Estrogen Regulates Interferon-gamma (IFN-γ) and IFN-γ-Inducible iNOS Gene Expression: Implications to Immunity and Autoimmunity

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ABSTRACT

Estrogen Regulates Interferon-gamma (IFN-γ) and IFN-γ-Inducible iNOS Gene: Implications to Immunity and Autoimmunity

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It is now clear that estrogen not only modulates the differentiation and function of reproductive systems, but it also profoundly regulates the immune system of normal and autoimmune individuals. An important mechanism by which estrogen regulates the immune system is by altering the secretion and/or response to cytokines. We hypothesized that estrogen may alter the levels and/or response to IFN-γ, a prototype Th1 cytokine, that plays a pivotal role in immunity against intracellular infections and in many autoimmune and inflammatory disorders. We found that estrogen treatment tended to upregulate the secretion of IFN-γ protein and mRNA expression from Concanavalin-A (Con-A)-activated splenic lymphocytes. Impressively, we found that splenocytes from estrogen-treated mice when activated with Con-A also resulted in increased release of nitric oxide compared to placebo-treated mice. Furthermore, Con-A-activated splenocytes from estrogen-treated mice also had upregulated iNOS mRNA, iNOS protein, and nitric oxide-regulated COX-2 protein when compared to control mice. Blocking co-stimulatory signals mediated through interactions of CD28 and B7 molecules by using CTLA-4Ig markedly decreased not only IFN-γ, but also nitric oxide, thereby implying an important role for CD28/B7 interactions in IFN-γ/nitric oxide. Estrogen-induced upregulation of iNOS/nitric oxide is mediated through IFN-γ since: (i) Estrogen alone did not upregulate iNOS/nitric oxide in IFN-γ knockout mice; (ii) addition of rIFN-γ to activated splenocytes from estrogen-treated mice further upregulated nitric oxide levels. We next investigated whether estrogen also upregulated IFN-γ-inducing cytokines and select IFN-γ-inducing transcription factors. Estrogen treatment resulted in increased mRNA and/or protein expression of IFN-γ inducing cytokines and their

receptors, including: IL-18, IL-15, IL-27, IL-12Rβ2, and IL-18Rβ. We also found that T-bet, a critical Th1 transcription factor, and STAT-4 phosphorylation, a key molecule in IL-12 signaling were both increased, while IRF-4, an important player in Th2 differentiation, was diminished in Con-A-activated splenocytes from mice treated with estrogen. Altogether, these studies are the first to demonstrate that estrogen regulates IFN-γ-dependent iNOS and describes the potential mechanisms of how estrogen alters IFN-γ-inducible genes, IFN-γ inducing cytokines, and transcription factors in normal C57BL/6 mice. These studies may have profound implications to many autoimmune and inflammatory disorders, where estrogen is known to regulate the course of these diseases. Since estrogen may promote inflammatory disorders by upregulating pro-inflammatory biomolecules including IFN-γ, nitric oxide, and COX-2, these studies may help in the design of therapeutic agents that regulate or block secretion and/or response to these inflammatory molecules.

To the best man I know!

To the man with a great heart to his family, his students, his patients, and everything God created!

To the best surgeon in technique, in logic, in knowledge, in ethics, and at heart!

To the pioneer of transplantation in Turkey and a founder of a University!

To the most extraordinaire, supporting, honest, and loving human being!

To My Dad!

To the smartest woman I know!

To the woman with a great heart!

To the best pathologist, professor, and a teacher in all aspects of life!

To the most protective, loving, honest, supporting, beautiful, and extraordinaire person!

To My Mom!

I wish everybody were as lucky as me to have a mom and a dad like you!

From the ashes a fire shall be woken, A light from the shadows shall spring; Renewed shall be blade that was broken; The crown less again shall be king.

J. R. R. Tolkien

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CHAPTER 1. Literature Review

Sex Hormones and the Immune System

Immune homeostasis requires that the immune system respond effectively to infectious agents. A constant challenge of the immune system is to respond effectively to infectious agents, yet at the same time, it must impose restraints to avoid induction of damaging immune responses to self-antigens. This "immune homeostasis balance" can be tilted by diverse immunomodulators including hormones such as estrogen.

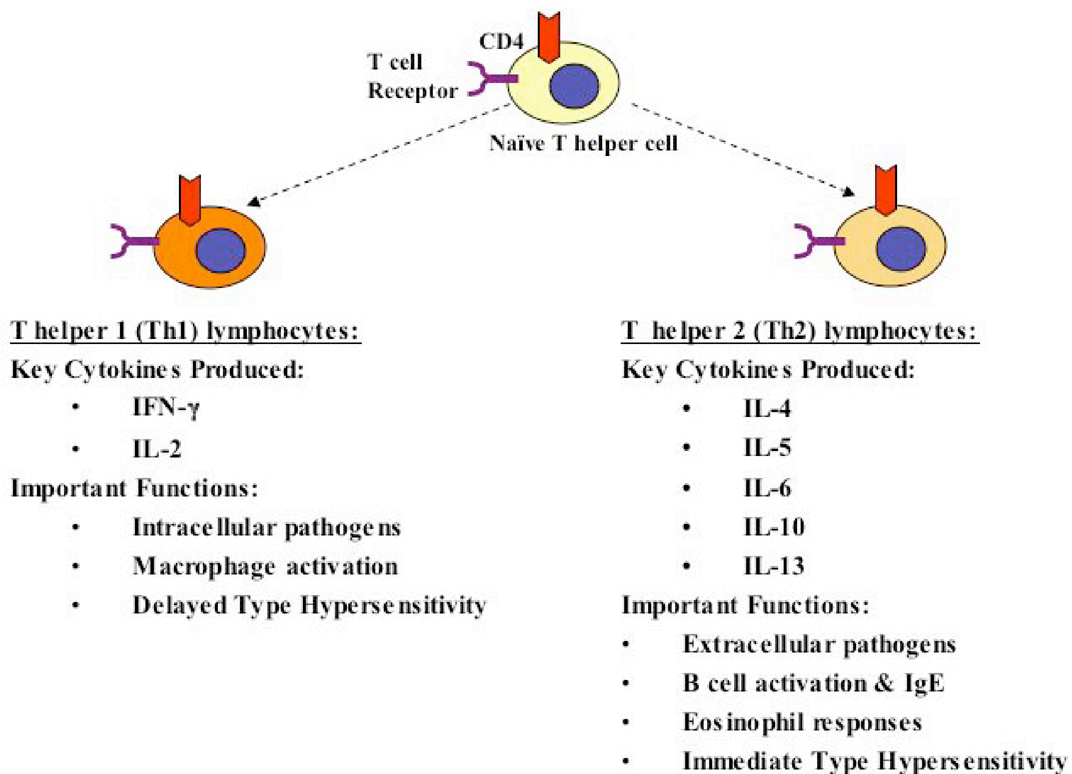
The following is a brief summary of the major types of immune responses and key cellular players.

1. The innate (non-adaptive) immune system is comprised of cell populations such as NK (natural killer cells), natural killer T cells and γδ T cells and soluble factors such as components of the complement system to counter invading microbes. The innate (natural) immune response, by the virtue of being non-specific, ensures rapid defense against a wide-range of microbes by recognizing the molecular pattern of foreign organisms. The recognition molecules expressed on self-cells allows them to be protected from being treated as foreign.

2. The adaptive (acquired) immune response is composed of a small number of responsive T and B cells that are specific to the invading pathogens. The adaptive response is a specific immune response resulting from close interactions of T, B, and antigen presenting cells (APC). T cells can be divided into sub-populations based on unique surface markers and/or functions. T cells expressing the CD4 marker are designated as ***helper cells***. These cells are crucial for providing help to T, and B cells, macrophages and other immune and non-immune cells. T cells expressing the CD8 marker are referred to as ***Cytotoxic T cells*** and are crucial to defense against intracellular pathogens, such as viruses and bacteria. Based on patterns of cytokine secretion, T helper cells ($CD4^+$) are further subdivided into two subsets, called T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells secrete mainly IFN-γ and IL-2 as their key cytokines, while Th2

cells predominantly secrete IL-4, IL-10, IL-5, and IL-6. The distinction between Th1 and Th2 cells is not absolute since several experimental and human studies demonstrate mixed Th1 and Th2 type cytokine expression. Cytokines produced by Th1 cells play a critical role in local inflammatory reactions and cytotoxicity against intracellular pathogens, bacteria and viruses. On the other hand, Th2 type cytokines are important in stimulating B cells to proliferate, differentiate, produce antibodies and provide protection against free antigens (**Figure 1**).

Figure 1. Th1 and Th2 Lympocytes



B lymphocytes have two major functions: (i) to produce antibodies and (ii) to serve as antigen presenting cells. When activated, naïve circulating B cells differentiate into plasma cells and secrete large amounts of antibodies. A third type of lymphocyte is natural killer cells (NK cells), which are primarily involved in cytotoxicity of defective or infected cells. It is now recognized that to effectively combat the invading microbes, there is significant "cross-talk" between the innate and adaptive immunity.

Sexual Dimorphism in Immune Responses: Effects of Estrogen

An important immunological observation is that there are marked physiological differences in the immunecapabilities between males and females (1-3). The gender dependent differences in females and males are largely attributed to sex hormones, such as estrogen and androgens. In addition to endogenous source of estrogen, exposure to estrogen occurs through several exogenous sources. Adult women are exposed to estrogen in therapeutic forms as oral contraceptives or estrogen replacement therapy (hormone replacement therapy, HRT). Furthermore, humans of all ages are exposed to both phytoestrogens that naturally occur in plants, and environmental endocrine distrupters such as DTT and dioxin, which mimic estrogenic activity in both humans and animals (2). This chapter focuses on the immunomodulatory effects of a natural estrogen (17-β estradiol or E2), a principal hormone employed in these dissertation studies.

There is limited information, however on the precise mechanisms of the actions of estrogen on immune system. Understanding the effects of estrogen on the immune system is critical since it has far-reaching implications, since exposure to estrogens occurs through multiple sources, and the facts that estrogens influence normal and autoimmune individuals.

This chapter, which reviews pertinent literature, is structured as follows:

(1) A brief review of estrogen and estrogen receptors
(2) Estrogen effects on key cells of the immune system
(3) Estrogen effects on autoimmunity
(4) Conclusions

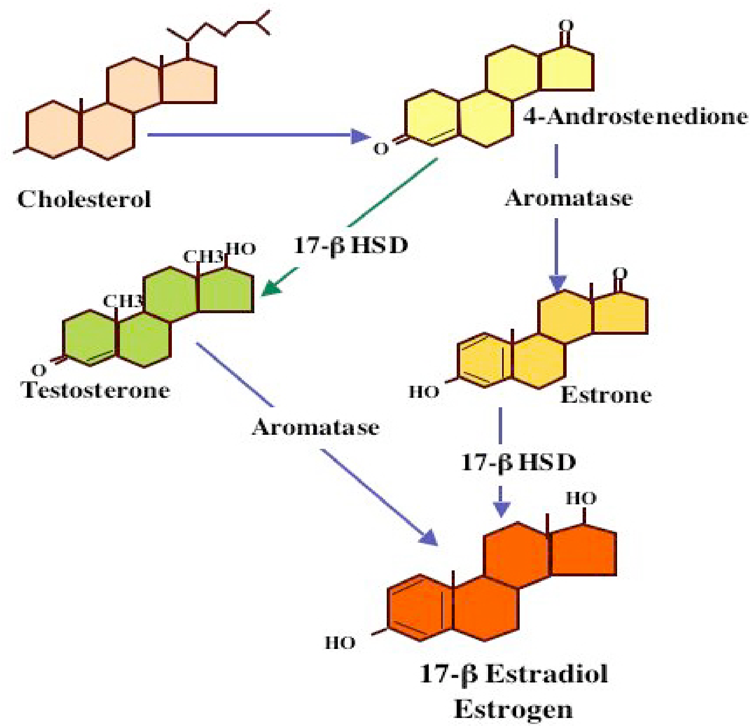
Review of Estrogen

Physiology and Sources of Natural Estrogen

"Estrogen" (17-β estradiol) comes from two Greek words; *oistros*, "mad desire" and *gennan* "to produce" (4). 17-β estradiol is a steroid hormone produced mainly by the ovaries in premenopausal women. In men and post-menopausal women, estrogen is also derived from androgen metabolism. Although it is responsible for many female sexual characteristics, it is also a key regulator of growth and differentiation of a variety of tissues in both males and females. Estrogen has wide-reaching physiological effects since it acts not only on classic target tissues such as reproductive system, nervous system and mammary glands, but also on the immune system, cardiovascular system and skeletal tissues.

Among estrogens, 17-β estradiol is the most abundant and potent form compared to estrone (E1) and estriol (E3) (**Figure 2**). In the ovaries, 17-β estradiol, is principally produced by theca and granulosa cells. Theca cells, stimulated by lutenizing hormone (LH), hydroxylate cholesterol into a C19 androgenic precursor under the influence of aromatase cytochrome P450 (5). The end-product of this reaction is androstenedione, which is converted into testosterone in theca cells. Testosterone and androstenedione taken up by granulosa cells are aromatized by aromatase and converted to 17-β estradiol in premeopausal women (6). In pregnant women, the hydroxylation of 17-β estradiol in fetal and maternal liver and placenta results in estriol (E3) (6). In addition to the ovary, extragonadal estrogen biosynthesis takes place in mesenchymal cells of adipose and breast tissue, osteoblasts and chondrocytes of bone, vascular endothelium and aortic smooth muscle cells and numerous sites in the brain (5). These sites are important sources of estrogen in postmenopausal women, as well as in men. Estrogen produced at these sites, unlike that secreted by the ovaries, tends to act locally at high concentrations (7, 8). Postmenopausal women and men produce predominantly estrone (E1) via the aromatization of androstenedione in non-reproductive tissues (6).

Figure 2. Synthesis of 17-β Estradiol



Estrogen and Estrogen Receptors

Estrogen exerts its biological functions on its target tissues by both estrogen receptor (ER)-dependent and ER-independent mechanisms. Estrogen binds to two specific, but distinct, receptors: ERα and ERβ, which belong to the nuclear hormone receptor family. Estrogen receptors (ERs) are ligand activated transcription factors, which are located intracellularly and to some degree on the cell surface (9). Each type of estrogen receptor appears to be differentially expressed in various tissues. ERα is expressed in moderately high concentrations in the uterus, mammary gland, placenta, testis, ovary, pituitary, kidney, epididymis, liver, bone and adrenal glands (10-12). ERα expression is very low or not detectable in the prostate, testis, ovary, pineal gland, thyroid gland, parathyroids, adrenals, pancreas, gallbladder, skin, urinary tract, and erythroid tissues (12). ERβ is expressed predominantly in the prostate, ovary, lung, bladder, spleen, thymus, brain, uterus, hypothalamus, and testis (10, 11). In addition to ERα and ERβ, a third subtype of

ER designated as ERγ was recently identified in fish (13) and mouse liver tissue (14). ERγ, also called putative estrogen receptor (pER), is detected in ovary, skeletal, neural, vascular and retinal cells, as well as in endometrial, prostate and mammary tumors (14). The exact function of this receptor is still not clear.

Estrogen Receptors and the Immune System

It is conceivable that, differences in the relative expression of ERs in various tissues may result in selective action of estrogen in organ systems, such as the immune system. Several earlier studies demonstrate the presence of estrogen receptors in many cells of lymphoid organs: murine and rat macrophages, human monocytic cell lines (15-17), human peripheral blood mononuclear cells (18), cytotoxic/ suppressor and memory T cells, and B cells (19, 20). Potential roles of ERα and ERβ in the immune system are investigated in respective gene-knockout mice. ERα is expressed on nearly all lymphocyte subsets from the thymus (21) and thymic stromal cells (22). ERα appears to be involved in thymic development since the absence of ERα in knockout mice leads to hypoplasia of the thymus, an increase in the frequency of immature double $CD4^{+}CD8^{+}$ thymocytes (23) and a decrease in mature type thymocytes, $CD4^{+}CD8^{-}$ cells (24). The studies show that ERα is obligatory for the functional development of the thymus (23). ERβ is also important in the regulation of the immune system. ERβ is expressed at low levels in thymic $CD4^{-}CD8^{-}$ double negative T cells in wild type mice (21). The expression of ERβ is essential for estrogen-mediated thymic cortex atrophy and to the shift in thymocyte subpopulations in female mice (23). Double positive $CD4^{+}CD8^{+}$ thymocytes and thymic stromal cells express ERβ (22).

ERα knockout mice also have decreased splenic size, while ERβ knockout mice result in splenomegaly suggesting the physiological importance of ERα in maintaining normal spleen size (23). The deletion of ERα results in hypoplasia of both the thymus and spleen (23). Estrogen receptors are also important for B cell lymphopoiesis and maintenance of the cytokine profile. Complete B lymphopoiesis in bone marrow and the spleen is

observed in wild type mice with intact ERα and ERβ when compared to ERα or ERβ knockout mice given estrogen (25). Estrogen treatment of ERα knockout mice do not increase the frequency of B cells actively secreting immunoglobulins both from the bone marrow and the spleen compared to wild type or ERβ knockout mice (25). ERα knockout mice given estrogen have fewer cells in all populations of B cell subsets in the bone marrow, whereas wild type mice given estrogen have decreased early hematopoietic B cells progenitors and a shift toward a mature B cell subpopulation (25, 26). These data indicate that ERα is not required for regulating B lymphopoiesis, but instead is predominantly responsible in regulating estrogen induced B cell changes and immunoglobulin secretion (25-27). The loss of ERβ results in hyperplasia in the bone marrow, where there is an increase in the number of granulocytes and B cells in the blood as well as lymphoadenopathy (28). Estrogen treatment of wild type and ERβ knockout mice increase the frequency of B cells actively secreting immunoglobulins (25), implying that ERα is involved in Ig secretion. These studies show that ERβ in combination with ERα is essential for mediating the B cell responses and spleen.

The expression of estrogen receptors is shown to be important for T helper cell activities. ERα expression, but not ERβ, is essential for enhanced estrogen induced Th1 cell responses such as increased IFN-γ secretion from hematopoietic cells (29).

Murine natural killer cells (NK cells) express both ERα and ERβ. Estrogen is known to reduce NK cell activity (30-32). NK cells from both wild type and ERα knockout mice given estrogen exhibit a significant decrease in NK cell activity suggesting that ERβ can be involved in estrogen mediated NK cell cytotoxicity (33).

Structure of Estrogen Receptors

ERα and ERβ belong to the steroid nuclear receptor family, which is composed of proteins with three functional domains. These domains are A/B domain (NH_2 terminal), C domain (DNA-binding domain) and D domain (hinge region), and E/F domain (ligand-

binding domain, COOH terminal) (**Figure 3**) (9, 34). ERα and ERβ share an amino acid homology of 97% in DNA-binding domain, while they have a homology of only 60% in the ligand-binding domain. The differential homology in these domains suggests that they could interact with the same gene but bind differentially to ligands (**Figure 4**) (10, 35). ERα has two transactivation functional regions, AF-1 (Activation Factor-1) in the DNA-binding domain and AF-2 (Activation Factor-2) in the Ligand-binding domain (36). These regions synergize with each other and provide a response to estrogen. The A/B domain includes the AF-1 region (ligand-independent activation function region) that is involved in protein to protein interactions such as coactivators, and transcriptional activation of target gene expression (9). This transactivator region is found to be active in the absence of estrogen (37). ERβ also has an AF-1 region but most of its activity comes from AF-2 (38). The hinge region contains a nuclear localization signal and links the C domain to the multi-functional carboxyl-terminus E/F domain. The hormone dependent AF-2 region in the E/F domain (ligand-binding domain-LBD) is important in ligand binding, in this case estrogen, dependent transcriptional activity and interaction with coactivators (36, 39). Besides ligand binding, this region is responsible for ligand-dependent activation by nuclear hormone receptors (40-42), heat shock protein (hsp) interactions, nuclear translocalization (43), and transactivation of target gene expression (9, 44). Heat shock proteins, for example hsp90, can help other proteins to fold and prevent aggregation. They act as chaperones in the absence of ligand, binding to unliganded ERs, and thereby maintaining the receptors in an inactive, but functional and ready state for ligand binding (45, 46).

Figure 3. Structure of Estrogen Receptor

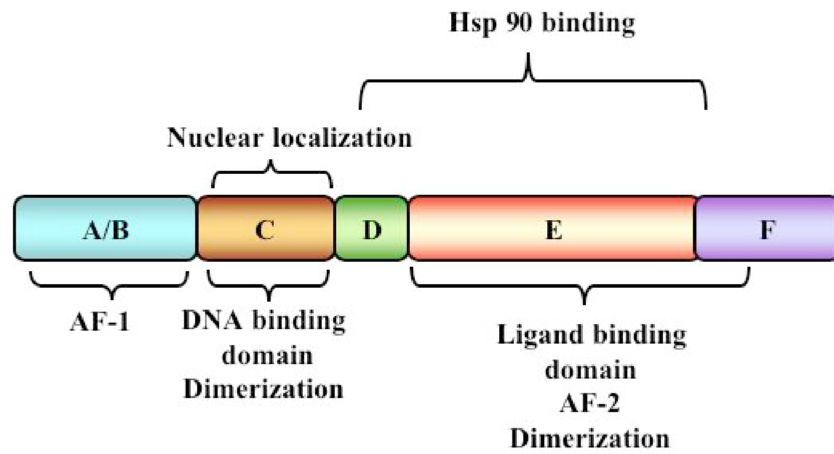
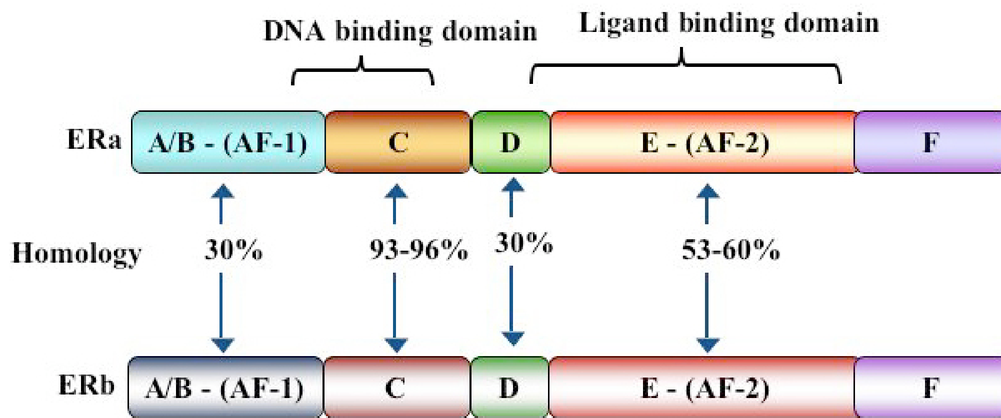


Figure 4. Homology between Estrogen Receptors



Transcriptional cofactors, such as corepressor or coactivator proteins, are associated with inhibition or activation of gene expression. Coactivator proteins enhance gene expression by remodeling chromatin and allowing interactions with the basal transcription machinery (39). One important member of the coactivator protein family is p160/SCR. SCR coactivators function predominantly by recruiting chromatin modifying enzymes such as histone acetyltransferase (HAT) to the ligand activated estrogen receptors (9). This enzyme results in histone acetylation and chromatin decondensation, which leads to increased transcription of estrogen regulated genes. The AF-1 region is thought to interact with coactivator proteins such as p160 to influence the magnitude of transactivation of the target genes in combination with the AF-2 region (47). The hydrophobic surface of the AF-2 region can bind coactivator proteins such as GRIP1/TIF2 (transcriptional intermediary factor-2) (48-50), and SRC-1 (steroid receptor coactivator-1) (51), which belongs to the p160 family. Coactivators of the p160 family interact with other coactivator proteins such as CREB-binding protein (CBP)/p300 (50, 52, 53) and CBP-associated factor (P/CAF) (54). The ability of AF-2 to stimulate gene transcription is based on this cluster of coactivator proteins, which in turn bind to the basal transcription mechanism (55) and turn on histone acetyl-transferase activities (HAT) to activate transcription (54, 56, 57).

On the other hand, negative corepressors and coregulators inhibit gene activation or turn off the activated genes. Negative coregulatory factors, RIP140 (58) and SHP (59, 60), compete with SRC-1 coactivator proteins such as TIF2 (60) for the AF-2 region and may recruit deacetylases to estrogen target genes. Transcription of estrogen target genes are predominantly enhanced by the binding of estrogen receptors to estrogen response elements (ERE) (9). Increased signaling through ERs mediated by estradiol may lead to augmented expression of estrogen target genes. The binding of negative coregulators or corepressors leads to histone deacetylation, chromatin condensation, and suppression of overexpression of the estrogen-induced genes (61).

