. So far I have discussed the importance of IL-17 and the different genetic and epigenetic mechanism which regulate the induction of IL-17. In next section, I will briefly review IL-17-mediated signaling mechanism and the mechanisms by which IL-17 exerts its pro-inflammatory role in different physiological and pathological condition.

IL-17-mediated proinflammatory (IL-17 Signaling and Response)

IL-17 is a powerful proinflammatory cytokine and is known to induce the release of potent proinflammatory biomolecules that aid in the influx of neutrophils to the site of tissue damage. IL-17 receptor family consists of five different receptors (IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE). The IL-17 receptors are glycosylated Type 1 transmembrane multimeric proteins that are expressed ubiquitously in various cells of the body. The molecular mass of nascent IL-17R protein is approximately 112kDa [184]. Of these known receptors, IL-17RA and IL-17RC heterodimerize, and act as a common receptor for IL-17A and IL-17F [185] (Figure 6). This receptor is expressed on epithelial cells, fibroblasts, B and T lymphocytes, myelomonocytic cells and marrow stromal cells [186, 187]. To-date, most of the IL-17 response studies have been performed on fibroblasts and cells of epithelial origin.

Engagement of IL-17RA and IL-17RC receptor with IL-17A or IL-17F triggers signaling through Act1-TRAF6 (TNF-receptor associated factor 6) pathway (Figure 6) [188-190]. Act1 is confirmed to be critical for IL-17 signaling as an adaptor molecule [191], since Act1-deficient mice have decreased IL-17-dependent EAE and colitis [192]. Cytoplasmic domain of IL-17R is referred as SEFIR (SEF and IL-17R) domain (at C terminus), which interacts with SEFIR domain on Act1 adaptor protein. Act1 is a U-box Type E3 ubiquitin ligase and mediates lysine-

63 linked ubiquitination of TRAF6 [193]. This lysine-63 linked ubiquitin aids in protein-protein interaction of TRAF6 with TGFβ Activated Kinase 1 (TAK1) and subsequent activation of IκB kinase (IKK). IKK then phosphorylates IκB inhibitory protein (which sequesters NF-κB) and allows translocation of NF-κB into nucleus and induction of NF-κB dependent genes [194]. NF-κB-inducing kinase acts as a mediator in IL-17 triggered NF-κB signaling cascade [195]. IL-17 induces NF-κB consensus sequence binding in mouse fibroblasts [196] and human macrophages [197] and favors the induction of genes such as IL-6, IL-8 which have NF-κB recognition sites [196]. In addition, IL-17 signaling has been shown to use pathways regulated by p38 MAPK, C/EBP, ERK-1/ERK-2 and JNK-1/JNK-2 [198-203]. JAK/STAT family, including, Tyk2, JAK1, 2, 3 and STAT 1, 2, 3, 4 have also been reported to be involved in signal transduction of IL-17-induced genes [204, 205]. A mechanism has been recently identified that controls Th17-mediated inflammatory diseases. In this pathway, TRAF3 binds to IL-17R and inhibits Act1/TRAF6-NF-κB activation [206].

It has been shown that IL-17 also regulates gene expression by enhancing mRNA stability by Act1 dependent, but TRAF6 independent pathway [207, 208]. This pathway is triggered when IKKi kinase phosphorylates Act1 on Ser311 and promotes TRAF2/5 interaction with Act1, which activates MAPK signaling. This leads to stabilization of chemokines mRNAs [209]. Increased IL-17-mediated mRNA stability has been reported for COX2 [210], KC, MIP-2 [207].

IL-17 stimulates the production of various inflammatory mediators such as IL-6, IL-8, GM-CSF, growth related oncogene (Gro alpha), epithelial cell-derived neutrophils activating protein 78 (ENA-78) and PGE2, C-reactive proteins [203], CXCL1, 2, 3, 5, 6 [211], IL-6, CXCL8, MCP-1 [212]. Studies in mouse embryonic fibroblasts and in fibroblastoid L929 cells have shown that IL-17 also induces MCP-1, KP, macrophage inflammatory protein (MIP)-2, TIMP-1, granulocyte chemotactic protein-2 (GCP-2) and matrix metalloproteinases (MMP) -3, 9, and 13 [213]. Stimulation of human colonic subepithelial myofibroblasts with either IL-17A or IL-17F increased the expression of proinflammatory IL-6, IL-8, leukemia inhibitory factor (LIF), MMP-1 and MMP-3 by rapid phosphorylation of ERK 1/2, p38 MAPKs, c-Jun-NH_2-terminal kinase (JNK) [214]. It is believed that IL-17 could potentially contribute to cartilage degradation and

synovial inflammation in osteoarthritis patients by upregulating chemokines secretion. Stimulation of synovial fibroblasts and chondrocytes isolated from osteoarthritis patients with rIL-17 upregulated IL-8 and GRO-alpha from synovial fibroblasts and MCP-1 from chondrocytes [215]. IL-17 stimulation of lung stromal fibroblasts releases various proangiogenic factors such as NO, HGF, MCP-1, KC, MIP-2, PGE1, PGE2, and VGEF in a dose-dependent manner [216]. Additionally, IL-17A has been shown to promote osteoblastogenesis by suppressing leptin in estrogen-deficiency induced bone loss [217]. IL-17 triggered IL-6 induction from fibroblasts results in inhibition of negative regulation by suppressor of cytokine signaling 3 (SOCS3), which leads to promotion of inflammation and autoimmunity [130].

IL-17 has also been shown to synergize with other cytokines/factors to affect biological function. IL-17 alone or in combination with B cell-activating factor (BAFF) has been shown to regulate B cell survival, proliferation and their differentiation into immunoglobulin-producing plasma cell by activating NF-κB regulated-Twist-1 [218]. IL-17 has also been found to cosynergize with TLR ligands, IFNγ, IL-1β, CD40-ligand and TNFα to fine-tune inflammatory responses [219]. IFNγ synergistically increases IL-17-induced MCP-1 and IL-8 from fetal intestinal epithelial cells [220]. In renal epithelial cells, IL-17 and CD40-ligand enhance production of IL-6, IL-8, and RANTES synergistically, while increasing MCP-1 in an additive fashion [221].

Concluding Comments: These studies indicate the IL-17 plays critical role in inflammation. Overall many studies have also established that abnormal regulation of IL-17 levels, including IL-23-mediated expansion of Th17 cells; have been strongly associated with inflammatory responses and a variety of autoimmune diseases, which are female predominant. The central objective of my Ph.D. dissertation work is to investigate whether estrogen upregulates IL-17.

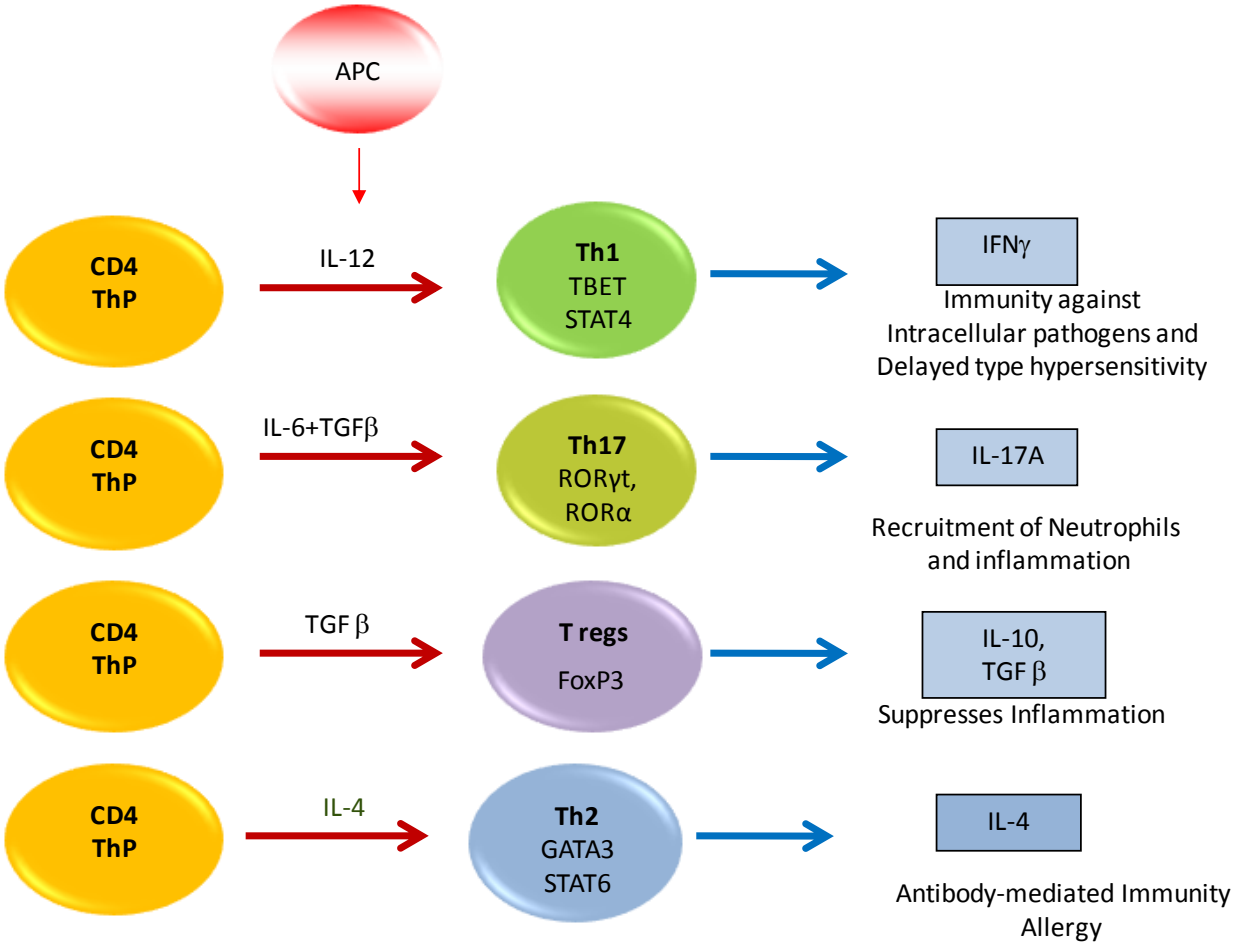

Figure 1: T helper (Th) differentiation

Exposure of naïve CD4 $^{+}$ T helper precursor (Thp) cells to different cytokines released from neighboring tissues and antigen presenting cells (APCs) activates the differentiation pathway which commits these cells into distinct CD4 $^{+}$ lineages. These distinct lineages express specific transcription factors and cytokines and have different functions.

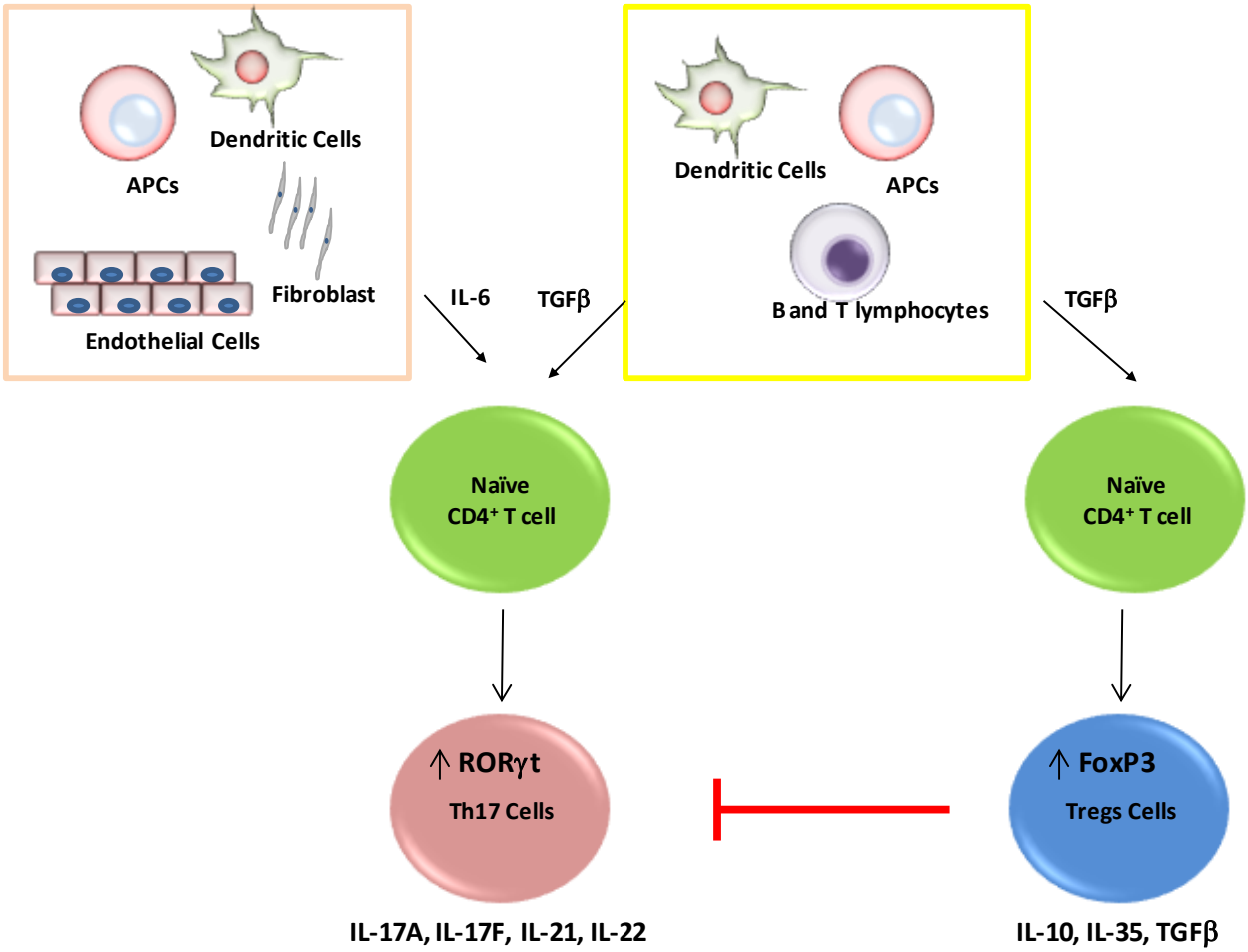

Figure 2: Th17 differentiation

IL-6 secreted by variety of cells such as endothelial cell, Antigen presenting cells (APCs), fibroblasts etc is presence of low amounts of TGFβ (secreted by immune cells including T cells, B cells, etc) commits naïve $CD4^+$ T cells to IL-17-secreting Th17 cells. In absence of IL-6, high amounts of TGFβ favor commitment of naïve $CD4^+$ T cells to iTregs.

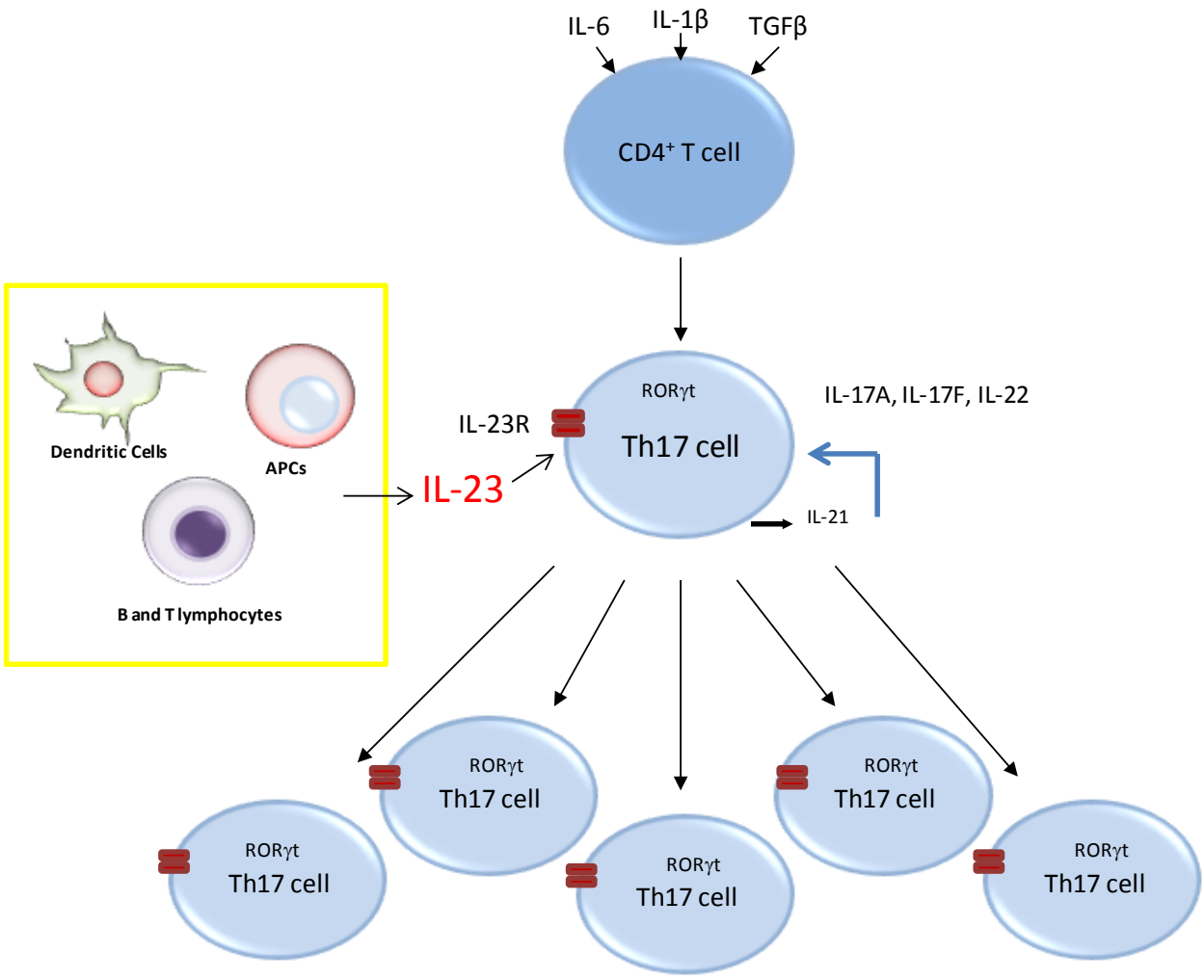

Figure 3: Th17 proliferation

Th17 proliferation: IL-6 is presence of low amounts of TGFβ commits naïve $CD4^+$ T cells to IL-17 secreting Th17 cells. Th17 cells secrete bunch of other cytokines such as IL-17F, IL-22, IL-21 etc. Once committed, there is increased expression of IL-21 and IL-23R. IL-21 acts as in autocrine fashion and promotes IL-17 induction. IL-23 secreted by a variety of cells such as endothelial cell, antigen presenting cells, lymphocytes, etc activates IL-23R and amplifies and stabilizes the proliferation of IL-17-producing cells.

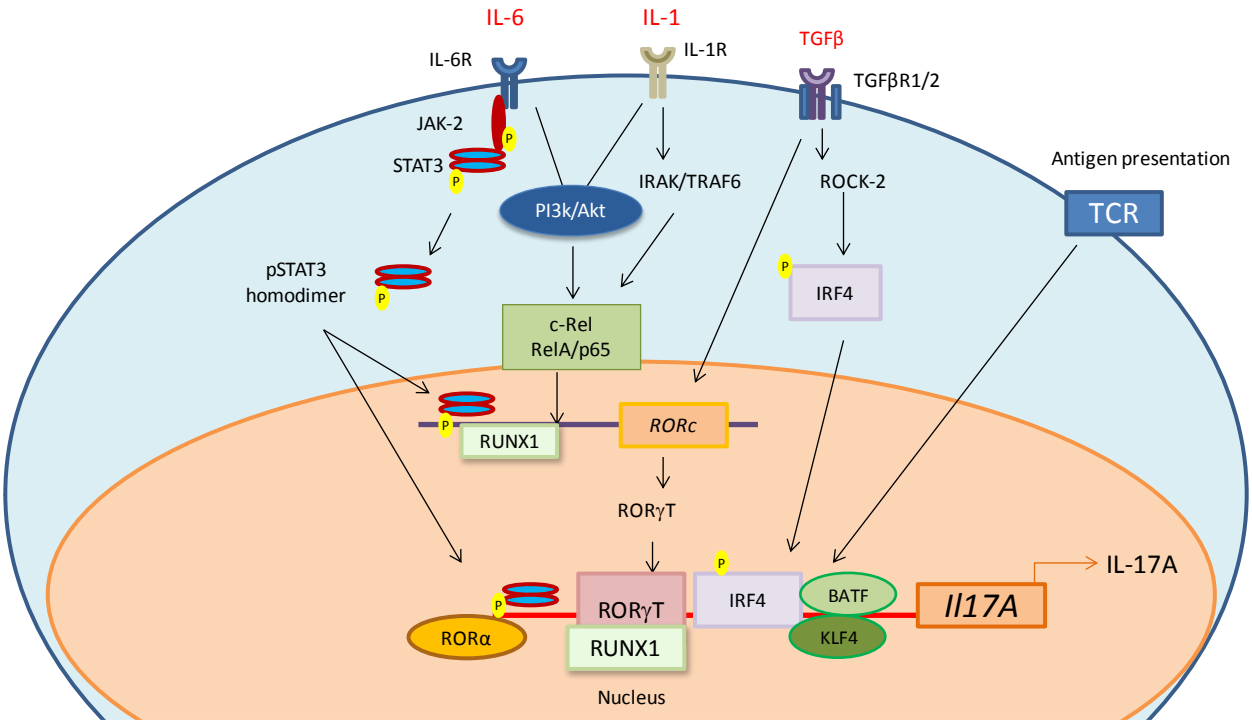

Figure 4: Positive Transcriptional Regulators of IL-17 induction

Different cytokines and antigen specific stimuli trigger different signaling cascades for activation of *RORc* and consequently *Il17* gene. TCR, T cell receptor; BATF, B cell-activating transcription factor; IL, interleukin; TGFβ, transforming growth factor β; RORγt, retinoic acid-related orphan receptor γt; STAT, signal transducer and activator of transcription; IRF-4, interferon-inducible factor-4; RUNX1, Runt-related transcription factor 1; IRAK, IL-1 receptor-associated kinase; TRAF6, TNF receptor associated factor-6 ; ROCK, Rho-associated serine/threonine kinases.