Estrogen Receptor Signaling

Ligand-bound ERα and ERβ interact with DNA response elements (estrogen response elements, ERE) in the promoters of many estrogen dependent-target genes and regulate the rates of transcription (9, 62). Intense research in many laboratories reveals that estrogen-responsive genes can be activated/transcribed by several mechanisms, including ligand and ER-dependent as well as ER-independent mechanisms.

1. The Ligand Dependent Estrogen Action (Figure 5):

In this classical pathway, the estrogen receptor is stored in the nucleus in an inactive state bound to heat shock proteins (63, 64). Binding of the ER to estrogen results in a conformational change involving dimerization and release of the ER from heat shock proteins. Dimerization of the ER ensures/provides stability in binding to the ERE in the promoters of estrogen-responsive genes(65). The transcription of the target gene is mediated by the binding of the DNA-binding domain in the receptor to the estrogen response element (ERE) and by the ligand-binding domain in the AF-2 region of the receptors(66, 67). The ER dimer bound to EREs (an inverted repeat sequence separated by three non-specific nucleotides, 5-GGTCAnnnTGACC-3) interacts with coactivators to promote chromatin remodeling and to recruit general transcription factors (68). This results in the up or down regulation of target gene expression. Interestingly, both ERα and ERβ receptors can both exist intracellularly as homodimers or heterodimers (69). The different transcriptional activities via homo or heterodimers formed by ERα and ERβ could explain the selective actions of estrogen in different cell types and genes (70). Although these ERs are both expressed in tissues and form functional heterodimers; when co-expressed, ERβ inhibits the transcriptional activity of ERα at saturating hormone levels and modulate the transcriptional activity of ERα (71, 72). Therefore, overall estrogen responsiveness may be determined via ERα versus ERβ ratio in the cells where both receptors are expressed.

2. The Ligand Independent Pathway: It is noteworthy that estrogen receptors can be activated even in the absence of ligand (**Figure 6**). ERs can be activated by many non-estrogenic physiological molecules, such as growth factors, epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), cell cycle proteins, protein kinases, thereby demonstrating “cross-talk” among ER and other key signaling proteins. Growth factors, such as EGF and IGF-1, are able to bind to the AF-1 region but not to the AF-2 region (73, 74). EGF binding can activate ERs by phosphorylation via signaling through the MAP kinase pathway (73). ERs can also be phosphorylated by cyclins or general regulators such as Protein Kinase C (PKC) (75), or Protein Kinase A (PKA) (73).

3. The ERE Independent Pathway: (**Figure 7**). ERs can physically interact and “cross-talk” with key transcription molecules. Both ERα and ERβ when complexed with estrogen, bind to Jun and Fos located on the AP-1 site, a non-ERE site. The coactivators (p160) recruited by Jun/Fos bind to the ERs, which are bound to the complex and trigger the ERs to elicit increased transcription of the target gene. In the presence of ERα, estrogen acts as an agonist and activates transcription, while in the presence of ERβ estrogen acts as an antagonist and inhibits transcription (76). Another way that ERs can affect through transcription of estrogen target genes is the interaction with NF-κB. The NF-κB family consists of NF-κB1/ p50, NF-κB2/p52, c-Rel/p75, RelA/ p65, and RelB proteins (77). The interaction between ERα and c-Rel protein prevents NF-κB from binding the IL-6 promoter (77) and inhibits the protein expression of IL-6 (78, 79). ERα (bound or unbound to estrogen) is also known to interact with transcription factor, Sp-1.

4. The Non-genomic Pathway, Cell Surface ER Signaling Pathway: It is now recognized that estrogen can also induce non-genomic changes by rapid signaling through binding to ERs at the cell surface (**Figure 8**). The estrogen:ER complexes in the caveolae of cell membrane may participate in signaling by activating G-proteins. Activation of G-proteins leads to rapid and specific signaling through the activation of PI3K (phosphoinositol 3-kinase/Akt) and MAPK pathways, or p21ras (80), B-Raf (81), and Src (82) resulting in rapid gene transcription and biological effects on cells (83-85).

It is apparent that multiple actions of estrogen are mediated by alpha and beta estrogen receptors with many different mechanisms that shed light on the distinct tissue patterns of estrogen receptors and new estrogen targets in the body. It is likely that all of the above mechanisms may be acting in different cells of the immune system. It is possible that estrogen may differentially affect the transcription of genes in various tissues by affecting the coactivators, corepressors, and coregulators.

Figure 5. Ligand Dependent Classical Estrogen Receptor Pathway

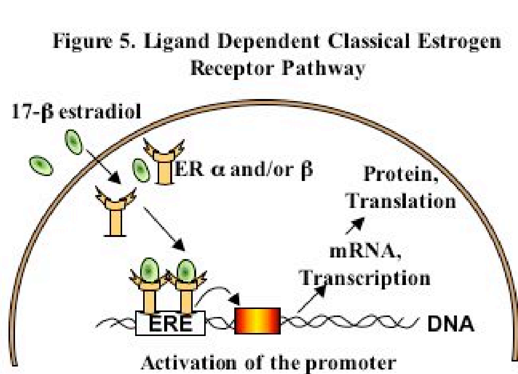


Figure 7. Ligand Dependent, but Estrogen Receptor Element (ERE) Independent Pathway

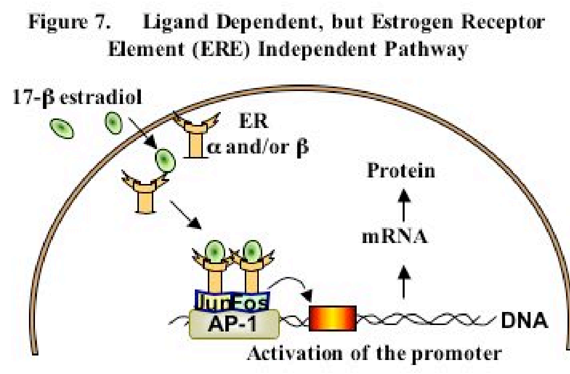


Figure 6. Non-ligand Dependent Activation of Estrogen Receptor

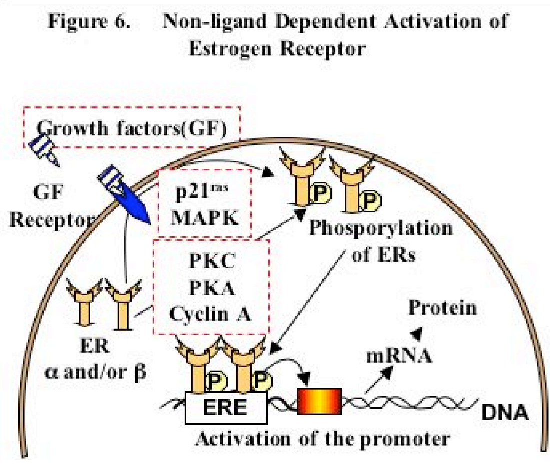
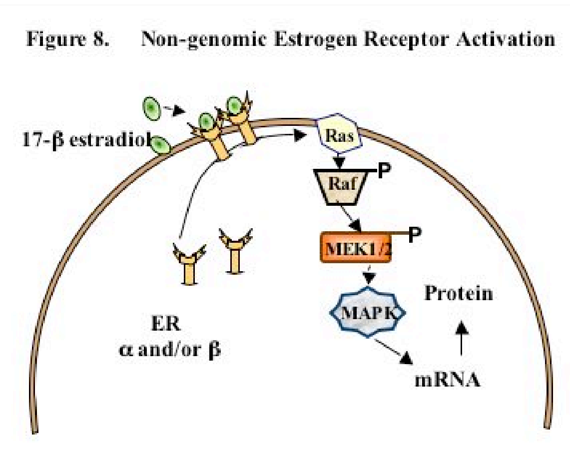


Figure 8. Non-genomic Estrogen Receptor Activation



Estrogen Effects on Key Cells of the Immune System

Estrogens are known to affect all major cells of the immune system including T and B cells, macrophages, dendritic (DC) and natural killer cells (NK).

Thymus and T cells: Sex hormones have been shown to modulate thymic and T cells function. The function of T cells can be affected by estrogens at many levels including alteration in synthesis and/ or responses to cytokines, activation of cells, and structural changes in the thymus. Estrogen treatment of gonadectomized mice results in a dose-dependent decrease in thymus weight, thymus involution, thymocyte numbers, and $CD4^+CD8^+$ cells (86, 87), an increase in $CD4^+$ and $CD8^+$ T cells, and in $CD3^-CD4^-CD8^-$ triple negative cells (88, 89). Estrogen blocks the development of the thymus by decreasing triple negative T cells ($CD3^-CD4^-CD8^-$) and depleting the rest of the maturational stages such as $CD44^+$, $CD25^+$; $CD44^-$, $CD25^+$; $CD44^-$, $CD25^-$ T cell subsets. Ovariectomy causes a significant decrease in the number of intermediate $CD3^+$ extrathymic T cells, which is countered by estrogen administration (90). In the spleen, the relative percentages of $CD8^+$ SP splenocytes are decreased and the $CD4^+/CD8^+$ single positive cell ratio is increased after estrogen treatment in ovariectomized female C3H mice with estrogen injection (91). In humans, women and hypogonadal men have an increased $CD4^+/CD8^+$ ratio and the number of circulating $CD4^+$ T cells are increased in peripheral blood (92). Postmenopausal women have reduced numbers of $CD4^+$ T lymphocyte subpopulations compared to fertile women (93).

Besides affecting the development of T lymphocytes, estrogen is capable of affecting the functionality of naïve or activated T cells as well by altering both pro and anti-inflamatory cytokines that markedly influence the outcome of T cell mediated immunity. For example, the administration of 17-β estradiol increases antigen-specific $CD4^+$ T cell responses and the number and development of IFN-γ-producing cells in lymph nodes of gonadectomized estrogen treated female mice (29), whereas it decreases T cell dependent

delayed type hypersensitivity (DTH) in MRL, C57B6, C3H, NZB/W and DBA/1 mice but not in BALB/c and NFR/N mice (94, 95).

Antibodies and B cells: In general, females as a group have enhanced antibody-mediated immune responses to both foreign antigens and self-antigens and therefore, it is not suprising that majority of autoimmune diseases occur predominantly in females(2, 32, 96-98). Women have significantly higher levels of immunoglobulins, such as IgM and IgG (99-101), but there is no apparent difference in IgE levels (102). Women also have higher immune responses to several, but not all, infectious agents including such as cytomegalovirus (103), hepatitis C (104), and HIV infection (105). Estrogen, affects the development and activity of B lymphocytes at different stages of development, differentiation and function, which can also vary among lymphoid organs. In the bone marrow, estrogen decreases the numbers of B cell precursors by negatively affecting differentiation, proliferation and viability of early B cell precursors (106-108), however, in the mature lymphoid organs such as spleen; estrogen-treated gonadectomized male mice have increased splenic weight, enlarged volumes of white and red pulp, and increased erythroblasts size (109). Although, studies show that there is an increase in spleen weight after estrogen treatment, the number of splenic lymphocytes is decreased after estrogen treatment (110). Estrogen promotes a decrease in $B220^+$ splenic lymphocytes (25, 110), but results in a 10-fold increase in plasma cells suggesting enhanced differentiation of B cells by estrogen (110). Estrogen also affects the survival of B cells. It has been shown that estrogen increases the expression of the anti-apoptotic gene, *bcl-2*, and other genes such as *cd22, shp-1*, and *vcam-1* in B cells (111, 112) that results in decreased apoptosis (2, 113).

Estrogen treatment also modulates the functionality of B cells by increasing immunoglobulin production such as IgG (mostly IgG2a) and IgM (114, 115) as well as. spontaneous secretion of antibodies against bromelain-treated mouse erythrocytes (Br-ME) (116) or induced antibodies to oxazolone in sera (95). ER-α is very important in the augmentation of immunoglobulin production from bone marrow and spleen after estrogen treatment (25). Estrogen treatment induces antibodies to IgG_2, IgG_3, and IgM, as well as

to various auto-antigens (101, 117) such as increase the levels of anti-DNA serum titers (118), in immunoglobulin depositions in renal glomeruli (113), anti-cardiolipin (101, 110, 117, 119, 120), anti-phosphatidylserine, and anti-phoshatidylinositol auto-antibodies (101, 117, 121). The effect of estrogen treatment on immunoglobulin production is also observed in humans. Human peripheral blood mononuclear cells cultured with estrogen *in vitro* have enhanced IgG and IgM secretion (99, 120); on the other hand, testosterone has suppressive effects on immunoglobulin levels (122).

Macrophages and Dendritic Cells: Antigen presenting cells, such as macrophages, which are crucial for induction of immune response in particular are another important target of estrogen (2). There is limited data on estrogen effects on innate immunity, an important arm of immunity. However, there is evidence in the literature that estrogen affects macrophages, dendritic and Natural Killer (NK) cells. It has been shown that estrogen increases murine and human macrophage phagocytic activity (3, 123-125), enhances Fc-γ Receptor expression on the surface of macrophages, which is important in host defense against infection (126), and estrogens alter cytokines (TNF-α, IL-1, IL-10) at mRNA and/ or protein levels (127-130).

Dendritic cells (DC) are potent antigen presenting cells, and are known to activate naïve T lymphocytes, to assist in regulation of Th1 and Th2 cytokines, and to conduct phagocytosis /degradation of unwanted material. While the splenic dendritic cells induce mainly Th1 cytokines, dendritic cells from Peyer's patches induce predominantly Th2 cytokines (131). 17-β estradiol promotes differentiation of functional DC from murine bone marrow precursor cells. Inhibition of DC differentiation in a hormone-deficient medium is restored by the addition of physiological concentrations of estrogen. These estrogen-treated DCs have the ability to stimulate proliferation of naive $CD4^+$ T cells. Estrogen leads to differentiation of a $CD11c^+$ CD11b (intermediate) DC population with high levels of cell surface expression of MHC class II, B7.2, CD40 and B7.1 (132, 133). Human monocyte derived immature DCs have increased IL-6, IL-8 and MCP-1 secretion after short-term *in vitro* estrogen treatment (134). These data suggest that estrogen can

modulate the differentiation, maturation, numbers and function of active APCs, thereby resulting in much more potent immune responses.

Natural Killer Cells (NK): Natural killer cells (NK cells) are large granular lymphocytes attributed with the ability to lyse certain tumor cells. Sustained high levels of estrogen treatment have been shown to reduce natural killer cell activity both *in vivo* and *in vitro* (30, 135-140). In mice, administration of estrogen reduces NK cell cytotoxicity in a dose-dependent manner. This suppression of NK cell activity is observed in all mouse strains with varying sensitivity to estrogen treatment. While estrogen decreases the NK cell activity in C3H/N, DBA/1, and NZB/W strains of mice (>50%), this effect is less pronounced (< 30%) in C57BL/6 and MRL lpr/lpr strains of mice (115). The duration of estrogen treatment differentially affects NK cell activity. One month of *in vivo* treatment with estrogen enhances NK cell activity while prolonged exposure to estrogen decreases NK activity (135). NK cells may have regulatory effects on B cells since, the administration of anti-asialo GM1, an antibody to NK cells, to estrogen-treated mice results in a significant increase in IgG producing B cells (115). Down-regulation of this natural killer cell activity occurs in both orally and transcutaneously estradiol-treated patients and in postmenopausal women receiving estrogen replacement therapy (141).

Effects of Estrogen on IFN-γ and Other Cytokines

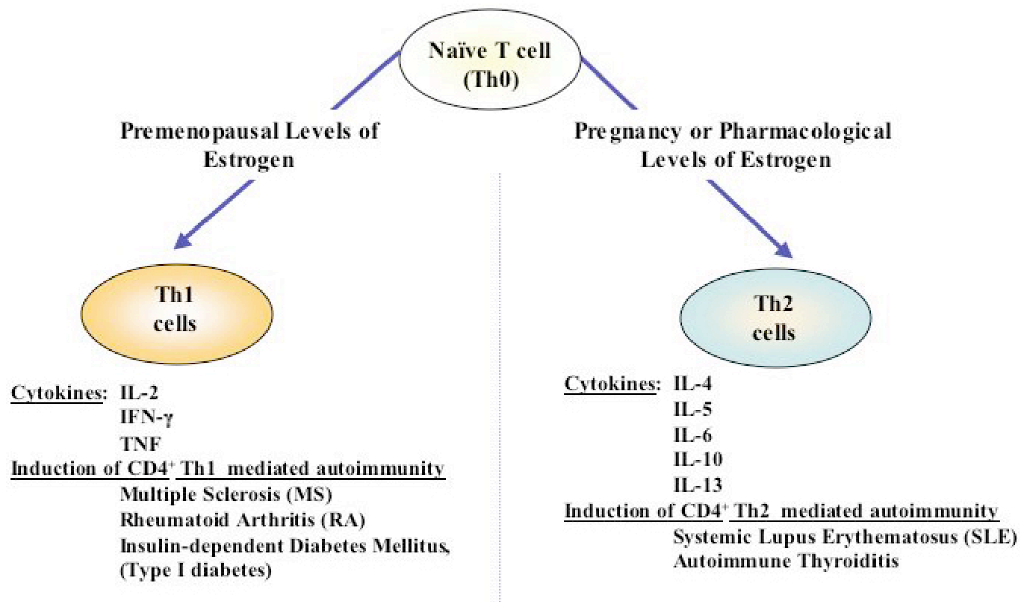
The immune system uses cytokines to orchestrate and coordinate functions of diverse cells of lymphoid and non-lymphoid organs. Cytokines, derived from antigen presenting cells (APC: macrophages, dendritic cells, or B cells) promote cellular infiltration and the first wave of activation of immune cells. The effect of APC-derived cytokines such as IFN-γ on naïve T helper (Th) cells is a significant event since the presence of IFN-γ markedly influences the differentiation of Th cells into Th1 or Th2 type lymphocytes. IFN-γ is one of the most important cytokines in modulating cell-mediated immunity (142, 143). Th1 cytokines such as IFN-γ is upregulated by cytokines produced by APCs, which

include TNF (tumor necrosis factor), IL-1, IL-6, IL-12, IL-18, IL-15 (144), and IL-27 (145).

IFN-γ, a major cytokine involved in Th1 responses, inflammation, autoimmune diseases, and intracellular immunity (146), is exquisitely sensitive to estrogen regulation (147). The effect of endogenous estrogen on IFN-γ is apparent in gender related studies. IFN-γ protein is produced more in cell cultures from female BALB/c, C57BL/6J, (NZB X NZW) F1 and CBA/Ca strains of mice compared to males in response to stimulation by mycobacteria, Con-A, and viruses (148-151) compared to males. Transcription of IFN-γ is also increased in unstimulated T cells from females of the (NZB X NZW) F1 strain of mice (152). It has been shown that IFN-γ is increased by physiological doses of estrogen in both humans (153) and mice (147). Administration of physiological doses of 17-β estradiol to castrated female mice results in a significant increase of antigen-specific $CD4^+$ T cell responses and in the selective development of IFN-γ-producing cells from lymph nodes (29). ERα, but not ERβ, is necessary for the enhanced estrogen-driven Th1 cell responsiveness and estrogen administration promotes Th1 responses due to functional expression of ERα expression in hematopoietic cells (29).

Studies demonstrated that estrogen (17-β estradiol) during pregnancy, a state where estrogen levels are higher than the non-pregnant premenopausal state, was skewed towards Th2 type response (154, 155). The cytokine profile from the supernatants of whole human blood cells skews from Th1 to Th2 with increasing concentrations of estrogen (156) (**Figure 9**). IFN-γ, IL-12 and IL-10 levels are increased in cultures that are incubated with preovulatory doses of estrogen (156). Stimulated whole blood cells cultured with estrogen concentration typical of these found during pregnancy suppresses IFN-γ, IL-12, and the IFN-γ/IL-10 ratio, while increasing IL-10 secretion (156). Elevation in IL-10 levels switches the Th1 profile to Th2 cytokines, which is important for successful pregnancy.

Figure 9. Effect of Estrogen on Th1 and Th2 Cytokines and Autoimmunity



In addition to IFN-γ, there are other cytokines that are profoundly affected by estrogen. 17-β estradiol decreases IL-1α, IL-6, and TNF-α production, but not IL-10, IL-12, and macrophage inflammatory protein (MIP) production in supernatants of LPS-induced splenic macrophages (157). In the spinal cord, estrogen inhibits the expression of LT-β, TNF-α, and IFN-γ in spinal cords (SC), but has no effect on IL-4 or IL-10 (158). The concentration of estrogen affects the pattern of secretion of different types of cytokines. At lower physiological concentrations (~10^{-8} M), estrogen decreases the spontaneous secretion of IL-6, TNF-α, IL-1ra, IL-1β, and the ratio of IL-1β/IL-1rα in whole blood cultures from postmenopausal women (159). At higher physiological level or at pharmacological doses, estrogen enhances the production of IL-1 and IL-6 (159, 160). The effect of high doses of estrogen is also observed in other systems. Estrogen treatment up-regulates IL-6 production in a dose-dependent manner in cultured fibroblast-like synoviocytes (161) and restores the levels of IL-1β and IL-6 secretion from peritoneal and splenic macrophages, which are significantly depressed in vehicle-treated ovariectomized female CBA/J mice after trauma-hemorrhage (162).

Regulation of cytokines by estrogen varies depending upon a number of factors including types of cells, activation status of the cells, stage of estrus cycle, and level of the hormone. Therefore, it is not suprising that the administration of estrogen to different types of cells results in different outcomes. The expression levels of IL-1, IL-6, and TNF-α mRNA, but not IL-1ra, vary with stage of the cycle (163, 164). The levels of IL-1α, IL-1β, and TNF-α are significantly increased following ovulation both in unstimulated and LPS-stimulated monocytes (164). Protein levels of each cytokine in the uterus are highest during proestrus and/or estrus (163). Estrogen increases IL-1α and TNF-α mRNA in the thymus and macrophage-like PMA-differentiated U937 cells (130, 165). Estrogen suppresses IL-10 synthesis in macrophage-like PMA-differentiated U937 cells (130), and in PHA-LPS activated whole blood cultures (156), and decreases IL-6 production from macrophages of ethanol/burn-injured male mice (166), as well as stimulates IL-6 and TNF-α secretion from male peripheral blood mononuclear cells (PBMC) or IL-10 secretion from post-menopausal women. Although estrogen-treated and LPS-stimulated murine splenocytes and bone marrow derived-macrophages treated with estrogen have reduced IL-6 and TNF-α levels (157, 167, 168), the peritoneal rat macrophages treated with estrogen have increased TNF-α levels (129, 130, 169).

Sex differences are also noted with regard to type of cytokine induction and have strong implications to the induction of autoimmune diseases. It is thus conceivable that alterations in cytokine and/or responses to these cytokines by estrogen can profoundly affect the outcome of immune responses both physiologically and pathologically.

Estrogen and Autoimmunity

In addition to understanding the physiological and biological effects of estrogen on the various immune cells, the importance of understanding estrogen effects are further highlighted by the fact that estrogen may have a significant role in autoimmune diseases. A notable feature of many, but not all, autoimmune diseases in that despite differences in anatomic pathology, a common feature is that females are more susceptible to autoimmune diseases compared to their male counterparts (2, 170, 171). A survey of only

24 autoimmune diseases suggested that 3% of the North American and European population suffer from autoimmune diseases (172). This amounts to approximately 1 in 31 Americans being affected with an autoimmune disease. These numbers could be much higher if several more diseases with possible autoimmune components are included. Graves'/hyperthyroidism, IDDM, pernicious anemia, rheumatoid arthritis, thyroiditis, and vitiligo are the most prevalent autoimmune diseases. The second group with the highest rate included glomerulonephritis, MS, and SLE. Significantly, most diseases are more common in women with a risk of 2.7 times more than men to acquire an autoimmune disease (172). Consistent with previous literature, at least 85% of thyroiditis, systemic sclerosis, systemic lupus erythematosus, and Sjögren disease patients are female (173). It is also shown that young post-pubescent women are 10 times more likely to develop autoimmune diseases than men (174). Furthermore, as discussed in subsequent sections, a striking feature of most autoimmune diseases in diverse animal models is that these diseases occur predominantly in females compared to males. The female predominance in the manifestation of autoimmune diseases both in humans and animal models is shown in **Table 1** (2, 175). The precise underlying reasons for these marked gender differences in susceptibility to autoimmune diseases is a subject of intense investigation. Clearly, gender differences in autoimmune diseases include (i) the effects of sex hormones, (ii) regulation by sex chromosomal genes, (iii) microchimerism. Of these factors, much work has been done with regard to the effects of sex hormones in autoimmune diseases. Since the effects of sex hormones on the immune system are complex, it is therefore, not suprising that the effects of sex hormones on various autoimmune diseases can vary. A strong argument for the role of sex hormones such as estrogen in autoimmune diseases comes from a number of animal models of autoimmune disease and is discussed in subsequent sections. It is known that females and castrated males with much higher estrogen levels have altered immune responses (2, 3, 176), which are related to the outcome of autoimmune diseases.

Table 1. Susceptibility of Females to Autoimmune Diseases Compared to Males

Humans

Disease	**Female to Male Ratio**
Multiple Sclerosis (MS)	9-13 : 1
Hashimoto Thyroiditis	25 : 1
Sjogren's Syndrome	13 : 1
Rheumatoid Arthritis	2-4 : 1
Scleroderma	3-4 : 1

Animal Models

Disease	**Female to Male Ratio**
SLE (MS in humans; B/W, SNF1,SWRxSJL , NZBxDBA, MRL/lpr)	Earlier expression of the disease or autoantibody formation in females
Autoimmune Thyroiditis	3-4 : 1
Polyarthritis	6 : 1
NOD	Higher incidence in females

(2, 3)

Estrogen, Autoimmunity, and IFN-γ

Since, cytokines are chemical molecular messages that affect the functioning of diverse immune and non-immune cells, it is not suprising that significant alterations in the patterns of cytokines are evident in different autoimmune diseases. It is beyond the scope of this dissertation to address all of these immunological changes. This section will focus on IFN-γ, because it has been shown to play an important role in autoimmune diseases and also because it is uniquely sensitive to estrogenic compounds. Moreover, the primary focus of this thesis is on estrogen effects on IFN-γ and IFN-γ mediated events. Estrogens may mediate immunological responses during the onset of the autoimmune diseases, in part, by regulating the secretion of several cytokines. Studies have shown that estrogens markedly augment secretion of IFN-γ (177-179). These observations are of biological and clinical importance since IFNγ secreted by Th1, $CD8^+$ T, and NK cells, plays a central role in host defenses against intracellular infections (180). IFN-γ is known to: upregulate the expression of major histocompatibility complex (MHC) class II molecules on target cells (181), markedly activate macrophages (182), promote differentiation of naïve $CD4^+$ T cells into Th1 subclass (183), induce isotype switching of IgM to IgG2a or IgG3 antibody subclasses, and enhance immunoglobulin secretion in resting B cells (184, 185). IFN-γ has been associated with the pathogenesis of several female-predominant autoimmune diseases (146, 186-188).

One model of autoimmune diseases, in which IFN-γ has been extensively studied, is SLE, the experimental model murine lupus. Estrogen acts on target cells and alters the rate of transcription of target genes such as IFN-γ, which is relevant to SLE. The gender effect on IFN-γ in the experimental murine lupus model is observed in female B/W mice, which demonstrate an earlier display of lupus (148). They also have a higher secretion of IFN-γ protein than male B/W mice (186). During the course of the disease, it is found that the level and the expression of IFN-γ is increased suggesting a Th1 predominance in SLE patients (189-192). Data also show that as the severity of the disease increases, the level of IFN-γ decreases and the cytokine profile skews towards Th2 type cytokine profile

(193, 194). This decrease in IFN-γ is in parallel with an increase in Th2 cytokines such as IL-6 and IL-10, which can result in the hyperactivation of B cells to produce auto-antibodies (194). The same pattern of Th1 response is observed in experimental animal models of SLE. In MRL-lpr mice, increased expression and protein levels of IFN-γ in lymphoid organs and their cultures correlate with active lupus (195-198). In addition to increased IFN-γ, an elevation in the production of IgG2a and IgG3 is observed as in human SLE patients (196). Studies performed using IFN-γ knockout mice also demonstrate the importance of IFN-γ in the onset of SLE. In the absence of the IFN-γ gene, levels of auto-antibodies (anti-ds and ss-DNA auto-antibodies) and immune complex mediated pathologies such as, glomerulonephritis are decreased in murine models of SLE (199-202). The significant reduction in the severity of the disease in the absence of IFN- γ gene and the importance of IFN-γ, is also shown by using an IFN-γ Receptor Fc encoding plasmid that is injected into MRL-lpr mice (203). The soluble IFN-γ receptor Fc captures IFN-γ protein (203). Administration of this plasmid at pre-disease stages results in decreased IFN-γ in the sera and decreased hallmarks of lupus, auto-antibodies, hyperplasia, glomerulonephritis, and mortality (203). Signaling through the IFN-γ receptor is essential for the initiation and the progression of murine lupus in MRL-lpr ^{fas} mice (204). IFN-γ receptor knockout mice have none of the hallmarks of lupus nephritis, such as lymphadenopathy, splenomegaly, or kidney destruction, but exhibit decreased TNF-α and increased survival (204). The deletion of the IFN-γ receptor gene also results in decreased anti-double stranded DNA (dsDNA), anti-histone auto-antibodies, suppressed IgM and IgG antibodies, and IgG switching, increased B cell lymphomas due to suppressed auto-antibody production, and no glomerulonephritis in the kidneys of (NZB x NZW) F1 female (BW) mice (186). A similar pattern of suppression in the onset of the disease is also observed in IFN-γ receptor knockout mice with MRL-lpr ^{fas} background (205, 206). IFN-γ receptor MRL-lpr knockout mice have no expansion of CD4-CD8- double negative T cells (206).

Other factors in relation to IFN-γ in active SLE patients are the IFN-γ-inducing cytokines: IL-12 and IL-18 (207-210). In the absence of the IL-12 p40 gene, levels of

IFN-γ expression are decreased and the survival of lupus is increased (205). The transcription factor, T-bet, regulates naïve T cells to commit and become Th1 type cells. T-bet deficient B cells have impaired IgG2a, IgG2b, and IgG3 production and switching to IgG2a in response to IFN-γ (211). The absence of T-bet reduces auto-antibody production and hyperglobulinemia (211). These data show the importance of IFN-γ inducing cytokines and the transcription factor, T-bet, in secretion of IFN-γ and the pathogenesis of the autoimmune disease. Estrogen treatment of IL-12 stimulated T cells from female NOD mice have increased phosphorylation of STAT4 suggesting increased susceptibility to diabetes in female mice can be due to enhancement of Th1 responses via activation of IFN-γ inducing cytokines and their respective signaling mechanisms via estrogen (212).

Estrogen treatment of T cells from female SLE patients also results in increased expression of CD40L on the surface (213). The increased expression of CD40L on activated T cells is shown to be an important player in the *in vivo* priming of Th1 cells and IFN-γ production (214, 215). These data provide evidence for the estrogen-IFN-γ-SLE connection. Although there are not many studies about the effect of estrogen on the progression of SLE or on cytokine profiles, the data so far implies that estrogen primarily induces a Th1 profile.

Interferon-γ plays a critical role in other autoimmune diseases and experimental animal models such as MS (Multiple Sclerosis; animal model: experimental autoimmune encephalomyelitis, EAE), RA (Rheumatoid Arthirtis; animal model: Collagen-induced arthritis, CIA), MG (Mystenia Gravis; animal model: experimental autoimmune myasthenia gravis, EAMG), and autoimmune diabetes (IDDM). The significance of IFN-γ in these diseases has also been shown using knockout mouse models. IFN-γ receptor knockout mice with experimental autoimmune myasthenia gravis exhibit decreased anti-AChR auto-antibodies and severity of the disease compared to wild type mice (188, 216). The same profile is observed in the absence of the IFN-γ gene in EMG mice (217). In experimental autoimmune encephalomyelitis and collagen-induced arthritis, deletion of

the IFN-γ and IFN-γ receptor genes results in augmentation of the disease incidence and severity (216, 218-220).

Estrogen treatment in the experimental murine models of autoimmune diseases shows different responses in terms of IFN-γ. For example, T cells or dendritic cells from EAE mice have decreased IFN-γ secretion in response to estrogen treatment (221, 222). This downregulation of IFN-γ by estrogen can cause the suppression of EAE that is induced by Th1 responses. Autoimmune diabetes developing female NOD mice have higher IFN-γ levels than male mice (212).

CONCLUSION

Despite intense research in endocrinology over decades, the complexity of estrogen action on its target tissues is only now being comprehended. Estrogen effects on target genes are critically influenced by factors such as the presence of ERα, ERβ (ERαα, ERββ, or ERαβ ratios), cofactors, coactivators, and coregulators. Further, estrogen-dependent genes can be activated by both ligand-dependent and independent mechanisms. ERs inturn can cross-talk with other transcriptional molecules such as NF-κB and AP-1 that are crucial in immune regulation and thus influence the transcription of genes. Therefore, it is not suprising that the effects of estrogen on the immune system and on autoimmune diseases can vary in different experimental settings and in different animal models.

The effect of estrogen on the normal immune system is dependent on various factors such as the level of the hormone, the type of the tissues and lymphocytes, the stage of lymphocyte differentiation, and the differences in expression of estrogen receptors and their signaling pathways. Given that, one important mechanism of estrogen modulation of the immune system is by altering the secretion and/or response to cytokines. One such cytokine is IFN-γ, a pro-inflammatory cytokine that plays a pivotal role in many autoimmune diseases. Although evidence from other laboratories also suggests that estrogen regulates IFN-γ, the consequences of estrogen exposure on immune cells,

mainly in view of IFN-γ and IFN-γ mediated events such as expression of IFN-γ inducing cytokines and/or IFN-γ induced molecules such as iNOS are not known. This dissertation focuses on whether estrogen modulates IFN-γ and this alteration results in the regulation of IFN-γ-inducible iNOS. This dissertation also explores the molecular mechanisms involved in estrogen-induced increase in IFN-γ, thus whether estrogen regulation of IFN-γ is due to a response to IFN-γ-promoting cytokines, transcription factors, and/or costimulatory signals.

The data provided in this study will cover most of the vital gaps and the shortage of thorough knowledge in the effects of estrogen on IFN-γ and Th1 responses. This study is extremely important due to the fact that estrogen-induced expression of IFN-γ, IFN-γ responsive genes and molecules may alter normal immune and susceptibility to autoimmunity.

f.) References

1. Ansar Ahmed, S., W. J. Penhale, and N. Talal. 1985. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol 121:531*.
2. Ansar Ahmed, S., B. D. Hissong, D. Verthelyi, K. Donner, K. Becker, and E. Karpuzoglu-Sahin. 1999. Gender and risk of autoimmune diseases: possible role of estrogenic compounds. *Environ Health Perspect 107 Suppl 5:681*.
3. Ansar Ahmed, S., and N. Talal. 1990. Sex hormones and the immune system--Part 2. Animal data. *Baillieres Clin Rheumatol 4:13*.
4. Ruggiero, R. J., and F. E. Likis. 2002. Estrogen: physiology, pharmacology, and formulations for replacement therapy. *J Midwifery Womens Health 47:130*.
5. Simpson, E. R. 2003. Sources of estrogen and their importance. *J Steroid Biochem Mol Biol 86:225*.
6. Williams R.H. , F. D. W., Kronenberg H. M., Larsen P. R., . Wilson J. Md. 1998. *Williams Textbook of Endocrinology, 9th Edition*.
7. Labrie, F., A. Belanger, V. Luu-The, C. Labrie, J. Simard, L. Cusan, J. L. Gomez, and B. Candas. 1998. DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging. *Steroids 63:322*.
8. Labrie, F., A. Belanger, L. Cusan, and B. Candas. 1997. Physiological changes in dehydroepiandrosterone are not reflected by serum levels of active androgens and estrogens but of their metabolites: intracrinology. *J Clin Endocrinol Metab 82:2403*.
9. Nilsson, S., S. Makela, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, E. Enmark, K. Pettersson, M. Warner, and J. A. Gustafsson. 2001. Mechanisms of estrogen action. *Physiol Rev 81:1535*.
10. Kuiper, G. G., B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, and J. A. Gustafsson. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology 138:863*.

11. Mosselman, S., J. Polman, and R. Dijkema. 1996. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett 392:49*.
12. Weihua, Z., S. Andersson, G. Cheng, E. R. Simpson, M. Warner, and J. A. Gustafsson. 2003. Update on estrogen signaling. *FEBS Lett 546:17*.
13. Sabo-Attwood, T., K. J. Kroll, and N. D. Denslow. 2004. Differential expression of largemouth bass (Micropterus salmoides) estrogen receptor isotypes alpha, beta, and gamma by estradiol. *Mol Cell Endocrinol 218:107*.
14. Rao, B. R. 1998. Isolation and characterization of an estrogen binding protein which may integrate the plethora of estrogenic actions in non-reproductive organs. *J Steroid Biochem Mol Biol 65:3*.
15. Frazier-Jessen, M. R., and E. J. Kovacs. 1995. Estrogen modulation of JE/monocyte chemoattractant protein-1 mRNA expression in murine macrophages. *J Immunol 154:1838*.
16. Gulshan, S., A. B. McCruden, and W. H. Stimson. 1990. Oestrogen receptors in macrophages. *Scand J Immunol 31:691*.
17. Danel, L., M. Menouni, J. H. Cohen, J. P. Magaud, G. Lenoir, J. P. Revillard, and S. Saez. 1985. Distribution of androgen and estrogen receptors among lymphoid and haemopoietic cell lines. *Leuk Res 9:1373*.
18. Carbone, A., M. Piantelli, P. Musiani, L. M. Larocca, F. B. Aiello, N. Maggiano, C. Scoppetta, F. Crucitti, and F. O. Ranelletti. 1986. Estrogen binding sites in peripheral blood mononuclear cells and thymocytes from 2 myasthenia gravis patients. *J Clin Lab Immunol 21:87*.
19. Cutolo, M., S. Accardo, B. Villaggio, P. Clerico, M. Bagnasco, D. A. Coviello, G. Carruba, M. lo Casto, and L. Castagnetta. 1993. Presence of estrogen-binding sites on macrophage-like synoviocytes and CD8+, CD29+, CD45RO+ T lymphocytes in normal and rheumatoid synovium. *Arthritis Rheum 36:1087*.
20. Cohen, J. H., L. Danel, G. Cordier, S. Saez, and J. P. Revillard. 1983. Sex steroid receptors in peripheral T cells: absence of androgen receptors and restriction of estrogen receptors to OKT8-positive cells. *J Immunol 131:2767*.

21. Tornwall, J., A. B. Carey, R. I. Fox, and H. S. Fox. 1999. Estrogen in autoimmunity: expression of estrogen receptors in thymic and autoimmune T cells. *J Gend Specif Med 2:33*.

22. Mor, G., A. Munoz, R. Redlinger, Jr., I. Silva, J. Song, C. Lim, and F. Kohen. 2001. The role of the Fas/Fas ligand system in estrogen-induced thymic alteration. *Am J Reprod Immunol 46:298*.

23. Erlandsson, M. C., C. Ohlsson, J. A. Gustafsson, and H. Carlsten. 2001. Role of oestrogen receptors alpha and beta in immune organ development and in oestrogen-mediated effects on thymus. *Immunology 103:17*.

24. Yellayi, S., C. Teuscher, J. A. Woods, T. H. Welsh, Jr., K. S. Tung, M. Nakai, C. S. Rosenfeld, D. B. Lubahn, and P. S. Cooke. 2000. Normal development of thymus in male and female mice requires estrogen/estrogen receptor-alpha signaling pathway. *Endocrine 12:207*.

25. Erlandsson, M. C., C. A. Jonsson, U. Islander, C. Ohlsson, and H. Carlsten. 2003. Oestrogen receptor specificity in oestradiol-mediated effects on B lymphopoiesis and immunoglobulin production in male mice. *Immunology 108:346*.

26. Thurmond, T. S., F. G. Murante, J. E. Staples, A. E. Silverstone, K. S. Korach, and T. A. Gasiewicz. 2000. Role of estrogen receptor alpha in hematopoietic stem cell development and B lymphocyte maturation in the male mouse. *Endocrinology 141:2309*.

27. Islander, U., M. C. Erlandsson, B. Hasseus, C. A. Jonsson, C. Ohlsson, J. A. Gustafsson, U. Dahlgren, and H. Carlsten. 2003. Influence of oestrogen receptor alpha and beta on the immune system in aged female mice. *Immunology 110:149*.

28. Shim, G. J., L. Wang, S. Andersson, N. Nagy, L. L. Kis, Q. Zhang, S. Makela, M. Warner, and J. A. Gustafsson. 2003. Disruption of the estrogen receptor beta gene in mice causes myeloproliferative disease resembling chronic myeloid leukemia with lymphoid blast crisis. *Proc Natl Acad Sci U S A 100:6694*.

29. Maret, A., J. D. Coudert, L. Garidou, G. Foucras, P. Gourdy, A. Krust, S. Dupont, P. Chambon, P. Druet, F. Bayard, and J. C. Guery. 2003. Estradiol enhances primary antigen-specific CD4 T cell responses and Th1 development in vivo.

Essential role of estrogen receptor alpha expression in hematopoietic cells. *Eur J Immunol 33:512*.

30. Seaman, W. E., and T. D. Gindhart. 1979. Effect of estrogen on natural killer cells. *Arthritis Rheum 22:1234*.

31. Seaman, W. E., T. C. Merigan, and N. Talal. 1979. Natural killing in estrogen-treated mice responds poorly to poly I.C despite normal stimulation of circulating interferon. *J Immunol 123:2903*.

32. Ansar Ahmed, S., M. J. Dauphinee, A. I. Montoya, and N. Talal. 1989. Estrogen induces normal murine CD5+ B cells to produce autoantibodies. *J Immunol 142:2647*.

33. Curran, E. M., L. J. Berghaus, N. J. Vernetti, A. J. Saporita, D. B. Lubahn, and D. M. Estes. 2001. Natural killer cells express estrogen receptor-alpha and estrogen receptor-beta and can respond to estrogen via a non-estrogen receptor-alpha-mediated pathway. *Cell Immunol 214:12*.

34. Katzenellenbogen, J. A., and B. S. Katzenellenbogen. 1996. Nuclear hormone receptors: ligand-activated regulators of transcription and diverse cell responses. *Chem Biol 3:529*.

35. McDonnell, D. P., D. L. Clemm, T. Hermann, M. E. Goldman, and J. W. Pike. 1995. Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. *Mol Endocrinol 9:659*.

36. Tora, L., J. White, C. Brou, D. Tasset, N. Webster, E. Scheer, and P. Chambon. 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell 59:477*.

37. Lees, J. A., S. E. Fawell, and M. G. Parker. 1989. Identification of constitutive and steroid-dependent transactivation domains in the mouse oestrogen receptor. *J Steroid Biochem 34:33*.

38. McInerney, E. M., K. E. Weis, J. Sun, S. Mosselman, and B. S. Katzenellenbogen. 1998. Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. *Endocrinology 139:4513*.

39. McInerney, E. M., M. J. Tsai, B. W. O'Malley, and B. S. Katzenellenbogen. 1996. Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. *Proc Natl Acad Sci U S A 93:10069*.

40. Danielian, P. S., R. White, J. A. Lees, and M. G. Parker. 1992. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *Embo J 11:1025*.

41. Ham, J., and M. G. Parker. 1989. Regulation of gene expression by nuclear hormone receptors. *Curr Opin Cell Biol 1:503*.

42. Meier, C. A. 1997. Regulation of gene expression by nuclear hormone receptors. *J Recept Signal Transduct Res 17:319*.

43. Picard, D., and K. R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *Embo J 6:3333*.

44. Tsai, M. J., and B. W. O'Malley. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem 63:451*.

45. Pratt, W. B., and D. O. Toft. 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev 18:306*.

46. Godowski, P. J., and D. Picard. 1989. Steroid receptors. How to be both a receptor and a transcription factor. *Biochem Pharmacol 38:3135*.

47. Webb, P., P. Nguyen, C. Valentine, G. N. Lopez, G. R. Kwok, E. McInerney, B. S. Katzenellenbogen, E. Enmark, J. A. Gustafsson, S. Nilsson, and P. J. Kushner. 1999. The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. *Mol Endocrinol 13:1672*.

48. Hong, H., K. Kohli, A. Trivedi, D. L. Johnson, and M. R. Stallcup. 1996. GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc Natl Acad Sci U S A 93:4948*.

49. Hong, H., K. Kohli, M. J. Garabedian, and M. R. Stallcup. 1997. GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol Cell Biol 17:2735*.

50. Voegel, J. J., M. J. Heine, M. Tini, V. Vivat, P. Chambon, and H. Gronemeyer. 1998. The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and -independent pathways. *Embo J 17:507*.

51. Henttu, P. M., E. Kalkhoven, and M. G. Parker. 1997. AF-2 activity and recruitment of steroid receptor coactivator 1 to the estrogen receptor depend on a lysine residue conserved in nuclear receptors. *Mol Cell Biol 17:1832*.

52. Torchia, J., D. W. Rose, J. Inostroza, Y. Kamei, S. Westin, C. K. Glass, and M. G. Rosenfeld. 1997. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature 387:677*.

53. Chakravarti, D., V. J. LaMorte, M. C. Nelson, T. Nakajima, I. G. Schulman, H. Juguilon, M. Montminy, and R. M. Evans. 1996. Role of CBP/P300 in nuclear receptor signalling. *Nature 383:99*.

54. Korzus, E., J. Torchia, D. W. Rose, L. Xu, R. Kurokawa, E. M. McInerney, T. M. Mullen, C. K. Glass, and M. G. Rosenfeld. 1998. Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science 279:703*.

55. Swope, D. L., C. L. Mueller, and J. C. Chrivia. 1996. CREB-binding protein activates transcription through multiple domains. *J Biol Chem 271:28138*.

56. Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell 87:953*.

57. Spencer, T. E., G. Jenster, M. M. Burcin, C. D. Allis, J. Zhou, C. A. Mizzen, N. J. McKenna, S. A. Onate, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley. 1997. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature 389:194*.

58. Cavailles, V., S. Dauvois, F. L'Horset, G. Lopez, S. Hoare, P. J. Kushner, and M. G. Parker. 1995. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *Embo J 14:3741*.

59. Johansson, L., A. Bavner, J. S. Thomsen, M. Farnegardh, J. A. Gustafsson, and E. Treuter. 2000. The orphan nuclear receptor SHP utilizes conserved LXXLL-

related motifs for interactions with ligand-activated estrogen receptors. *Mol Cell Biol 20:1124*.

60. Johansson, L., J. S. Thomsen, A. E. Damdimopoulos, G. Spyrou, J. A. Gustafsson, and E. Treuter. 1999. The orphan nuclear receptor SHP inhibits agonist-dependent transcriptional activity of estrogen receptors ERalpha and ERbeta. *J Biol Chem 274:345*.
61. Hart, L. L., and J. R. Davie. 2002. The estrogen receptor: more than the average transcription factor. *Biochem Cell Biol 80:335*.
62. Nilsson, S., and J. A. Gustafsson. 2002. Biological role of estrogen and estrogen receptors. *Crit Rev Biochem Mol Biol 37:1*.
63. Rossini, G. P. 1994. The quaternary structures of untransformed steroid hormone receptors: an open issue. *J Theor Biol 166:339*.
64. King, W. J., and G. L. Greene. 1984. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature 307:745*.
65. Hall, J. M., J. F. Couse, and K. S. Korach. 2001. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem 276:36869*.
66. Watanabe, T., S. Inoue, H. Hiroi, A. Orimo, H. Kawashima, and M. Muramatsu. 1998. Isolation of estrogen-responsive genes with a CpG island library. *Mol Cell Biol 18:442*.
67. Hyder, S. M., G. L. Shipley, and G. M. Stancel. 1995. Estrogen action in target cells: selective requirements for activation of different hormone response elements. *Mol Cell Endocrinol 112:35*.
68. Huang, J., X. Li, P. Yi, R. Hilf, R. A. Bambara, and M. Muyan. 2004. Targeting estrogen responsive elements (EREs): design of potent transactivators for ERE-containing genes. *Mol Cell Endocrinol 218:65*.
69. Levin, E. R. 2001. Cell localization, physiology, and nongenomic actions of estrogen receptors. *J Appl Physiol 91:1860*.
70. Katzenellenbogen, B. S. 1996. Estrogen receptors: bioactivities and interactions with cell signaling pathways. *Biol Reprod 54:287*.
71. Matthews, J., and J. A. Gustafsson. 2003. Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol Interv 3:281*.