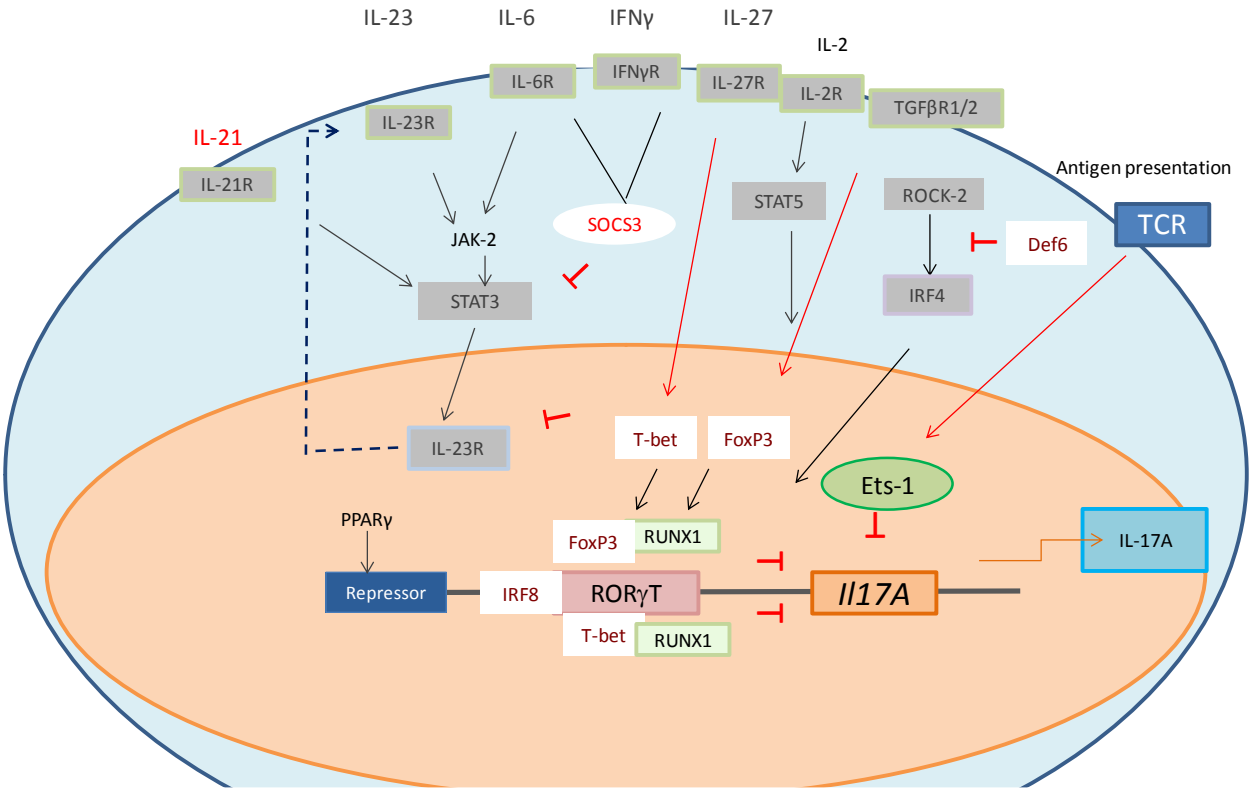

Figure 5: Negative Regulators/Suppressors of IL-17 induction

IL-17 induction is tightly regulated by multiple repressors and transcription factors. T-bet or FoxP3 interaction with RUNX1 prevents RORγt-RUNX1 interaction which prevents RORγt-mediated IL-17 induction. Def6 binding to IRF4 prevents ROCK2-mediated IRF4 phosphorylation and subsequent IL-17 induction. PPARγ, peroxisome proliferator activated receptor γ; SOCS, suppressors of cytokine signaling.

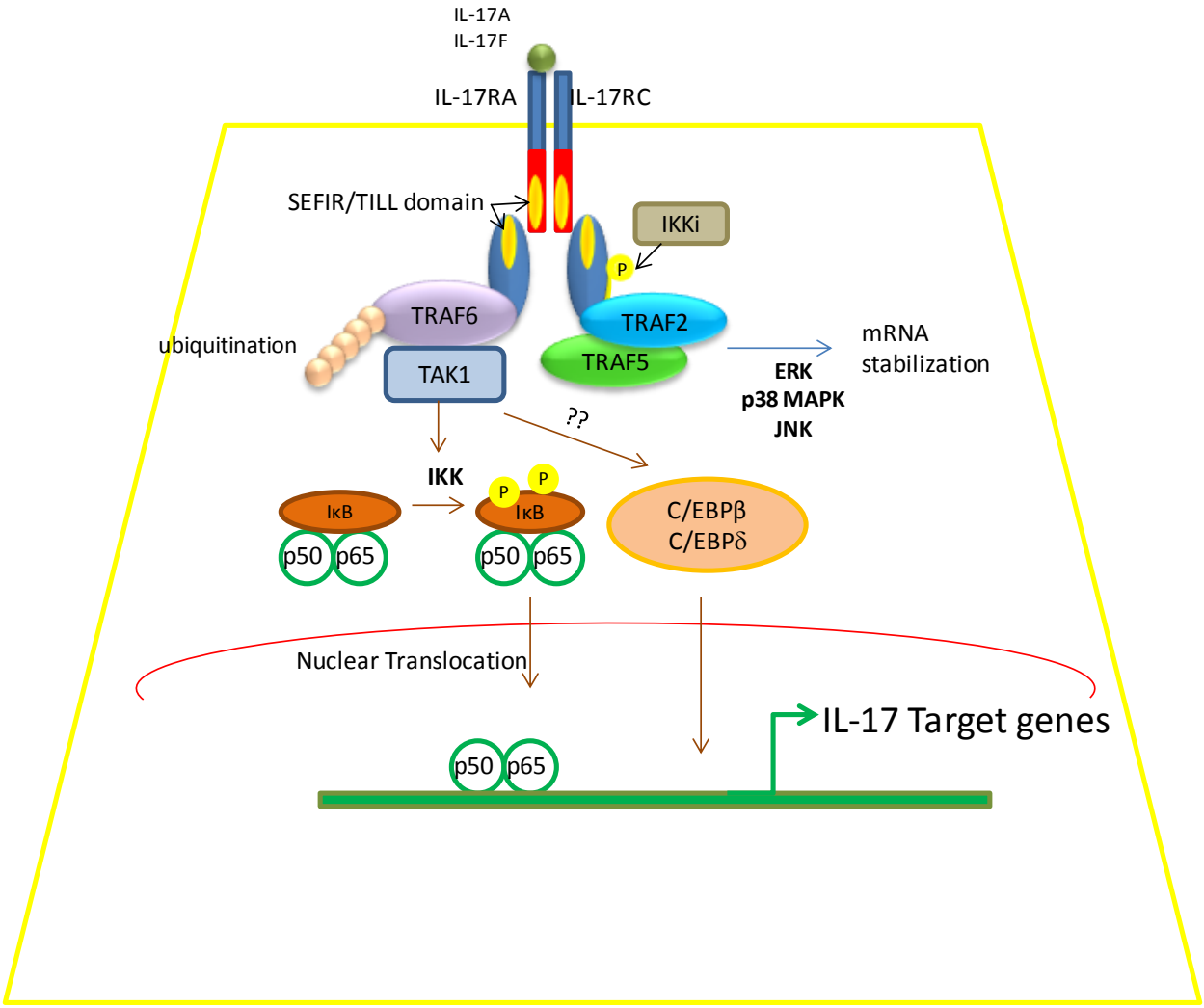

Figure 6: IL-17 Signaling Pathway

IL-17A and IL-17F signal through common IL-17RA/RC receptor complex which interacts with Act-1 adaptor protein by SEFIR domain. Act1, an E3 ubiquitin ligase then binds to TRAF6 and ubiquitinates it to allow TRAF-TAK1 interaction and subsequent activation of NF-κB pathway. Alternately, Act-1 is phosphorylated at Ser311 by IKKi which allows Act-1 and TRAF2/5 interaction and MAPK mediated chemokine mRNA stabilization.

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Chapter 3: Rationale and Hypothesis

Sex hormone especially estrogens have been shown to regulate the "cross-talk" between all major cells of the innate and adaptive immune system. Estrogen has been shown to affect the activation of macrophages, neutrophil and NK cells. Growing list of literature suggest that estrogen has profound effect on the commitment, differentiation of Th1/Th2/Tregs and also on levels, biological roles and effector functions of various cytokines such as IL-27, IFNγ, IL-4 etc in both healthy and disease models such as autoimmune diseases. The main focus of our laboratory over past 2 decades is to decipher the role of sex hormone, estrogen in particular, in immune regulation. We have shown that estrogen upregulates induction of proinflammatory IFNγ (Th1 protoype cytokine) and modulates IFNγ-mediated induction of proinflammatory biomolecules such as MCP-1, iNOS, NO, Cox-2 [1-5]. We have also shown that estrogen enhances LPS-mediated IFNγ induction by activating NF-κB signaling pathway [3, 6]. In addition, estrogen has been shown to regulate the expression of microRNA (miRNA), novel gene regulators, in both lymphoid and non-lymphoid tissue [7]. Interestingly, miRNAs are now known to regulate cytokines such as IFNγ, and IL-17. However, little is known whether estrogen has any effect on IL-17 induction, a hallmark cytokine for both Th17 and Tc17 cells. It is now evident that IL-17 plays a major role in inflammation by regulating the induction of various proinflammatory genes, which aid in the recruitment and activation of neutrophils. Although IL-17 is considered to be protective in infection, overproduction of IL-17 in conditions like autoimmune diseases has been shown to aggravate these diseases and contribute to tissue injury. Since estrogen modulates the immune system and is known to regulate inflammatory disorders, it is conceivable that estrogen will also affect IL-17 induction and response. Therefore, I will test the following central hypothesis: **Estrogen will enhance IL-17 induction by splenic lymphocytes by regulating transcription factors and miRNAs and augment IL-17-mediated proinflammatory events**. The central hypothesis is divided in 2 main Aims.

Hypothesis for Aim 1: Estrogen will increase the ability of lymphocytes to secrete IL-17 by (i) upregulating the transcription factors involved in IL-17 induction (e.g. RORγT); (ii) by altering the epigenetic miRNA regulation of IL-17 induction.

This aspect is discussed in Chapter 4, Chapter 5, and Chapter 6.

Hypothesis for Aim 2: Estrogen-treatment of splenic lymphocytes will increase their responsiveness for IL-17 to induce proinflammatory biomolecules such as MCP-1.

This aspect is discussed in Chapter 6.

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Chapter 4: Estrogen increases, whereas IL-27 and IFN-γ decrease, splenocyte IL-17 production in WT mice

Deena Khan, Rujuan Dai, Ebru Karpuzoglu, Sattar Ansar Ahmed

European Journal of Immunology, 2010, 40 (9): 2549-2556

DOI: 10.1002/eji.201040303, John Wiley and Sons Inc.

Abstract

Estrogen-mediated regulation of Th1, Th2 and Tregs effector functions are well documented, surprisingly it is still not known whether estrogen modulates IL-17, a powerful proinflammatory cytokine which plays a pivotal role in several inflammatory and autoimmune diseases. To address this critical gap in literature, in the present study, we determined whether estrogen regulates IL-17 in wildtype (C57BL/6) and autoimmune lupus prone (NZB/W) mice. By comprehensive analysis, we report that estrogen upregulates not only IL-17 levels in the supernatants of activated splenocytes, but also IL-17-specific transcription factor, retinoic acid-related orphan receptor gamma T (RORγt). Exposure to IL-23 further enhances IL-17 levels in cells from estrogen-treated mice. Addition of IL-27 or IFNγ at the time of initiation of culture decreased IL-17 dramatically and RORγt partially. However, delay in addition of IL-27 or IFNγ decreased IL-17 (albeit less profoundly) but not RORγt. Furthermore, inhibition of JAK-2 inhibited IL-17 induction but not RORγt expression suggesting that other transcription factors are also critical in estrogen-mediated upregulation of IL-17.

European Journal of Immunology

SHORT COMMUNICATION

Estrogen increases, whereas IL-27 and IFN-γ decrease, splenocyte IL-17 production in WT mice

Deena Khan[1], Rujuan Dai[1], Ebru Karpuzoglu[2] and Sattar Ansar Ahmed[1]

[1] Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
[2] Institute of Genes and Transplantation (E.K.), Baskent University, Ankara, Turkey

Estrogen-mediated regulation of Th1, Th2 and Treg effector functions are well documented but, surprisingly, there is little information whether estrogen modulates IL-17, a powerful proinflammatory cytokine that plays a pivotal role in several inflammatory and autoimmune diseases. Therefore in the current study, we determined whether estrogen regulates the expression levels of IL-17 in WT C57BL/6 mice. By ELISA, ELISPOT and/or flow cytometric analyses, we found that estrogen upregulated the levels of not only IL-17, but also the IL-17-specific transcription factor retinoic acid-related orphan receptor γ t (RORγt), in activated splenocytes. IL-17 levels were further enhanced by exposure of activated splenocytes to IL-23, particularly in cells from estrogen-treated mice. Exposure of splenocytes to IL-27 or IFN-γ at the time of activation markedly inhibited the levels of IL-17 and RORγt. Interestingly, a delay of 24 h in exposure of activated splenocytes to IL-27 or IFN-γ decreased IL-17 levels (albeit less profoundly) but not RORγt. These findings imply that the suppressive effects of IL-27 and IFN-γ are more effective prior to the differentiation and commitment of IL-17-secreting cells. Furthermore, inhibition of JAK-2 by AG490 suppressed IL-17 but not RORγt expression, suggesting that other transcription factors are also critical in estrogen-mediated upregulation of IL-17.

Key words: Estrogen · IFN-γ · IL-17 · IL-27 · Lupus · RORγt

Introduction

A recent paradigm shift in inflammation is the discovery of a novel lineage of $CD4^+$ Th17 cell, which secrete a potent proinflammatory cytokine, IL-17A (referred to as IL-17) [1]. IL-17 promotes inflammation by recruiting neutrophils, monocytes and macrophages to the site of inflammation and also by acting on target cells to stimulate a broad range of strong inflammatory molecules such as CXCL1, 2, 3, 5, 6 [2], IL-6, CXCL8, MCP1 [3]. IL-17 has also been found to synergize with TLR ligands, IFN-γ, IL-1β and TNF-α to fine-tune inflammatory responses [4]. Additionally, IL-17A has been shown to promote osteoblastogenesis by suppressing leptin in estrogen-deficiency-induced bone loss [5]. Recently, a flurry of reports have indicated that proinflammatory IL-17 is involved in various chronic debilitating autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, psoriasis and multiple sclerosis [6–8]. IL-17 has been shown to increase production of total IgG, anti-dsDNA IgG and IL-6 by peripheral blood mononuclear cells of patients with lupus nephritis [9].

Studies from our laboratory as well other have reported that estrogen, a known immunomodulator, regulates several proinflammatory mediators including IFN-γ, MCP-1, MCP-5, Cox-2, iNOS [10–12]. Estrogen-induced upregulation of pro-inflammatory molecules is noteworthy since estrogen has been implicated in many inflammatory autoimmune diseases such

Correspondence: Prof. Sattar Ansar Ahmed
e-mail: ansrahmd@vt.edu

as SLE [13]. Although estrogen-induced regulation of Th-1 and Th-2-mediated cytokines and Treg activation is now well established, to date there are no reports on estrogen regulation of proinflammatory Th17 cells in WT mice [10, 14–16]. Given the importance of IL-17 and estrogen in autoimmune diseases, we wanted to investigate whether estrogen also modulates IL-17 induction in both lupus-prone NZB/W mice and WT C57BL/6 mice. Our novel finding in this report is that estrogen promotes IL-17 levels and upregulates IL-17-specific transcription factor, retinoic acid-related orphan receptor γ t (RORγt). Addition of IL-23 upregulates IL-17 induction; however the frequency of IL-17-producing cells remains the same. Further we demonstrate that IL-17 levels are inhibited by the addition of IL-27 or IFN-γ and JAK-2 inhibitor. Together, these findings have important implications for understanding and pharmacological manipulation of IL-17-associated and estrogen-modulated pathologies.

Results and discussion

Estrogen upregulates IL-17 induction in autoimmune mice

There is growing observation that IL-17 levels and IL-17-secreting cells are increased in SLE patients and in animal models [17–20]. Since estrogen has been shown to promote murine lupus, we hypothesized that estrogen may also promote the induction of IL-17 in lupus-prone mice. Towards this end, splenocytes from estrogen and placebo-treated NZB/W lupus-prone autoimmune mice were stimulated with known IL-17-inducing stimuli (IL-6+TGF-β+anti-CD3 antibodies) and IL-17 levels determined in the supernatants collected. As shown in Fig. 1A, our preliminary studies suggest that the levels of IL-17 were found to be increased in splenocytes from estrogen-treated (26663±12120) when compared with placebo-NZB/W mice (8804±1353 pg/mL; at 72 h. The levels of IL-17A in culture supernatants from gonadal-intact mice (9530±2372 pg/mL; $n = 5$) were similar to that in placebos. Further, flow cytometric analysis also revealed that IL-17$^+$ cells in estrogen-treated NZB/W mice were increased when compared with placebos in stimulated cells (Fig. 1B and C). The numbers of IL-17$^+$ cells/million splenocytes were also higher in estrogen-treated NZB/W mice (61 350±1550) when compared with placebo controls (14 100±1167). These initial results suggest that estrogen-treated NZB/W mice have greater propensity to induce IL-17 when compared with placebo-treated mice. The frequency of IL-17$^+$ cells was also increased in unstimulated cells from estrogen-treated NZB/W mice, suggesting that estrogen promotes differentiation of IL-17-secreting cells *in vivo* (data not shown).

Estrogen enhances IL-17 levels and intracellular IL-17$^+$ cells in normal C57BL/6 mice

Since estrogen increased IL-17 induction in lupus-prone mice, we next determined whether estrogen could also promote IL-17 in normal mice (C57BL/6). Exposure of cells to IL-6 alone or TGF-β alone did not noticeably induce IL-17 levels. Activation of splenocytes with combination of IL-6 and TGF-β demonstrated low, but detectable levels of IL-17 particularly in cells from estrogen-treated male mice. Impressively, addition of anti-CD3 antibody to IL-6 and TGF-β cocktail robustly increased IL-17 levels in cultures from estrogen-treated cells when compared with cells from placebo-treated male mice (Fig. 2A). Anti-CD3 antibodies alone induced low levels of IL-17. Kinetics analysis revealed that estrogen promotion of IL-17 induction was evident as early as 3 h (although not statistically significant) and the levels progressively increased by 72 h of culture (Fig. 2B). Similar studies were performed in female C57BL/6 mice and splenocytes were cultured in presence of IL-17-inducing stimuli for 48 and 72 h. The levels of IL-17 were significantly increased in

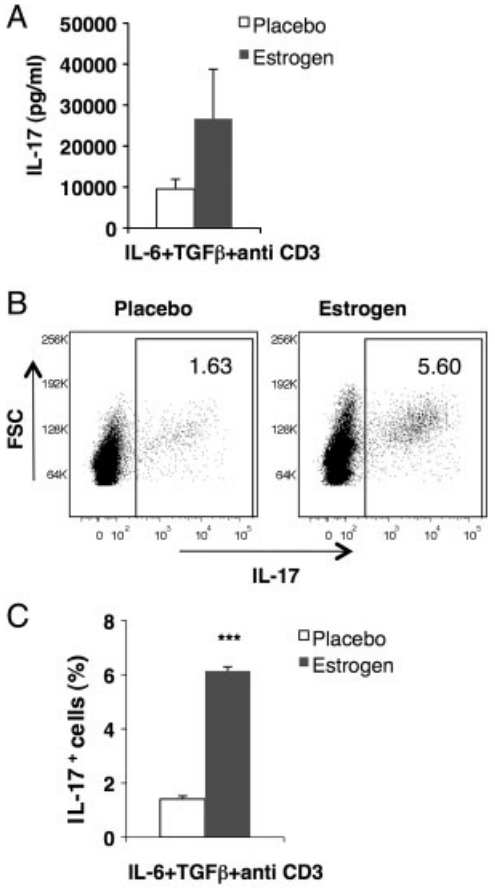
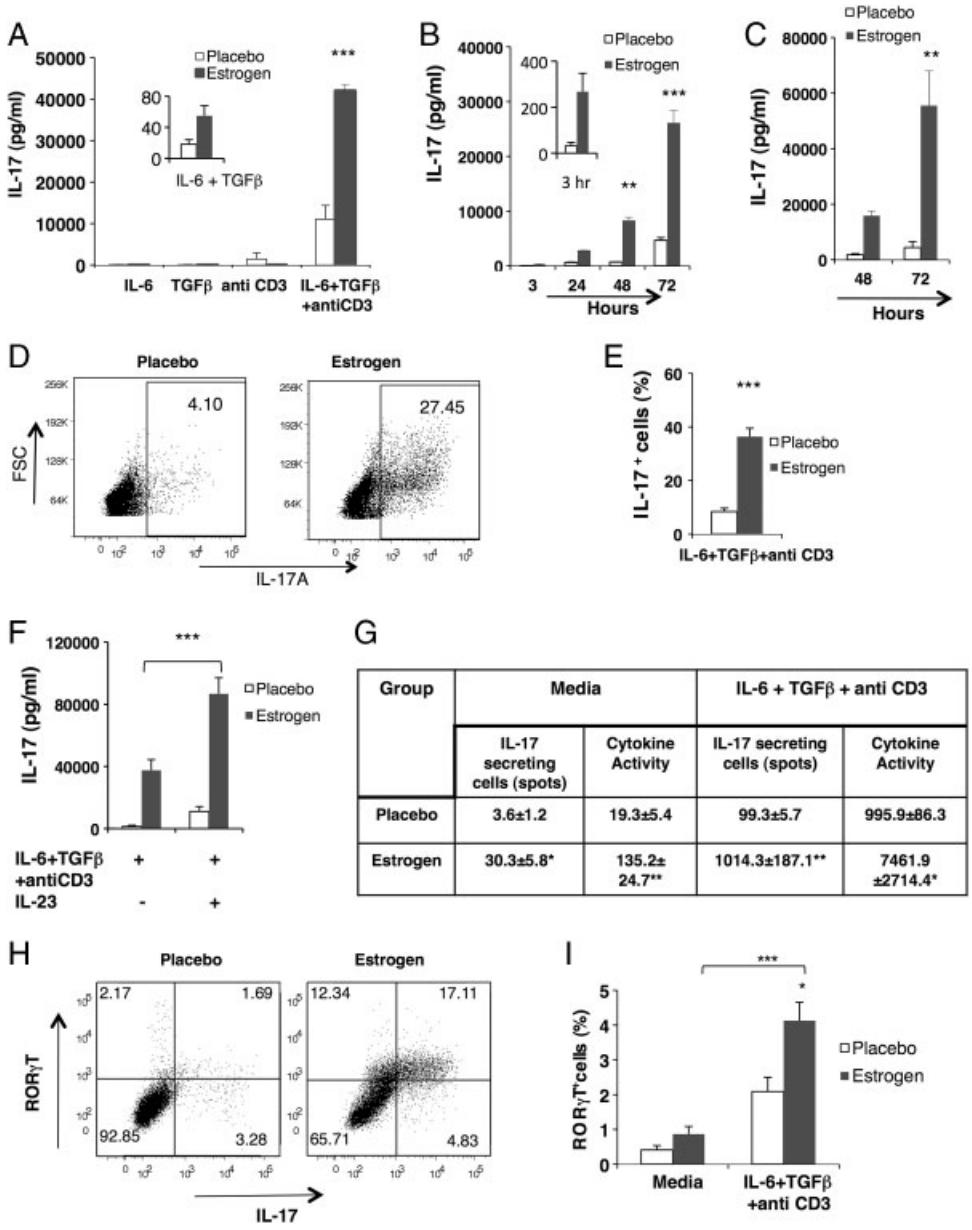


Figure 1. IL-17 levels and IL-17$^+$ cells are increased in estrogen-treated autoimmune lupus mice. Splenic lymphocytes (2.5×10^6/mL) from estrogen- and placebo-treated lupus-prone male NZB/W mice were either (A) cultured in the presence of IL-6+TGF-β+anti-CD3 antibodies for 72 h and IL-17 levels in the culture supernatants were analyzed by ELISA or (B and C) were stimulated with IL-6+TGF-β+anti-CD3 antibodies for 21 h followed by stimulation with PMA (100 ng/mL), ionomycin (1 μg/mL) and brefeldin A (1 μL/mL) for 3 h and stained for intracellular IL-17 expression. (B) Representative flow cytometry plots of IL-17$^+$ cells (percentages indicated). (C) Mean percentage of IL-17$^+$ cells. (A and C) Means±SEM (estrogen = 2; placebo = 7); ***$p<0.001$, Tukey–Kramer multiple comparison test.