72. Hall, J. M., and D. P. McDonnell. 1999. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology 140:5566*.
73. Bunone, G., P. A. Briand, R. J. Miksicek, and D. Picard. 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *Embo J 15:2174*.
74. Smith, C. L. 1998. Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biol Reprod 58:627*.
75. Lahooti, H., T. Thorsen, and A. Aakvaag. 1998. Modulation of mouse estrogen receptor transcription activity by protein kinase C delta. *J Mol Endocrinol 20:245*.
76. Paech, K., P. Webb, G. G. Kuiper, S. Nilsson, J. Gustafsson, P. J. Kushner, and T. S. Scanlan. 1997. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science 277:1508*.
77. Galien, R., and T. Garcia. 1997. Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site. *Nucleic Acids Res 25:2424*.
78. Ray, P., S. K. Ghosh, D. H. Zhang, and A. Ray. 1997. Repression of interleukin-6 gene expression by 17 beta-estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-kappa B by the estrogen receptor. *FEBS Lett 409:79*.
79. Ray, A., K. E. Prefontaine, and P. Ray. 1994. Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor. *J Biol Chem 269:12940*.
80. Migliaccio, A., M. Di Domenico, G. Castoria, A. de Falco, P. Bontempo, E. Nola, and F. Auricchio. 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *Embo J 15:1292*.
81. Singh, M., G. Setalo, Jr., X. Guan, M. Warren, and C. D. Toran-Allerand. 1999. Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. *J Neurosci 19:1179*.

82. Migliaccio, A., G. Castoria, M. Di Domenico, A. de Falco, A. Bilancio, M. Lombardi, M. V. Barone, D. Ametrano, M. S. Zannini, C. Abbondanza, and F. Auricchio. 2000. Steroid-induced androgen receptor-oestradiol receptor beta-Src complex triggers prostate cancer cell proliferation. *Embo J 19:5406*.
83. Mendelsohn, M. E. 2000. Nongenomic, ER-mediated activation of endothelial nitric oxide synthase: how does it work? What does it mean? *Circ Res 87:956*.
84. Simoncini, T., A. Hafezi-Moghadam, D. P. Brazil, K. Ley, W. W. Chin, and J. K. Liao. 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature 407:538*.
85. Pedram, A., M. Razandi, M. Aitkenhead, C. C. Hughes, and E. R. Levin. 2002. Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. *J Biol Chem 277:50768*.
86. Safadi, F. F., I. R. Dissanayake, G. G. Goodman, R. A. Jago, A. E. Baker, A. R. Bowman, D. A. Sass, S. N. Popoff, and S. Epstein. 2000. Influence of estrogen deficiency and replacement on T-cell populations in rat lymphoid tissues and organs. *Endocrine 12:81*.
87. Kuhl, H., M. Gross, M. Schneider, W. Weber, W. Mehlis, M. Stegmuller, and H. D. Taubert. 1983. The effect of sex steroids and hormonal contraceptives upon thymus and spleen on intact female rats. *Contraception 28:587*.
88. Rijhsinghani, A. G., K. Thompson, S. K. Bhatia, and T. J. Waldschmidt. 1996. Estrogen blocks early T cell development in the thymus. *Am J Reprod Immunol 36:269*.
89. Screpanti, I., S. Morrone, D. Meco, A. Santoni, A. Gulino, R. Paolini, A. Crisanti, B. J. Mathieson, and L. Frati. 1989. Steroid sensitivity of thymocyte subpopulations during intrathymic differentiation. Effects of 17 beta-estradiol and dexamethasone on subsets expressing T cell antigen receptor or IL-2 receptor. *J Immunol 142:3378*.
90. Yahata, T., T. Kurabayashi, A. Honda, Y. Tojo, K. Tanaka, and T. Abo. 1996. Physiological dose of estrogen regulates extrathymic T cells in female mice. *Cell Immunol 171:269*.

91. Deshpande, R. R., J. C. Chapman, and S. D. Michael. 1997. The anovulation in female mice resulting from postnatal injections of estrogen is correlated with altered levels of CD8+ lymphocytes. *Am J Reprod Immunol 38:114*.

92. Amadori, A., R. Zamarchi, G. De Silvestro, G. Forza, G. Cavatton, G. A. Danieli, M. Clementi, and L. Chieco-Bianchi. 1995. Genetic control of the CD4/CD8 T-cell ratio in humans. *Nat Med 1:1279*.

93. Giglio, T., M. A. Imro, G. Filaci, M. Scudeletti, F. Puppo, L. De Cecco, F. Indiveri, and S. Costantini. 1994. Immune cell circulating subsets are affected by gonadal function. *Life Sci 54:1305*.

94. Carlsten, H., R. Holmdahl, A. Tarkowski, and L. A. Nilsson. 1989. Oestradiol suppression of delayed-type hypersensitivity in autoimmune (NZB/NZW)F1 mice is a trait inherited from the healthy NZW parental strain. *Immunology 67:205*.

95. Carlsten, H., R. Holmdahl, A. Tarkowski, and L. A. Nilsson. 1989. Oestradiol- and testosterone-mediated effects on the immune system in normal and autoimmune mice are genetically linked and inherited as dominant traits. *Immunology 68:209*.

96. Olsen, N. J., and W. J. Kovacs. 1996. Gonadal steroids and immunity. *Endocr Rev 17:369*.

97. Grossman, C. J. 1984. Regulation of the immune system by sex steroids. *Endocr Rev 5:435*.

98. Ansar Ahmed, S., and N. Talal. 1989. Sex hormones and autoimmune rheumatic disorders. *Scand J Rheumatol 18:69*.

99. Weetman, A. P., A. M. McGregor, B. R. Smith, and R. Hall. 1981. Sex hormones enhance immunoglobulin synthesis by human peripheral blood lymphocytes. *Immunol Lett 3:343*.

100. Giltay, E. J., J. C. Fonk, B. M. von Blomberg, H. A. Drexhage, C. Schalkwijk, and L. J. Gooren. 2000. In vivo effects of sex steroids on lymphocyte responsiveness and immunoglobulin levels in humans. *J Clin Endocrinol Metab 85:1648*.

101. Ansar Ahmed, S., and D. Verthelyi. 1993. Antibodies to cardiolipin in normal C57BL/6J mice: induction by estrogen but not dihydrotestosterone. *J Autoimmun 6:265*.

102. Mathur, S., R. S. Mathur, J. M. Goust, H. O. Williamson, and H. H. Fudenberg. 1977. Sex steroid hormones and circulating IgE levels. *Clin Exp Immunol 30:403*.

103. Froberg, M. K., N. Seacotte, and E. Dahlberg. 2001. Cytomegalovirus seropositivity and serum total cholesterol levels in young patients. *Ann Clin Lab Sci 31:157*.

104. Yamakawa, Y., M. Sata, H. Suzuki, S. Noguchi, and K. Tanikawa. 1996. Higher elimination rate of hepatitis C virus among women. *J Viral Hepat 3:317*.

105. Des Jarlais, D. C., and S. R. Friedman. 1988. Gender differences in response to HIV infection. *Adv Biochem Psychopharmacol 44:159*.

106. Medina, K. L., and P. W. Kincade. 1994. Pregnancy-related steroids are potential negative regulators of B lymphopoiesis. *Proc Natl Acad Sci U S A 91:5382*.

107. Kincade, P. W., K. L. Medina, and G. Smithson. 1994. Sex hormones as negative regulators of lymphopoiesis. *Immunol Rev 137:119*.

108. Medina, K. L., A. Strasser, and P. W. Kincade. 2000. Estrogen influences the differentiation, proliferation, and survival of early B-lineage precursors. *Blood 95:2059*.

109. Sasaki, K., and T. Ito. 1981. Effects of estrogen and progesterone on the spleen of the mouse: a light and electron microscopic study. *Arch Histol Jpn 44:203*.

110. Verthelyi, D. I., and S. Ansar Ahmed. 1998. Estrogen increases the number of plasma cells and enhances their autoantibody production in nonautoimmune C57BL/6 mice. *Cell Immunol 189:125*.

111. Jenkins, J. K., S. Suwannaroj, K. B. Elbourne, K. Ndebele, and R. W. McMurray. 2001. 17-beta-estradiol alters Jurkat lymphocyte cell cycling and induces apoptosis through suppression of Bcl-2 and cyclin A. *Int Immunopharmacol 1:1897*.

112. Grimaldi, C. M., J. Cleary, A. S. Dagtas, D. Moussai, and B. Diamond. 2002. Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest 109:1625*.

113. Bynoe, M. S., C. M. Grimaldi, and B. Diamond. 2000. Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc Natl Acad Sci U S A 97:2703*.

114. Han, D., M. S. Denison, H. Tachibana, and K. Yamada. 2002. Effects of estrogenic compounds on immunoglobulin production by mouse splenocytes. *Biol Pharm Bull 25:1263*.

115. Nilsson, N., and H. Carlsten. 1994. Estrogen induces suppression of natural killer cell cytotoxicity and augmentation of polyclonal B cell activation. *Cell Immunol 158:131*.

116. Ansar Ahmed, S., T. B. Aufdemorte, J. R. Chen, A. I. Montoya, D. Olive, and N. Talal. 1989. Estrogen induces the development of autoantibodies and promotes salivary gland lymphoid infiltrates in normal mice. *J Autoimmun 2:543*.

117. Verthelyi, D., and S. Ansar Ahmed. 1997. Characterization of estrogen-induced autoantibodies to cardiolipin in non-autoimmune mice. *J Autoimmun 10:115*.

118. Grimaldi, C. M., D. J. Michael, and B. Diamond. 2001. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *J Immunol 167:1886*.

119. Forsberg, J. G. 2000. Neonatal estrogen treatment and its consequences for thymus development, serum level of autoantibodies to cardiolipin, and the delayed-type hypersensitivity response. *J Toxicol Environ Health A 60:185*.

120. Kanda, N., and K. Tamaki. 1999. Estrogen enhances immunoglobulin production by human PBMCs. *J Allergy Clin Immunol 103:282*.

121. Verthelyi, D., and S. Ansar Ahmed. 1994. 17 beta-estradiol, but not 5 alpha-dihydrotestosterone, augments antibodies to double-stranded deoxyribonucleic acid in nonautoimmune C57BL/6J mice. *Endocrinology 135:2615*.

122. Kanda, N., T. Tsuchida, and K. Tamaki. 1996. Testosterone inhibits immunoglobulin production by human peripheral blood mononuclear cells. *Clin Exp Immunol 106:410*.

123. Vernon-Roberts, B. 1969. The effects of steroid hormones on macrophage activity. *Int Rev Cytol 25:131*.

124. Baranao, R. I., A. Tenenbaum, M. E. Sales, and L. S. Rumi. 1992. Functional alterations of murine peritoneal macrophages during pregnancy. *Am J Reprod Immunol 27:82*.

125. Baranao, R. I., A. Tenenbaum, and L. S. Rumi. 1991. Effects of sexual steroid hormones on the functionality of murine peritoneal macrophages. *Steroids 56:481*.

126. Gomez, F., P. Ruiz, J. A. Bernal, M. Escobar, A. Garcia-Egido, and J. J. Lopez-Saez. 2001. Enhancement of splenic-macrophage Fcgamma receptor expression by treatment with estrogens. *Clin Diagn Lab Immunol 8:806*.

127. Mori, H., M. Nakagawa, N. Itoh, K. Wada, and T. Tamaya. 1990. Danazol suppresses the production of interleukin-1 beta and tumor necrosis factor by human monocytes. *Am J Reprod Immunol 24:45*.

128. Mori, H., M. Sawairi, N. Itoh, T. Hanabayashi, and T. Tamaya. 1992. Effects of sex steroids on cell differentiation and interleukin-1 beta production in the human promyelocytic leukemia cell line HL-60. *J Reprod Med 37:871*.

129. Chao, T. C., P. J. Van Alten, J. A. Greager, and R. J. Walter. 1995. Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cell Immunol 160:43*.

130. Carruba, G., P. D'Agostino, M. Miele, M. Calabro, C. Barbera, G. D. Bella, S. Milano, V. Ferlazzo, R. Caruso, M. L. Rosa, L. Cocciadiferro, I. Campisi, L. Castagnetta, and E. Cillari. 2003. Estrogen regulates cytokine production and apoptosis in PMA-differentiated, macrophage-like U937 cells. *J Cell Biochem 90:187*.

131. Everson, M. P., D. S. McDuffie, D. G. Lemak, W. J. Koopman, J. R. McGhee, and K. W. Beagley. 1996. Dendritic cells from different tissues induce production of different T cell cytokine profiles. *J Leukoc Biol 59:494*.

132. Paharkova-Vatchkova, V., R. Maldonado, and S. Kovats. 2004. Estrogen preferentially promotes the differentiation of CD11c+ CD11b(intermediate) dendritic cells from bone marrow precursors. *J Immunol 172:1426*.

133. Zhang, Q. H., Y. Z. Hu, J. Cao, Y. Q. Zhong, Y. F. Zhao, and Q. B. Mei. 2004. Estrogen influences the differentiation, maturation and function of dendritic cells

in rats with experimental autoimmune encephalomyelitis. *Acta Pharmacol Sin 25:508*.

134. Bengtsson, A. K., E. J. Ryan, D. Giordano, D. M. Magaletti, and E. A. Clark. 2004. 17-{beta}-estradiol (E2) modulates cytokine and chemokine expression in human monocyte-derived dendritic cells. *Blood*.

135. Screpanti, I., A. Santoni, A. Gulino, R. B. Herberman, and L. Frati. 1987. Estrogen and antiestrogen modulation of the levels of mouse natural killer activity and large granular lymphocytes. *Cell Immunol 106:191*.

136. Seaman, W. E., T. D. Gindhart, J. S. Greenspan, M. A. Blackman, and N. Talal. 1979. Natural killer cells, bone, and the bone marrow: studies in estrogen-treated mice and in congenitally osteopetrotic (mi/mi) mice. *J Immunol 122:2541*.

137. Hanna, N., and M. Schneider. 1983. Enhancement of tumor metastasis and suppression of natural killer cell activity by beta-estradiol treatment. *J Immunol 130:974*.

138. Baral, E., E. Nagy, and I. Berczi. 1995. Modulation of natural killer cell-mediated cytotoxicity by tamoxifen and estradiol. *Cancer 75:591*.

139. Styrt, B., and B. Sugarman. 1991. Estrogens and infection. *Rev Infect Dis 13:1139*.

140. Ferguson, M. M., and F. G. McDonald. 1985. Oestrogen as an inhibitor of human NK cell cytolysis. *FEBS Lett 191:145*.

141. Albrecht, A. E., B. W. Hartmann, C. Scholten, J. C. Huber, W. Kalinowska, and C. C. Zielinski. 1996. Effect of estrogen replacement therapy on natural killer cell activity in postmenopausal women. *Maturitas 25:217*.

142. Farrar, M. A., and R. D. Schreiber. 1993. The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol 11:571*.

143. Schreiber, R. D., and M. A. Farrar. 1993. The biology and biochemistry of interferon-gamma and its receptor. *Gastroenterol Jpn 28 Suppl 4:88*.

144. Borish, L. C., and J. W. Steinke. 2003. 2. Cytokines and chemokines. *J Allergy Clin Immunol 111:S460*.

145. Takeda, A., S. Hamano, A. Yamanaka, T. Hanada, T. Ishibashi, T. W. Mak, A. Yoshimura, and H. Yoshida. 2003. Cutting edge: role of IL-27/WSX-1 signaling

for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol 170:4886*.

146. Billiau, A. 1996. Interferon-gamma in autoimmunity. *Cytokine Growth Factor Rev 7:25*.

147. Fox, H. S., B. L. Bond, and T. G. Parslow. 1991. Estrogen regulates the IFN-gamma promoter. *J Immunol 146:4362*.

148. McMurray, R. W., R. W. Hoffman, W. Nelson, and S. E. Walker. 1997. Cytokine mRNA expression in the B/W mouse model of systemic lupus erythematosus--analyses of strain, gender, and age effects. *Clin Immunol Immunopathol 84:260*.

149. Huygen, K., and K. Palfliet. 1984. Strain variation in interferon gamma production of BCG-sensitized mice challenged with PPD II. Importance of one major autosomal locus and additional sexual influences. *Cell Immunol 85:75*.

150. Huygen, K., and K. Palfliet. 1983. Strain variation in interferon gamma production of BCG-sensitized mice challenged with PPD. I. CBA/Ca mice are low producers in vivo, but high producers in vitro. *Cell Immunol 80:329*.

151. Satoskar, A., and J. Alexander. 1995. Sex-determined susceptibility and differential IFN-gamma and TNF-alpha mRNA expression in DBA/2 mice infected with Leishmania mexicana. *Immunology 84:1*.

152. Sato, M. N., P. Minoprio, S. Avrameas, and T. Ternynck. 1995. Defects in the regulation of anti-DNA antibody production in aged lupus-prone (NZB x NZW)F1 mice: analysis of T-cell lymphokine synthesis. *Immunology 85:26*.

153. Grasso, G., and M. Muscettola. 1990. The influence of beta-estradiol and progesterone on interferon gamma production in vitro. *Int J Neurosci 51:315*.

154. Sabahi, F., M. Rola-Plesczcynski, S. O'Connell, and L. D. Frenkel. 1995. Qualitative and quantitative analysis of T lymphocytes during normal human pregnancy. *Am J Reprod Immunol 33:381*.

155. Shimaoka, Y., Y. Hidaka, H. Tada, T. Nakamura, N. Mitsuda, Y. Morimoto, Y. Murata, and N. Amino. 2000. Changes in cytokine production during and after normal pregnancy. *Am J Reprod Immunol 44:143*.

156. Matalka, K. Z. 2003. The effect of estradiol, but not progesterone, on the production of cytokines in stimulated whole blood, is concentration-dependent. *Neuroendocrinol Lett 24:185*.

157. Deshpande, R., H. Khalili, R. G. Pergolizzi, S. D. Michael, and M. D. Chang. 1997. Estradiol down-regulates LPS-induced cytokine production and NFkB activation in murine macrophages. *Am J Reprod Immunol 38:46*.

158. Matejuk, A., K. Adlard, A. Zamora, M. Silverman, A. A. Vandenbark, and H. Offner. 2001. 17 beta-estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nervous system of female mice with experimental autoimmune encephalomyelitis. *J Neurosci Res 65:529*.

159. Rogers, A., and R. Eastell. 2001. The effect of 17beta-estradiol on production of cytokines in cultures of peripheral blood. *Bone 29:30*.

160. Li, Z. G., V. A. Danis, and P. M. Brooks. 1993. Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells in vitro. *Clin Exp Rheumatol 11:157*.

161. Kawasaki, T., T. Ushiyama, K. Inoue, and S. Hukuda. 2000. Effects of estrogen on interleukin-6 production in rheumatoid fibroblast-like synoviocytes. *Clin Exp Rheumatol 18:743*.

162. Knoferl, M. W., D. Jarrar, M. K. Angele, A. Ayala, M. G. Schwacha, K. I. Bland, and I. H. Chaudry. 2001. 17 beta-Estradiol normalizes immune responses in ovariectomized females after trauma-hemorrhage. *Am J Physiol Cell Physiol 281:C1131*.

163. De, M., T. R. Sanford, and G. W. Wood. 1992. Interleukin-1, interleukin-6, and tumor necrosis factor alpha are produced in the mouse uterus during the estrous cycle and are induced by estrogen and progesterone. *Dev Biol 151:297*.

164. Willis, C., J. M. Morris, V. Danis, and E. D. Gallery. 2003. Cytokine production by peripheral blood monocytes during the normal human ovulatory menstrual cycle. *Hum Reprod 18:1173*.

165. Screpanti, I., D. Meco, S. Morrone, A. Gulino, B. J. Mathieson, and L. Frati. 1991. In vivo modulation of the distribution of thymocyte subsets: effects of

estrogen on the expression of different T cell receptor V beta gene families in CD4-, CD8- thymocytes. *Cell Immunol 134:414*.

166. Messingham, K. A., S. A. Heinrich, and E. J. Kovacs. 2001. Estrogen restores cellular immunity in injured male mice via suppression of interleukin-6 production. *J Leukoc Biol 70:887*.

167. Ito, A., B. F. Bebo, Jr., A. Matejuk, A. Zamora, M. Silverman, A. Fyfe-Johnson, and H. Offner. 2001. Estrogen treatment down-regulates TNF-alpha production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. *J Immunol 167:542*.

168. Zhang, X., L. Wang, H. Zhang, D. Guo, Z. Qiao, and J. Qiao. 2001. Estrogen inhibits lipopolysaccharide-induced tumor necrosis factor-alpha release from murine macrophages. *Methods Find Exp Clin Pharmacol 23:169*.

169. Chao, T. C., H. H. Chao, M. F. Chen, J. A. Greager, and R. J. Walter. 2000. Female sex hormones modulate the function of LPS-treated macrophages. *Am J Reprod Immunol 44:310*.

170. Ansar Ahmed, S., and Talal, N. 1993. Importance of sex hormones in lupus. In *Dubois' Lupus Erythematous*. W. D. a. H. BH, ed. Lea Fibiger, p. 148.

171. Ansar Ahmed, S., and T. N. 1988. Sex Steroids, Sex Steroid receptors and Autoimmune disorders. In *Steroid receptors and Disease: Cancer, Autoimmune, Bone and Circulatory Disorders*. B. K. Sheridan P.J., Trachtenberg M.C., ed. Marcel-Dekker Inc, NY, p. 289.

172. Jacobson, D. L., S. J. Gange, N. R. Rose, and N. M. Graham. 1997. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol 84:223*.

173. Cooper, G. S., and B. C. Stroehla. 2003. The epidemiology of autoimmune diseases. *Autoimmun Rev 2:119*.

174. Beeson, P. B. 1994. Age and sex associations of 40 autoimmune diseases. *Am J Med 96:457*.

175. Ansar Ahmed, S., M. J. Dauphinee, and N. Talal. 1985. Effects of short-term administration of sex hormones on normal and autoimmune mice. *J Immunol 134:204*.

176. Cutolo, M., A. Sulli, B. Seriolo, S. Accardo, and A. T. Masi. 1995. Estrogens, the immune response and autoimmunity. *Clin Exp Rheumatol 13:217*.

177. Karpuzoglu-Sahin, E., B. D. Hissong, and S. Ansar Ahmed. 2001. Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. *J Reprod Immunol 52:113*.

178. Karpuzoglu-Sahin, E., Y. Zhi-Jun, A. Lengi, N. Sriranganathan, and S. Ansar Ahmed. 2001. Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. *Cytokine 14:208*.

179. Hayashi, Y., N. Haneji, H. Hamano, K. Yanagi, M. Takahashi, and N. Ishimaru. 1996. Effector mechanism of experimental autoimmune sialadenitis in the mouse model for primary Sjogren's syndrome. *Cell Immunol 171:217*.

180. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu Rev Immunol 15:749*.

181. Wong, G. H., I. Clark-Lewis, L. McKimm-Breschkin, A. W. Harris, and J. W. Schrader. 1983. Interferon-gamma induces enhanced expression of Ia and H-2 antigens on B lymphoid, macrophage, and myeloid cell lines. *J Immunol 131:788*.

182. Pace, J. L., S. W. Russell, B. A. Torres, H. M. Johnson, and P. W. Gray. 1983. Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. *J Immunol 130:2011*.

183. Ruemmele, F. M., C. Gurbindo, A. M. Mansour, R. Marchand, E. Levy, and E. G. Seidman. 1998. Effects of interferon gamma on growth, apoptosis, and MHC class II expression of immature rat intestinal crypt (IEC-6) cells. *J Cell Physiol 176:120*.

184. Leibson, H. J., M. Gefter, A. Zlotnik, P. Marrack, and J. W. Kappler. 1984. Role of gamma-interferon in antibody-producing responses. *Nature 309:799*.

185. Sidman, C. L., J. D. Marshall, L. D. Shultz, P. W. Gray, and H. M. Johnson. 1984. Gamma-interferon is one of several direct B cell-maturing lymphokines. *Nature 309:801*.

186. Haas, C., B. Ryffel, and M. Le Hir. 1998. IFN-gamma receptor deletion prevents autoantibody production and glomerulonephritis in lupus-prone (NZB x NZW)F1 mice. *J Immunol 160:3713*.

187. Campbell, I. L., T. W. Kay, L. Oxbrow, and L. C. Harrison. 1991. Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J Clin Invest 87:739*.

188. Balasa, B., C. Deng, J. Lee, L. M. Bradley, D. K. Dalton, P. Christadoss, and N. Sarvetnick. 1997. Interferon gamma (IFN-gamma) is necessary for the genesis of acetylcholine receptor-induced clinical experimental autoimmune myasthenia gravis in mice. *J Exp Med 186:385*.

189. Chan, R. W., L. S. Tam, E. K. Li, F. M. Lai, K. M. Chow, K. B. Lai, P. K. Li, and C. C. Szeto. 2003. Inflammatory cytokine gene expression in the urinary sediment of patients with lupus nephritis. *Arthritis Rheum 48:1326*.

190. Sugimoto, K., S. Morimoto, H. Kaneko, K. Nozawa, Y. Tokano, Y. Takasaki, and H. Hashimoto. 2002. Decreased IL-4 producing CD4+ T cells in patients with active systemic lupus erythematosus-relation to IL-12R expression. *Autoimmunity 35:381*.

191. Soltesz, P., M. Aleksza, P. Antal-Szalmas, G. Lakos, G. Szegedi, and E. Kiss. 2002. Plasmapheresis modulates Th1/Th2 imbalance in patients with systemic lupus erythematosus according to measurement of intracytoplasmic cytokines. *Autoimmunity 35:51*.

192. Schwarting, A., G. Tesch, K. Kinoshita, R. Maron, H. L. Weiner, and V. R. Kelley. 1999. IL-12 drives IFN-gamma-dependent autoimmune kidney disease in MRL-Fas(lpr) mice. *J Immunol 163:6884*.

193. Chen, S., D. Hu, X. Shi, N. Shen, Y. Gu, and C. Bao. 2000. The relationship between Th1/Th2-type cells and disease activity in patients with systemic lupus erythematosus. *Chin Med J (Engl) 113:877*.

194. Jones, B. M., T. Liu, and R. W. Wong. 1999. Reduced in vitro production of interferon-gamma, interleukin-4 and interleukin-12 and increased production of interleukin-6, interleukin-10 and tumour necrosis factor-alpha in systemic lupus

erythematosus. Weak correlations of cytokine production with disease activity. *Autoimmunity 31:117*.

195. Fan, X., and R. P. Wuthrich. 1997. Upregulation of lymphoid and renal interferon-gamma mRNA in autoimmune MRL-Fas(lpr) mice with lupus nephritis. *Inflammation 21:105*.

196. Reininger, L., M. L. Santiago, S. Takahashi, L. Fossati, and S. Izui. 1996. T helper cell subsets in the pathogenesis of systemic lupus erythematosus. *Ann Med Interne (Paris) 147:467*.

197. Prud'homme, G. J., D. H. Kono, and A. N. Theofilopoulos. 1995. Quantitative polymerase chain reaction analysis reveals marked overexpression of interleukin-1 beta, interleukin-1 and interferon-gamma mRNA in the lymph nodes of lupus-prone mice. *Mol Immunol 32:495*.

198. Shirai, A., J. Conover, and D. M. Klinman. 1995. Increased activation and altered ratio of interferon-gamma: interleukin-4 secreting cells in MRL-lpr/lpr mice. *Autoimmunity 21:107*.

199. Carvalho-Pinto, C. E., M. I. Garcia, M. Mellado, J. M. Rodriguez-Frade, J. Martin-Caballero, J. Flores, A. C. Martinez, and D. Balomenos. 2002. Autocrine production of IFN-gamma by macrophages controls their recruitment to kidney and the development of glomerulonephritis in MRL/lpr mice. *J Immunol 169:1058*.

200. Pollard, K. M., P. Hultman, and D. H. Kono. 2003. Using single-gene deletions to identify checkpoints in the progression of systemic autoimmunity. *Ann N Y Acad Sci 987:236*.

201. Balomenos, D., R. Rumold, and A. N. Theofilopoulos. 1998. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. *J Clin Invest 101:364*.

202. Peng, S. L., J. Moslehi, and J. Craft. 1997. Roles of interferon-gamma and interleukin-4 in murine lupus. *J Clin Invest 99:1936*.

203. Lawson, B. R., G. J. Prud'homme, Y. Chang, H. A. Gardner, J. Kuan, D. H. Kono, and A. N. Theofilopoulos. 2000. Treatment of murine lupus with cDNA encoding IFN-gammaR/Fc. *J Clin Invest 106:207*.

204. Schwarting, A., T. Wada, K. Kinoshita, G. Tesch, and V. R. Kelley. 1998. IFN-gamma receptor signaling is essential for the initiation, acceleration, and destruction of autoimmune kidney disease in MRL-Fas(lpr) mice. *J Immunol 161:494*.

205. Kikawada, E., D. M. Lenda, and V. R. Kelley. 2003. IL-12 deficiency in MRL-Fas(lpr) mice delays nephritis and intrarenal IFN-gamma expression, and diminishes systemic pathology. *J Immunol 170:3915*.

206. Haas, C., B. Ryffel, and M. Le Hir. 1997. IFN-gamma is essential for the development of autoimmune glomerulonephritis in MRL/Ipr mice. *J Immunol 158:5484*.

207. Uhm, W. S., K. Na, G. W. Song, S. S. Jung, T. Lee, M. H. Park, and D. H. Yoo. 2003. Cytokine balance in kidney tissue from lupus nephritis patients. *Rheumatology (Oxford) 42:935*.

208. Robak, E., T. Robak, A. Wozniacka, M. Zak-Prelich, A. Sysa-Jedrzejowska, and H. Stepien. 2002. Proinflammatory interferon-gamma--inducing monokines (interleukin-12, interleukin-18, interleukin-15)--serum profile in patients with systemic lupus erythematosus. *Eur Cytokine Netw 13:364*.

209. Amerio, P., A. Frezzolini, D. Abeni, P. Teofoli, C. R. Girardelli, O. De Pita, and P. Puddu. 2002. Increased IL-18 in patients with systemic lupus erythematosus: relations with Th-1, Th-2, pro-inflammatory cytokines and disease activity. IL-18 is a marker of disease activity but does not correlate with pro-inflammatory cytokines. *Clin Exp Rheumatol 20:535*.

210. Tokano, Y., S. Morimoto, H. Kaneko, H. Amano, K. Nozawa, Y. Takasaki, and H. Hashimoto. 1999. Levels of IL-12 in the sera of patients with systemic lupus erythematosus (SLE)--relation to Th1- and Th2-derived cytokines. *Clin Exp Immunol 116:169*.

211. Peng, S. L., S. J. Szabo, and L. H. Glimcher. 2002. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci U S A 99:5545*.

212. Bao, M., Y. Yang, H. S. Jun, and J. W. Yoon. 2002. Molecular mechanisms for gender differences in susceptibility to T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Immunol 168:5369*.

213. Rider, V., S. Jones, M. Evans, H. Bassiri, Z. Afsar, and N. I. Abdou. 2001. Estrogen increases CD40 ligand expression in T cells from women with systemic lupus erythematosus. *J Rheumatol 28:2644*.

214. Stuber, E., W. Strober, and M. Neurath. 1996. Blocking the CD40L-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. *J Exp Med 183:693*.

215. Hirohata, S. 1999. Human Th1 responses driven by IL-12 are associated with enhanced expression of CD40 ligand. *Clin Exp Immunol 115:78*.

216. Zhang, G. X., B. G. Xiao, X. F. Bai, P. H. van der Meide, A. Orn, and H. Link. 1999. Mice with IFN-gamma receptor deficiency are less susceptible to experimental autoimmune myasthenia gravis. *J Immunol 162:3775*.

217. Karachunski, P. I., N. S. Ostlie, C. Monfardini, and B. M. Conti-Fine. 2000. Absence of IFN-gamma or IL-12 has different effects on experimental myasthenia gravis in C57BL/6 mice. *J Immunol 164:5236*.

218. Espejo, C., M. Penkowa, I. Saez-Torres, J. Hidalgo, A. Garcia, X. Montalban, and E. M. Martinez-Caceres. 2002. Interferon-gamma regulates oxidative stress during experimental autoimmune encephalomyelitis. *Exp Neurol 177:21*.

219. Chu, C. Q., Z. Song, L. Mayton, B. Wu, and P. H. Wooley. 2003. IFNgamma deficient C57BL/6 (H-2b) mice develop collagen induced arthritis with predominant usage of T cell receptor Vbeta6 and Vbeta8 in arthritic joints. *Ann Rheum Dis 62:983*.

220. Vermeire, K., H. Heremans, M. Vandeputte, S. Huang, A. Billiau, and P. Matthys. 1997. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *J Immunol 158:5507*.

221. Liu, H. Y., A. C. Buenafe, A. Matejuk, A. Ito, A. Zamora, J. Dwyer, A. A. Vandenbark, and H. Offner. 2002. Estrogen inhibition of EAE involves effects on dendritic cell function. *J Neurosci Res 70:238*.

222. Ito, A., A. Matejuk, C. Hopke, H. Drought, J. Dwyer, A. Zamora, S. Subramanian, A. A. Vandenbark, and H. Offner. 2003. Transfer of severe experimental autoimmune encephalomyelitis by IL-12- and IL-18-potentiated T cells is estrogen sensitive. *J Immunol 170:4802*.

CHAPTER 2. Immunomodulatory Effects of Estrogen: Implications to IFN-γ

This chapter deals with addressing the central question " *Does estrogen (17-β estradiol) alter interferon-γ (IFN-γ) secretion?*" This chapter will present evidence to show the activated splenic lymphocytes from estrogen-treated mice upregulate IFN-γ.

This chapter is divided into 3 subsections and has resulted in two publications.

(1) **Subsection 1: "Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice.** Karpuzoglu-Sahin E, Zhi-Jun Y, Lengi A, Sriranganathan N, Ansar Ahmed S. Cytokine. 2001 May 21;14 (4):208-17." This publication shows that estrogen regulates the expression of IFN-γ and IL-2, but not IL-4. Studies in orchiectomized males demonstrated that estrogen increased the relative expression of IFN-γ mRNA expression and IFN-γ protein secretion by splenic lymphocytes.

(2) **Subsection 2: "Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol.** Karpuzoglu-Sahin E, Hissong BD, Ansar Ahmed S. J Reprod Immunol. 2001 Oct-Nov;52(1-2):113-27."This publication demonstrated that estrogen upregulated IFN-γ secretion not only in males, but also in females. Kinetic studies revealed that higher level of IFN-γ secreted was evident as early as 6 hours of culture. Estrogen also upregulates CD80 expression on B cells, and may be involved in co-stimulation of T cells to secrete increased IFN-γ.

(3) **Subsection 3: "A Simpler Method to Quantify Gene Expression by Real-Time PCR: Analysis of Estrogen-Induced IFN-γ Expression**. Karpuzoglu-Sahin, E., Hardy C., Lengi, A., Ansar Ahmed, S." This manuscript will be submitted shortly. This manuscript demonstrated a method in quantitating the target and house-keeping genes in Real Time-PCR using a standard curve based on the target gene or house-keeping gene itsef. The results obtained from standard curve method were compared

to the Delta Delta CT method that is used for the relative calculation of target mRNA, and also to relative densities obtained from RT-PCR to demontrate the validity of the method. This method is easy, efficient, and more economical compared to other quantitative Real Time-PCR methods such as Taqman Probe method. In addition to the in-house development of the standard curve method, the manuscript also demonstrates the quantitative increase in IFN-γ mRNA expression upon estrogen treatment.

2.1. Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. Karpuzoglu-Sahin E, Zhi-Jun Y, Lengi A, Sriranganathan N, Ansar Ahmed S. Cytokine. 2001 May 21;14(4):208-17

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EFFECTS OF LONG-TERM ESTROGEN TREATMENT ON IFN-γ, IL-2 AND IL-4 GENE EXPRESSION AND PROTEIN SYNTHESIS IN SPLEEN AND THYMUS OF NORMAL C57BL/6 MICE

E. Karpuzoglu-Sahin, Y. Zhi-Jun, A. Lengi, N. Sriranganathan, S. Ansar Ahmed

Estrogens have been shown to markedly modulate the immune system. One mechanism by which estrogens could modulate the immune system is by regulating cytokines, an aspect not well-studied thus far. To address this issue, normal C57BL/6 orchiectomized mice were given estrogen and its effects on selected cytokines, interferon-gamma (IFN-γ), interleukin 2 (IL-2) and IL-4 in lymphocytes from a developmental organ (thymus) and a mature lymphoid organ (spleen) examined. Estrogen significantly increased IFN-γ and IL-2 mRNA in concanavalin-A (Con-A) activated thymocytes, splenic lymphocytes, and in enriched splenic T cells. Estrogen had no marked effect on IL-4 mRNA. While estrogen increased IFN-γ mRNA in Con-A activated unseparated splenic lymphocytes and enriched splenic T cells, a numerical increase in IFN-γ was noticed only in the supernatants of Con-A activated unseparated splenic lymphocytes, but not in enriched splenic T cells. This suggests that for optimal secretion of IFN-γ in estrogen-treated mice, co-stimulatory signals from antigen presenting cells are needed. Gender differences in IFN-γ and IL-2 mRNA were also evident. Con-A activated splenic lymphocytes from gonadal-intact, untreated female had a pattern of numerical increase in IFN-γ mRNA, and IFN-γ and IL-2 protein levels compared to their male counterparts. Taken together, our data suggests that estrogens regulate the expression of cytokines, which could account in part, for the gender differences in immune capabilities.

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a.) ABSTRACT:

Estrogens have been shown to markedly modulate the immune system. One mechanism by which estrogens could modulate the immune system is by regulating cytokines, an aspect not well studied thus far. To address this issue, normal C57BL/6 orchiectomized mice were given estrogen and its effects on selected cytokines, interferon-gamma (IFN-γ), interleukin-2 (IL-2) and IL-4 in lymphocytes from a developmental organ (thymus) and a mature lymphoid organ (spleen) examined. Estrogen significantly increased IFN-γ and IL-2 mRNA in concanavalin-A (Con-A) activated thymocytes, splenic lymphocytes, and in enriched splenic T cells. Estrogen had no marked effect on IL-4 mRNA. While estrogen increased IFN-γ mRNA in Con-A activated unseparated splenic lymphocytes and enriched splenic T cells, a numerical increase in IFN-γ was noticed only in the supernatants of Con-A activated unseparated splenic lymphocytes, but not in enriched splenic T cells. This suggests that for optimal secretion of IFN-γ in estrogen-treated mice, co-stimulatory signals from antigen presenting cells are needed. Gender differences in IFN-γ and IL-2 mRNA were also evident. Con-A activated splenic lymphocytes from gonadal-intact, untreated female had a pattern of numerical increase in IFN-γ mRNA, and IFN-γ and IL-2 protein levels compared to their male counterparts. Taken together, our data suggests that estrogens regulate the expression of cytokines, which could account in part, for the gender differences in immune capabilities.

b.) INTRODUCTION

Physiological gender differences in immune capabilities are now well recognized[1,2,3,4]. In general, females have stronger B-cell mediated responses, have higher levels of serum immunoglobulins and are more resistant to certain infections compared to males[1,4,5]. At a cellular level, females have increased $CD4^+$ T cell numbers and CD4/CD8 ratio[6]. Gender differences are also evident in immunopathological conditions such as autoimmune diseases and hypersensitivity[1,2,7]. Women have a higher incidence of organ-specific and non-organ specific autoimmune diseases[1,8,9]. In many animal models of human autoimmune diseases, female animals have a higher incidence and/or greater severity of autoimmune diseases than

their male counterparts[1,2,3,10]. In large part, gender differences in the immune system have been attributed to the effects of sex steroid hormones such as estrogens.

Understanding the effects of estrogen on the immune system is becoming increasingly important since, humans and animals are exposed to the estrogenic compounds from a variety of sources[3,7]. These include natural endogenous exposure, administration of synthetic estrogenic pharmaceuticals (such as estrogen replacement therapy and oral contraceptives), intake of phytoestrogens (legumes), and unintentional exposure to estrogenic endocrine disrupters (found in pesticides, industrial byproducts and contaminants). Some of these exposures can be of chronic duration. For example, postmenopausal women are given prolonged, often life-long, estrogen replacement therapy to prevent osteoporosis and coronary diseases. Estrogen-containing oral contraceptives are consumed recurrently throughout a woman's reproductive life. Chronic exposure of humans to estrogenic chemicals could conceivably occur through drinking water that is contaminated with sewage water containing metabolites of estrogen-containing contraceptives (ethinyl estradiol), or prolonged occupational contact with estrogenic pesticides or chemicals in plastics. It is now recognized that estrogens regulate not only the reproductive system, but can also affect multiple aspects of the immune system[1,2,3,4,8,10,11]. It is likely that estrogens may mediate these effects, in part, by regulating the secretion of cytokines, which are molecular messengers that coordinate and regulate multiple types of immune cells. The effects of sustained exposure to estrogens on cytokine gene/protein expression in lymphocytes are not known. Therefore, in this study, as a model for other estrogens, we investigated the consequence of chronic administration of 17-β-estradiol on kinetics of expression of major cytokines (IFN-γ, IL-2 and IL-4) at both mRNA and protein levels in the splenic and thymic lymphocytes.

In this initial study, we targeted three cytokines, IFN-γ, IL-2 and to less extent IL-4, that are known to have broad effects on diverse types of cells of the immune system. Interferon-gamma (also termed as Type II IFN or Immune IFN) secreted by T-helper 1 (Th1), $CD8^+$ cells and NK cells, plays a central role in host defenses against intracellular infections[12]. Biologically, IFN-γ is known to: upregulate the expression of major histocompatibility complex (MHC) class II molecules on target cells, potently activate macrophages, promote

differentiation of naïve $CD4^+$ T cells into Th1 subclass[12,13,14], and induce isotype switching of IgM to IgG2a or IgG3 antibody subclasses. This cytokine has been implicated in pathogenesis of several diseases such as septic shock or several female-predominant autoimmune diseases[12,14]. Interleukin-2 (a Th-1 cytokine) has both autocrine and paracrine function. It amplifies several aspects of T cell responses, augments IFN-γ production, supports long-term growth, enhances activation-induced apoptosis, and is a stimulus for antibody production[15]. Interleukin-4 (a Th-2-type cytokine) induces isotype switching from IgM to IgG1 or IgE antibodies, inhibits macrophage activation and blocks most of the macrophage activating effects on IFN-γ[12,14].

Thus far, the long-term effects of natural estrogen in C57BL/6 mice on selected cytokine gene expression and protein synthesis in lymphocytes from representative developmental lymphoid organ (thymus) and in a mature lymphoid organ (spleen) have not been comprehensively studied. In the present studies, we find that estrogen regulates the gene expression of IFN-γ and IL-2, but not IL-4 in C57BL/6J mice.

c.) MATERIALS AND METHODS

Mice: Thirty two 3-week-old male C57BL/6J inbred mice were purchased from the Charles River Animal Vendor (Wilmington, MA). Three to four-months old male and female C57BL/6J mice were also purchased from the Charles River Animal Facility (Wilmington, MA). These mice were gonadal-intact and are referred to as untreated male and female mice. All mice were fed on a commercial pellet diet and water *ad libitum* and housed 3-5 animals per cage in our laboratory animal facility. They were kept on a 12 h light, 12 h dark cycle.

Estrogen Treatment: Four to six week-old mice were anesthetized with sodium pentobarbital and orchiectomized as described in our previous studies[2,10]. Silastic capsules containing six to eight mg of 17-β-estradiol were surgically implanted on the dorsal side of the neck as standard procedures that are described previously[2,10,16]. These implants release estrogen gradually for several months. Control mice received empty silastic implants (placebo

implants). Mice were euthanized by cervical dislocation after 4-6 months after estrogen/placebo treatment.

Isolation of lymphocytes: As has been noted in previous studies[2,10], the thymuses of E2-treated mice were atrophied. Thymocytes from placebo and estrogen-treated mice were isolated by gently meshing on a sterile 60 mesh screen and washed repeatedly before use[2,17,18]. Splenic lymphocytes were isolated according to our previously reported procedures[2,17]. Briefly, spleens were gently teased on sterile 60 mesh steel screen (Sigma, St Louis, MO) and washed in RPMI 1640 media (Cell Gro, Mediatech, Herndon,VA). Lymphocytes were enriched by either depletion of erythrocytes by ACK lysis or by centrifugation over Lympholyte-M according to manufacturer's instructions (1,500 Xg at room temperature for 25 minutes) (Accurate Chemical & Scientific Corp. NY). Lympholyte-M procedure yielded fewer cells, the pattern of response, however, in relation to estrogen effects on cytokines was similar in lymphocytes derived from both isolation procedures.

Splenic T cells were enriched by negative selection using complement-mediated lysis of non-T cells. B cells and residual RBCs in unseparated splenic cells were targeted for removal by anti-heat-stable antigen monoclonal antibody [J11d.2 cell line produces monoclonal antibodies (IgM isotype) against mouse cortical thymocytes, erythrocytes, neutrophils and B (TIB-183, ATCC, MD)], while macrophages and B cells were targeted for elimination by the binding to anti-Class II antibody [mouse anti-mouse monoclonal antibody against I-A^d (IgG2a isotype), purified from cell line 25-9-17S II (HB-26, ATTC, MD), and mouse anti-mouse monoclona antibody against I-A^d (IgG2a isotype), purified from cell line MK-D6, (HB-3, ATTC, MD)]. One ml of 1:20 diluted complement (Pel-Freez Biologicals, AK) was added to washed 10^7 cells and incubated at room temperature for 15 minutes. Cells were washed and purified T cells were resuspended in complete RPMI. Alternatively, splenic T cells were enriched by positive selection using MACS columns. Splenic lymphocytes isolated from Lympholyte-M method were resuspended in 90μl of MACS buffer (0.5% BSA, 0.5 mM EDTA in PBS). Ten μl of the magnetically labeled anti-CD90 (Thy 1.2) (Miltenyi Biotec, Auburn, CA) antibodies were added per 10^7 cells and incubated on ice for 15 minutes, according to the manufacturer's instructions. The MACS separation columns were washed

with 5 ml of MACS buffer and cells labeled with MACS anti-CD90 were added to column reservoir and subjected to magnetic field. Effluent was collected as the negative fraction. The trapped anti-CD90-labeled T-lymphocytes were removed by gentle washing in MACS buffer, washed in complete RPMI, adjusted to 5 X 10^6 cells/ml, and kept on ice until further analysis. Lymphocytes were counted and viability was assessed by trypan blue exclusion. The purity of cells was assessed by staining an aliquot of cells with FITC-anti-CD90 or a cocktail of FITC-anti-CD8 and PE-anti-CD4 antibodies and subsequently subjecting to flow cytometry analysis. Both procedures of splenic T cell enrichment (between 85 to 93 % purity) yielded similar patterns of expression of cytokines.

<u>Cultures of Lymphocytes</u>: One hundred μl of cells at a density of 5x10^6 cells/ml were added to 96-well sterile plates containing 100 μl of complete RPMI-1640 or Concanavalin A (Con A, 10 μg/ml). Cells were incubated at 37^0C, 5% CO_2 for defined time points. Thymocytes were cultured for 6, 12, 24 hours, while unseparated splenic lymphocytes and enriched T cells were cultured for 24, 48, and 72 hours. We chose to examine thymocytes at early time points compared to splenic lymphocytes, because a significant percentage of thymocytes are known to die by 48 hours of culture. In some studies, Con-A-stimulated splenic lymphocytes were collected at 6, 12, 24, and 48 hours of culture. After incubation of cultures, cells were collected and used for isolation of total RNA. Supernatants were collected for analysis of cytokine protein levels and stored at -20^0C.

<u>Cytokine Specific ELISA:</u> Sandwich ELISA assay was used to detect IFN-γ, IL-2, and IL-4 protein levels. Ninety-six well Maxisorp high binding immunoassay plates were coated overnight with anti-cytokine antibodies (anti-IFN-γ clone R4-6A2, ATCC, HB-170; anti-IL-2, clone JES6-1A12, Pharmingen; anti-IL-4 antibody, clone 11B11, ATCC, HB-188) in PBS and then blocked with 2% BSA for 1 hour. Plates were overlaid with 50 μl of 2% BSA and 50 μl of supernatants with necessary dilutions or 50 μl of recombinant cytokines with serial dilutions for the development of a standard curve (r-IL2, r-IL4, r-IFN-γ, Pharmingen, San Diego, CA). Plates were incubated at room temperature for 2 hours and then washed with (50mM Tris-HCl, 2% Tween20) washing solution. Biotin conjugated anti-cytokine antibodies

(anti-IL-2, anti-IL-4, anti-IFN-γ, Pharmingen, San Diego, CA) were applied to the plates and incubated for 1 hour. After 1 hour incubation, plates were washed again and overlaid with avidin-horseradish peroxidase solution (Vector Labs) for 1 hour. After a final wash, substrate TMB was added for 30 minutes and 0.18 M sulfuric acid was added to stop the reaction. Plates were read at 450 nm by ELISA reader (Molecular Devices). Protein amount was calculated by the formula obtained from standards using SoftMax Pro software from Molecular Devices Inc.