Group	Media		IL-6 + TGFβ + anti CD3	
	IL-17 secreting cells (spots)	Cytokine Activity	IL-17 secreting cells (spots)	Cytokine Activity
Placebo	3.6±1.2	19.3±5.4	99.3±5.7	995.9±86.3
Estrogen	30.3±5.8*	135.2± 24.7**	1014.3±187.1**	7461.9 ±2714.4*

Figure 2. Estrogen upregulates IL-17 levels, IL-17$^+$ cells and RORγt expression in splenocytes from WT mice. Splenic lymphocytes (2.5×10^6/mL) from (A and B) male (placebo = 3 and 2; estrogen = 3 and 2; representative of three independent experiments) or (C) female C57BL/6 mice (placebo = 3; estrogen = 2) were cultured in the presence of either IL-6, TGF-β or anti-CD3 antibody alone or in combination for (A) 72 h or (B and C) with IL-6+TGF-β+anti-CD3 antibody for the indicated time points. IL-17 levels in the culture supernatants were determined by ELISA. (D–F) Splenic lymphocytes from estrogen- and placebo-treated WT male C57BL/6 mice were stimulated with IL-6+TGF-β+anti-CD3 antibody for 72 h and (D and E) stained for flow cytometry or (F) were cultured with/without IL-23 for a further 24 h. (D) Representative flow cytometry plots (indicating the percentages) of IL-17$^+$ cells (E) Mean percentage of total IL-17$^+$ cells (placebo = 4; estrogen = 5; representative of two independent experiment) and (F) IL-17 levels (pg/mL) (placebo = 4; estrogen = 5). (G–H) Splenic lymphocytes from estrogen- and placebo-treated WT male C57BL/6 mice were activated with IL-6+TGF-β+anti-CD3 antibody for (G and I) 48 h and (H) 72 h. (G) The number of IL-17-secreting cells was determined by ELISPOT assay (placebo = 3; estrogen = 3). (H) Representative flow cytometry plots of RORγt$^+$ IL-17$^+$ cells (percentages indicated; placebo = 4; estrogen = 5) and (I) Mean percentage (placebo = 3; estrogen = 3). All data, with the exception of representative plots are mean ± SEM; *p<0.05; **p<0.01 and ***p<0.001; (E and G), two-tailed t-test; (A–C, F, I), Tukey–Kramer multiple comparison test.

estrogen-treated females at 72 h (Fig. 2C). Given that estrogen promoted IL-17 both in males and females, subsequent studies were conducted in gonadectomized male mice. Male C57BL/6 mice were chosen to avoid the confounding effects of endogenous estrogens from extra-gonadal tissues in females.

Flow cytometric analyses also showed that estrogen-treated mice have increased IL-17$^+$ cells (4-6 fold). Figure 2D is the representative dot plot of IL-17$^+$ cells in placebo- and estrogen-treated mice at 72 h. The relative numbers of IL-17$^+$ cells and total numbers of IL-17$^+$ cells/million splenocytes were found to

be significantly higher in splenocytes from estrogen-treated mice after 72 h of stimulation with IL-17 inducing stimuli (363 700 ± 31 701) when compared with placebos (83 975 ± 12 658) (Fig. 2E). The trends were similar at earlier time points (3 and 24 h) also; however the total percentage of IL-17$^+$ cells was less. Our results differ with that of Wang *et al.* [21], in which estrogen treatment significantly reduced IL-17 induction from MOG_{35-55}-(myelin oligodendrocyte glycoprotein) activated lymphocytes from WT mice; with EAE; however, the same treatment increased estrogen-mediated IL-17 induction in PD-1-deficient mice with EAE. In addition to the differences in estrogen treatment and levels (they used 2.5 mg slow release pellets for 60 days, which achieved serum estrogen levels of 1500–2000 pg/mL that are comparable to pregnancy), there are several notable differences between this study and ours including differences in stimuli (MOG *versus* IL-17-inducing stimuli), culture conditions, animal model (EAE versus normal) and autoimmune states (EAE *versus* lupus). As expected, antigen (MOG)-specific IL-17 levels were markedly lower than in our study, where we employed standard IL-17-inducing stimuli. It is thus not surprising that there are differences in IL-17 induction patterns in these two studies. However, both studies suggest that IL-17 is regulated by estrogen.

Additionally, stimulation of splenocytes with IL-17-inducing stimuli yielded very weak IFN-γ levels (705 ± 244 pg/mL at 24 h). The IFN-γ levels were at least 10–12-fold lesser than what we observe when cells were stimulated with optimal dose (10 μg/mL) of ConA or anti-CD3 antibodies [12] implying the type of stimulation is critical for IFN-γ induction.

Since IL-23 is well documented to be involved in the maintenance and sustenance of IL-17-producing cells [22], we determined whether IL-23 is also increased in IL-17-inducing conditions. We found that the levels of IL-23 were comparable in placebo- and estrogen-treated mice (data not shown). We next determined whether IL-23 has any effect on IL-17 levels and frequency. We stimulated splenocytes from estrogen- and placebo-treated mice for 72 h with IL-6+TGF-β+anti-CD3 antibodies and added IL-23 and cultured for additional 24 h. We found that addition of IL-23 to the culture significantly increased IL-17 induction from estrogen-treated mice (Fig. 2F). Interestingly, flow cytometric analysis of IL-17$^+$ cells revealed that there was no increase in the number of IL-17$^+$ cells (data not shown) even though IL-17 levels were increased in supernatants. This suggests that IL-23 promotes secretion of IL-17 levels in estrogen-treated mice.

IL-17-secreting cells are increased in estrogen-treated mice

Since estrogen promoted IL-17 in both male and female WT and male autoimmune mice, for detailed analysis of subsequent studies, only WT male C57BL/6 mice were utilized. Next we determined whether the increased IL-17 levels in estrogen-treated mice were due to the increased numbers of IL-17-secreting cells. The frequency analysis of IL-17A-secreting cells done by ELISPOT assay confirmed that estrogen increased numbers of IL-17-secreting cell as well as cytokine activity (Fig. 2G).

Intracellular expression of RORγt is increased in estrogen-treated mice

We next determined whether estrogen also upregulates the expression of RORγt, an IL-17-specific transcription factor. Flow cytometric analysis indicated that the percentage of RORγt$^+$ IL-17$^+$ cells was nearly four times in estrogen-treated mice when compared with placebo mice at 72 h after culture (Fig. 2H). Total RORγt expression was also increased in activated splenocytes from estrogen-treated mice when compared with placebos at 48 h (Fig. 2I). This suggests that estrogen-mediated upregulation of IL-17 levels correlates with increased expression of RORγt expression in estrogen-treated cells.

IL-27 and IFN-γ suppresses IL-17 induction

Recent advances in IL-27 biology have shown that IL-27 is not only an initial inducer of Th1 differentiation, but it is also a potent downregulator of cytokines [23, 24]. IL-27 suppress inflammation by: (i) inhibiting IL-17 induction in EAE [23] and/or (ii) inducing Th2 cytokines (*e.g.* IL-10) [25]. Conversely, IL-27 has also been shown to downregulate Treg [26]. IFN-γ has also been shown to inhibit the differentiation of naïve CD4 precursors to Th17 cell type [27]. Therefore, we next determined whether estrogen-induced IL-17 could be downregulated by IL-27 or IFN-γ. It was found that IL-27, when added at the time of culture, markedly diminished the induction of IL-17 even at a low dose (1 ng/mL) in both placebo- and estrogen-treated mice at 48 h (Fig. 3A). Interestingly, suppression of IL-17 by IL-27 was higher in cells from estrogen-treated mice when compared with placebo-treated mice (*e.g.* at 72 h the average inhibition at 10 ng/mL by IL-27 was 79 and 49% in estrogen- and placebo treated mice, respectively; data not shown). Interestingly, IFN-γ effectively suppressed IL-17 in cells from estrogen-treated mice (Fig. 3B) and had minimal suppressive effect in cells from placebo-treated mice (at 72 h the average inhibition at 10 ng/mL of IFN-γ was 76 and 6% in estrogen- and placebo-treated mice, respectively; data not shown). This may be due to our earlier observations that cells from estrogen-treated mice had enhanced IFN-γ-induced responses compared with placebos (*e.g.* iNOS, MCP-1) [10, 11].

Furthermore, presence of IL-27 and IFN-γ in the culture decreased RORγt expression in both estrogen- and placebo-treated splenocytes cultured in presence or absence of either IL-27 or IFN-γ for 48 h (Fig. 3C and D). This suggests that IL-27 and IFN-γ suppress IL-17 induction by inhibiting RORγt expression. Our findings are in agreement with a recent finding, which suggests that IL-27 inhibits IL-17 induction by suppressing RORγt expression [28]. It is interesting to note that while addition

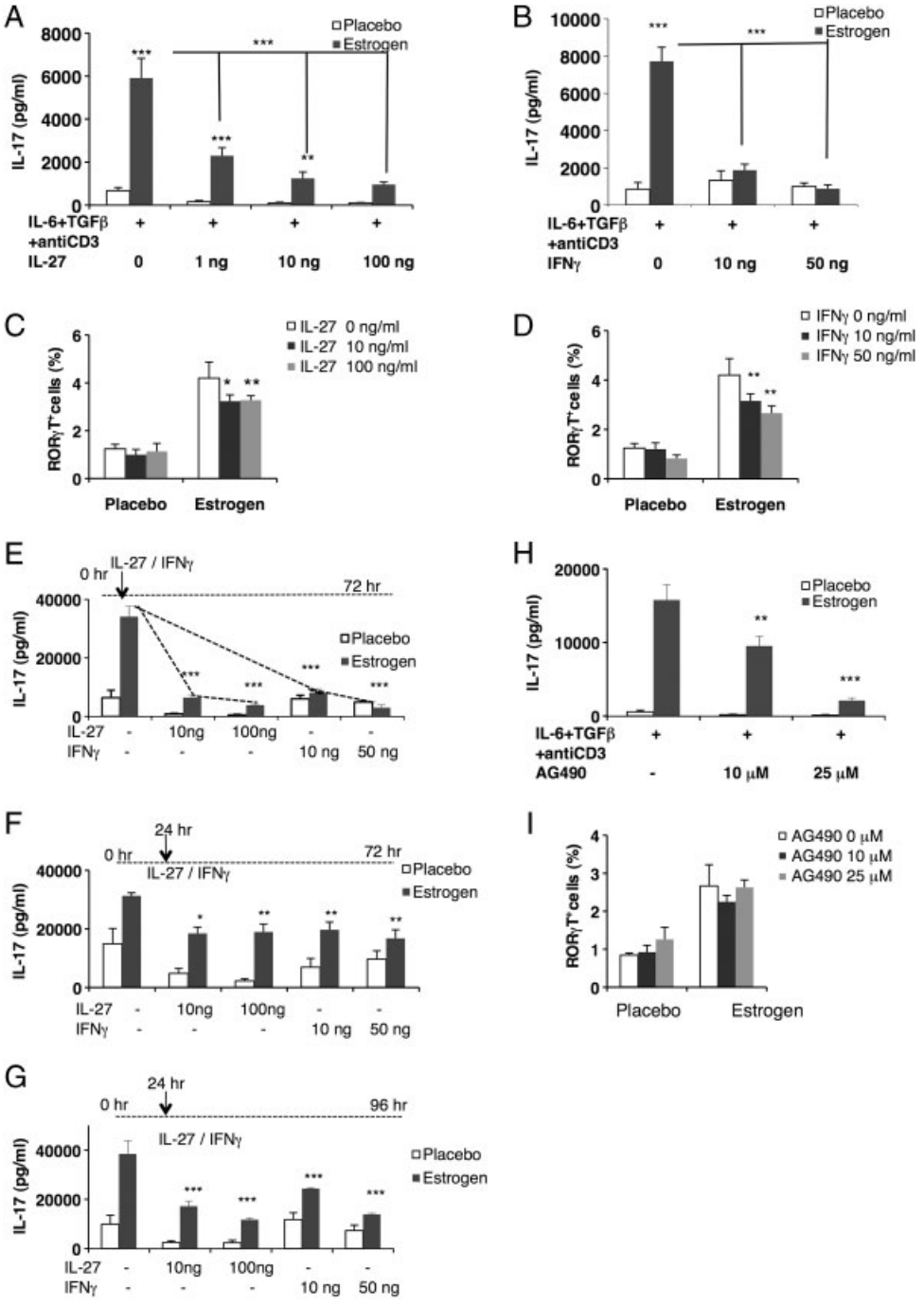


Figure 3. IL-27 and IFN-γ suppress IL-17 induction and RORγt expression. (A–D) Splenic lymphocytes (2.5×10^6/mL) from estrogen- and placebo-treated male C57BL/6 mice were activated with IL-6+TGF-β+anti-CD3 antibody in the presence or absence of (A) rIL-27 (placebo = 3; estrogen = 3; representative of two independent experiment) or (B) rIFN-γ for 48 h (placebo = 4; estrogen = 6; representative of two independent experiment), and IL-17 levels determined by ELISA. (C and D) Splenic lymphocytes from estrogen- and placebo-treated male C57BL/6 mice were activated with IL-6+TGF-β+anti-CD3 antibody in the presence or absence of (C) rIL-27 or (D) rIFN-γ (placebo = 3; estrogen 3) for 48 h and the mean percent RORγt$^+$ expression in splenocytes determined by flow cytometry. (E–G) Splenic lymphocytes from estrogen- and placebo-treated male C57BL/6 mice were activated with IL-6+TGF-β+anti-CD3 antibody, and rIL-27 or rIFN-γ were added either together with IL-6+TGF-β+anti-CD3 antibody stimulation or at the indicated time points. IL-17 levels were measured by ELISA after 72 or 96 h (placebo = 4; estrogen = 6). (H) Splenocytes from estrogen- and placebo-treated male C57BL/6 mice were cultured in the presence of the JAK2 inhibitor AG490 for 48 h and IL-17 levels analyzed (placebo = 3; estrogen = 5). (I) Splenocytes from estrogen- and placebo-treated male C57BL/6 mice were activated with IL-6+TGF-β+anti-CD3 antibody in the presence or absence of AG490 and the percent RORγt$^+$ cells determined after 24 h (placebo = 3; estrogen = 4). Data are means ± SEM; $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; (A, B, E and H), Tukey–Kramer test; (C, D, F and G), Student Newman–Keuls test.

of 100 ng/mL of IL-27 markedly decreased IL-17 levels (83%) (Fig. 3A) there was a less dramatic reduction in RORγt (51%) in estrogen-treated mice. This implies that other transcription factors (RORα, STAT3; or yet undiscovered) may be involved in IL-17 induction. It is also possible that a modest decrease in RORγt is sufficient to markedly diminish the induction of IL-17.

Interestingly, delaying the addition of IL-27 or IFN-γ after 24 h of start of culture did suppress IL-17 induction in 72 and 96 h (Fig. 3F and G) culture. However, the degree of reduction of IL-17 was not as marked as noted when IL-27 or IFN-γ were added at initiation of culture (Fig. 3E). Interestingly, the expression of RORγt$^+$ cells was not decreased by delaying the addition of IL-27 and IFN-γ by 24 h (data not shown). These findings suggest that once the cell is committed to IL-17-secreting cell then the magnitude of inhibitory effect of IL-27 and IFN-γ is lowered as has been reported previously [29]. Impressively, the addition of JAK2 inhibitor AG490 also decreased IL-17 induction (Fig. 3H), without modulating the expression of RORγt (Fig. 3I). These results further strengthen our view that upstream signaling proteins, *e.g.* JAK2- STAT3, are also critical for IL-17 induction.

Concluding remarks

Overall, this is the first study that documents estrogen-treated mice have propensity to induce powerful proinflammatory IL-17 in activated splenocytes of WT mice. Interestingly, estrogen treatment alone (*i.e.* in absence of stimuli) is not sufficient to induce IL-17 at high levels. However, when appropriately stimulated with IL-17-inducing stimuli, splenocytes from estrogen-treated mice have robust IL-17 induction response. Exposure of cells to IL-23 further enhances IL-17 levels in cells from estrogen-treated mice. This suggests that estrogen exposure presets conditions that favor IL-17 induction upon activation of cells. The estrogen-promotion of IL-17 adds new knowledge as to how this hormone regulates inflammatory conditions. Our studies also show that both IL-27 and IFN-γ can downregulate IL-17, potentially by in part suppressing RORγt expression. These studies have implications to not only a better understanding of estrogen-induced inflammatory cytokines but also provide new possibility of downregulation of this response. Future studies are required to study in detail the signaling events, which favor IL-17 induction in estrogen-treated mice.

Materials and methods

Animals

At 4–5 wk of age, male and female WT C57BL/6 (Charles River Laboratories) and lupus-prone male NZB/W mice (Jackson Laboratories) were gonadectomized and surgically implanted with silastic capsules containing 17β-estradiol (estrogen; 3–5 mg; Sigma-Aldrich) or empty (placebo) implants by standard procedures that have been extensively described in our previous studies [10–12, 30]. These implants are designed to slowly release sustained levels (156–220 pg/mL) of estrogen [11, 30]. WT mice were terminated at 2 months. Lupus-prone NZB/W mice were terminated 6 months after estrogen treatment at a time when mice develop lupus (as evidenced by high proteinuria). NZB/W mouse was chosen as an autoimmune susceptible strain since this is a classic model for lupus and the effects of estrogen in promotion of lupus are well established. Since estrogen worsens lupus disease and increases mortality [31], by 6 months of estrogen treatment, we were able to utilize only two mice (with the loss of five) in this particular group for our preliminary experiment. All animal-related procedures were in accordance with Virginia Tech Institutional Animal Care guidelines, and were approved by the Institutional Animal Care and Use Committee. Mice were fed a commercial pellet diet devoid of estrogenic hormones (7013 NIH-31 Modified 6% Mouse/Rat Sterilizable Diet; Harlan-Teklad).

Isolation and culture of splenic lymphocytes

IL-17 was induced in splenic lymphocytes (2.5×10^6 cells/mL) by culturing with previously reported [32, 33] recombinant cytokines rIL-6 (20 ng/mL; Ebiosciences) plus TGF-β (3 ng/mL; R&D Systems, Minneapolis, MN) and anti-CD3 antibody (1 μg/mL; Ebiosciences). Control cells were cultured in the absence of these stimuli. In selected experiments, splenocytes were also cultured with rIL-23 (10 ng/mL), rIL-27 (1, 10, 100 ng/mL; Ebiosciences), rIFN-γ (10 and 50 ng/mL; BD PharMingen, San Diego, CA), JAK2 inhibitor AG490 (10, 25 μM) for defined time points. Exposure of cells to the above reagents did not affect the viability of the cells as demonstrated by Alamar Blue assay and 7-AAD-flow cytometric assay (data not shown).

Cytokine ELISA

Protein levels of IL-17 in culture supernatants were determined with IL-17A ELISA kit *per* manufacturer's instructions (Ebiosciences) using Vmax microplate reader (Molecular Devices, Sunnyvale, CA).

Flow cytometric analysis of intracellular expression of IL-17 and RORγt

Percent IL-17 expressing cells and RORγt subset were quantified by flow cytometric analysis. Splenocytes (1×10^6/100 μL) were cultured for defined time points with additional 3 h activation with PMA, ionomycin and brefeldin A and then subjected to intracellular staining (antibodies from Ebiosciences) by using BD Cytofix/Cytoperm Kit according to the manufacturers' instructions. Stained cells were visualized using a FACS Aria flow

cytometer (BD Biosciences) and data analyzed using FlowJo version 7 software. Data were expressed as percent IL-17$^+$ or RORγt$^+$ cells.

IL-17 ELISPOT assay

The numbers of IL-17-secreting cells were determined by using mouse IL-17A ELISPOT kit according to manufacturer's instructions (Ebiosciences). Splenic lymphocytes (5×10^5/mL) were cultured in presence or absence of IL-6+TGF-β+anti-CD3 antibodies for 48 h. The spots were counted using automated AID ELISpot plate reader (Autoimmun Diagnostika, Strassberg, Germany).

Statistical analysis

The significance of differences between placebo- and estrogen-treated samples was assessed as indicated using GraphPad InStat version 3.0a for Macintosh (GraphPad Software). The significance level is indicated as asterisk ($^*p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$, respectively).

Acknowledgements: This work was supported in part by the National Institutes of Health (1 RO1 AI051880-04A1) and Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). Intramural Research Competition (IRC) Grant (441303) and Lupus Foundation of America. The authors want to thank Ms. Melissa Makris for flow cytometric analysis and Mr. Peter Jobst, Ms. Connie Kingrea and the animal care staff.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **MOG:** myelin oligodendrocyte glycoprotein · **ROR:** retinoic acid-related orphan receptor · **SLE:** systemic lupus erythematsus

Full correspondence: Prof. Sattar Ansar Ahmed, Head Biomedical Sciences and Pathobiology, 1410 Prices Fork Road, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24060-0342, USA
Fax: +1-540-231-3426
e-mail: ansrahmd@vt.edu

Received: 5/1/2010
Revised: 14/5/2010
Accepted: 22/6/2010
Accepted article online: 7/7/2010

Chapter 5: Estrogen induces IL-17 by regulating multiple transcription factors and microRNAs -326 and -223

Abstract

Aberrant levels of IL-17 are evident in various inflammatory and female-predominant autoimmune diseases. We found that IL-17 levels can be markedly enhanced from splenocytes of mice by merely administering estrogen to orchiectomized C57BL/6 mice. To understand the molecular basis, we comprehensively investigated various transcription factors and signaling pathways involved in estrogen-mediated upregulation of IL-17 in splenocytes. Estrogen fine tunes the balance of transcription factors by upregulating those that positively regulate the induction of IL-17 (RORγt, RORα, NF-κB, JAK-2), and decreasing those that inhibit IL-17 (IRF8, ETS-1). MAPK and P13K pathways are not involved in estrogen-mediated upregulation of IL-17. NF-κB was found to be important in IL-17 induction as demonstrated by the use of NF-κB inhibitor, and p65siRNA experiments. Estrogen induces serine-protease mediated truncation of NF-κB, which is associated with increased IL-17. Estrogen also upregulates microRNA 326 and 223, which are known to epigenetically target IL-17-inhibitors, ETS-1 and Roquin, respectively. Inhibition of either miR326 or miR223 with specific antagomir inhibited estrogen-mediated IL-17 induction. Together, this is the first study to comprehensively show estrogen calibrates multiple transcription factors and microRNAs to enhance IL-17 induction.

Introduction

Although IL-17 was discovered nearly 20 years ago, it was not until 2005, this cytokine gained attention of immunologists when two groups independently identified a new subtype of $CD4^+$-Th cell sub-population (Th17) that secretes IL-17A (IL-17) [1, 2]. Subsequently, there was a flurry of reports documenting not only the existence of this sub-population but also the role of IL-17 in mediating pro-inflammatory events in different infectious and autoimmune diseases. Upregulation of IL-17 has been reported in a variety of infections including *Klebsiella*

pneumoniae [3], *Porphyromonas gingivalis* [4], *Nippostrongylus brasiliensis* [5] etc. Although IL-17 is known to have protective effects in infection, overproduction of IL-17 and/or aberrant response to IL-17 has been shown to aggravate disease conditions and contribute to tissue injury as observed in different autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), psoriasis, inflammatory bowel disease (IBD) among others [6-10]. IL-17 has potential to mobilize, recruit, and activate neutrophils, thus linking adaptive and innate immunity [11]. Cytokines and chemokines released in response to IL-17 promote granulopoiesis and accumulation of neutrophils; protection of mucosal membrane by mucin secretion and tight junction formation [12, 13]. Together, these reports confirm the importance of IL-17 in regulating inflammation.

Estrogen, a female sex steroid, *is* known to modulate the immune system of normal and autoimmune individuals [14-16]. We and others have shown that estrogen treatment markedly regulates various cytokine (IL-1, IL-6, IFNγ etc) from a number of different cell types. Recently, our laboratory was the first to show that in vivo estrogen treatment upregulates IL-17 induction on activation of splenocytes from normal C57BL/6 and autoimmune lupus prone NZB/W mice [17]. This was accompanied with increased expression of the unique Th17 lineage-specific transcription factor retinoic acid-related orphan receptor gamma t (RORγt). We also showed that known IL-17-suppressors, IFNγ and IL-27, inhibited IL-17 induction from estrogen-treated cells when added at an early time point along with inhibition of RORγt expression. Inhibition of JAK-2 activity by using AG490 decreased IL-17 levels markedly without affecting the levels of RORγt [17]. This suggests that even though RORγt is important for IL-17 induction, there are other transcription factors involved in estrogen-mediated IL-17 induction. Although several independent studies have examined the role of independent transcription factors in the regulation of IL-17, to our knowledge, there is no study that has comprehensively investigated the role of various transcription factors/signaling pathways that positively and negatively regulate IL-17, especially in an estrogen model of inflammation. To address this critical gap in literature, in this study we have identified key estrogen-regulated transcription factors and epigenetic factors which affect the induction of IL-17 from activated splenocytes.

Materials and Methods

Animals: As is the standard procedures in our laboratory for over two decades, at 4–5 wks of age, male wildtype C57BL/6 (Charles River Laboratories) were gonadectomized and surgically implanted with silastic capsules containing 17β-estradiol (estrogen; 3-5 mg; Sigma-Aldrich) or empty (placebo) implants for 7-8 weeks, that have been extensively described in our previous studies [18-20]. These implants are designed to slowly release sustained levels (156-220 pg/ml) of estrogen [19, 20]. All animal-related procedures were in accordance with Virginia Tech Institutional Animal Care guidelines, and were approved by the Institutional Animal Care and Use Committee. Mice were fed a commercial pellet diet devoid of estrogenic hormones (7013 NIH-31 Modified 6% Mouse/Rat Sterilizable Diet; Harlan-Teklad).

Isolation and culture of Splenic Lymphocytes: Splenocytes from estrogen and placebo-treated mice were isolated and cultured as previously published [18, 21]. IL-17 was induced in splenic lymphocytes (2.5×10^6 cells/ml) by culturing with previously reported [22, 23] standardized final concentrations of recombinant cytokines rIL-6 (20 ng/ml; eBiosciences) plus TGF-β (3 ng/ml; R&D Systems, Inc., MN) and low dose of anti CD3 antibody (1 μg/ml; eBiosciences). Control cells were cultured in the absence of these stimuli. In selected experiments, splenocytes were also cultured for defined time points with various inhibitors including: NF-κB inhibitor, A77 1726 (10 μM, Axxora, CA, USA), MAPK kinase inhibitor PD98059 (10 μM; Sigma-Aldrich), JAK2 inhibitor AG490 (10, 25 μM, Sigma-Aldrich), PI3kinase inhibitor Wortmannin (50 nM), serine protease inhibitor, AEBSF (50 nM, 100 nM; Calbiochem, CA). Exposure of cells to the above reagents did not affect the viability of the cells as demonstrated by our previously reported Alamar Blue assay [24] and the 7-AAD-flow cytometric assay [25, 26] (data not shown).

IL-17A ELISA: Protein levels of IL-17 in culture supernatants were determined with IL-17A homodimer ELISA kit (eBiosciences) using Vmax microplate reader (Molecular Devices, Sunnyvale, CA) as published in our previous studies [17].

Flow Cytometric Analysis of Intracellular Expression of IL-17 and RORγT: Splenocytes ($1x10^6$/100 µl) were cultured for defined time points with additional 3 hr activation with PMA, ionomycin and brefeldin A and then subjected to intracellular staining (antibodies from eBiosciences) by using BD Cytofix/Cytoperm Kit essentially per our previous report [17]. Stained cells were visualized using a FACS Aria flow cytometer (BD Biosciences) and data analyzed using FlowJo version 7 software. Data is expressed as percent IL-17^+ or RORγT^+ cells.

siRNA Transfection A nucleofector device and mouse macrophage nucleofector kit (Amaxa) were used to transfect siRNA oligonucleotides (Dharmacon) to mouse splenocytes as we described previously [27]. Briefly, 1.5×10^7 freshly isolated splenocytes were pelleted, resuspended in 100 µl of mouse macrophage nucleofector solution, and mixed with 3.5 µg of siRNA oligonucleotides. The sample was transferred to a cuvet and then transfected using the optimal nucleofector program m001. Twenty-four hours after transfection, the splenocytes were stimulated with IL-17-inducing stimuli for 24, 48 and 72 hrs. The cells were collected for Western blot and flow cytometric analysis. Supernatants were used for measurement of IL-17.

Western Blotting**:** Western blotting was performed to analyze the expression of proteins in nuclear and whole cell extracts as per our previous publications [19, 21, 27]. Nuclear protein extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). For whole-cell extracts, cell pellets were lysed with CellLyticM Cell Lysis Reagent (Sigma-Aldrich). The blot images were captured and analyzed using a Kodak Image Station 4000M [27]. Different antibodies used were phosphorylated and total STAT3 (Cell Signaling, Danvers, MA), NF-κB p65 (c-20), total STAT1 and ETS-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and loading control β-actin (Sigma-Aldrich). NF-κB p65 (c-20) is specific for COOH terminus of p65, and detects full-length p65, but not C-terminal truncated p65.

DNA Binding Assay- To analyze the DNA binding activity of STAT3 in nuclear extracts from placebo and estrogen-treated mice, TransAM STAT3 kit from Active Motif (CA, USA) was used as per manufacturers' instructions and as reported in our previous study [27].

***TaqMan Real-time PCR*:** Total mRNA from splenic lymphocytes was isolated by miRNeasy mini kit (QIAGEN Bioscience, MA) and real-time PCR performed by TaqMan RNA to Ct kit (Applied Biosystems, Carlsbad, CA) using IRF8, ETS-1, Roquin, RORα , RORγt with Act B (endogenous control) TaqMan gene expression assays from Applied Biosystems. miR-326 and miR-223 expression was determined by using TaqMan miRNA assay kit and normalized to snoRNA202 endogenous control. The relative expression level of miRNA and mRNA was calculated using the $2^{-\Delta\Delta Ct}$ (Livak) method after normalization to controls as previously described [28].

***Antagomir transfection*:** miRNA antagomirs against miR-326 and miR-223 (single-stranded chemically modified oligonucleotides) were designed based on previous report [29], and synthesized by Dharmacon RNA Technologies (Lafayette, CO). To transfect with specific antagomir or control/negative inhibitors oligonucleotide, 1.5×10^7 freshly isolated mouse splenocytes were washed with PBS (containing 0.5% BSA) and incubated at 37^0C for 2 hrs in the presence of 1 µM antagomir in 1.5 ml Accell siRNA delivery media (Dharmacon), as described before with slight modifications [30]. The cells were then pelleted and resuspended with 0.1 µM specific antagomir or control oligonulceotide in complete RPMI and subjected to stimulation and culture for indicated time points.

***Statistical analysis**:* The significance of differences between placebo and estrogen-treated samples was assessed as indicated using GraphPad InStat version 3.0a for Macintosh (GraphPad Software). Paired *t* tests were performed between negative control and specific inhibitor transfected cells. The significance level is indicated as asterisk (* for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$ respectively).

Results

RORα and RORγt mRNA expression is increased in Estrogen-treated mice

In our previous study, we have shown that RORγt is upregulated by estrogen [17]. We therefore examined if estrogen also alters RORα, another orphan nuclear receptor transcription factor

known to upregulate IL-17 synergistically with RORγt [31]. We found that although RORα mRNA levels (Figure 1A) were lower than RORγt mRNA levels at 48 hrs (nearly 10 fold; Figure 1B); the levels were significantly higher in activated splenocytes from estrogen-treated mice when compared to placebo-treated mice. The data has been normalized to the levels of RORα and RORγt levels in freshly isolated (time 0 hr) splenocytes from placebo treated group, respectively.

NF-κB and JAK-2 signaling pathways are required for IL-17 production in estrogen-treated mice

We have previously reported that exposure of splenic lymphocytes from estrogen-treated mice to IL-6+TGFβ (plus low dose of anti CD3 antibodies) results in a robust induction of IL-17 [17]. Kinetics of IL-17 induction revealed that high levels of IL-17 were induced after 48 hrs of activation [17]. We have previously shown that inhibiting JAK-2 (by a specific JAK-2 inhibitor, AG490) decreases IL-17 induction from estrogen-treated mice [17]. In this study, we wanted to extend this observation to determine the role of downstream signaling pathways such as NF-κB, PI3K and MEK-1 in the induction of IL-17 in splenocytes from estrogen-treated mice. To test this, we pretreated splenic lymphocytes with inhibitors targeting specific pathways (PI3K, MEK-1, NF-κB, and JAK-2) for 1 hr and then activated cells with IL-17-inducing stimuli and subsequently determined IL-17 levels. IL-17 levels were markedly decreased by NF-κB inhibitor (A77 1726), less profoundly by JAK-2 inhibitor (AG490), but not by either PI3K or MEK-1 inhibitors, suggesting the importance of NF-κB and JAK2 in IL-17 induction (Figure 2A).

NF-κB inhibitor but not JAK-2 inhibitor decreases IL-17 and RORγt expression in cells

We next performed flow cytometric assays to determine whether NF-κB and JAK-2 specific inhibitors will affect RORγt$^+$ cells from estrogen-treated mice. NF-κB inhibitor markedly decreased the percentage of RORγt$^+$IL-17$^+$ cells and RORγt$^+$ IL-17$^-$ cells. On the other hand, even though JAK-2 inhibitor decreased secretion of IL-17 levels, it did not affect the percentage of IL-17 $^+$ cell and/or RORγt expression (Figure 2B).

Serine protease-mediated truncation of p65/Rel A is involved in estrogen-mediated IL-17 induction

We have recently reported that NF-κB p65/RelA and STAT-1 are truncated in the nuclei of splenocytes of estrogen-treated mice [27]. This truncated NF-κB is associated with increased transcriptional activity and enhanced output of IFNγ and nitric oxide [27]. Our initial studies suggested that this truncation is attributed to serine protease activity [27]. Inhibition of these serine protease activity in cells from estrogen-treated mice by 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), prevents truncation of p65/RelA (i.e. retains the normal size) and importantly decreases inflammatory IFNγ and iNOS expression [27]. Since we found that inhibition of NF-κB markedly decreased IL-17 levels, we next determined whether truncation of p65 is involved in estrogen-mediated IL-17 induction and whether prevention of this truncation by AEBSF will decrease IL-17. To test this, we cultured cells from estrogen and placebo-treated mice with IL-17-inducing stimuli with or without serine protease inhibitor, AEBSF (Figure 2C and D). Estrogen truncates NF-κB p65 at the COOH end through serine proteases [27]. Therefore, anti p65 antibody that recognize to COOH end was able to bind NF-κB p65 only in cells from placebo but not from estrogen-treated mice (Figure 2C). Exposure to serine protease inhibitor (AEBSF) prevents truncation in cells from estrogen-treated mice and thus, restores normal size NF-κB, which now allows binding and detection by the specific p65 antibody that binds to COOH end of p65. AEBSF treatment decreased IL-17 induction from estrogen-treated mice (Figure 2D). These results indicated that truncation of p65 is associated with estrogen-mediated IL-17 induction.

p65 siRNA inhibits IL-17 induction from estrogen-treated cells

Transfection of placebo- and estrogen-treated cells with p65 siRNA decreased IL-17 induction when compared to cells transfected with control matched oligonucleotide (Figure 2E and F, respectively). The decreased p65 levels in Western blot from estrogen and placebo-treated mice indicate the efficiency of transfection. These results further confirm the importance of p65/NF-κB signaling in induction of IL-17 from estrogen-treated mice, even though p65 is partially proteolyzed in estrogen-treated mice.

Estrogen treatment has delayed phosphorylation of STAT3 levels and decreases DNA binding activity of pSTAT3

Activation of IL-6Rα/gp130 by IL-6 results in autophosphorylation and transphosphorylation of receptor-associated JAK-family proteins, Tyk and Jak2 and tyrosine moieties on the receptor [32]. These phosphorylated tyrosine act as the recruiting/docking site for STAT3 molecules, which in turn get phosphorylated. Phosphorylated STAT3 proteins then homodimerize and migrate to the nucleus and activate different proinflammatory genes [32-34]. Since JAK-2 inhibitor AG490 decreased IL-17 induction but not RORγt$^+$ IL-17$^+$ cells (Figure 2) [17], we next determined whether or not downstream events of JAK-2 (i.e. phosphorylation of STAT3) are altered in estrogen-treated mice. Surprisingly, we found that estrogen-treatment delayed STAT3 phosphorylation when compared to controls (Figure 3A). Activated cells from estrogen-treated mice had decreased pSTAT3 levels even at early time points. Not only the levels of pSTAT3 were decreased in estrogen-treated mice, we also found that at both early (30 min and 1 hr) and late time points (24 hr, 48 hr), there was decreased STAT3 DNA binding activity in cells from estrogen-treated mice when compared with placebo-treated mice (Figure 3B and C). These results indicate that the kinetics of STAT3 phosphorylation and DNA binding activity is modulated by *in vivo* estrogen treatment. Even though the levels of pSTAT3 and pSTAT3 DNA binding activity is lower in estrogen-treated mice, there is increased IL-17 induction, which implies that JAK-2-STAT-3 signaling may not be as important as other transcription factors/signaling pathways in estrogen-mediated IL-17 regulation.

Estrogen downregulates IRF8, an inhibitor of IL-17

It is likely that estrogen upregulates IL-17 by not only upregulating IL-17 promoting transcription factors but also by downregulating the transcription factors that inhibit IL-17. We next examined whether estrogen prevents the expression of IL-17 inhibitors. Recently, IRF8 has been shown to regulate IL-17 by silencing Th17 differentiation and downregulating Th17-associated genes [35]. By 48 hrs of culture, by real-time PCR assay we noted that there was decreased IRF8 mRNA expression in stimulated cells from estrogen-treated mice when compared with placebo-treated mice (Figure 4).

Estrogen treatment decreases negative regulator ETS-1 while increases miR-326 expression

Studies in ETS-1 deficient mice established that ETS-1 is a negative regulator of IL-17 [36]. It was also shown that ETS-1 does not bind directly to the IL-17 promoter [36]. We found the ETS-1 mRNA (Figure 5A) and protein expression (Figure 5B) was decreased in cells from estrogen-treated mice when compared with placebo-treated mice. This was evident even in unstimulated freshly-isolated cells from estrogen-treated mice suggesting that *in vivo* estrogen exposure modulates the change in ETS-1 expression.

Since recent study has shown that ETS-1 is targeted by miR-326 (a microRNA which post-transcriptional regulates genes) [37], we next investigated whether miR-326 levels were increased by estrogen. Real-time PCR analysis revealed that miR-326 expression was markedly increased in cells from estrogen-treated mice compared to controls (Figure 5C). Interestingly, time kinetics revealed that decreased ETS-1 correlated with increased miR-326 and IL-17 levels (Figure 5D). Together these results suggest that induction of IL-17 in cells from estrogen-treated mice is possibly controlled by miR-326 mediated suppression of ETS-1.

Furthermore, to confirm that miR-326 is important in IL-17 induction, we inhibited miR-326 expression in cells from estrogen-treated mice by using specific antagomir and analyzed the effect of miR326 antagomir on miR-326 expression (to test transfection efficiency), and ETS-1 and IL-17 levels. We found that transfection of miR-326 antagomir decreased miR-326 expression (Figure 6A) and importantly IL-17 levels (Figure 6B) when compared to cells transfected with scramble/negative control. In addition, we observed increase in levels of ETS-1 protein expression in nuclear extracts of cells treated with miR-326 antagomir at 48 hr (Figure 6C, densitometry data not shown).

Estrogen increases miR-223 levels which promote IL-17 induction

In addition to miR-326, miR-223 is also known to upregulate IL-17 induction by targeting 3' untranslated region of Roquin, another indirect inhibitor of IL-17 [38]. We have previously reported that miR-223 is increased in freshly-isolated cells from estrogen-treated mice and that

miR-223 regulates IFNγ expression [28], therefore, we next determined the miR-223 levels in cells activated with IL-17 inducing stimuli for 24 and 48 hrs and compared with miR-223 mRNA levels in freshly isolated placebo control. We found significant increase in miR-223 levels in cells from estrogen-treated mice (Figure 6D). Further, suppressing expression of miR-223 in estrogen-treated cells by miR-223 antagomir (Figure 6E) also decreased IL-17 levels when compared to control transfected cells (Figure 6B). However, miR-223 target Roquin mRNA was not found to be altered by miR-223 antagomir in cells from estrogen-treated mice (data not shown).