Determination of Cytokine Gene Expression: Cells were subjected to RNA isolation using TRIzol reagent (Gibco BRL, Life Technologies) as described in our previous studies[19]. One ml of TRIzol was added to pellets of $5x10^6$ cells. Homogenized samples were incubated for 5 minutes and 0.2 ml of chloroform added. Samples were centrifuged at 12,000 Xg for 15 minutes at 2 to 8^0C. After centrifugation, the upper phase was transferred to a fresh tube and RNA precipitated with 0.5 ml of isopropanol by centrifugation at 12,000 Xg for 10 min at 2 to 8^0C. The RNA pellet was washed with 75% ethanol and centrifuged at 7,500 Xg for 5 minutes. The RNA pellet was dried and resuspended in 50 μl of RNase free sterile water. The RNA samples were read at 260 and 280 nm, and the amount of RNA was calculated and stored at -70^0C. The RT-PCR reaction was used to detect β-actin, IL-2, IL-4 and IFN-γ mRNA expression. For the synthesis of cDNA, total RNA was suspended in 40 μl reaction solution containing 2.0 μg oligo $(dT)_{12-18}$ (Gibco BRL, Gaithersburg, MD), 1.25 mM of dNTP, 20 units of Rnasin (Promega Corp., Maddison, WI), 1X RT buffer, 4.0 μl of 0.1 M DTT and 1.5 μl (300 units) of moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD). The reaction mixture was incubated at 37^0C for 1 hour. The PCR reactions were performed in 25 μl volume, containing 5 μl of cDNA samples. The reaction mixture had 2.5 mM $MgCl_2$, 0.25 mM of dNTP, 1X PCR buffer, 1 μl of each specific primer, and 0.2 μl (1.0 unit) of Taq DNA Polymerase (Gibco BRL, Gaithersburg, MD). The PCR reactions were performed under the following conditions: 30 cycles of 94^0C for 1 minute, annealing at 55^0C and amplification at 72^0C for 1.5 minutes. The final extension was done for 10 minutes at 72^0C. Twelve microliters of PCR products were electrophoresed in 1.5 % agarose gel containing ethidium bromide (0.45 μg/ml). For analysis of cytokine mRNA

expression, photographs were taken and RT-PCR products were evaluated using scanning densitometry (Molecular Dynamics, CA). The data was normalized to β-actin and expressed as relative densities.

Determination of intracellular IFN-γ protein: Western blot assay was used to detect the IFN-γ protein level in whole cell lysates of placebo and estrogen-treated enriched T and unseparated splenic lymphocytes. Enriched T and unseparated lymphocytes were cultured with Con-A (10 μg/ml) at 37^0C, 5% CO_2 for defined time points. Enriched T cells and unseparated splenic lymphocytes were cultured for 24 or 48hours, respectively. At the end of the incubation period, cells were harvested and resuspended in 25μl of lysis buffer (50mM Tris pH: 7.4, 300mM NaCl, 2mM EDTA pH:8, 0.5% Triton X-100, freshly added mammalian protease inhibitor cocktail (10μg/ml, Sigma), vortexed, and incubated on ice for 15 minutes. Ten μl of 3x sample buffer (150 mM Tris pH: 6.8, 30% glycerol, 6% SDS, 50mM DTT, bromophenol blue) was added to 20 μl of lysate and heated at 95^0C for 5 minutes. Samples were electrophoresed on a 12.5% SDS-PAGE gel at 25mA constant current until dye runs of the bottom. The protein was transferred to nitrocellulose membrane (BioRad, CA) by blotting for 1.5 hours at 250mA constant current. After transferring the proteins to nitrocellulose, the membrane was blocked in TBST-5% milk for 1 hour at room temperature. The blot was incubated with IFN–γ primary antibody (goat polyclonal IgG (D-17), Santa Cruz Inc., CA) diluted in blocking buffer (1:1000) for 1 hour on the rocking platform. The blot was rinsed briefly in TBST, then washed 2 times for 10 minutes with TBST. The secondary HRP-conjugated antibody (anti-goat IgG, 1:1000, Santa Cruz Inc., CA) was applied in blocking buffer to each blot and incubated for 1 hour. After blots were washed, they were labeled using ECL protocol (Amersham Pharmacia Biotech, NJ).

Statistics: The data were analyzed by using SAS software (SAS Institute, Cary NC, Version 6.12) at the Laboratory for Study Design and Statistical Service, VMRCVM. The ANOVA for SPLIT-PLOT DESIGN was used to assess the statistical validity of the data. Estrogen/placebo-treatment was considered as whole plot and time as sub-plot. Statistical significant interactions were further investigated using SLICE option to test the simple main

effects. A similar analysis was also performed for impact of gender on cytokine expression profile. All values are expressed as mean and SEM.

d.) RESULTS

Estrogen increases IFN-γ and IL-2 mRNA in activated thymocytes: Thymocytes activated by Con-A had an overall increase in IFN-γ mRNA and IL-2 mRNA ($p<0.05$) (Fig 1A). Kinetic studies revealed that this tendency for increased IFN-γ and IL-2 mRNA was evident at all time points studied, especially by 24 hours (Fig 1B and C). In contrast to IFN-γ and IL-2 mRNA, estrogen did not appear to have any noticeable effects on IL-4 mRNA gene expression (Fig 1A and D).

Estrogen also increased IFN-γ and IL-2 mRNA in splenic lymphocytes: Since estrogen increased IFN-γ mRNA and IL-2 mRNA in a primary lymphoid organ (thymus), we next investigated whether this hormone has similar effects in a secondary mature lymphoid organ (spleen). Con-A activated splenic lymphocytes from estrogen-treated mice had increased IFN-γ mRNA and IL-2 mRNA expression compared to placebo treated mice ($p<0.05$) (Fig 2A). As noticed in thymocytes estrogen did not appear to affect IL-4 mRNA (Fig 2A). Since IFN-γ is primarily secreted by T cells, we next investigated whether estrogen augments IFN-γ and IL-2 mRNA in splenic enriched T cells. This pattern of increased IFN-γ mRNA and IL-2 mRNA in estrogen-treated mice was also noted in enriched splenic T cells ($p<0.05$), (Fig 2B). IL-4 mRNA in Con-A activated splenic enriched T lymphocytes was not augmented by estrogen (Fig 2B).

Estrogen treatment increases IFN-γ protein levels: Supernatants of Con-A stimulated thymocytes from placebo or estrogen-treated mice did not have detectable levels of IFN-γ, IL-2 or IL-4 cytokines (data not shown). In the supernatants of Con-A-stimulated splenic lymphocyte from estrogen treated mice, the levels of IFN-γ protein were appreciably increased (Fig 3A). IL-2 protein levels, however did not differ between estrogen treated and placebo treated mice (Fig 3A). The IFN-γ protein levels in the supernatants of enriched T cells versus unseparated splenic lymphocytes from placebo-treated mice were comparable (Mean

IFN-γ in enriched T cells was 1765.87 pg/ml, while the mean IFN-γ in unseparated splenic lymphocytes was 1405.6 pg/ml). However, the IFN-γ in the supernatants of enriched T cells from estrogen-treated mice was noticeably decreased compared to the IFN-γ levels in the supernatants of unseparated splenic lymphocytes after estrogen treatment (Mean IFN-γ in enriched T cells was 2009.11 pg/ml, mean IFN-γ in unseparated splenic lymphocytes was 3666.1 pg/ml), (Fig 3A and B). Estrogen promotion of IFN-γ and IL-2 was not evident in enriched T cells (Fig 3B). IL-4 protein levels were not detectable in supernatants of all cultures.

Gender differences in cytokine profile: To investigate the physiological effects of estrogen on IFN-γ, we next analyzed whether there are gender differences in the cytokine profiles of untreated males and female C57BL/6 mice. In this part of the study, we also investigated whether endogeneous female sex hormones modulate cytokine gene/ protein expression earlier than 24 hours (6 and 12 hrs). This rationale is supported by earlier studies, which showed that intracellular IFN-γ in Con-A-stimulated splenic lymphocytes from C57BL/6 mice was evident at low levels by 8 hrs, peak levels between 24-30 hrs and low levels at 72 hrs[20]. Untreated intact female C57BL/6 mice tended to have higher IFN-γ mRNA than their male counterparts (Fig 4A). Pronounced gender differences were not evident with regards to IL-2 mRNA (Fig 4A and C). IL-4 was not studied since in our previous experiments, estrogen treatment did not significantly affect IL-4 either at mRNA level or at protein level. The levels of IFN-γ protein secretion was appreciably higher in the supernatants of untreated female C57BL/6 mice compared to the males in the supernatants of Con-A stimulated splenic lymphocytes (Fig 4D). A numerical difference in IFN-γ protein levels was apparent by 24 hours of culture. By 48 hours, both sexes had high levels of Con-A induced IFN-g (data not shown). Supernatants of Con-A-activated splenic lymphocytes from intact, untreated female C57BL/6 mice had higher levels of IL-2 secretion by 24 hours of culture ($p<0.05$, Fig 4F). A similar pattern was evident at 48 hours of culture (data not shown).

e.) DISCUSSION

The immunological consequences of chronic estrogen exposure, particularly with regards to the expression of cytokines are not known. To address this issue, our laboratory is actively engaged in conducting a series of studies where 17-β-estradiol (as a model of other types of estrogen) is given to normal mice for 4-6 months. The present report focused on whether estrogens modulate the expression of three major cytokines (IFN-γ, IL-2 and IL-4) at both mRNA and protein levels in the splenic and thymic lymphocytes, an aspect not comprehensively studied thus far.

Our data shows that estrogen significantly increased the level of expression of IFN-γ and IL-2 mRNA, but not IL-4 mRNA, in Con-A-stimulated lymphocytes. This pattern of estrogen-induced increase in IFN-γ and IL-2 mRNA was evident in Con-A activated lymphocytes from both developmental lymphoid organ (thymocytes), as well as mature lymphoid organ (unseparated lymphocytes and enriched T cells from spleen (Figures 1A, 2A and 2B). This increase in IFN-γ and IL-2 mRNA levels may suggest a skew towards T helper 1 cells. The supernatants of Con-A-stimulated splenic lymphocyte from estrogen-treated C57BL/6 mice also had increased levels of IFN-γ indirectly suggesting increased translation of IFN-γ gene. The IL-4 protein was not detectable in the supernatants of Con-A-stimulated splenic lymphocytes from either sham or estrogen treated C57BL/6 mice. Our observations of estrogenic modulation of cytokines are supported by earlier studies. For e.g., direct exposure of T cell clones from multiple sclerosis patients to 17-β-estradiol increased the secretion of IFN-γ and IL-10, but not IL-4 and TGF-β1[21]. Short-term estrogen treatment of CD-1 mice also increased IFN-γ mRNA expression in Con-A stimulated unseparated splenic lymphocytes[22]. Human peripheral blood mononuclear cells (PBMC) from non-pregnant women that were cultured *in vitro* with estrogen secreted higher levels of spontaneous and staphylococcal enterotoxin A-induced IFN-γ protein compared to cultures from men[23].

As indicated above, our studies showed that unseparated splenic lymphocytes derived from estrogen-treated mice, when activated with Con-A, had significantly increased overall expression of IFN-γ mRNA and a numerical increase in the overall levels of IFN-γ. While Con-A activated enriched splenic T cells from estrogen treated C57BL/6 mice, had significantly increased expression of IFN-γ mRNA expression ($p<0.05$), the levels of IFN-γ protein in their supernatants after Con A stimulation were not increased. These findings suggest that for optimal induction of IFN-γ by estrogen, interaction of T cells with antigen presenting cells is necessary. In this regard, it is now well-known that optimal activation of naïve $CD4^+$ T cell requires a first signal through TCR-CD3 complex and a second co-stimulatory signal, which can be provided by the engagement of B7.1 or B7.2 on antigen presenting cells with CD28 on T cells. This signal results in the production of cytokines such as IL-2, which promotes the differentiation of cells[24] that secrete cytokines such as IFN-γ or IL-4[24,12]. Cytokines such as, IL-12 and/or IL-18 derived from antigen presenting cells are also known to promote IFN-γ. Further, studies showed that purified T cells from human peripheral blood mononuclear cells that are stimulated via TCR/CD3 complex and a P815 mastocytoma cell transfected with CD40 had enhanced secretion of IFN-γ and IL-2 proteins compared to purifed T cells stimulated only via TCR/CD3 complex[25]. A second APC-dependent signal such as CD40/CD40L ligand interaction is likely another pathway in optimal cytokine secretion. The effect of estrogen on co-stimulatory signals that regulate IFN-γ is currently under investigation in our laboratory. In this study, Con-A (the first signal) activation of enriched T cells from estrogen treated mice may increase the expression of IL-2 and IFN-γ mRNA. A second APC-dependent signal (presumably through co-stimulatory molecules) is needed for optimal IFN-γ secretion, suggesting that estrogen may facilitate APC-derived positive signals.

Estrogen increased IL-2 mRNA expression in activated lymphocytes, but had no significant effects on protein levels of IL-2. One possibility is that secreted IL-2 may be biologically used by T cells, which inturn induces the production of IFN-γ. Alternatively, the possibility that IL-2 is bound to soluble IL-2 receptors cannot be excluded. Statistically, estrogen had no influence on IL-4 mRNA, although on occasion we noticed a decreased IL-4 mRNA

expression in estrogen-treated mice. Further, IL-4 protein levels could not be detected in the supernatants of Con-A-activated splenic lymphocytes of C57BL/6 mice**.** Others have also shown that there were no gender differences in IL-4 expression in (NZB X NZW)F1 and DBA/2 strains of mice[26]. Other studies have also reported that C57BL/6 splenic lymphocytes stimulated by Con-A induced only low levels of IL-4 protein in the cytoplasm[20]. However, IL-4 can be induced in C57BL/6 under certain conditions such as during pregnancy and/or infection with *Leishmania major*[27,28], or in lymph nodes of adult mice after diethylstilbestrol treatment (unpublished observations).

Previous studies from our laboratory have shown that estrogen administration to normal mice induces hyperactivity of splenic B lymphocytes, characterized by increased production of autoantibodies to mouse erythrocytes[2], dsDNA[16], phospholipids[10,18,29] and an increased output of autoantibodies from plasma cells[18,30]. Based on these findings, we had anticipated that Th-1-type cytokines (IFN-γ and/or IL-2) to be decreased or unaltered, while a Th-2-type cytokine (IL-4) to be increased by estrogen. It is likely that estrogen may increase other Th-2-type cytokines such as IL-6 or IL-5 that affect B cells, an aspect that needs to be investigated in future studies. In this regard, estrogen has been shown to increase IL-5 mRNA in a T cell line but decrease IL-6 in bone marrow stromal cells, osteoblastic cells, and uterine epithelial cells[31]. The possibility that estrogen may promote IL-4 in strains other than in C57BL/6 strain of mouse cannot be discounted. Recent studies have ascribed a new role for IFN-g effects on B cells. For example, IFN, including IFN-γ, has been shown to increase the viability of B cells[32,33]. In separate studies, we find that estrogen-treated B cells, upon activation, are resistant to apoptosis and tend to have increased viability (unpublished studies). It is likely that estrogen-induced increase in IFN-γ may, in part, increase the survival of B cells including autoimmune clones, an area currently being examined in our laboratory. The increase in IFN-γ by estrogen may be of significant value since, this cytokine has been incriminated in a number of autoimmune conditions, which occur predominantly in the female gender group. In several animal models of autoimmunity, IFN-γ appears to play a role in the development of lupus nephritis[34], Sjogren's syndrome[35], autoimmune arthritis[36], and autoimmune insulitis[37].

It is relevant that gender differences in levels of cytokines have also been noted. Female mice, BALB/c, C57BL/6J, (NZB X NZW)F1 and CBA/Ca strains, produce more IFN-γ protein compared to males in response to stimulation by mycobacteria, Con-A or viruses[26,38,39,40,41]. Unstimulated T cells of (NZB X NZW)F1 females also showed increased *in situ* expression of IFN-γ transcripts even at very early age and throughout their life[42]. Interestingly, in one study, higher IL-2 levels in supernatants of Con-A-stimulated lymphocytes were evident in 26-month old female Fischer 344 rats compared to its male counterparts, but not in 3-month old rats[43]. The synthesis of IL-2 receptor (IL-2R) on unfractionated spleen cells followed a course essentially similar to that of IL-2, i.e. higher in female rats compared to males[44]. The secretion of IL-4, a prototype Th-2 cytokine, was found to be higher in female C3H/HeN strain of normal mice compared to males[45]. Our studies also show that there was a noticeable trend of increase in IFN-γ mRNA, and IL-2 and IFN-γ proteins in untreated female C57BL/6 mice compared to males, implying that female sex hormones, such as estrogen, may play a physiological role in regulating these cytokines. Taken together, our studies are a first report to show that estrogen can regulate immunoregulatory cytokines, notably IFN-γ and IL-2, in both mature and developmental lymphoid organs.

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f.) REFERENCES

1. Ansar Ahmed S, Penhale WJ, and Talal N (1985) Sex hormones, immune responses and autoimmune diseases. Am.J. Pathol. 121: 531-551.
2. Ansar Ahmed S, Dauphinee MJ, Montoya AL, Talal N (1989) Estrogen induced normal murine CD5+ B cells to produce autoantibodies. J. Immunol. 142:2647-2653.
3. Ansar Ahmed S, Hissong BD, Verthelyi D, Donner K, Becker K, Karpuzoglu-Sahin E. (1999) Gender and risk of autoimmune diseases: possible role of estrogenic compounds. Environ Health Perspect ;107 Suppl 5:681-6.
4. Olsen NJ, Kovacs WJ (1996) Gonadal steroids and immunity. Endocr Rev. 17:369-84
5. Butterworth M, McClellan R, Allensmith M (1967) Influence of sex on immunoglobulin levels. Nature 214: 1224-1225.
6. Amadori A, Zamarchi R, Desilvestro G, Forza G, Cavatton G, Danielli GA, Clementi M, Chieco-Bianchi L (1995) Genetic control of the CD4/CD8 T cell ratio in humans. Nature Med.1: 1279-1283.
7. Ansar Ahmed S. (2000) The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology. 7;150:191-206.
8. Lahita RG, Bradlow HL, Ginzler E, Pang S, New M (1987) Low plasma androgens in women with systemic lupus erythematosus. Arthritis Rheum. 30:241-8
9. Beeson PB (1994) Age and sex associations of 40 autoimmune diseases. Am J Med. 96: 457-462.
10. Ansar Ahmed S, and Verthelyi D (1993) Anticardiolipin autoantibodies in normal C57BL/6J mice: Induction by estrogen but not dihydrotestosterone. J Autoimmun. 6: 265-279.
11. Ansar Ahmed S, Talal N (1993) Importance of sex hormones in lupus. In: *Dubois' Lupus Erythematous* (Eds.) Wallace DJ and Hahn BH, Lea Fibiger 148-156
12. Boehm U, Klamp T, Groot M, Howard JC (1997) Cellular responses to interferon-gamma. Annu Rev Immunol. 15:749-95.
13. Ruemmele FM, Gurbindo C, Mansour AM, Marchand R, Levy E, Seidman EG (1998) Effects of interferon gamma on growth, apoptosis, and MHC class II expression of immature rat intestinal crypt (IEC-6) cells. J Cell Physiol. 176:120-6.

14. Cohen JJ, Gupta S (1996) Mechanisms of lymphocyte activation and immune regulation VI : cell cycle and programmed cell death in the immune system Advances In Experimental Medicine and Biology, Vol 406. New York : Plenum Press
15. Sugamura K, Asao H, Kondo M, Tanaka N, Ishii N, Ohbo K, Nakamura M, Takeshita T. (1996) The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. Annu Rev Immunol. 14:179-205.
16. Verthelyi D, Ansar Ahmed S. (1994) 17 beta-estradiol, but not 5 alpha-dihydrotestosterone, augments antibodies to double-stranded deoxyribonucleic acid in nonautoimmune C57BL/6J Endocrinology. 135:2615-22.
17. Ansar Ahmed S, Gogal JrRM, and Walsh JE (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]thymidine incorporation assay. J Immunol Meth. 170: 211-224.
18. Verthelyi D, Ansar Ahmed S. (1997) Characterization of estrogen-induced autoantibodies to cardiolipin in non-autoimmune mice. J Autoimmun. 10:115-25.
19. Zhi-Jun Y, Sriranganathan N, Vaught T, Arastu SK, Ansar Ahmed S. (1997) A dye-based lymphocyte proliferation assay that permits multiple immunological analyses: mRNA, cytogenetic, apoptosis, and immunophenotyping studies. J Immunol Methods. 210:25-39.
20. Sander B, Cardell S, Heremans H, Andersson U, Moller G (1989) Detection of individual interleukin-4 and gamma interferon-producing murine spleen cells after activation with T-cell mitogens. Scand J Immunol. 30:315-20.
21. Gilmore W, Weiner LP, and Correale J (1997) Effect of estradiol on cytokine secretion by proteolipid protein-specific T cell clones isolated from multiple sclerosis patients and normal control subjects. J. Immunol. 158: 446-451.
22. Fox HS, Bond BL, Parslow TG (1991) Estrogen regulates the IFN-gamma promoter. J Immunol. 146:4362-4367.
23. Grasso G, Muscettola M (1990) The influence of beta-estradiol and progesterone on interferon gamma production in vitro. Int J Neurosci. 51:315-7.
24. Lenschow DJ, Walunas TL, Bluestone JA (1996) CD28/B7 system of T cell costimulation. Annu Rev Immunol. 14:233-58.

25. Peng X, Kasran A, Warmerdam PA, de Boer M, Ceuppens JL (1996) Accessory signaling by CD40 for T cell activation: induction of Th1 and Th2 cytokines and synergy with interleukin-12 for interferon-gamma production. Eur J Immunol. 26:1621-7.
26. McMurray RW, Hoffman RW, Nelson W, Walker SE (1997) Cytokine mRNA expression in the B/W mouse model of systemic lupus erythematosus--analyses of strain, gender, and age effects. Clin Immunol Immunopathol. 84:260-8.
27. Krishnan L, Guilbert LJ, Russell AS, Wegmann TG, Mosmann TR, Belosevic M (1996) Pregnancy impairs resistance of C57BL/6 mice to Leishmania major infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines. J Immunol 156:644-52
28. Krishnan L, Guilbert LJ, Wegmann TG, Belosevic M, Mosmann TR (1996) T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. J Immunol. 156:653-62
29. Ansar Ahmed S., Boone J., and Verthelyi D (1993) Anticardiolipin antibodies in autoimmune-prone BXSB and MRL/LPR mice, and estrogen-treated normal C57BL/6 mice crossreact with other phospholipids. Int. J Immunopath and Pharm. 6:135-147.
30. Verthelyi D, Ansar Ahmed S. (1998) Estrogen increases the number of plasma cells and enhances their autoantibody production in nonautoimmune C57BL/6 Cell Immunol. 189:125-34.
31. Sullivan DA. (1997) Sex hormones and Sjogren's J Rheumatol. Sep;24 Suppl 50:17-32.
32. Johnson-Leger C, Hasbold J, Holman M, Klaus GG (1997) The effects of IFN-gamma on CD40-mediated activation of B cells from X-linked immunodeficient or normal mice. J Immunol. 159:1150-9.
33. Snapper CM, Rosas F, Moorman MA, Jin L, Shanebeck K, Klinman DM, Kehry MR, Mond JJ, Maliszewski CR (1996) IFN-gamma is a potent inducer of Ig secretion by sort-purified murine B cells activated through the mIg, but not the CD40, signaling pathway. Int Immunol. 8:877-85.

34. Haas C, Ryffel B, Le Hir M (1997) IFN-gamma is essential for the development of autoimmune glomerulonephritis in MRL/Ipr mice. J Immunol. 158:5484-91.

35. Hayashi Y, Haneji N, and Hamano H (1996) Cytokine gene expression and autoantibody production in Sjogren's syndrome of MRL/lpr mice. Autoimmunol. 23:269-277.

36. Billiau A (1996) 4Interferon-gamma in autoimmunity. Cytokine Growth Factor Rev. 7:25-3

37. Campbell IL, Kay TW, Oxbrow L, Harrison LC (1991) Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. J Clin Invest. 87:739-42.