Discussion

It is well established that the differentiation of naïve $CD4^+$ cells into *bona fide* distinct T-cell lineages, Th1, Th2 and Th17, is regulated by specific transcription factors. T-bet has been shown to be important for Th1, while GATA-3 is critical for Th2 differentiation. *In vitro* and *in vivo* studies have shown that the differentiation of Th17 cells require TGFβ1 and IL-6-induced upregulation of unique lineage-specific transcription factor, RORγt, encoded by *RORc* gene [31, 39, 40]. The transcription factor RUNX1 regulates Th17 differentiation by upregulating RORγt expression and by directly binding with RORγt to the IL-17 promoter [41]. Transcription factors such as RelA (p65) and c-Rel, members of NF-κB family have been recently shown to bind and activate promoters of RORγ and RORγt, respectively [42]. The positive role of NF-κB in IL-17 induction was further substantiated by the findings that activation of NF-κB increases secretion of IL-17 [43, 44]. Cyclosporine A inhibition of phosphorylation of IkappaB and Akt and consequently decreased the binding of NF-κB to the IL-17 promoter also results in decreased IL-17 induction [45]. In addition, inhibition of NF-κB by the specific inhibitor, BAY 11-7082, decreased IL-17 induction [46]. Interestingly, inhibitors of the MAP kinase ERK (UO126) and p38 MAPK (SB203580) did not inhibit IL-17 induction [46], thereby further suggesting the positive role of NF-κB in the induction of IL-17. In our previous publication [17], we had reported that *in vivo* estrogen-mediated enhancement of IL-17 induction from activated splenocytes was accompanied with increased RORγt expression and influenced by JAK-2

activity. In this study, we have further expanded these observations to examine the role of various transcription factors involved in estrogen-mediated IL-17 induction. RORγt-related transcription factor RORα was also found to be increased in cells from estrogen-treated mice. We also found that NF-κB pathway is essential for IL-17 induction from estrogen-treated cells and inhibition of NF-κB decreased percent RORγt expressing cells (Figure 2B). These results are in agreement with a recent finding that the NF-κB activates RORγt promoter [42] and is important for IL-17 induction [43, 44]. Decreasing the expression of p65 by using p65-specific siRNA also reduced the levels of IL-17 from activated cells. Further, truncation of p65 by serine proteases has a stimulatory effect on IL-17 induction by estrogen. These results are in agreement with our previous report that truncation of p65 by serine protease proteolysis induced by estrogen plays an important role in estrogen-mediated post-translational modification of NF-κB, and promotion of IFNγ and iNOS [27].

Although, JAK-2/STAT3 pathway has been shown to be critical for IL-17 commitment and RORγt induction [32, 47], we observed that JAK-2 inhibitor AG490 decreased IL-17 levels but not the percent of IL-17^{+} and/or RORγt^{+} cells. This could possibly be due to AG490-mediated suppression of other STAT pathways (STAT1; STAT5) [48, 49], which have shown to negatively regulate IL-17 induction [47, 50]. Alternatively, in estrogen-model of inflammation, JAK-2 signaling may affect the output/secretion of IL-17 but not expansion of Th17 cells. It is also noteworthy that MAPK and PI3K pathways are not involved in estrogen-mediated promotion of IL-17. Interestingly, pSTAT3 levels and DNA binding activity was unexpectedly found to be higher in placebo even at early time points than in splenocytes from estrogen-treated mice. At 72 hr STAT3 DNA binding activity in estrogen-treated mice was comparable (but not higher than) to that of placebo-treated mice. This suggests that delaying of phosphorylation of STAT3 in estrogen-treated mice may influence the induction of IL-17.

It is essential to control the induction of IL-17 since dysregulated overproduction of IL-17 results in extensive tissue damage and inflammation. Recent studies in different experimental models have identified different transcription factors that negatively regulate IL-17 including, IRF8, T-bet and ETS-1. IRF8, also known as ICSBP, (product of macrophage, B cells, DCs and activated T cells) has been shown to downregulate IL-17 by silencing Th17 differentiation and

downregulating Th17-associated genes [51]. IRF8 physically interacts with RORγt and inhibits *IL-17* transcription by binding to its promoter [35]. We also found that estrogen-treated cells have decreased IRF8 mRNA expression, which may be one of the potential targets for increased IL-17 induction from estrogen-treated cells.

T-bet, a Th1 lineage specific transcription factor, also suppresses Th17 development by binding to the transcription factor Runx1 via tyrosine 304 of T-bet. This T-bet-Runx1 binding has been shown to block the transactivation of *RORc* gene and therefore IL-17 induction [52]. We have previously shown that exposure of cells to IL-27 or IFNγ (which upregulate T-bet [53]), markedly decreased IL-17 levels in cells from estrogen-treated mice [17]. Future studies need to be conducted to confirm whether IL-27-mediated T-bet induction results in inhibition of RORγt and Runx-1 interaction. Another T-bet interacting transcription factor, v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1), has been shown to inhibit Th17 differentiation. ETS-1-deficient mice have increased IL-17 levels (without affecting RORγt), suggesting that ETS-1 is a negative regulator of IL-17. So far it has not been defined whether or not there is direct interaction between ETS-1 and *IL-17* gene [36]. In the present study we found that *in vivo* estrogen treatment decreases ETS-1 mRNA and protein levels when compared with placebo-treated mice (Figure 5A and B), which negatively correlates with upregulated IL-17 levels from estrogen-treated mice (Figure 5D). Intriguingly, we found that by microRNA, miR-326, known to target ETS-1 is upregulated in cells from estrogen-treated mice. The kinetic pattern of upregulation of miR-326 (and downregulation of ETS-1) correlated with enhanced IL-17 levels in cells from estrogen-treated mice (Figure 5C). In addition, suppression of miR-326 expression by transfecting estrogen-treated cells with antagomir decreased IL-17 levels but increased ETS-1 levels (Figure 6A, B, and C). Our findings are in agreement with a recent report that showed that miR-326 expression correlated with disease severity in human MS patients and experimental autoimmune encephalomyelitis (EAE) mice [37]. These workers also surmised that miR-326 played a role in MS pathogenesis by regulating Th-17 cell differentiation through translational inhibition of ETS-1, a negative regulator of Th17 differentiation since miR-326 correlated with IL-17 levels [37]. By FISH assay, it was confirmed that IL-17A and miR-326 are present particularly in $CCR6^{+}CD4^{+}$ T cells from the peripheral blood of patients with relapsing MS and

in *in vitro* differentiation model [37]. In addition, another novel finding is that miR-223 is increased in cells from estrogen-treated mice. Inhibition of miR-223 upregulated IL17 and thus is a positive regulator for IL-17 induction (Figure 6B, D and E).

There are other reported transcription factors which have either positive or negative effect on IL-17 induction such as IRF4 [54], suppressor of cytokine signaling (SOCS3) [55], peroxisome proliferator-activated receptor γ (PPARγ) [56] etc. These aspects were not investigated with respect to estrogen induction of IL-17 and are a subject of a separate study. Although other studies have identified independent transcription factors that regulate IL-17, this is the first study that comprehensively investigates multiple transcription factors in one model, i.e. estrogen-induced promotion of IL-17 and inflammation. We report that estrogen-treatment preferentially fine tunes the balance of positive and negative regulators of IL-17 to favor promotion of this cytokine. We also report that at least two miRNAs (miR-326 and miR-223) also epigenetically regulate IL-17. It is likely that there may be yet other untested miRNAs and/or transcription factors that estrogen may alter to regulate IL-17. Identification of these molecular targets of estrogen involved in IL-17 induction enhances our understanding of estrogen-regulation of inflammation and hopefully, will assist in designing specific strategies to downregulate IL-17 in inflammatory disorders.

Acknowledgements

Funding source: NIH (RO1 AI051880-04A5), NIHT35-RR021311 and Lupus Foundation of America, Inc. VMRCVM Intramural Research Competition (IRC) Grant (441303) and departmental funds. We want to thank Ms Melissa Makris for flow cytometry, and the animal care staff for taking care of animals. Deena Khan was supported by graduate stipend from College of Veterinary Medicine and Office of Research and Graduate Studies.

Legends

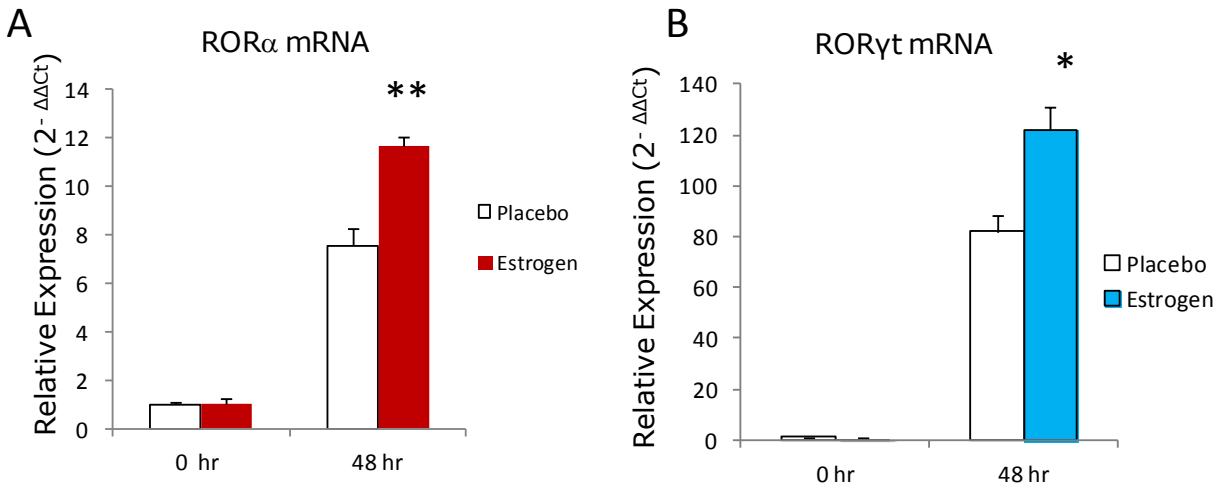

Figure 1: RORα and RORγt mRNA are increased in estrogen-treated mice.

A and *B*, Splenocytes from estrogen- and placebo-treated mice were stimulated with IL-17-inducing stimuli (IL-6 (20 ng/ml) +TGFβ (3 ng/ml) +antiCD3 antibodies (1 µg/ml)) and total RNA extracted at 48 hrs. Relative expression of RORα (*A*) and RORγt (*B*) determined and compared with 0 hr (freshly isolated splenocytes) mRNA expression by using TaqMan gene expression assays (n=3/group). The data represent means ± SEM (* $p<0.05$), Student-t test was performed.

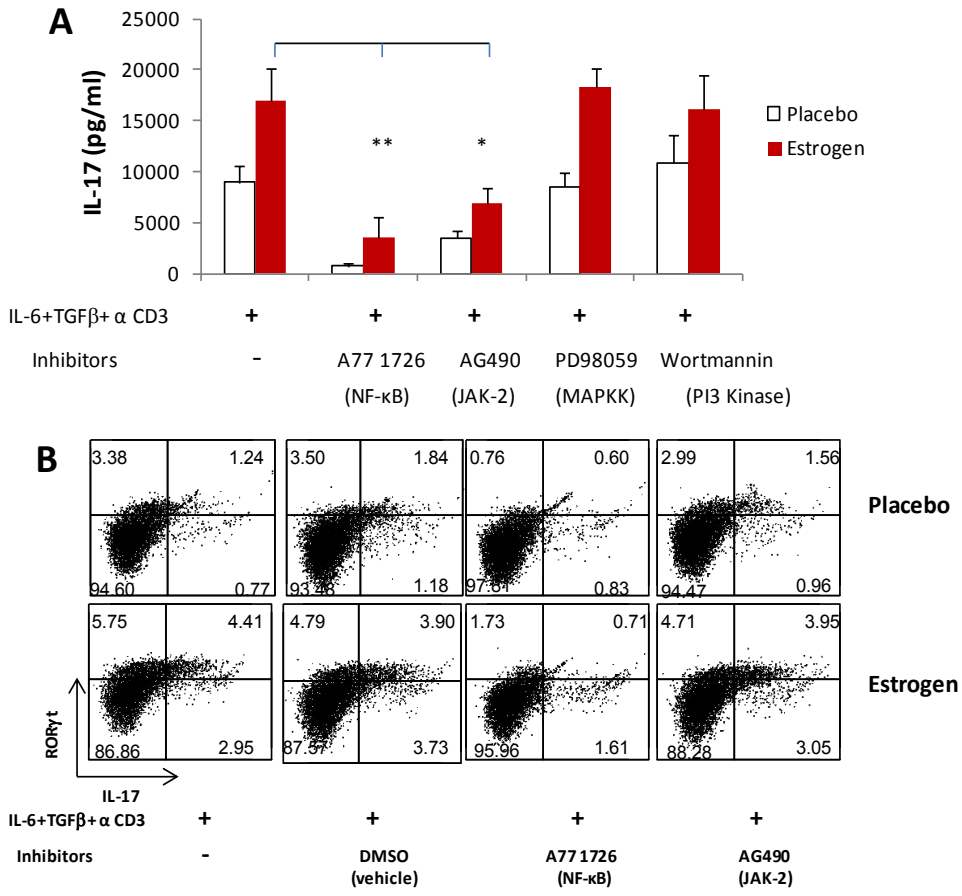
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Figure 2: Estrogen mediated IL-17 induction is dependent on NF-κB signaling pathway.

A and *B,* Cells from estrogen- and placebo-treated mice were cultured in presence or absence of inhibitors or vehicle control (DMSO) for 1 hr followed by activation with IL-6+TGFβ+antiCD3 antibodies for 72 hrs. IL-17 levels were measured by ELISA (*A*), n=4-6/group, repeated twice and representative dot plot of IL-17^{+}RORγT^{+} cells were analyzed by flow cytometric analysis (*B*). *C,* Whole cell extract of cells treated with or without AEBSF (100 μM) for 3 hrs were analyzed for p65 levels and compared with loading control (β-actin) by Western blotting. *D*, Splenocytes from estrogen- and placebo-treated mice were cultured in presence of serine protease inhibitor, AEBSF (50 μM) and IL-17-stimulating cocktail for 48 hrs. The levels of IL-17 were determined by ELISA after 48 hrs (n=3/group; repeated three times). *E* and *F*, Transfection of cells with p65 siRNA or control siRNA was performed and relative levels of IL-17 in stimulated culture supernatants were determined in placebo (*E*) and estrogen (*F*) mice by ELISA. (A total of 3 mice/treatment group were used and representative data has been shown). The levels of IL-17 in control siRNA group were normalized to 1 and compared with the levels of IL-17 in p65 siRNA-treated cells from same mouse. The levels of IL-17 (at 72 hr) from placebo cells treated with control and p65 siRNA transfected were 114.905 pg/ml and 56.298 pg/ml, respectively. For estrogen mouse, it was 1106.811 pg/ml and 366.857 pg/ml, respectively. p65 transfection efficiency in whole cell extracts at 48 hrs after transfection (24 hr stimulation) was determined by Western blotting. The data represent means ± SEM (* $p<0.05$), Student-Newman-Keuls Multiple Comparisons Test was performed.

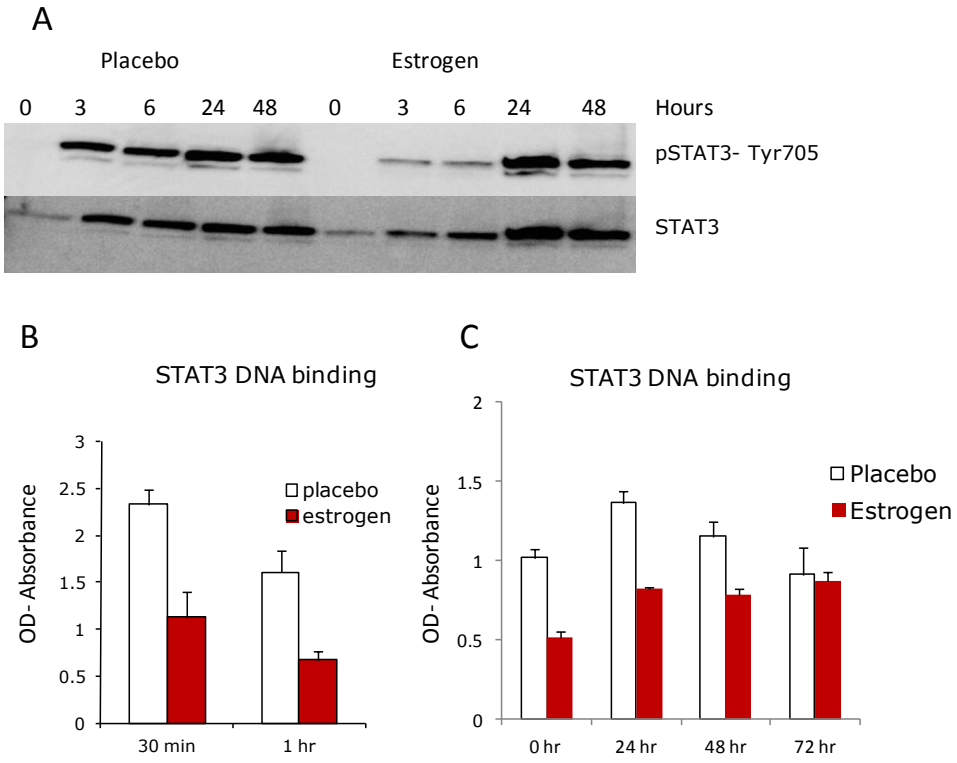

Figure 3: pSTAT3 levels and DNA binding activity is delayed by estrogen treatment.

A, pSTAT3-tyr705 and total STAT3 levels in nuclear extracts of splenocytes from estrogen- and placebo-treated mice activated with IL-17-inducing stimuli for indicated times were determined by Western Blotting (repeated 3 times). *B* and *C*, STAT3 DNA binding activity was determined in nuclear extracts of placebo and estrogen-treated mice by using TransAM STAT3 DNA binding ELISA kit for early time points, 30 min and 1 hr (*B*) n=4-6/group, and late time points 24 -72 hr (*C*) n=3/group.

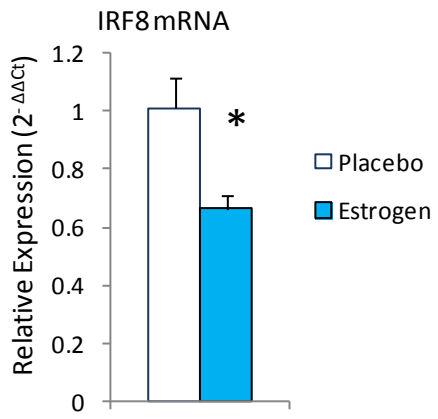

Figure 4: IRF8 mRNA is decreased in estrogen-treated mice.

Splenocytes from estrogen- and placebo-treated mice were stimulated with IL-17-inducing stimuli and total RNA were extracted at 48 hrs and real time PCR performed. Relative expression of IRF8 was determined by using TaqMan gene expression assays (n=3/group). The data represent means ± SEM (* $p<0.05$), Student-t test was performed to determine statistical validity.

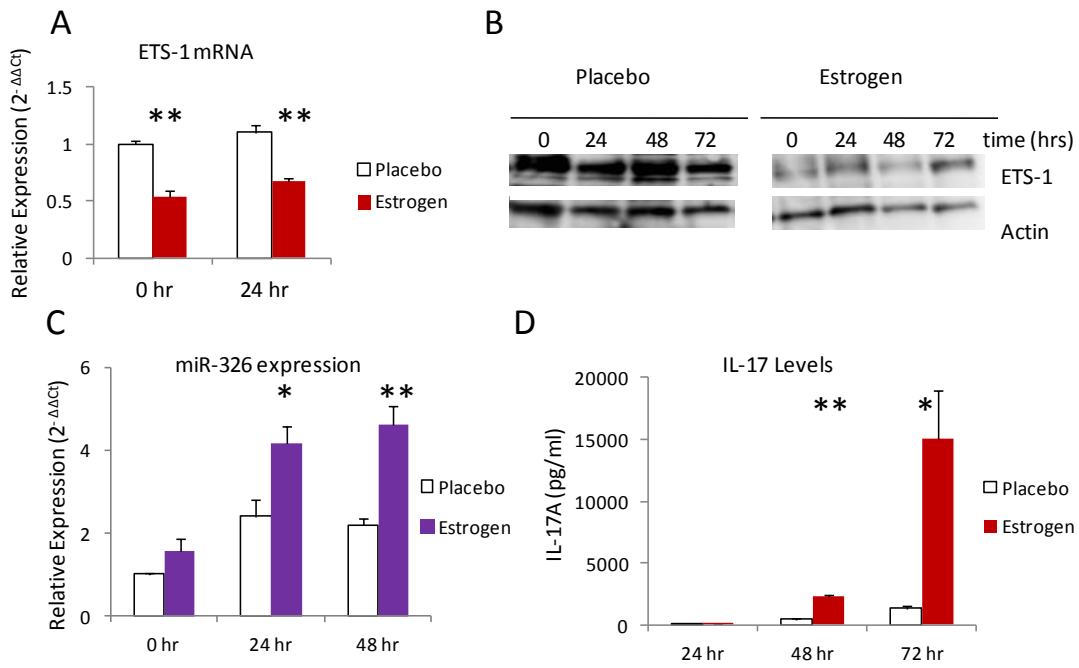

Figure 5: Estrogen downregulates IL-17-inhibitory transcription factor (ETS-1) via miR-326

A, *B*, *C*, *D*, Splenocytes from estrogen- and placebo-treated mice were stimulated with IL-17-inducing stimuli for indicated times and cells and supernatants collected for analysis. *A*, *ETS-1* mRNA levels were determined by real time PCR and *B*, protein levels in nuclear extracts were analyzed by Western blotting. *C*, Relative miR326 mRNA levels were measured by TaqMan real time PCR *D*, IL-17 protein levels in culture supernatants of the activated cells from estrogen- and placebo- treated mice were measured by ELISA, (n=3/group).