38. Savertnik N, Fox HS (1990) Interferon gamma and the sexual dimorphisim of autoimmunity. Mol. Biol. Med. 7:323-331.

39. Huygen K, Palfliet K (1984) Strain variation in interferon gamma production of BCG-sensitized mice challenged with PPD II. Importance of one major autosomal locus and additional sexual influences. Cell Immunol. 85:75-81.

40. Huygen K, Palfliet K (1983) Strain variation in interferon gamma production of BCG-sensitized mice challenged with PPD. I. CBA/Ca mice are low producers in vivo, but high producers *in vitro*. Cell Immunol. (2):329-34.

41. Satoskar A, Alexander J (1995) Sex-determined susceptibility and differential IFN-gamma and TNF-alpha mRNA expression in DBA/2 mice infected with Leishmania mexicana. Immunology. 84:1-4.

42. Sato MN, Minoprio P, Avrameas S, Ternynck T (1995) Defects in the regulation of anti-DNA antibody production in aged lupus-prone (NZB x NZW)F1 mice: analysis of T-cell lymphokine synthesis. Immunology. 85:26-32.

43. Davila DR, Kelley KW (1988) Sex differences in lectin-induced interleukin-2 synthesis in aging rats. Mech Aging Dev. 44:231-40.

44. Ramseier H, Lichtensteiger W, Schweidler R, Schlumpf MM (1993) Release of IL-1 and IL-2 and expression of IL-2 receptors as a function of postnatal age and sex. Eur Cytokine Netw. 4:453-9.

45. Araneo BA, Dowell T, Diegel M, Daynes RA (1991) Dihydrotestesterone exerts a depressive influence on the production of interleukin-4, interleukin-5, Interferon gamma but not interleukin-2 by activated murine cells. Blood 77:688-699.

g.) FIGURES

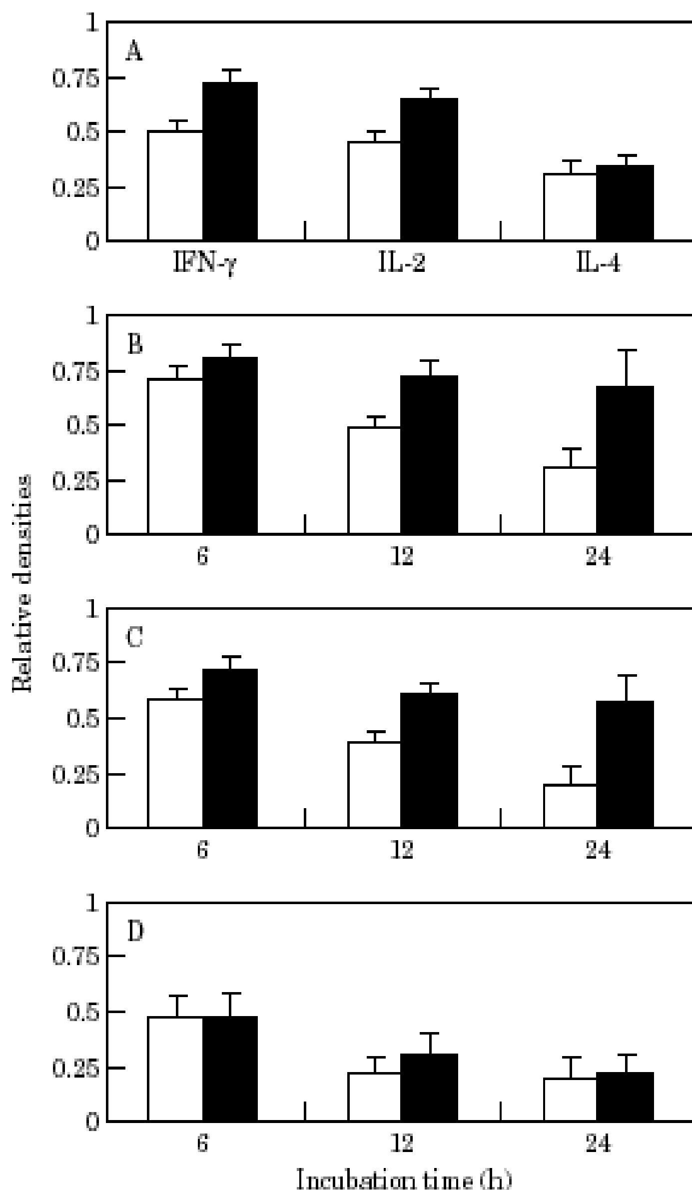

Figure 1. Estrogen affects IFN-γ and IL-2 mRNA expression in thymocytes of C57BL/6 mice. Thymocytes, from age-matched C57BL/6 orchiectomized mice that were implanted with17-β-estradiol or control (placebo) for 4–6 months, were stimulated with Con-A (10 μg/ml). The expression of IFN-γ, IL-2, and IL-4 specific mRNA in thymocytes cultured at 6, 12, and 24 h was detected by using RT-PCR. The PCR products were quantified by scanning densitometer and normalized using β-actin (a housekeeping gene), and were expressed as relative densities. Panel A depicts comparison of overall level of cytokine specific mRNA between estrogen- (■) and placebo-treated (□) thymocytes. The IFN-γ and IL-2 mRNA (Panel A, $P<0.05$) were significantly different in long-term estrogen-treated mice. IL-4 mRNA expression was essentially the same in both treatments (Panel A, $P>0.05$, not significant), (placebo $n=21$, estrogen $n=18$ for Panel A). Panels B and C, respectively, demonstrate that IFN-γ and IL-2 gene expression from estrogen-treated mice showed a tendency to increase especially at 24 h of incubation with Con-A. The expression of IL-4 mRNA in thymocytes from estrogen-treated mice was similar to thymocytes from placebo-treated mice at all times examined (Panel D, $P>0.05$), (placebo $n=7$, estrogen $n=6$ at each time point for Panel B, C, and D).

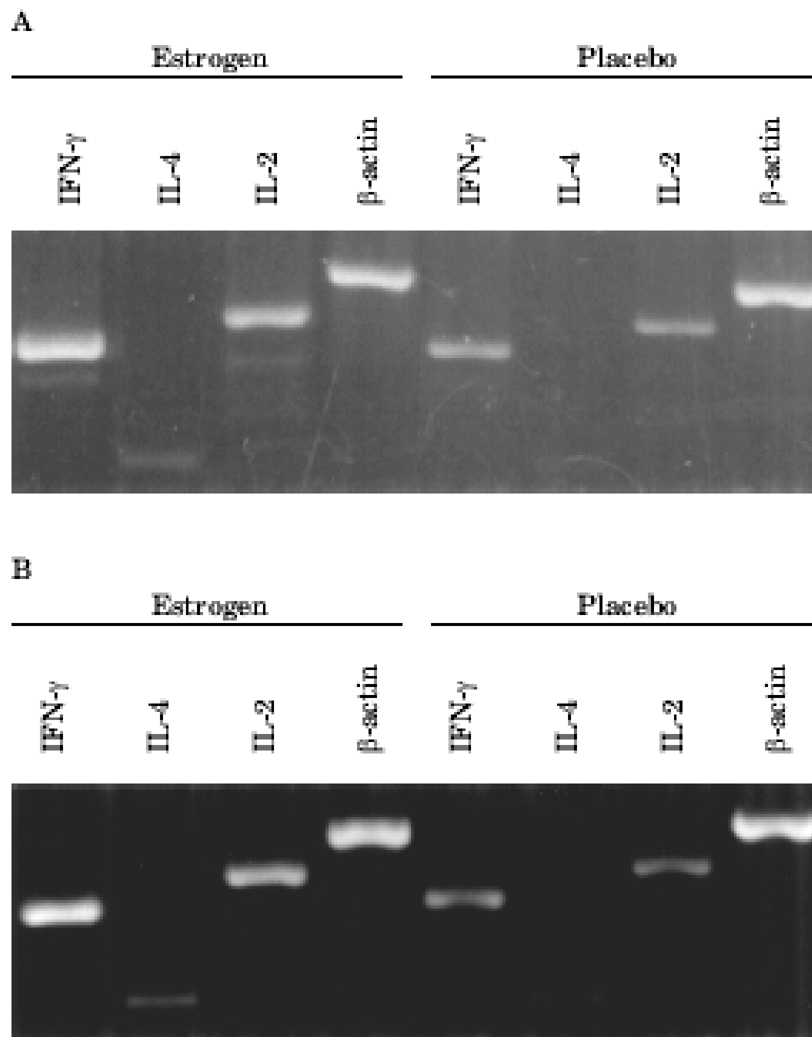

Figure 2. RT-PCR products from estrogen-treated C57BL/6 mice. Thymocytes, from age-matched C57BL/6 orchiectomized mice that were implanted with17-β-estradiol or control (placebo) for 4–6 months, were stimulated with Con-A (10 μg/ml). The expression of β-actin, IFN-γ, IL-2, and IL-4 specific mRNA in thymocytes and enriched splenic T lymphocytes cultured at 24 h was detected by using RT-PCR. The RT-PCR products were run on 1.5% agarose gel. The picture was a representative of seven experiments. Panel A represents the di•erences in gene expression of IFN-γ, IL-2, IL-4 and β-actin (housekeeping gene) between estrogen and placebo-treated cells thymocytes. Panel B shows the differences in gene expression of IFN-γ, IL-2, IL-4 and β-actin (housekeeping gene) in enriched T cells isolated from the spleens of estrogen- and placebo-treated mice. The IFN-γ and IL-2 mRNA were increased in long-term estrogen-treated mice. IL-4 mRNA expression was essentially the identical in both treatments.

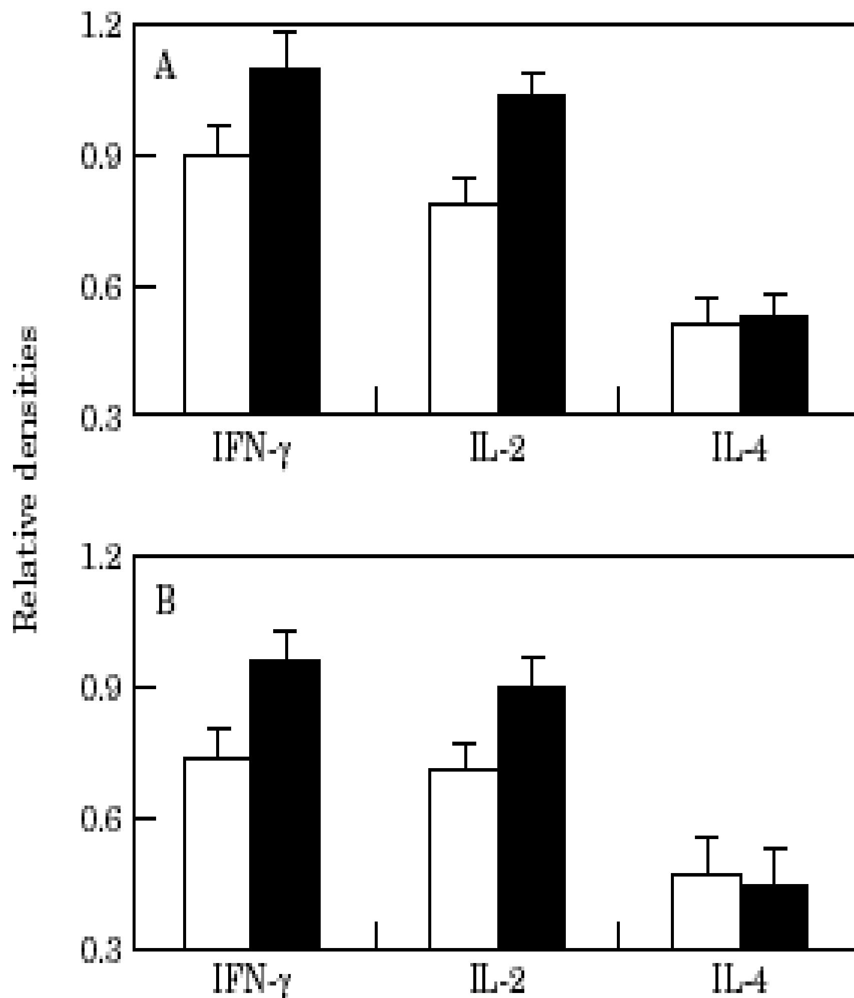

Figure 3. Splenic lymphocytes from estrogen treated C57BL/6 mice have increased IFN-γ and IL-2 mRNA expression. Unseparated splenic lymphocytes from age-matched C57BL/6 orchiectomized mice that were given 17-β-estradiol, (black-filled bars), or placebo-treated (empty bars) for 4–6 months were stimulated with Con-A. The expression of IFN-γ, IL-2, and IL-4 specific mRNA in lymphocytes cultured at 24, 48, and 72 h was detected by using RT-PCR. The PCR products were quantified by scanning densitometer, and normalized using β-actin (a housekeeping gene), and expressed as relative densities. Panel A represents combined data from Con-A-activated splenic lymphocytes at 24, 48, and 72 h of culture. The level of IFN-γ and IL-2 mRNA expression was significantly increased in unseparated splenic cells of estrogen-treated mice (Panel A, $P<0.05$). The expression of IL-4 mRNA was not significantly altered by estrogen (Panel A, $P>0.05$), (placebo $n=24$, estrogen $n=24$ for Panel A). Panel B shows the expression of IFN-γ and IL-2 mRNA in Con-A-stimulated splenic enriched T cells was also significantly increased ($P<0.05$) As noticed in unseparated splenic lymphocytes, estrogen did not markedly alter the overall levels of IL-4 mRNA in Con-A-activated splenic enriched T cells ($P>0.05$, not significant), (placebo $n=18$, estrogen $n=18$) for Panel B.

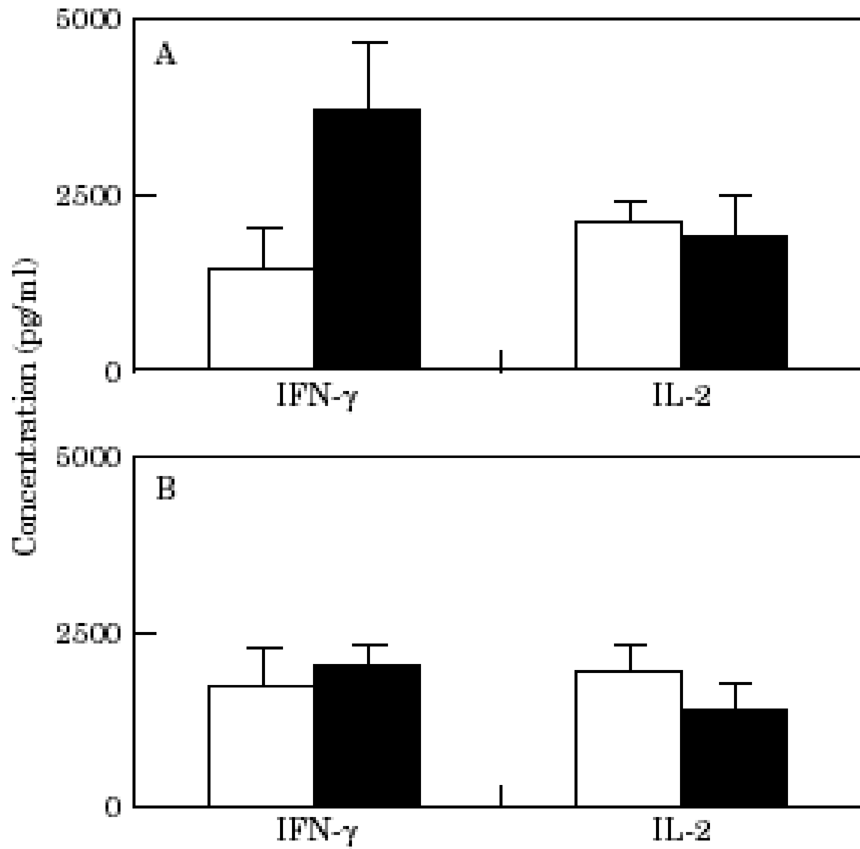

Figure 4. Effects of estrogen on IFN-γ and IL-2 protein production from splenic lymphocytes. Total unseparated splenic lymphocytes from age-matched C57BL/6 mice that were implanted with 17-β-estradiol, (black-filled bars) or control (placebo) (empty bars) for 4–6 months were stimulated with Con-A and supernatants were analyzed by ELISA as described in Materials and Methods. A pattern of increased levels of IFN-γ protein in the supernatants of estrogen-treated splenic lymphocytes was evident compared to placebo-treated cells (Panel A). Panel B shows the IFN-γ and IL-2 protein levels in the supernatants of enriched T lymphocytes from estrogen- and placebo-treated mice. No significant increase in IFN-γ was evident in splenic T cells from estrogen-treated mice. The P value was above 0.05 (placebo n=30, estrogen n=30 for Panel A and B).

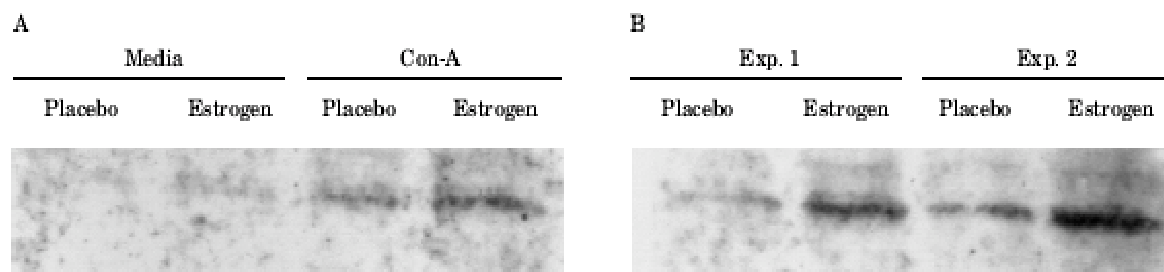

Figure 5. Intracellular IFN-γ protein levels. Unseparated splenic lymphocytes from age-matched C57BL/6 orchiectomized mice that were given 17-β-estradiol or placebo treatment for 4–6 months were stimulated with Con-A. The intracellular protein levels of IFN-γ in enriched T lymphocytes and unseparated lymphocytes cultured for 48 h were detected by using Western blotting. Panel represents data from unseparated (media) or Con-A-stimulated splenic lymphocytes at 48 h of culture. The level of IFN-γ was increased in unseparated splenic cells of estrogen treated mice (Panel A), representative of three experiments). Panel B shows the levels of IFN-γ protein in Con-A-stimulated enriched splenic T cells. The IFN-γ protein levels were appreciably elevated in estrogen-treated mice compared to placebo (control) mice (representative of three experiments).

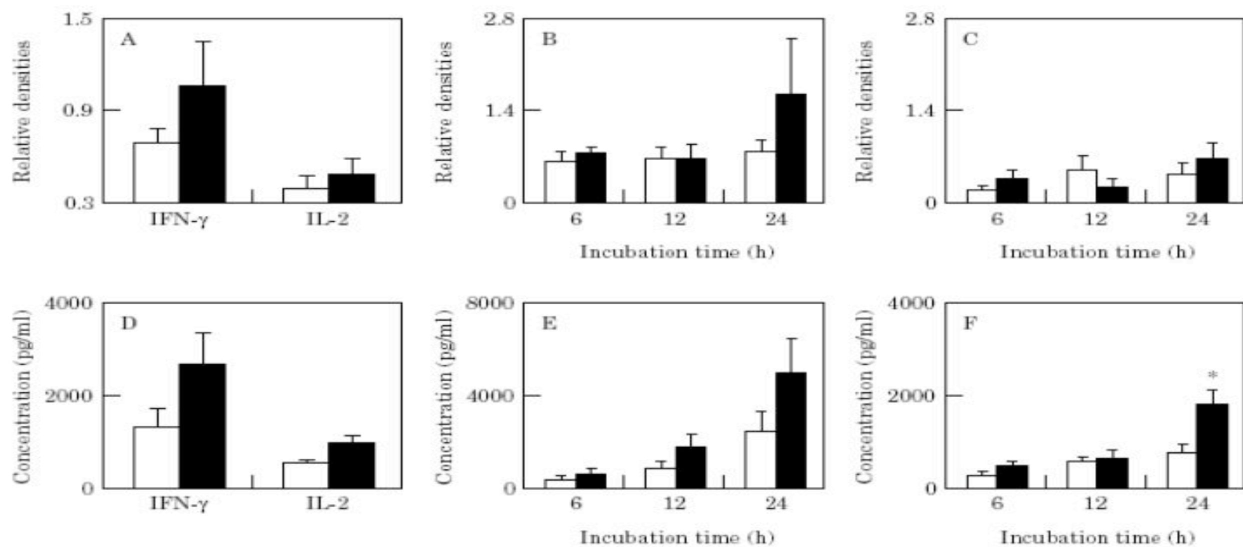

Figure 6. Effect of gender on cytokine gene expression. Total unseparated splenic lymphocytes from 3–4 months old male and female C57BL/6 mice were stimulated with Con-A. IFN-γ, and IL-2 mRNA expression was detected using RT-PCR as described in Materials, (empty bars) and Methods, (black-filled bars) section. The quantified PCR products were normalized using β-actin.The expression of IFN-γ mRNA in females showed a tendency to increase compared to males (Panel A) (male *n*=23, female *n*=23). The kinetic profile of the expression of IFN-γ showed a pattern of increase at 24 h in females (Panel B) IL-2 expression levels were nearly the same in both genders (Panel A and C), (*P*>0.05). Total unseparated splenic lymphocytes from 3–4 months old male and female C57BL/6 mice were stimulated with Con A and supernatants were analyzed by ELISA as described in Materials and Methods section [Male mice (6 h *n*=11, 12 h *n*=6, 24 h *n*=6), female mice (6 h *n*=9, 12 h *n*=6, 24 h *n*=6)]. Panel D represents combined data from Con-A-activated splenic lymphocytes at 6, 12 and 24 h of culture. The protein expression of IFN-γ in females was higher than in males (Panel D; male *n*=29, female *n*=29). The protein levels of IFN-γ in the supernatants of females demonstrated an increase especially after 24 h of incubation with the T cell stimulant (Panel E). The *P* value was above 0.05. The increase in IL-2 protein secretion in Con-A-activated splenic lymphocytes from females over time points may illustrate the efect of physiological levels of female sex hormones (Panel F). By 24 h of culture, IL-2 protein was significantly increased in the supernatants of untreated C57BL/6 female mice (Panel F, *P*<0.05), [male mice (6 h *n*=9, 12 h *n*=9, 24 h *n*=11), female mice (6 h *n*=9, 12 h *n*=9,24 h *n*=11) for Panels E and F].

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Interferon-γ levels are upregulated by 17-β-estradiol and diethylstilbestrol☆

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Abstract

Gamma-interferon (IFN-γ) plays an important role in the maintenance of immune homeostasis by regulating the functions of all key cells of the immune system. Pathologically, IFN-γ has been implicated in several autoimmune diseases. Since estrogens affect autoimmunity, we investigated whether immunomodulatory estrogenic hormones affects IFN-γ. Concanavalin-A-stimulated splenic lymphocytes from orchiectomized or ovariectomized C57BL/6 mice exposed to estrogen for 3–5 months secreted higher levels of IFN-γ protein compared to controls. This increase is, in part, due to increased levels of IFN-γ mRNA. Kinetic studies suggested that splenic lymphocytes from estrogen-treated gonadectomized mice had increased IFN-γ mRNA and protein as early as 6–12 h of culture. Estrogen also increased the expression of co-stimulatory CD80 (B7-1) molecules on B cells. Since natural estrogen increases IFN-γ, it became important to test whether diethylstilbestrol (DES, a synthetic estrogen which was given to millions of women) also alters IFN-γ levels. Our initial investigatory studies show that prenatal mice exposed to DES had a normal ability to secrete IFN-γ. However, a second exposure of these mice to DES (single dose of 1 μg/g.b.w), as late as 1–1.5 years of age, led to a pronounced increase in the number of IFN-γ secreting cells and augmented secretion of IFN-γ. Increased IFN-γ secretion by splenic lymphocytes from these mice was noted even after stimulation with a submitogenic concentration of anti-CD3 antibodies with or without anti-CD28 antibodies. Cell mixing experiments suggested that the

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a.) ABSTRACT:

Gamma-interferon (IFN-γ) plays an important role in the maintenance of immune homeostasis by regulating the functions of all key cells of the immune system. Pathologically, IFN-γ has been implicated in several autoimmune diseases. In this study, we investigated whether immunomodulatory estrogenic hormones affects IFN-γ. Concanavalin-A-stimulated splenic lymphocytes from orchiectomized or ovariectomized C57BL/6 mice exposed to estrogen for 3-5 months secreted higher levels of IFN-γ protein compared to controls. This increase is, in part, due to increased levels of IFN-γ mRNA. Kinetic studies suggested that splenic lymphocytes from estrogen-treated gonadectomized mice had increased IFN-γ mRNA and protein as early as 6-12 hours of culture. Estrogen also increased the expression of co-stimulatory CD80 (B7-1) molecules on B cells. Since natural estrogen increases IFN-γ, it became important to test whether diethylstilbestrol (DES, a synthetic estrogen which was given to millions of women) also alters IFN-γ levels. Prenatally DES-exposed mice had normal ability to secrete IFN-γ. However, a second exposure of these mice to DES (single dose of 1 μg/g.b.w), as late as 1-1.5 years of age, led to pronounced increase in the number of IFN-γ secreting cells and augmented secretion of IFN-γ. Increased IFN-γ secretion by splenic lymphocytes from these mice was noted even after stimulation with a submitogenic concentration of anti-CD3 antibodies with or without anti-CD28 antibodies. Cell mixing experiments suggested that DES-induced increase in IFN-γ secretion is due to hormonal effects on T cells but not on APC. Together our studies show that: (1) estrogens upregulate IFN-γ secretion, a vital immunoregulatory cytokine, and (2) inappropriate exposure of developing fetus to DES permanently alters the "cytokine programming" of lymphocytes.