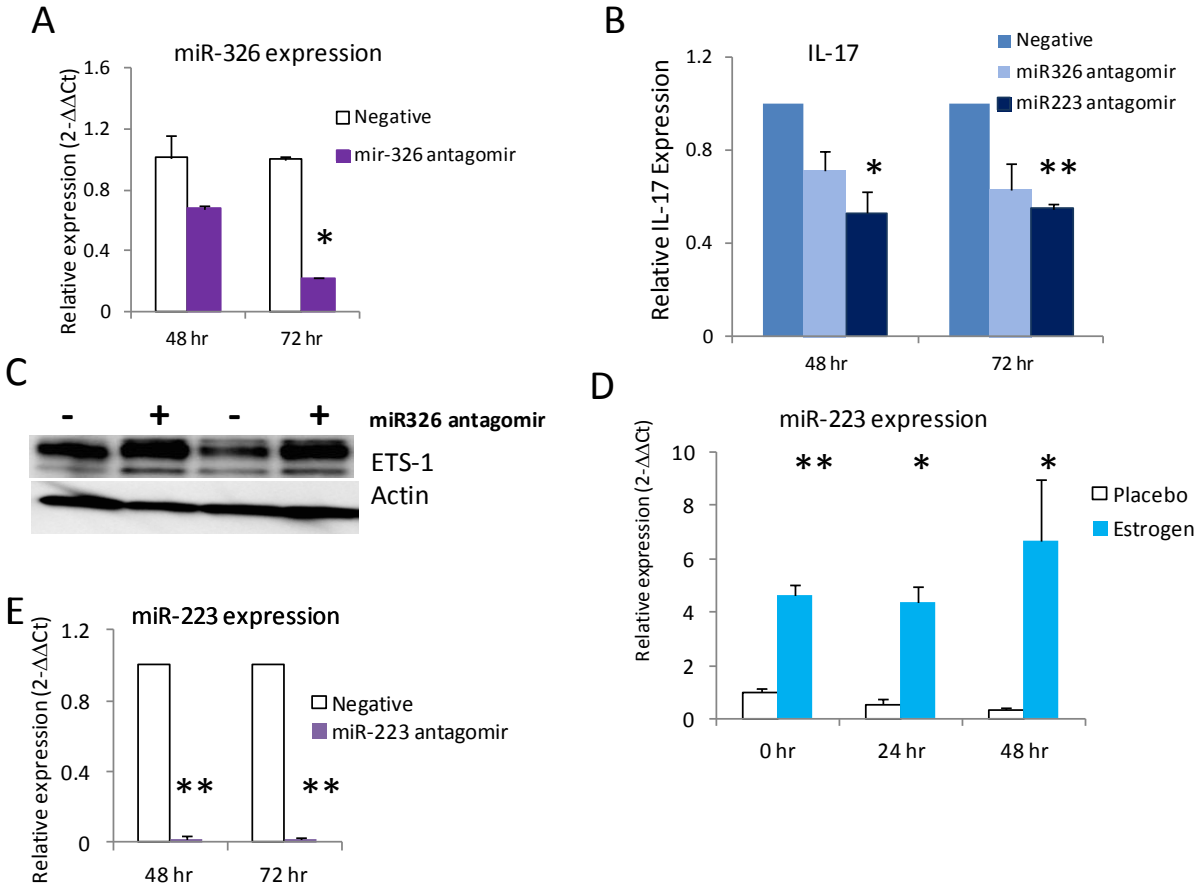

Figure 6: Estrogen upregulates miR-223 and upregulates miR-223 and miR326 mediated IL-17 induction.

A. miR-326 antagomir transfection efficiency at 48 and 72 hr was determined by analyzing miR-326 expression (TaqMan real time PCR) in transfected cells from estrogen-treated mice and compared with cells transfected with control antagomir (n=2/group). *B,* The levels of IL-17 from control transfected cells at 48 and 72 hr were normalized to 1 and compared with miR-326 antagomir transfected cells or miR223 antagomir transfected cells (n=3/group). *C*, ETS-1 levels in nuclear extracts of cells transfected (48 hr) with either miR-326 antagomir or control was determined by Western blotting. *D*. Relative miR-223 mRNA levels were measured by TaqMan real time PCR (n=3/group). *E.* miR-223 antagomir transfection efficiency in at 48 and 72 hr was determined by analyzing miR-223 expression in transfected cells from estrogen-treated mice and

compared with control cells (n=2/group; repeated twice). The data represent means ± SEM (* $p<0.05$, ** $p<0.01$), Student-t test was performed.

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Chapter 6: Estrogen induces IL-17-producing Tc17 cells and increases responsiveness to IL-17

Abstract

IL-17A and its isoforms IL-17F share similar biological functions and signal via common IL-17RA and IL-17RC receptor. The importance of these proinflammatory cytokines in different inflammatory and autoimmune diseases is well evident now with increasing reports demonstrating positive correlation between the presence of these cytokines and disease pathogenesis. In our previous publication, we have demonstrated that IL-17A levels and its specific transcription factor, RORγt, are markedly increased from activated splenocytes from mice exposed to estrogen *in vivo*. Given the importance of IL-17 in the disease progression and pathogenesis, in this study we have determined whether estrogen-treatment has any effect on other IL-17A related isoforms and the proinflammatory events-mediated by these isoforms. We found that estrogen upregulates IL-17F and IL-17A/F isoforms of IL-17A in activated splenocytes. We also found that majority of IL-17 positive cells are $CD8^+$ suggesting that estrogen-mediated IL-17 induction is predominantly from Tc17 cells with increased proliferation demonstrated by CFSE cell proliferation assay. Furthermore, we show that estrogen enhances proinflammatory MCP-1 levels from IL-17A and IL-17F-activated splenocytes. To our knowledge, this is the first study to demonstrate that estrogen induces various isoforms of IL-17, primarily from Tc17 cells, and demonstrate enhanced responsiveness to IL-17.

Introduction

The IL-17 cytokine isoforms are glycoproteins of 155 amino acids and range from 20 to 30 kDa in size. These IL-17 isoforms have overlapping, but not identical biological activities. They share 4-conserved cysteine residues at C-terminal region. So far, major focus has been on IL-17A (a founding member of IL-17, hence referred as IL-17) and IL-17F because of their important functional and biological properties. IL-17A and IL-17F are nearly 50% homologous and map to

the same chromosomal loci, 6p12 [1]. They exist as either homodimers of IL-17A or IL-17 or as IL-17A/F heterodimers [2]. IL-17A is a powerful proinflammatory cytokine and is now known to be secreted by many cell types including: $CD4^+$ cells (Th17), $CD8^+$ cells (Tc17), γδ T cells, natural killer cells and mast cells, neutrophils, among others [3-6]. IL-17F largely has similar action as IL-17A and is produced by $CD4^+$ cells, $CD8^+$ cells, monocytes, basophils, mast cells, γδ T cells, NKT cells, etc [7-10]. IL-17 induced by cells of innate immune system such as γδ T cells, Lti cells, Paneth cells, iNKT cells, neutrophils etc [11], is rapid and in absence of antigenic stimuli, while those from the cells of adaptive immune system (CD4 or CD8) is delayed and requires proper antigenic stimuli for IL-17 production.

IL-17-producing $CD8^+$ T cells (Tc17), a recently defined subpopulation of effector T cells, appear to play an important role in a wide range of conditions, such as infection, autoimmune diseases, immune thromobocytopenia and cancer [12-14]. Tc17 cells differentiate from the same precursors that differentiate into Tc1 cells [15]. As is the case of $CD4^+$ T cells (Th17 cells), activation of $CD8^+$ T cells by IL-6 or IL-21 plus TGFβ, leads to the differentiation into IL-17-producing cells. These cells express hallmark molecules of Th17 program including retinoic acid receptor-related orphan receptor (ROR) γT, RORα, IL-21 and IL-23R. In the presence of IL-6 and IL-21, overexpression of the master regulator RORγT is linked to IL-17 production and Tc17generation [14]. An interesting feature of Tc17 cells compared to classic Tc1 cells is that these cells have greatly suppressed cytotoxic function together with manifestation of low levels of the classic cytotoxic lymphocyte markers: T-box transcription factor (T-bet), GATA-3, eomesodermin [14]. Tc17 are negative for granzyme B, perforin and cytolytic activity [16]. It has been shown that Tc17 can protect against lethal influenza infection accompanied with enhanced expansion of Tc17 cells and increased neutrophil influx into the lung [16]. Interestingly, a study shows that Tc17 cells clear vaccinia virus infection by increased FasL expression *in vivo* [17]. IL-17-secreting Tc17 cells are also important in controlling tumor growth by aiding in rapid recruitment of neutrophils. These activated neutrophils attract Th1 and Tc1 lymphocytes and neutrophils by secreting chemokines such as CCL3, CCL4, CCL5, CXCL9 and CXCL10 [18]. Together, these reports suggest that IL-17 and its isoforms play important role in disease development or progression, clearance of pathogens, and tumor surveillance.

Estrogen modulation of both innate and adaptive immune system is well evident. For past two decades our laboratory had extensively studied the role of sex hormone, estrogen in particular, in immune regulation [19-22]. We have recently reported that estrogen upregulates IL-17A induction in normal wildtype C57BL/6 mice. In addition to IL-17 levels, we also found that the numbers of IL-17-secreting cells and $ROR\gamma T^{+}IL\text{-}17^{+}$ cells were also increased in splenocytes from estrogen-treated mice when compared to placebo-treated mice [23]. In this study we investigated whether: (i) estrogen promotes other isoforms of IL-17; (ii) CD8 contributes to IL-17 induction; and (iii) response to recombinant IL-17 is enhanced in cells from estrogen-treated mice.

Materials and Methods

Animals: At 4–5 wks of age, male and female wildtype C57BL/6 (Charles River Laboratories and Harlan Laboratories) were gonadectomized and surgically implanted with silastic capsules containing 17beta-estradiol (estrogen; 3-5 mg; Sigma-Aldrich) or empty (placebo) implants by standard procedures for 2 months that have been extensively described in our previous studies [21, 24-26]. These implants are designed to slowly release sustained levels (156-220 pg/ml) of estrogen [24, 25]. All animal-related procedures were in accordance with Virginia Tech Institutional Animal Care guidelines, and were approved by the Institutional Animal Care and Use Committee. Mice were fed a commercial pellet diet devoid of estrogenic hormones (7013 NIH-31 Modified 6% Mouse/Rat Sterilizable Diet; Harlan-Teklad).

Isolation and culture of Splenic Lymphocytes: IL-17 was induced in splenic lymphocytes (2.5 x 10^6 cells/ml) by culturing with previously reported [27, 28] recombinant cytokines rIL-6 (20 ng/ml; Ebiosciences) plus TGF-β (3 ng/ml; R&D Systems, Inc., Minneapolis, MN) and anti CD3 antibody (1 μg/ml; Ebiosciences). Control cells were cultured in the absence of these stimuli. In selected experiments, splenocytes were also cultured with rIL-27 (10 ng/ml; Ebiosciences), JAK2 inhibitor AG490 (10, 25 μM) for defined time points. For IL-17 response study, splenic lymphocytes from estrogen and placebo-treated mice were stimulated with different doses of recombinant IL-17A and its isoforms (Ebiosciences), and endpoints determined. Exposure of

cells to the above reagents did not affect the viability of the cells as demonstrated by the Alamar Blue assay [29] and 7-AAD-flow cytometric assay [30](data not shown).

IL-17 isoform and MCP-1 ELISA: Protein levels of IL-17A IL-17F, IL-17A/F in culture supernatants were determined with ELISA kits per manufacturer's instructions (Ebiosciences) using Vmax microplate reader (Molecular Devices, Sunnyvale, CA). MCP-1 protein levels in culture supernatants were analyzed as previously described [31].

***RayBiotec Dot Blot*:** Splenocytes from estrogen- and placebo-treated mice were cultured in presence or absence of recombinant IL-17A (200 ng/ml), IL-17F (200 ng/ml) and IL-17A/F (100 ng/ml) for 24 hrs. The supernatants were collected and used for simultaneous detection of 32 cytokines and chemokines by using membrane based RayBiotech Mouse Cytokine Array 2 (Norcross, GA) was used according to our previously published report [25]. The blots were developed and analyzed on Kodak Image Station 4000MM.

***Taqman Real-time PCR*:** Total mRNA from splenic lymphocytes was isolated by miRNeasy mini kit (QIAGEN Bioscience, Maryland, USA) and real-time PCR performed by using TaqMan Universal Master Mix II (Applied Biosystems, Carlsbad, CA) using T-bet, eomesodermin, perforin and granzyme TaqMan gene expression assays from Applied Biosystems.

CFSE staining and Flow Cytometric Analysis of Intracellular Expression of IL-17 and RORγT: Freshly-isolated splenic lymphocytes were stained with CFSE dye (5μM) for 10 min and then after washing cultured in presence of different stimuli as indicated. After 3 days of culture, cells were surface stained for either CD4 or CD8 antibody and fixed. Percent IL-17 expressing cells and RORγT subset were quantified by intra-cytoplasmic staining with specific antibodies. Splenocytes ($1x10^6$/100 μl) were cultured for defined time points with additional 3 hr activation with PMA, ionomycin and brefeldin A and then subjected to intracellular staining (antibodies from Ebiosciences) by using BD Cytofix/Cytoperm Kit according to the manufacturers' instructions. Stained cells were visualized using a FACS Aria flow cytometer

(BD Biosciences) and data analyzed using FlowJo version 7 software. CFSE data was expressed as percent $CD4^+$, $CD8^+$ cells and intracytoplasmic data as percent $IL\text{-}17^+$ or $ROR\gamma T^+$ cells.

Statistical analysis: The significance of differences between placebo and estrogen-treated samples was assessed as indicated using GraphPad InStat version 3.0a for Macintosh (GraphPad Software). The significance level is indicated as asterisk (* for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$ respectively).

Results

IL-17F and IL-17A/F levels are increased in estrogen-treated mice

In our previous publication, we reported that *in vivo* estrogen treatment primes the splenic lymphocytes to secrete IL-17A upon stimulation with IL-6+TGFβ+antiCD3 antibodies [23]. IL-17A and IL-17F are homologous, and also exist as heterodimers IL-17A/F. In addition, IL-17A and IL-17F are also known to signal through IL-17RA and IL-17RC receptor complex [32]. Since, IL-17A and IL-17F have overlapping biological functions due to shared receptor and common downstream signaling pathway; we next measured IL-17F and IL-17A/F levels in culture supernatants from estrogen and placebo-treated male and female mice. We found that stimulation of splenocytes from estrogen-treated male or female mice with IL-17-inducing stimuli (IL-6+TGFβ+antiCD3 antibodies) for 48 hrs enhanced the production of IL-17F levels in culture supernatants significantly (Figure 1A and B). In addition, the levels of IL-17A/F were also increased in culture supernatants from estrogen-treated male and female mice when compared with placebo-treated mice (Figure 1C and D). These results confirm that in addition to IL-17A, *in vivo* estrogen treatment activates the splenocytes to secrete more IL-17A/F and IL-17F isoforms of IL-17 family.

$CD8^+$ cells are major contributor of IL-17 in estrogen treated mice

We next determined which cell subset predominantly secretes IL-17 in estrogen treated mice. Since, mixed splenic lymphocyte culture were used for IL-17 induction, we surface stained the cells with either anti CD4 or CD8 antibodies to determine which population of T cell is $IL\text{-}17^+$

cells. Surprisingly, we found that the majority of IL-17$^+$ cells in estrogen-treated mice were CD8$^+$ (Figure 2A and B). Although, CD4$^+$IL-17$^+$ cells were also higher in estrogen-treated mice, there were more Tc17 than Th17 cells.

Estrogen treatment favors CD8+ cells proliferation

We next wanted to determine why more CD8$^+$ cells secrete IL-17 in estrogen-treated mice, is it because of increased proliferation of CD8$^+$ cells in estrogen-treated mice. To test this view, we pre-stained cells with CFSE and cultured as described above, stained with anti CD4 or CD8 antibodies and analyzed cell proliferation by flow cytometry. It was intriguing to see that *in vivo* estrogen-treatment favored marked proliferation of CD8$^+$ cells by 72 hrs and later (Figure 2C). Although, there was some proliferation in CD4$^+$ cells from estrogen-treated cells, but no daughter populations were observed in either CD8$^+$ or CD4$^+$ cells from placebo-treated mice. This suggests that estrogen modulates CD8$^+$ cell proliferation and could be the potential reason for the increased numbers of Tc17 cells in estrogen-treated mice.

Cytolytic markers are decreased in Tc17 cells

Perforin, granzyme are the main cytolytic markers expressed by Tc1 CD8$^+$ cells with cytolytic activity. It has been well documented that Tc17 cells lack these cytolytic markers and lack cytolytic activity [14, 33]; instead they mediate inflammation by activating neutrophils. We next analyzed whether estrogen treatment affects the expression of perforin and granzyme in Tc17 cells. By real-time RT-PCR we found that, splenocytes cells from estrogen-treated mice had increased perforin and granzyme mRNA expression (Figure 3A and B); however only slight difference were observed in the percentages of perforin and granzyme$^+$ cells from placebo and estrogen-treated mice (Figure 3C).

Eomesodermin mRNA levels are also decreased in estrogen treated mice

T-bet and eomesodermin (master regulators of Th1 and Tc1, respectively) are not essential for systemic cytotoxic T cell activity and are reported to be markedly decreased in Tc17 cells [34]. Therefore, we next analyzed mRNA levels of T-bet in estrogen-treated splenic lymphocytes and

cultured in presence of IL-17 inducing stimuli at 48 hr (Figure 3D). We found that T-bet mRNA levels were increased in activated cells from estrogen-treated mice. However, in freshly isolated splenocytes from estrogen- and placebo-treated mice there were no differences in the T-bet expression (data not shown).

It has been shown that Eomes directly bind to the proximal promoter regions of *Rorc* and *Il17a* inhibiting Th17 differentiation and TGFβ suppresses Eomes via the c-Jun-N-terminal kinase (JNK)-c-Jun signaling pathway [35]. For induction of IL-17, we use IL-6 and TGFβ, therefore we analyzed the expression of Eomes in activated splenocytes after 48 hrs. Eomes mRNA level were very low in the cells from estrogen-treated mice and placebo-treated mice (Figure 3D). The Eomes mRNA levels at 48 hr were lower than the levels in freshly isolated splenocytes from placebo-treated mice indicating possible inhibition of Eomes by TGFβ (data not shown).

Together, these results suggest that estrogen-modulates induction of IL-17A and its isoforms and increases proliferation of CD8 cells and therefore, has increased Tc17 cell population.

In vitro response to IL-17 is enhanced in estrogen treated mice

We have so far confirmed that in vivo estrogen treatment promotes induction of IL-17A and its isoforms from activated splenocytes. Since IL-17 is such an important pro-inflammatory cytokine and is involved in various autoimmune diseases especially in rheumatoid arthritis, multiple sclerosis, psoriasis, SLE etc, we next wanted to evaluate the response of estrogen-treated cells to IL-17. To determine, whether *in vivo* estrogen exposure regulates downstream signaling of IL-17 or not, we cultured splenic lymphocytes in presence or absence of varying concentrations of IL-17A or IL-17F and analyzed the expression of various cytokines by using semi-quantitative RayBiotech membranes (Figure 4A). In our preliminary screening, we found that MCP-1 and RANTES (CCL5; Regulated upon Activation, Normal T-cell Expressed, and Secreted) were increased in culture supernatants from estrogen-treated cells. We confirmed the levels of IL-17 induced MCP-1 by ELISA and found significant difference in MCP-1 induction from estrogen and placebo-treated mice at different IL-17 concentrations and time points (Figure 4B and C).

Discussion

In our previous study, we reported that estrogen upregulates induction of IL-17A from activated splenocytes when compared with cells from placebo-treated male and female mice. This study is an extension of our previous work and is focused on the effect of estrogen on other IL-17A-related isoforms, particularly IL-17F and IL-17A/F heterodimers. We found that there were increased levels of IL-17F and IL-17A/F heterodimers in activated splenocytes from estrogen treated male and female mice when compared with placebo-controls. The levels were markedly suppressed in cultures stimulated in presence of IL-17-suppressive IL-27 and JAK-2 inhibitor, AG490 (data not shown). This was similar to our earlier finding that IL-17A levels are decreased by IL-27 and JAK-2 inhibitor [23]. This further confirms that estrogen plays important role in immunomodulation. It was surprising that majority of IL-17^+ cells from estrogen are $CD8^+$ cells i.e. Tc17 cells. However, we still do not know the implications of increased Tc17 cells in estrogen-treated mice. The increased mRNA levels of perforin and granzyme could be due to increased proportion of $CD8^+$ cells in estrogen-treated mice. We still do not know if this increase is specific for Tc1 and/or Tc17 cell subsets in estrogen-treated mice. Future experiments in purified $CD8^+$ T cells cultured in presence of either Tc1 or Tc17 priming conditions are required to determine the expression of different cytolytic markers in estrogen- and placebo-treated mice.

There are increasing reports documenting the detection of Tc17 cells in various infections, tumor and other inflammatory diseases. Although, no expansion of Th17 or Tc17 cells was observed in the blood of acute Hepatitis C virus (HCV) patients, there were more Th17 and Tc17 cells in the liver of chronic HCV patient suggesting a localized role of this subset. These cells had a high expression of homing receptor CD161 but lowered inhibitory receptors, mucin domain containing molecule-3 (Tim-3) and programmed-death 1 [36]. It has been shown that Tc17 have a protective role against lethal influenza infection as evidenced by increased Tc17 cells and Tc17-mediated increased neutrophil influx in lungs. The protection afforded by Tc17 effectors is less perforin but more IFNγ-dependent, implying that different mechanisms are involved in Tc17 when compared to Tc1-mediated inflammatory events [16]. Interestingly, a

study shows that Tc17 cells clear vaccinia virus infection by increased FasL expression *in vivo* and this cytotoxic potential was independent of acquired Tc1-IFNγ phenotype [17].

In nasopharyngeal carcinoma patients, there is increased prevalence of Tcregs and Tc17 cells. While Tcregs secrete high levels of IL-10, IFNγ and low level of TGFβ, Tc17 cells express high levels of TNFα and IFNγ [37]. Although, Tc17 cells lack cytotoxic molecules, they are still found to be increased in cancer patients. In presence of IL-12, Tc17 cells convert to IFNγ-producing Tc17 cells which gain cytotoxic function and antitumor activity [38]. These observations demonstrate plasticity of Tc17 cells. For tumor immune surveillance, Th1 and $CD8^+$ T cells are considered to be important. It has been shown recently, that T-bet and eomesodermin, master regulators of Th-1 and $CD8^+$ T cells, are essential in regulating T-cell mediated immune responses against tumor by increasing the infiltration in tumor and by inhibiting differentiation of $CD8^+$ T cells into Tc17 cells. However, T-bet and eomesodermin are not essential for systemic cytotoxic T cell activity [34]. It has been observed that in hepatocellular carcinoma patients, tumor-activated monocytes promote expansion of Tc17 cells which lack perforin and granzyme but secrete IL-22, TNFα. Majority of these Tc17 cells are IFNγ positive [33].

It has been recently demonstrated that mice with impaired TGFβ receptor II signaling (TGF-βRIIDN mice) have multi-organ autoimmune disease contributed by spontaneous differentiation of $CD8^+$ cells into IL-17 producing cells. $CD4^+$ T cells from these mice were positive for Th1 and Th2 cytokine but not for Th17 cells. The inflammation was markedly decreased by neutralization of IL-17 or by depletion of $CD8^+$ T cells. This indicates that *in vitro* and *in vivo* differentiation into Tc17 cells is distinct since even in absence of TGFβ signaling in these mice, there was *in vivo* differentiation of $CD8^+$ cells into Tc17 cells [39]. IL-17A and IL-17F induced from Tc17 and IL-23 are involved in diabetes. Tc17 cells treated with TGFβ1 plus IL-6 or IL-23 likely differ in pathogenicity due to their disparate capacity to attract other immune cells and initiate inflammation. Tc17 cells treated with TGFβ1 plus IL-6 are not diabetogenic, whereas IL-23-treated cells potently induce the disease [12].

In murine model of atopic dermatitis, Tc17 cells play an important role in the development of skin lesion following CD4 depletion [40]. Additionally, there is an increased presence of Tc17 and Tc22 cells in skin lesions of psoriasis suggesting that $CD8^+$ T cells play an important role in psoriasis pathogenesis [41]. In the dermal infiltrates of allergic contact dermatitis patients, about 20% of the infiltrating cells were found to be IL-17-producing cells as they expressed RORC, and such RORC-expressing cells were detected in both $CD4^+$ (approximately 30%) and $CD8^+$ (approximately 20%) subsets in the elicitation phase of the disease. Compared with normal paired skin samples, gene expression of RORC, and IL-17A, IL-17F and IL-23 was significantly increased in positive patch test biopsies. The mRNA for interferon-gamma and IL-4 was also increased [42]. Tc17 cells add a new division to IL-17 secreting cells and much more needs to be determined with regards to the signaling events that favor the differentiation of Tc1 cells to Tc17 and the plasticity of these cells.

Since IL-17A and IL-17F are important proinflammatory cytokine with similar biological functions and signal via common receptor [43, 44], we next studied the effect of estrogen exposure on IL-17A- and IL-17F-mediated MCP-1 induction from splenocytes. We found that there was increased MCP-1 induction from IL-17A and IL-17F activated splenocytes from estrogen-treated mice when compared to placebo-treated mice. Our laboratory and others have previously demonstrated that estrogen exposure upregulates ConA-mediated MCP-1 induction from splenocytes [20] and LPS-mediated MCP-1 from dendritic cells [45]. This study further adds new dimension to estrogen-mediated MCP-1 induction since MCP-1 is an important chemokine in regulating macrophage and monocytes chemotaxis to the site of inflammation. The IL-17-mediated increase in MCP-1 from estrogen-treated mice may have clinical implications and needs to be confirmed in estrogen promoted disease condition.

Together, this study throws new light on the estrogen-mediated immunoregulation of IL-17 induction and response. It also improves our current understanding of role of estrogen in female-predominant autoimmune diseases and inflammatory conditions. Future studies in infection or autoimmune disease model and purified CD8 cells are needed to confirm the importance of estrogen in regulating Tc17 cell subset.

Acknowledgements

This work was supported in part by the National Institutes of Health (1 RO1 AI051880-04A1) and Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM), Intramural Research Competition (IRC) Grant (441303) and Lupus Foundation of America. We want to thank Ms Melissa Makris for flow cytometric analysis, Mr. Peter Jobst, Ms. Dana Reynolds, Ms. Connie Kingrea, and the animal care staff.

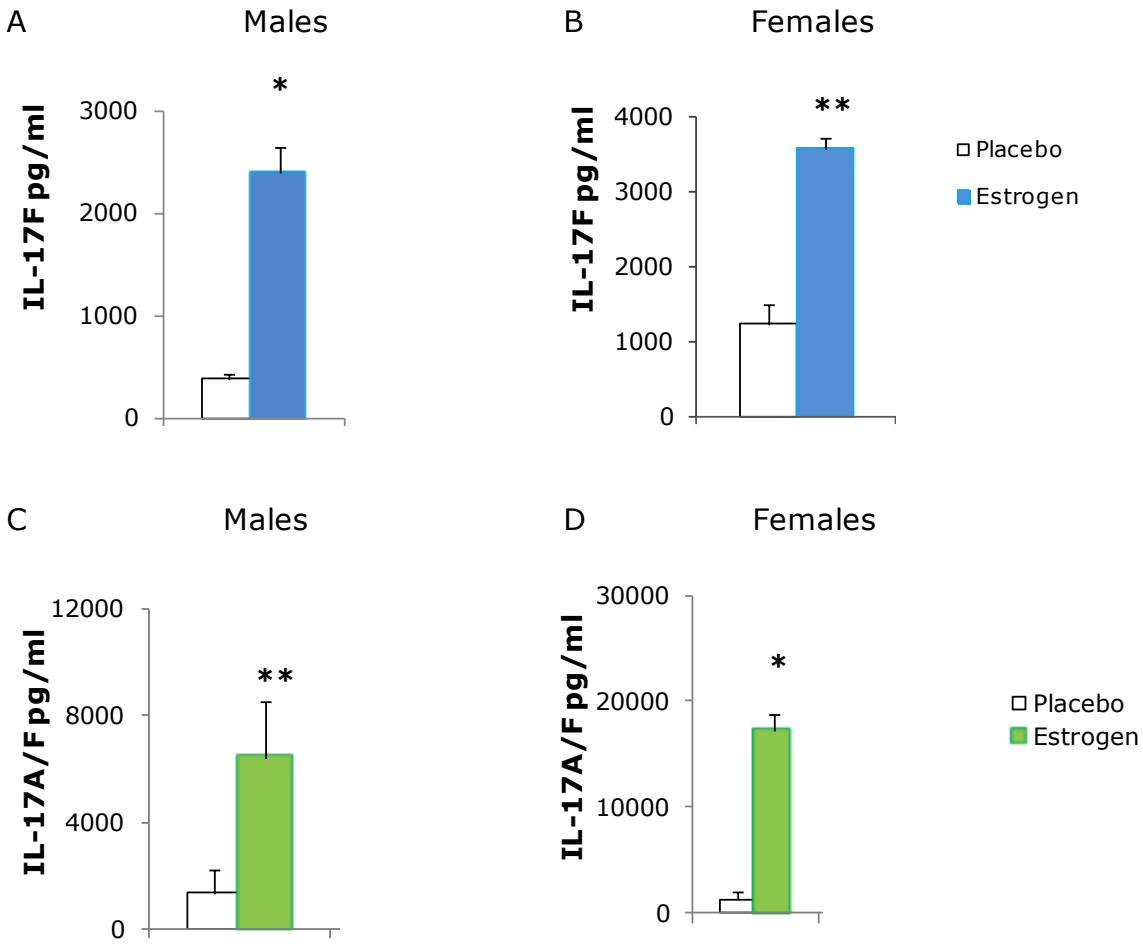

Figure 1: Estrogen upregulates IL-17F and IL-17A/F levels in activated splenocytes:

Splenocytes from estrogen and placebo-treated C57BL/6 male (*A* and *C;n=3-6/group*) and female (*B* and *D; n=2-3/group*) mice were activated with IL-17-inducing stimuli (IL-6 (20 ng/ml) +TGFβ (3 ng/ml) +antiCD3 antibodies (1µg/ml)) for 48 hrs. *A* and *B*, IL-17F levels; and *C* and *D*, IL-17A/F levels in supernatants were determined by ELISA. The data represent means ± SEM (* $p<0.05$, ** $p<0.01$), Tukey-Kramer Multiple Comparisons Test was performed.

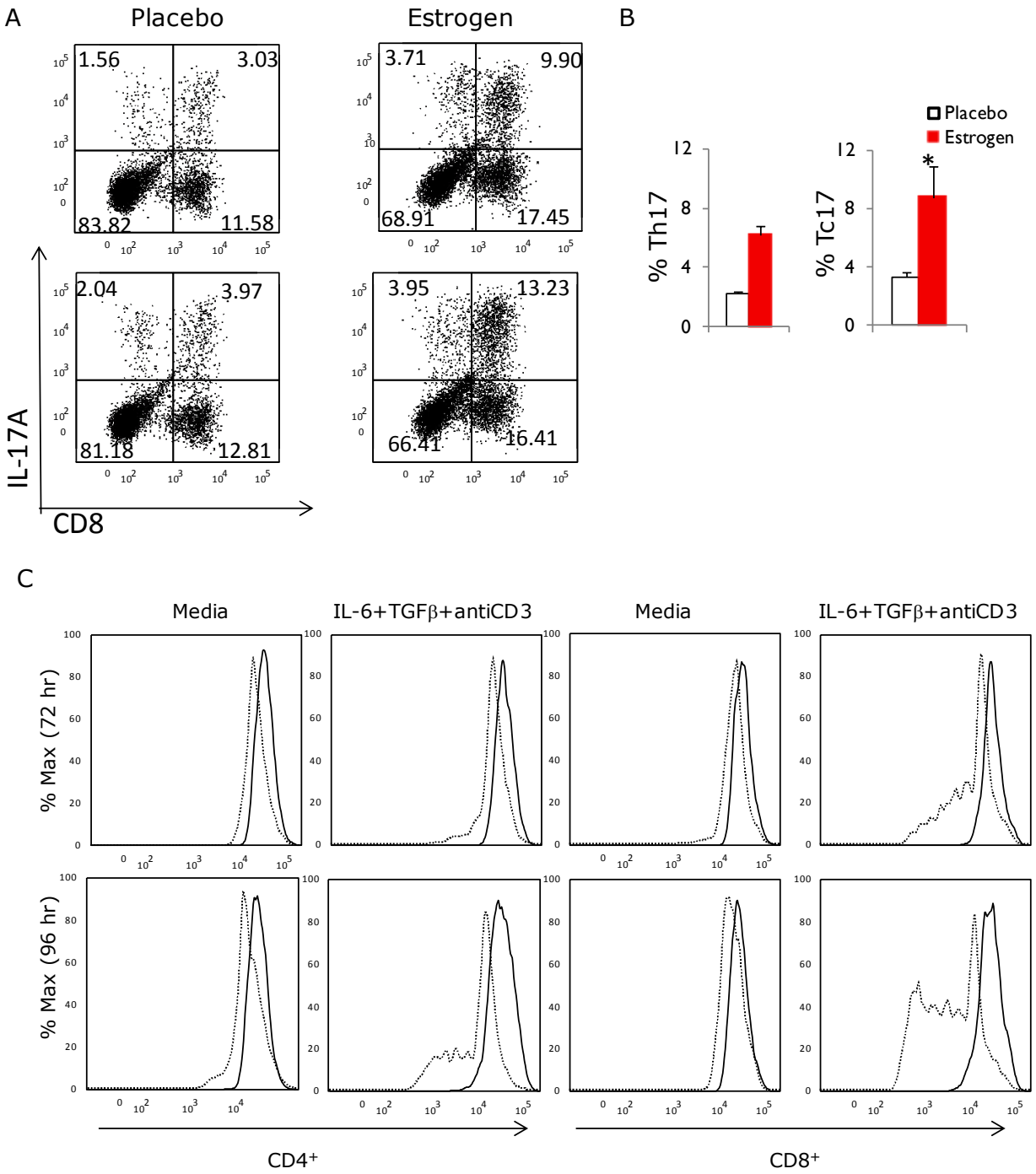

Figure 2: Estrogen increases Tc17 cells percentage and CD8+ cell proliferation.

A and *B*, Cells from estrogen- and placebo-treated mice were cultured in presence of IL-6+TGFβ+antiCD3 antibodies for a 72 hrs followed by stimulation with PMA+ionomycin and

Brefeldin A for additional 5 hrs. The cells were stained surface stained with fluorochrome-conjugated antiCD8 and antiCD4 antibodies and stained with anti IL-17A antibody after permeabilization. (*A*) Representative dot plot of $CD8^{+}IL\text{-}17^{+}$ cells from estrogen and placebo-treated mice. (*B*) The percentage of Th17 and Tc17 cells were analyzed by flow cytometric analysis (n=4-5/group). *C,* Splenocytes from estrogen- and placebo-treated mice were stained with CFSE and cultured in presence or absence of IL-17-stimulating cocktail for 72 and 96 hrs. The cells were surface stained with antiCD4 and antiCD8 antibody and analyzed for cell proliferation by flow cytometric analysis. Representative histogram shows proliferation of placebo cells (solid line) and estrogen cells (dotted/broken lines). The data represent means ± SEM (* $p<0.05$), Student-t Test was performed.

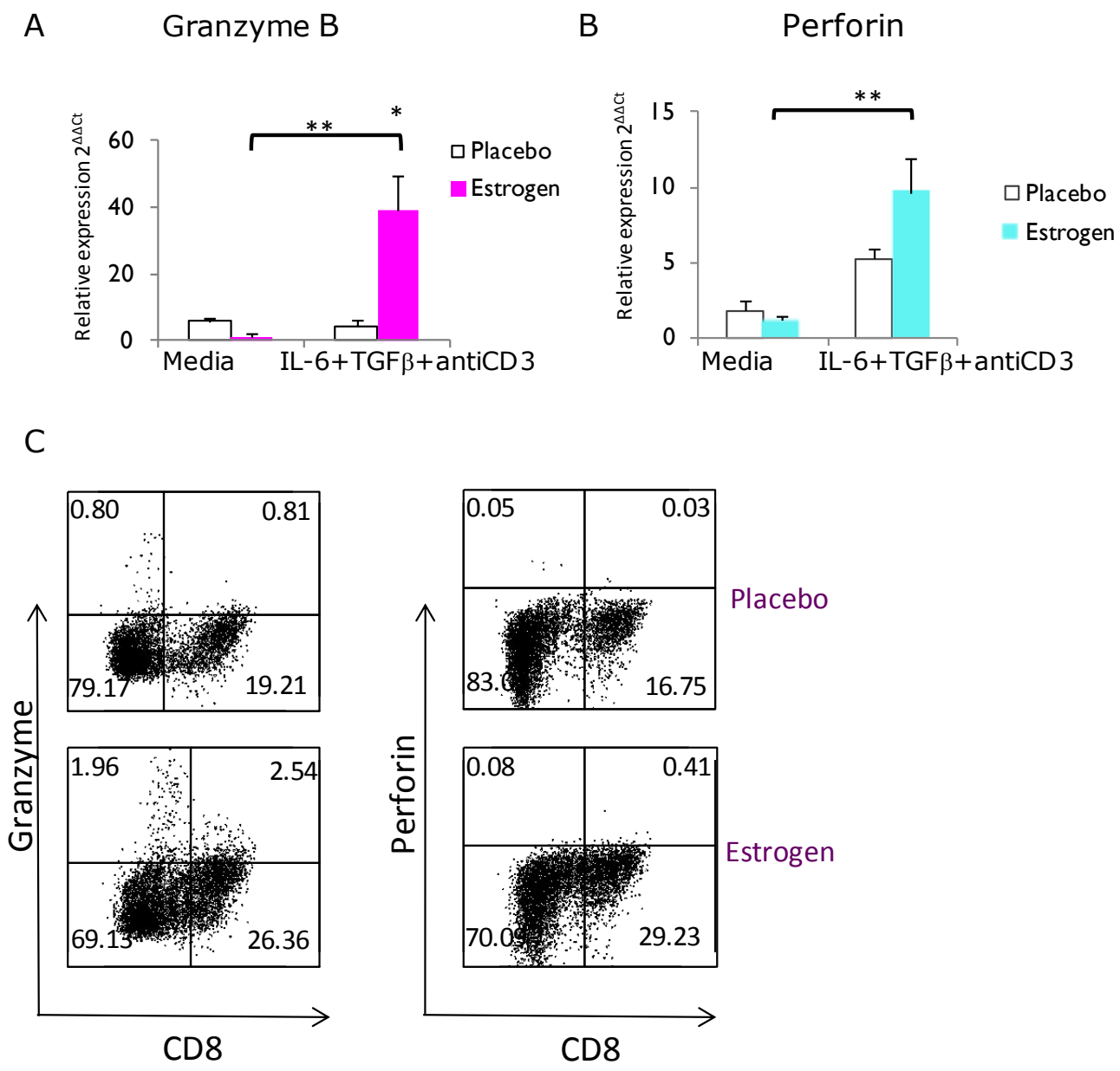
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Figure 3: Granzyme and perforin mRNA and flow cytometric analysis and T-bet and eomes mRNA levels in cells from estrogen and placebo-treated mice.

A and *B,* Cells from estrogen- and placebo-treated mice were cultured in presence or absence of IL-6+TGFβ+antiCD3 antibodies for a 72 hrs and analyzed for Granzyme B (*A*) and perforin (*B*) mRNA expression by TaqMan real time RT-PCR (n=3/group). (*C*) Splenocytes from placebo- and estrogen-treated mice were activated with IL-6+TGFβ+anti CD3 antibodies for 72 hr followed by PMA+ionomycin and Brefeldin A stimulation for additional 3 hrs. The cells were then stained for CD8 and granzyme/perforin. Representative dot plots of $CD8^{+}Perforin^{+}$ and $CD8^{+}Granzyme^{+}$ cells from placebo and estrogen mice are shown. (*D*), T-bet and Eomesodermin mRNA levels were measured in stimulated splenocytes from estrogen- and placebo-treated mice at 48 hrs (n=3/group). The data represent means ± SEM (* $p<0.05$; ** $p<0.01$), Tukey-Kramer Multiple Comparisons Test (*A* and *B*), Student-t Test (*D*) was performed.

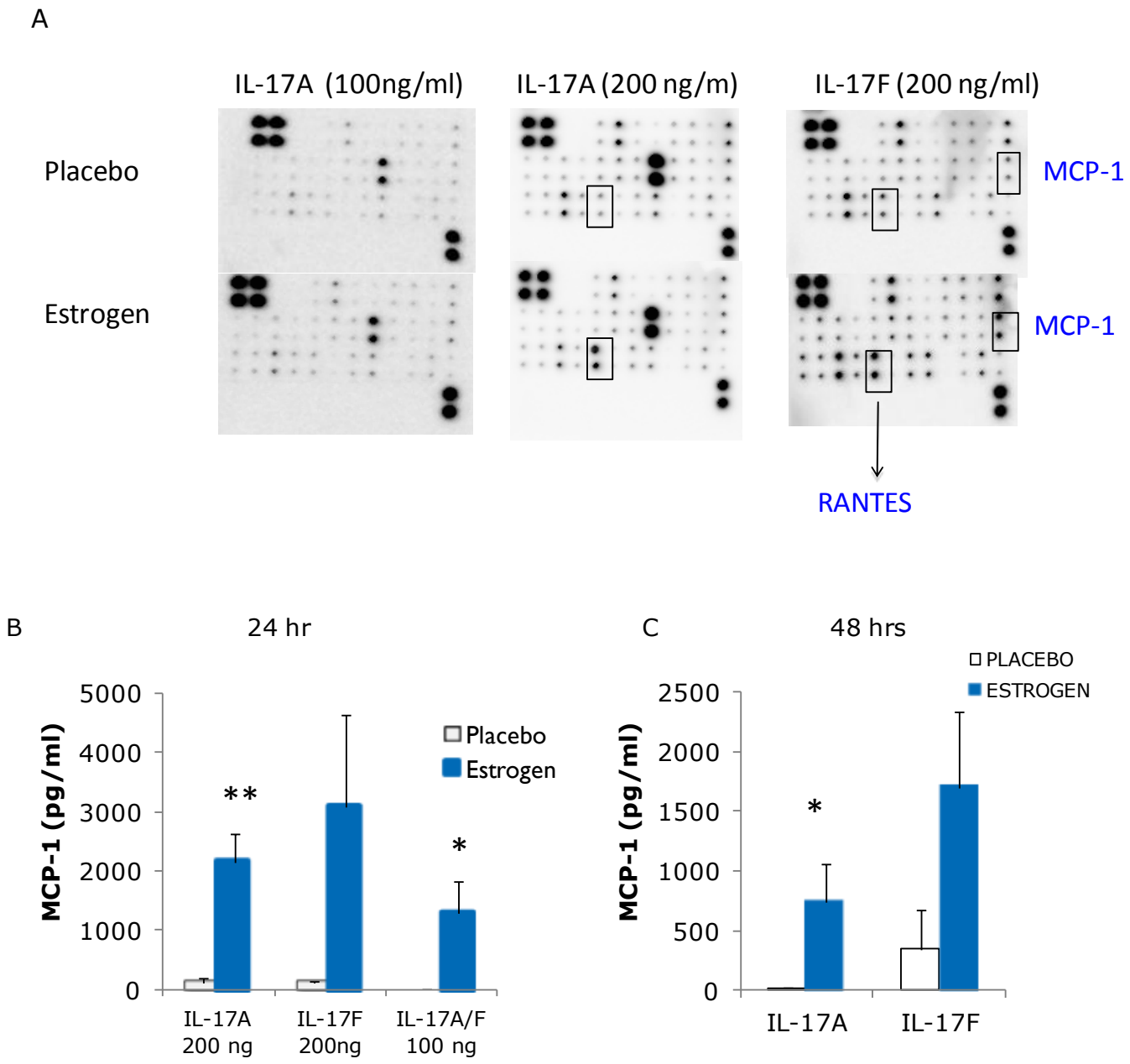

Figure 4: IL-17-mediated MCP-1 induction is upregulated in splenocytes form estrogen-treated mice.

Splenocytes from estrogen- and placebo-treated mice were stimulated with different concentrations of IL-17A and IL-17F for indicated time points and supernatants collected for analysis. (*A*) RayBiotech 32 cytokine array was used to screen for different proinflammatory genes upregulated by IL-17 in 24 hr culture supernatants. (*B* and *C*) The bar graphs show the

levels of MCP-1 protein in supernatants determined by ELISA at 24 hr, n=3-4/group (*B*) and at 48 hr with 100 ng/ml each of IL-17A and IL-17F, n=5-7/group (*C*). The data represent means ± SEM (* $p<0.05$; ** $p<0.01$), Student-t Test (*B* and *C*) was performed.

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Chapter 7: Conclusion

Estrogen regulation of the immune system is now well established [1-3]. The overall outcomes of estrogen effects on the innate and adaptive immune system is dependent on multiple variables including but not limited to cell type, route, dose and duration of estrogen, receptor expression etc. In addition, the effects of estrogen on immune responses are influenced by physiological, disease and immune status of the animal/human subject studied. Therefore, it is essential to keep the above mentioned variables in consideration while defining the role of estrogen in various immunological conditions. The variability is notably observed in female predominant autoimmune disease, MS and SLE, having diversely opposite mechanism of action of estrogen (discussed in detail in Chapter 1). Although a number of studies have focused on the role of estrogen and its derivatives in Th1 and Th2 cell type, thus far, there are no mechanistic studies conducted to determine the effect of estrogen on recently identified IL-17 secreted by Th17 cells and Tc17 cells of the adaptive immune systems. Therefore, in my Ph.D. project, I have focused on the role of estrogen in IL-17 induction and response.

Estrogen-induced inflammation murine model, which was chosen for this study has been well studied and established for nearly two decades in Dr. Ahmed's laboratory. The model mimics the exposure of females to extraneous estrogens in form of oral contraceptive treatment during child bearing ages, and as hormone replacement therapy (HRT) during post-menopausal state. In addition, this model also throws light on the effect of environmental estrogen on the immune function. In this model, C57BL/6 male mice are orchiectomized to remove in large part source of gonadal male hormones, and allows studying the effects of estrogen only, without complicating effects of other hormones (including female hormones). In this study, male mice are preferred over female mice, to avoid any extragonadal estrogens present in females to influence on the immune parameters. It has been shown before that these mice have marked changes in the morphology of the organs of immune system and also on the activity of immune cells [1, 4-6]. The levels of 17-β estradiol in serum are measured regularly to confirm that the levels of estrogen in treated mice are within physiological range (Figure 1).

The oral hormone replacement therapy came into question when it was reported that HRT is associated with increased risk of venous thromboembolism (VTE) especially in younger postmenopausal women and stroke in older women. In recent years, the use of transdermal HRT has replaced oral HRT and has not been linked to increased risk of VTE or stroke (doses≤50µg) and gall bladed diseases [7]. Increased risk to endometrial and breast cancer linked with long term estrogen therapy has also been reported [8, 9]. Therefore, it is essential to consider risk-benefit ratio for long term HRT in menopausal women. Furthermore, in young women with rheumatic diseases such as SLE, oral contraceptives are contraindicated especially in women with active and severe SLE, with a history of thrombosis, anticardiolipin antibody and lupus anticoagulant [10]. Similarly in Bechet's disease, oral contraceptive should be avoided since it increases the chances of fatal thromobosis [11]. Use of oral contraceptive has been linked with progression of Raynaud's phenomena to severe systemic sclerosis [12, 13]. On the contrary, it has been shown that there is improvement in rheumatoid arthritis with oral contraceptives and HRT, but may be associated with increased cardiovascular risk [14]. Therefore, it is not appropriate to compare the risk of different route and doses of estrogen used in HRT or oral contraceptive, without acknowledging the importance of distribution of estrogen receptors in different tissues and physiological state of the patient. The level of estrogen in circulation achieved during HRT are around $1/5^{th}$ of the levels of estrogen during the peak of menstrual cycle [15]. In our estrogen-inflammation model, the levels of estradiol in serum of mice are within physiological range, hence an optimal model to study effects of *in vivo* estrogen treatment on immune system. The most interesting aspect of this model is that by merely giving estrogen (i.e. without adjuvants or antigens), the cells of the immune system are in a "proinflammatory state''. Unstimulated cells from estrogen-treated mice do not secrete any inflammatory cytokines or exhibit any inflammation. However, exposure of immune cells from estrogen-treated mice to stimulants (e.g. ConA, anti-CD3 or LPS), induces a robust secretion of cytokines and chemokines [16-19].

For my research project, I focused on estrogen-regulation of IL-17. I found that *in vivo* estrogen treatment upregulates IL-17A, IL-17F, IL-17A/F levels from activated splenocytes when compared with splenocytes from placebo-treated mice (Figure 2 and 3). Importantly, this was

observed in both estrogen-treated male and female mice. Since, it is easier to perform surgeries in male mice (non-invasive orchiectomy and better recovery rate), and to avoid the risk of laparotomy and estrogens from extragonadal source in females, I performed the rest of the experiments on only male mice. Not only the levels of IL-17A were increased, there was increased IL-17 secretion from individual cell determined by ELISPOT and also increased percentages of IL-17^{+} cells in activated cells from estrogen-treated mice. It is noteworthy, that the levels of IL-17 were not evident in unstimulated (media alone) or freshly isolated splenocytes from estrogen-treated mice suggesting that IL-17 inducing conditions are essential for proper IL-17 production and that splenocytes from estrogen-treated mice are in primed state and release copious amounts of IL-17 upon stimulation (Figure 2).

It was found that estrogen upregulated RORγt mRNA and protein levels and RORα mRNA levels, which are known Th17 and Tc17-lineage specific transcription factors. The levels of IL-17 were found to be markedly decreased by IL-17-suppressive cytokines such as IL-27 and IFNγ, when added at early time of culture. This was accompanied with decreased RORγt expression. Interestingly, when IL-27 or IFNγ were added 24 hrs after start of culture, the decrease in IL-17 levels was not as marked as observed when added at start of culture. This confirms the previous reports that IL-27 inhibits *RORc* expression to inhibit lineage commitment of Th17 cell [20]. It was noteworthy that the inhibition of IL-17 by IFNγ was observed only in estrogen-treated mice, indicating that cells from estrogen-treated mice are more responsive to inhibitory action of IFNγ. Future experiments are required to determine why there is increased responsiveness of cells from estrogen-treated mice for IFNγ- mediated IL-17 inhibition.

Since estrogen regulates multiple transcription factors by genomic and non-genomic signaling pathways and IL-17- induction is tightly regulated by different signaling cascades, the next focus of this study was to identify different signaling pathways involved in estrogen-mediated IL-17 induction. NF-κB family is one of the key signaling molecules involved in IL-17 induction and I found by comprehensive analysis that p65/RelA is important in estrogen-mediated IL-17 induction. Inhibiting NF-κB signaling either by using specific inhibitor (A77 1726) in culture or by transfection with p65-specific siRNA, markedly inhibited IL-17 levels. In addition, decreasing serine protease activity by using AEBSF in culture, also decreased IL-17 protein

levels, by inhibiting serine protease mediated cleavage of p65. This serine protease-mediated cleavage of p65 has been earlier demonstrated to be necessary for estrogen-mediated IFNγ and iNOS induction [19, 21]. Together, these studies confirm the importance of NF-κB in estrogen-mediated IL-17 induction. Future studies are required to determine the interaction of NF-κB signaling molecules with estrogen receptors and also with *RORc* promoter.

Another important signaling pathway triggered by direct IL-6-IL-6R gp130 interaction is JAK-2-mediated STAT3 phosphorylation, followed by homodimerization and nuclear localization of phosphorylated STAT3. In addition, IL-21 and IL-23 (promoters of IL-17 induction) also activate STAT3 pathway. By using JAK-2 specific inhibitor AG490, it was confirmed that JAK-2 signaling is an important event in estrogen-mediated IL-17 induction. However, unlike NF-κB inhibitor-mediated decrease in IL-17^{+}RORγt^{+} percent cells, JAK-2 inhibitor did not affect the percentage of IL-17^{+}RORγt^{+} cells. Surprisingly, JAK-2 mediated downstream phosphorylation of STAT3 and STAT3 DNA binding was also found to be delayed in estrogen-treated mice when compared with placebo-treated mice. These findings suggest that while JAK-STAT3 pathway is important for IL-17 induction but may not be critical for estrogen regulation of IL-17.

There is a list of other transcription factors which negatively regulate IL-17 induction such as IRF8, Eomes, T-bet etc. I found that IRF8 mRNA was significantly decreased in activated splenocytes from estrogen-treated mice when compared with placebo-treated mice. Eomes mRNA levels were decreased in activated cells when compared to unstimulated cells. This was probably due to TGFβ-mediated inhibition of Eomes. This needs further confirmation by testing TGFβ-dose response on Eomes levels in the estrogen model. On the other hand, T-bet levels were found to be increased in activated cells when compared with unstimulated cells from estrogen-treated mice. It has been shown previously in our lab that stimulation of splenocytes from estrogen-treated mice with ConA or antiCD3 antibodies increased T-bet protein levels [22]. For optimal induction of IL-17 in culture, antiCD3 antibodies are used along with IL-6 and TGFβ [18], it is therefore possible that antiCD3 antibodies (in IL-17-inducing cocktail) are enhancing T-bet mRNA levels in estrogen-treated mice. Despite higher levels of T-bet mRNA in estrogen-treated cells, there is still enhanced IL-17 production from them.

ETS-1 is another known negative regulator of IL-17 production [23], and it was found that ETS-1 mRNA and protein levels were significantly lower in estrogen-treated mice. It has been shown that ETS-1 is epigenetically targeted by miR-326, which then promotes IL-17 induction. In the estrogen-model of inflammation, miR-326 levels were found to be increased. By specifically inhibiting miR-326 levels using antagomir, I confirmed that decreased miR-326 levels increased IL-17 protein levels and decreased ETS-1 protein expression.

Our lab has previously reported that miR-223 is increased in cells from estrogen-treated mice and regulates LPS-induced IFNγ levels but not iNOS and nitric oxide levels [24]. In addition, a recent study has shown that miR-223 regulates IL-17 induction by targeting the expression of Roquin, which negatively regulates IL-17 [25]. Roquin is an E3 ubiquitin ligase and has been shown to promote degradation of inducible costimulator (ICOS) mRNA and therefore, prevents development of autoimmunity and immune deregulation [26-29]. It was found that miR-223 levels are significantly increased in IL-6+TGFβ+antiCD3 antibodies stimulated and unstimulated cells. Inhibition of miR-223 levels by specific antagomir transfection inhibited IL-17 levels but not Roquin mRNA levels at 48 hr. The difference in results between my studies and the one reported earlier, may be attributed to different cells types (peripheral blood lymphocytes) and inflammation model (IL-10 knockout and colonic inflammation) used in the published report [25].

Since the studies were conducted on mixed splenic lymphocyte population, it was necessary to identify the main producer of IL-17 in estrogen-treated mice. Surprisingly, it was found that the majority of IL-17^+ cells in estrogen-treated mice were $CD8^+$. There was indeed small population of Th17 cells in estrogen-treated mice, which was also significantly higher than in placebos. It was found by CFSE staining that there was preferential CD8 cell growth in estrogen-treated mice when compared to placebos. These Tc17 lack perforin and granzyme B. Future studies using purified CD8 cells are required to confirm whether or not this subset is devoid of the cytolytic function and activity in response to infection or appropriate stimulation.

Estrogen treatment also altered the ability of splenocytes to respond to IL-17. It was found that estrogen upregulates IL-17A and IL-17F-mediated MCP-1 protein levels from splenocytes. By

preliminary screening by using 32 cytokine membrane arrays from RayBiotech, RANTES was also identified to be upregulated in cells from estrogen-treated mice. Further analysis in this direction is needed to confirm the finding. Future studies in synovial fibroblasts are being conducted since fibroblasts have been shown to be responsive to IL-17.

Thus far, this is the first study in which IL-17 regulation by estrogen has been investigated in depth. The current literature on IL-17 and estrogen is based mostly on different infectious and inflammatory disease conditions. There are no reports on estrogen-regulation of IL-17 and its different isoforms. Furthermore there have been no mechanistic studies on IL-17 induction. Though there are some studies on estrogen-regulation of IL-17-mediated proinflammatory response, e.g. estrogen level at estrus has been shown to impair dendritic cell function and as a consequence decreased IL-23-mediated Th17 regulation and increased susceptibility to *Candida albicans* infection [30]. On the other hand, a study shows that estrogen increases severity of *P. aeruginosa* strain PA508 infection by increasing IL-23 and IL-17 but decreasing lactoferrin response in lungs [31].

In EAE model, a study has demonstrated that estrogen receptor alpha is necessary for estrogen-mediated inhibition of Th1 and Th17 responses [32] and also for estrogen-mediated inhibition of $CD4^+$ cell homing in the CNS [33]. On the contrary, combination treatment of ERβ ligand and IFNβ in EAE has been shown to decrease IL-17 [34]. In absence of Tregs, estrogen protects against EAE by sequestering encephalitogenic IL-17 and IFNγ expressing T cells in the peripheral lymph organs [35]. Estrogen-mediated increased PD-1 in Tregs also protect against IL-17-mediated tissue damage in EAE [36]

Estrogen deficiency as observed during menopause has been shown to be related to increased osteoporosis. It has been shown that estrogen-deficient osteoporosis is further potentiated by increased IL-17-regulated Act1 signaling accompanied with increased RANK ligand levels [37]. In contrast, IL-17 decreases leptin levels and adipogenesis, which as a result protects from bone loss in ovariectomy-induced osteoporosis [38] .

Together, from the above studies it can be inferred that estrogen does affect IL-17 induction or IL-17-mediated proinflammatory events. Future studies are required to delineate the role of estrogen receptor in IL-17 induction. Thus far, there are no studies which have focused on ER interaction with IL-17 promoter. These studies will potentially help in designing the much needed therapies to target IL-17 and estrogen modulated inflammation and disease conditions.

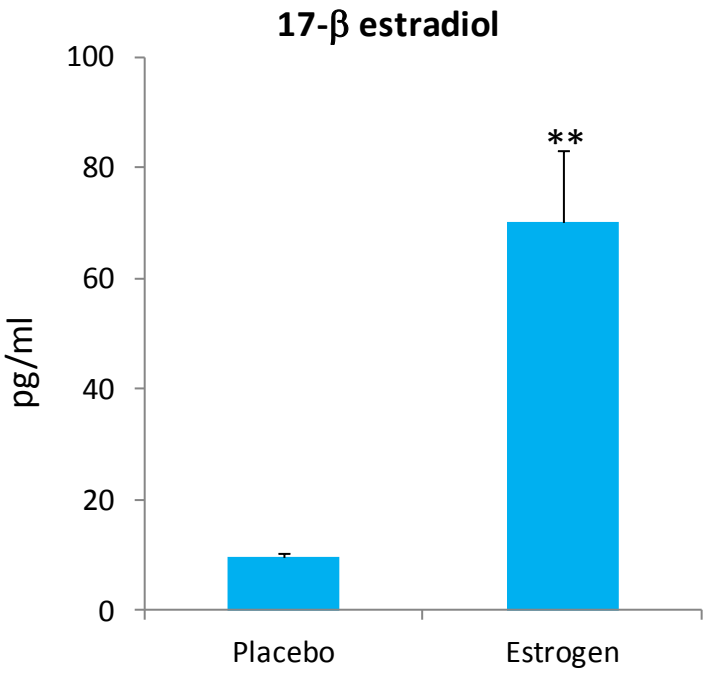

Figure 1: 17β-estradiol levels in mice serum treated for 8 weeks.

Blood from estrogen and placebo-treated mice (n=4/group) was collected at the time of termination. The levels of 17β-estradiol in serum were analyzed using Estradiol EIA kit from Cayman Chemicals (Ann Arbor, Michigan) and absorbance read at 405 nm. The data represents means ± SEM (* $p<0.05$), Student-t test was performed.

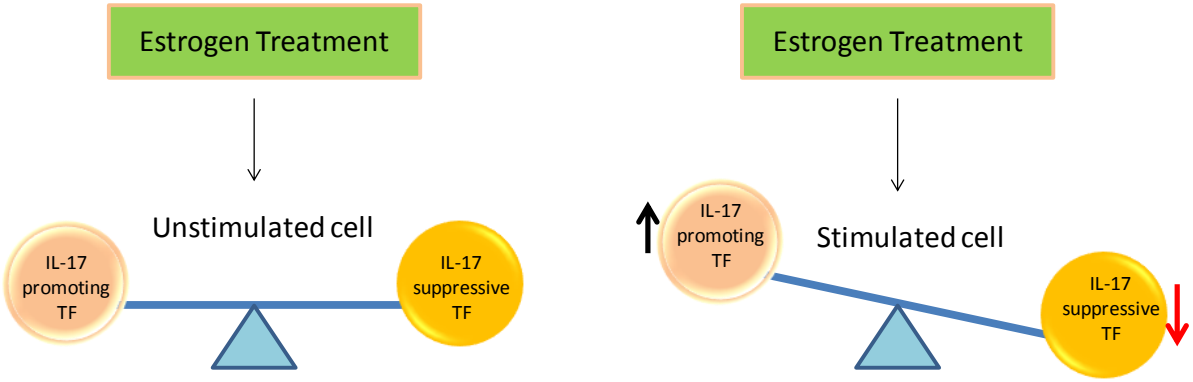

Figure 2: Estrogen fine tunes the balance between IL-17 promoting and IL-17 suppressive Transcription factors.

Upon stimulation of cells from estrogen-model of inflammation, there is upregulation or increased activity of IL-17 promoting transcription factors (TF) and downregulation or decreased activity of IL-17 suppressive TF.

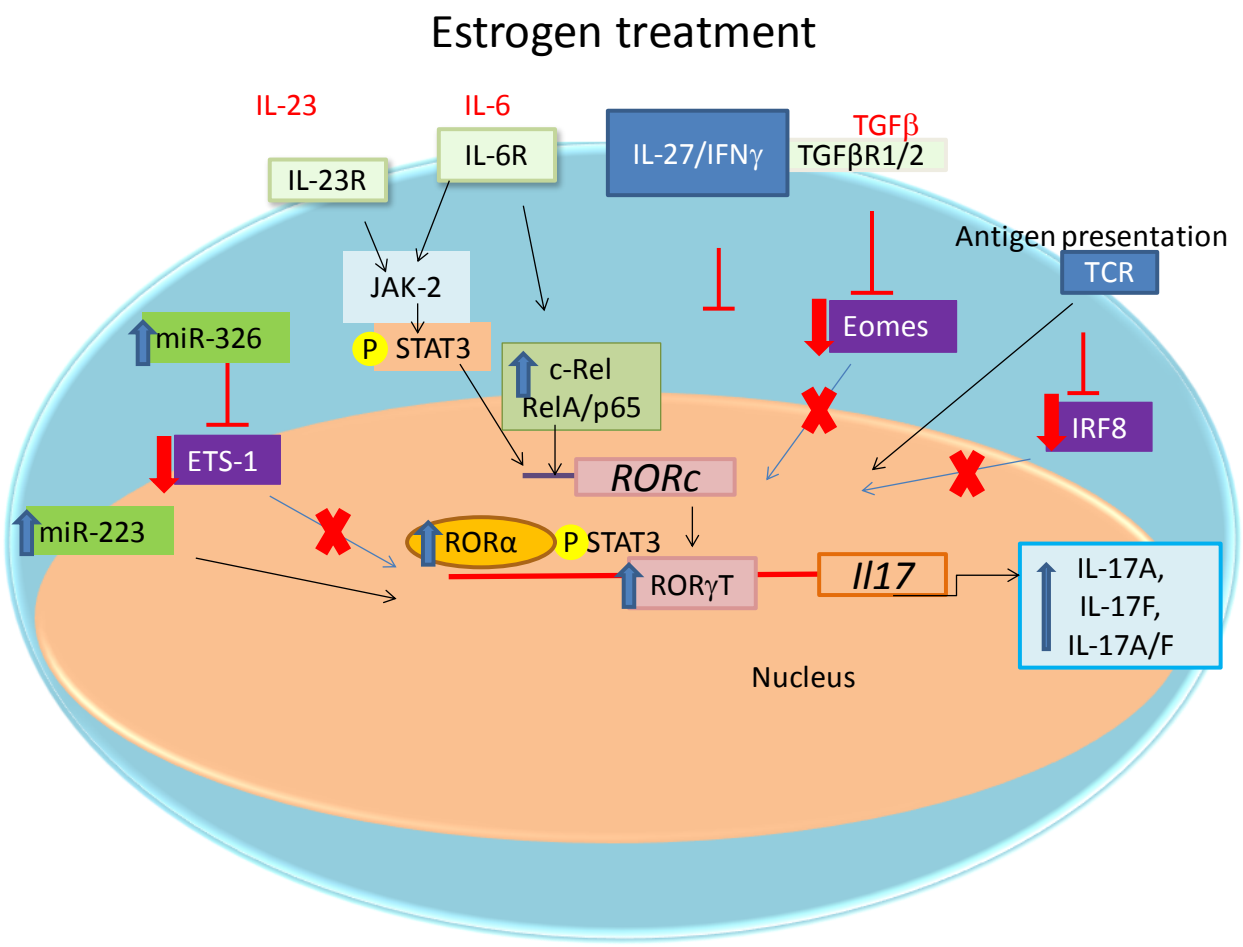

Figure 3: Estrogen-regulates IL-17 induction by regulating multiple transcription factors and by epigenetic miRNA regulation.

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