Key Words: Estrogen, Diethylstilbestrol, Interferon-gamma, lymphocytes, IFN-γ gene expression

b.) INTRODUCTION:

We and others have shown that estrogens have a marked influence on both normal and autoimmune responses (*Ansar Ahmed et al, 1985, 1999, Grossman, 1984, Olsen and Novacs, 1996*). Extensive studies have suggested that exposure to estrogenic compounds can occur through sources other than endogenous natural estrogenic hormones (17-β-estradiol and its metabolites) (*Colburn et al, 1993, Stancel et al, 1995)*. These include intake of estrogens for medical reasons, such as estrogen replacement therapy in postmenopausal women, diethylstilbestrol (DES) in pregnant women, and estrogen-based oral contraceptives in pre-menopausal women. The exposure to estrogenic compounds for medical reasons is believed to involve millions of women. The therapeutic use of DES is now limited and its use in pregnant women is banned in USA. Nevertheless, concerns of its health effects on the first and second generation remain. Other potential sources of estrogenic compounds include the inadvertent exposure through contact with environmental estrogens in detergents, pesticides, or industrial by-products. Additionally, human exposure could potentially occur through consumption of water contaminated with ethynil estradiol (derived from metabolites of estrogen-based contraceptive drugs), phyto- and mycoestrogens. It is therefore important to investigate the immunomodulatory effects of estrogens. The precise mechanisms underlying the immunomodulatory effects of estrogens are not apparent. We have reasoned that one mechanism by which estrogens may influence the immune system is by regulating cytokines such as interferon-γ (IFN-γ).

Investigations on the effects of estrogen on the induction of IFN-γ are clearly important since this cytokine is a key immunoregulatory cytokine that is secreted primarily by activated CD4 (Th-1), CD8 and NK cells. Intensive research over the past two decades on IFN-γ has revealed that this cytokine affects all major cellular players of the immune system including macrophages, T, B and NK cells. For example, IFN-γ directly or indirectly enhances the expression of molecules such as MHC I, II, and B7, which are central molecules in immune responses (*Boehm et al, 1997, Ruemmele et al., 1998*). Recent studies have shown that IFN-γ increases HLA-G mRNA and protein in transfected mouse fibroblasts (*Chu et al, 1999*). At a biological level, IFN-γ mediates resistance against viral, intracellular bacterial and protozoal

infections. Pathologically, IFN-γ has also been implicated in several autoimmune diseases (*Haas et al., 1997, Hayashi et al., 1996*).

Considering the important biological effects of IFN-γ, our initial goal was to determine whether or not estrogenic hormones can alter IFN-γ. The immunological consequences of exposure to various estrogenic compounds are likely to vary, depending upon the type of estrogenic compounds and the life-period of exposure. Therefore, we investigated the effects of two estrogenic compounds, a natural estrogen (17-β-estradiol) and a synthetic estrogen (diethylstilbestrol or DES), on the induction of IFN-γ. To simulate the human condition, we administered 17-β-estradiol to adult normal mice, while DES was given to pregnant animals (fetal exposure). We had hypothesized that mice prenatally exposed to DES may be more immunologically sensitive to subsequent exposure of estrogenic compounds later in life (*Ansar Ahmed, 2000*). To test this hypothesis, prenatal DES-exposed mice were given a second dose of DES during late-adult life. Our studies suggest that both estrogenic compounds induce IFN-γ.

c.) MATERIALS AND METHODS:

Experimental Design: Normal 4-5-week-old male and female mice were gonadectomized and subcutaneously given either an empty silastic implant (placebo control) or an implant containing estrogen as per our previously reported procedures *(Ansar Ahmed et al., 1989, Ansar Ahmed & Verthelyi , 1993)* . These standard 4 mm implants, silastic medical grade tubing 0.062" internal diameter X 0.125" outer diameter, containing 3-5 mg of powdered 17-β-estradiol are designed to slowly release estrogen over a period of 3-5 months (*Roubinian et al, 1978*). In our previous studies, we found that the serum levels of 17-β-estradiol in estrogen-treated orchiectomized males (211-425 pg/ml) at 3-5 months of treatment was comparable to female mice (345pg/ml) (*Ansar Ahmed et al, 1989*, *Verthelyi et al, 1994*). At the time of termination, 3-5 months after treatment, all mice were checked for the presence of estrogen implants and for biological evidence for estrogenic effects. These include an atrophied thymus, thick long-bones, an increased size of seminal vesicles in males and uteri in

females, and a distended urinary bladder. Two mice did not manifest the above estrogenic biological effects since they did not have estrogen implants, presumably implants were removed by these mice by self-biting, and hence were not included in the study.

Based on our preliminary studies, pregnant C57BL/6 mice were s/c given 7.5 μg of DES (Sigma, St. Louis, MO) in autoclaved corn oil (ICN, Aurora, OH), or corn oil only, at 14 and 17 days of gestation. The male and female offspring of these mice were given a second injection of DES, s/c, 1 μg/g.b.w. of DES (approx. 30 μg/ adult mouse) or oil at 12-16 months of age. Six days later, mice were terminated. Thus, there were four treatment groups in a 2x2 factorial array, which include: (1) prenatal DES/adult DES (DES/DES); (2) prenatal oil/adult DES (oil/DES); (3) prenatal DES/adult oil (DES/oil), and (4) prenatal oil/adult oil (oil/oil).

Splenic lymphocyte isolation and purification: Splenic lymphocytes from individual mice were gently isolated in RPMI-1640 media that is devoid of estrogenic phenol red (CellGro, Mediatech, Herndon, VA), as per our previous studies (*Ansar Ahmed et al., 1994, Donner et al., 1999*). The RBC were lysed by Tris-ammonium chloride lysis buffer (pH-7.2). The lymphocytes were washed in complete media, viability and numbers assessed by the trypan blue exclusion test and cells adjusted to a density of 5 X 10^6 cells/ml in complete RPMI. T cells were enriched by MACS magnetic microbeads conjugated to anti-mouse Thy1.2 antibodies (Miltenyi Biotec, Auburn, CA) and the eluted cells were enriched for antigen-presenting cells as described previously (*Karpuzoglu-Sahin et al. 2001*).

Phenotyping of lymphocytes by flow cytometric analysis: Splenic lymphocytes (100 ml of cells at 5 X 10^6 cells/ml) stimulated with an optimal dose of Con-A (10 μg/ml) for 48 hours were stained with 100 μl of an optimal concentration of PE-anti CD80 monoclonal antibodies plus FITC-anti-CD19, or species/isotype-matched control antibodies (BD-Pharmingen, San Diego, CA) according to our published procedures (*Ansar Ahmed et al., 1994, Donner et al., 1999*). Cells were analyzed on an EPICS XL-MXL flow cytometer (Coulter, Hialeah FL).

IFN-γ ELISA: To determine the kinetics of induction of IFN-γ, the supernatants of Con-A (10 mg/ml)-activated splenic lymphocytes (5 X 10^6 cells/ml) were collected at 6, 12, 24 and

48 hours of culture and frozen until use. In selected cultures, cells were stimulated with anti-CD3 antibodies (YCD3, a gift of Dr. Bottomly, Yale University) at a mitogenic (10μg/ml) or submitogenic (0.1 μg/ml) concentration). The cells were also stimulated with anti-CD3 (0.1 μg/ml) plus anti-CD28 antibodies (10 μg/ml). Subsequently, samples were thawed and levels of IFN-g assessed by a sandwich ELISA that was developed in our laboratory (*Karpuzoglu-Sahin et al., 2001*). Anti-IFN-γ antibodies (BD-Pharmingen), recombinant IFN-γ (as a positive control for obtaining the standard curve), were purchased from Pharmingen. The plates were read at 450 nm by an ELISA reader using Softmax software (Molecular Devices, CA).

<u>IFN-γ mRNA analysis by RT-PCR</u> The RNA was isolated by using TRIzol reagent (Gibco BRL, Life Technologies) and the absorbance at 260 and 280 nm was determined to estimate the amount of RNA and stored at -70°C, per our previous reports *(Karpuzoglu-Sahin et al., 2001, Yin Z-J et al. 1997*). The RT-PCR was performed by Ready-to-Go RT-PCR Beads (Pharmacia Biotech) using 25 pmol of primers of IFN-γ and house keeping gene, β-actin (Maxim Biotech Inc., San Francisco, CA.). Products were separated electrophoretically in a 2% agarose gel stained with ethidium bromide and visualized under UV light. Photographs of the gel were taken and the RT-PCR products evaluated using scanning densitometry (Molecular Dynamics, CA). The data was normalized to β-actin and expressed as relative densities.

<u>IFN-γ ELIspot</u>: A modification of the ELIspot procedure for antibodies, that we reported earlier (*Verthelyi & Ansar Ahmed 1998*), was performed. Briefly, Nunc Immulon 2 plates were coated with 50 μl of anti-IFN-γ (4 μg/ml, clone R4-6A2, BD-Pharmingen) in PBS, incubated at 4 C overnight and then blocked with 200 μl of 5% BSA in PBS for 2 hours at room temperature. After washing 5 times with 0.025% Tween-20, 100 μl of spleen cells (1 X 10^4 cell/well) from DES or oil treated mice and 100 μl of Con A (10 μg/ml) added. The plates were incubated at 37 C, 5% CO_2 for 6 h, washed and incubated with 100 μl of biotinylated anti-IFN-γ detecting antibody (0.5 μg/ml, clone XMG1.2, BD-Pharmingen) for 2 hours at room temperature. After washing, plates were incubated with 50 μl of streptavidin-

alkaline phosphatase (1:5000 dilution, BD-Pharmingen) for 2 hours at room temperature and washed. Finally, 50 µl of an agarose: 5-bromo-4-chloro-3-indolyl phosphate substrate solution was added, the plates incubated overnight, and the resulting spots counted over a light box using a magnifying lens.

Statistical analysis: The data were analyzed by using SAS software (SAS Institute, Cary NC, Version 6.12) at the Laboratory for Study Design and Statistical Service, VMRCVM. The ANOVA for SPLIT-PLOT DESIGN was employed. Estrogen/placebo-treatment was considered as whole plot and time as sub-plot. Statistics were performed after logarithmic transformation of the data.

d.) RESULTS:

Effects of 17-β-estradiol treatment of normal C57BL/6 mice on IFN-γ:

***(i) 17-β-estradiol treatment of orchiectomized male mice induces an earlier and sustained expression of IFN*-γ** . Supernatants of Con-A-activated splenic lymphocytes from 17-β-estradiol-treated orchiectomized males had increased levels of IFN-γ **(Figure 1A).** This increase was seen as early as 6-12 hours of culture, a pattern of estrogen-induced increase in IFN-γ also noted at 24 and 48 hours of culture. Analysis of data combined from 10 independent experiments, over the above time-points, showed that estrogen treatment of orchiectomized males significantly increased the levels of IFN-γ ($p=0.0421$) **(Figure 1B).** Estrogen also stimulated IFN-γ secretion by anti-CD3 antibody-stimulated splenic lymphocytes (data not shown).

(ii) 17-β-estradiol treatment of ovariectomized female mice induces an earlier and sustained expression of IFN-γ. We next performed kinetic studies to determine whether estrogen similarly increases IFN-γ in female ovariectomized mice. **Figure 1C** shows representative data from a representative experiment. The levels of IFN-γ in supernatants of splenic lymphocytes from estrogen-treated ovariectomized mice were increased at all time

points studied. This pattern of enhanced earlier and sustained increase in IFN-γ was similar to that noticed in male estrogen-treated mice. Since higher levels of IFN-γ were noted at 24 and 48 hours, subsequent studies were focused at these time points. As can be seen in **figure 1D,** estrogen treatment of ovariectomized mice significantly increased the IFN-γ levels at 24 and 48 hr (p=0.0105).

(iii) 17-β-estradiol increases IFN-γ mRNA. To investigate whether the estrogen-induced increase in IFN-γ is due to enhanced activation of the IFN-γ gene, the kinetics of relative levels of IFN-γ mRNA expression was determined. Con-A activated splenic lymphocytes from estrogen-treated orchiectomized mice exhibited increased expression of IFN-γ mRNA compared to placebo-treated mice. In general, high levels of IFN-γ mRNA were evident at 12 and 24 hours of culture **(Figure 2A and 2B)**. Similarly, Con-A-stimulated splenic lymphocytes from estrogen-treated ovariectomized mice had increased IFN-γ mRNA (data not shown).

(iv) 17-β-estradiol increases B7-1 expression: Since co-stimulatory molecules are thought to play a major role in the induction of IFN-γ, we next investigated whether 17-β-estradiol increases CD80 (B7-1) expression. Con-A-stimulated splenic lymphocytes from 17-β-estradiol-treated mice had increased percentages of B cells expressing B7-1 molecules **(Figure 2C)** (p<0.05). Ratios of CD80+ B cells to total number of lymphocytes are increased by estrogen treatment **(Figure 2D)** (p<0.05).

Effects of DES on IFN-γ

(i) Increased secretion of IFN-γ by splenic lymphocytes from prenatal DES-exposed mice becomes evident after a second exposure to DES is given during adult life: The ability of splenic lymphocytes from prenatal DES-exposed mice to secrete IFN-γ upon Con-A stimulation was largely unaltered during adult life (including at 1 to 1.5 years of age) compared to age and gender matched controls (data not shown). However, splenic lymphocytes from mice, which were prenatally exposed to DES and given a single DES

injection during adult life (DES/DES mice), had a markedly increased ability to secrete IFN-γ when exposed to Con-A **(Figure 3A)**. These mice had increased IFN-γ:IL-4 ratios, suggesting a bias towards development of a Th-1 phenotype **(Figure 3B)**. Our preliminary studies also suggested that this increased ability to secrete IFN-γ was also noted when splenic lymphocytes from DES/DES-exposed mice were stimulated with mitogenic or submitogenic concentration of anti-CD3 antibodies, with or without anti-CD28 (**Figure 3C**). These effects were not evident in mice prenatally exposed to oil but given DES during adult life (oil/DES), or in prenatally DES-exposed mice given oil in late adult-life (DES/oil). These central observations suggest that prenatal exposure to DES predispose these mice to respond aberrantly to a subsequent exposure of DES during adult life. Flow cytometric analysis of freshly isolated splenic lymphocytes from these mice suggested that key IFN-γ-secreting cells ($CD4^+$, $CD8^+$ or NK) were not selectively increased (data not shown). There were no differences in mitogen-induced lymphocyte proliferation in *adult* DES-treated mice. These immune effects were evident in DES/DES mice of both genders, particularly in males. Our initial studies suggest that DES/DES-exposed mice also had increased numbers of IFN-γ-secreting cells after 6 hours of stimulation with Con A (OIL/OIL: 20 ± 1; DES/DES: 51 ± 1) **(Figure 3D).** ELIspot results are presented as numbers of IFN-γ-secreting cells per 10^4 total lymphocytes.

(ii) Increased IFN-γ in DES/DES may be due to effects on T cells: Preliminary studies on reciprocal mixing of T and antigen presenting cells (APC) from oil/oil and DES/DES-exposed mice revealed that increased IFN-γ secretion in T cells from DES/DES-exposed mice was noticed regardless of co-culturing with APC from either DES/DES or oil/oil-exposed mice (**Figure 4**). This suggests an enhanced sensitivity of T cells, but not APC, to a second, late-life exposure to DES.

e.) DISCUSSION:

Previous studies have suggested that female autoimmune NZB/NZW mice have a higher ability to secrete IFN-γ compared to males (*McMurray et al, 1997*). The resistance of female ICR Swiss mice to the MM strain of encephalomyocarditis virus, compared to male mice, is attributed to their increased ability to secrete IFN-γ (*Curiel et al, 1998*). The present studies were designed to determine if estrogenic compounds could alter the expression of immunoregulatory IFN-γ. We find that estrogen increased the secretion of IFN-γ by Con-A activated splenic lymphocytes, which is in part due to enhanced expression of IFN-γ mRNA. The increased IFN-γ mRNA in activated splenic lymphocytes is in agreement with previous data which showed that estrogen increased IFN-γ promoter activity in lymphoid cells (*Sarvetnick and Fox., 1990*). We have recently observed that estrogen treatment of normal mice increased IFN-γ and IL-2 mRNA, but not IL-4 mRNA relative levels in activated thymocytes. This suggests that a bias towards Th-1 phenotype is also seen in developing T-cells (*Karpuzoglu-Sahin et al. 2001*). The present findings in both gonadectomized males and females suggest that the up-regulation of IFN-γ mRNA and protein is estrogen-dependent and not sex chromosomally determined.

Our laboratory is actively engaged in understanding the molecular mechanisms involved in estrogen-induced increase in IFN-γ . One possibility is that estrogen, in addition to having a direct effect on IFN-γ gene regulation, could enhance B7-1 expression, which interacts with co-stimulatory CD28 molecules to presumably increase IFN-γ. Our studies suggest that Con-A activated lymphocytes from estrogen-treated mice had an increased percentage of CD80+ (B7-1) B cells. In a related study, we found that co-stimulation of splenic lymphocytes from DES/DES-exposed mice with anti-CD28 plus anti-CD3 antibodies markedly enhanced the secretion of IFN-γ. An additional possibility is that estrogen-induced IFN-γ secretion may be due to a concurrent decrease in IL-10 or inhibitory IL-12 p40 homodimers and/or an increase in IL-12 p70. Our preliminary studies suggested that the supernatants of Con-A-stimulated lymphocytes did not have increased levels of IL-12 p40 homodimers. Estrogenic regulation of

IL-10 and IL-12 is suggested in a recent report which found that the antigen presenting cells from female SJL/J mice secreted less IL-10 and more IL-12 (*Wilcoxen et al, 2000*). One consequence of increased IFN-γ may be its effects on B cells. Recent studies have shown that IFN-γ enhanced the expression of the anti-apoptotic gene, *bcl-x_L* in B cells (*Hasbold et al, 1999)* and promoted the survival of B cells (*Johnson-Leger et al. 1997)*. We have noted that in other studies that B cells from estrogen-treated mice were resistant to apoptosis, especially when stimulated with anti-CD40 antibodies (*Ansar Ahmed et al 1999).* It is likely that IFN-γ may account, at least in part, for the increased survival of anti-CD40-stimulated B cells from estrogen-treated mice. While the present studies clearly show that estrogen chronic administration promotes the induction of IFN-γ, it is not known whether estrogen administered on a short-term basis will also similarly induce IFN-γ.

Diethylstilbestrol (DES), a synthetic estrogen was initially used as a therapeutic agent in a variety of clinical situations in women including threatened abortions and whenever estrogen-replacement therapy was indicated (*Golden et al., 1998, Herbst et al., 1971*). Five to 10 million Americans are believed to received DES during pregnancy or were exposed to the drug in utero (*Colburn et al., 1993*). Prenatal exposure of women to DES has been associated not only with neoplastic and reproductive disorders, but also immune abnormalities (*Golden et al., 1998, Herbst et al., 1971*). Limited studies in humans have suggested that DES exposed women developed a variety of autoimmune diseases (*Noller et al., 1998*). Mothers, daughters and sons exposed to DES reported more frequent respiratory infections (flu, colds), asthma, arthritis, and lupus (*Wingard and Turiel 1988*). Animal studies have confirmed that DES has profound immunomodulatory effects, including the induction of autoantibodies to cardiolipin (*Frosberg, 2000*). Our provocative findings suggest that high levels of IFN-γ secretion are noticed in prenatal DES-exposed individuals only when a second dose of DES is given during adult life. Prenatal DES exposure appears to pre-program the highly sensitive fetal immune system for augmented IFN-γ secretion when the individual is exposed to a second dose of an endocrine disrupting chemicals (EDC) later in life. This is termed "EDC-induced immunological imprinting". We recognize that it is unlikely that individuals prenatally exposed to DES will be subjected again to DES in adult life. It is emphasized that

DES is merely utilized as a model EDC, and given in a relatively low dose. The rationale for our studies that prenatal DES-exposed mice may respond abnormally to subsequent exposure of estrogenic compounds is also supported by observations in reproductive tissues. First, vaginal adenocarcinomas and immune abnormalities in prenatal DES-exposed females are noticed primarily in women, but not in prepubescent girls, suggesting that prenatal DES-exposure presets conditions, which are driven towards neoplasia, or immune abnormalities only when their endogenous female sex hormones are present at maturity. Uterine tissue from neonatally DES treated mice that were subsequently given estrogen had a significantly higher percentage of estrogen receptor mRNA, c-fos and c-jun, compared to controls, implying that prior exposure to DES alters the response to estrogen (*Kamiya et al., 1996*). Reproductive pathology was noticed only in uteri transplanted from prenatal DES, but not from control animals, into neonatal DES-treated Syrian hamsters (*Hendry et al., 1997*).

Overall, our studies clearly show that both natural and synthetic estrogens markedly up-regulate IFN-γ mRNA expression and protein secretion. These studies also suggest that splenic lymphocytes from DES/DES mice secrete high levels of IFN-γ after co-stimulation through CD28 molecules. Similar results were also obtained from splenic lymphocytes stimulated with Con-A and CD28 (data not shown). Preliminary studies suggested that the increased IFN-γ secretion by DES/DES lymphocytes is due to effects on T cells rather than on APC. These studies also suggest that immunological alterations in prenatal DES-exposed mice are not easily detectable by conventional immunological assays (proliferation, lymphocyte phenotyping), but become apparent by using sensitive IFN-γ analysis when T cells of DES-exposed mice are stimulated through specific molecules (e.g. TCR/CD3, CD28).

f.) REFERENCES:

Ansar Ahmed, S., Hissong, B.D., Verthelyi D., Donner, K.J., Becker, K., Karpuzoglu-Sahin, E., 1999. Gender and Risk of Autoimmune Disease. *Environ. Health Perspect*. 107,681-686,.

Ansar Ahmed S., Penhale W.J., 1985. Talal N. Sex hormones, immune responses and autoimmune diseases: *Am.J.Pathol.* 121:531-559

Grossman C., 1984. Regulation of the immune system by sex steroids. *Endocrinol. Review* 5: 435-454.

Olsen N.J., Kovacs W.J, 1996. Gonadal steroids and immunity. *Endocr Rev;* 17(4):369-84.

Colburn T.,vomSaal F.S., and Soto A.M. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Env. Health. Perspect.* 101: 378-384.

Stancel G.M., Boettger-Tong H.L., Chiappetta C., Hyder S.M., Kirkland J.L., Murthy L., Loose-Mitchell D.S. 1995. Toxicity of endogenous and environmental estrogens: what is the role of elemental interactions? *Environ Health Perspect*;103 Suppl 7:29-33,.

Boehm U, Klamp T, Groot M, Howard JC 1997. Cellular responses to interferon-gamma. *Annu Rev Immunol*. 15:749-95.

Ruemmele FM, Gurbindo C, Mansour AM, Marchand R, Levy E, Seidman EG 1998. Effects of interferon gamma on growth, apoptosis, and MHC class II expression of immature rat intestinal crypt (IEC-6) cells. *J Cell Physiol*. 176:120-6.

Chu W, Yang Y, Geraghty DE, Hunt , JS.,1999. Interferons enhance HLA-G mRNA and protein in transfected mouse fibroblasts. J Reprod Immunol 42:1-15

JS Haas C, Ryffel B, Le Hir M 1997. IFN-gamma is essential for the development of autoimmune glomerulonephritis in MRL/Ipr mice. *J Immunol*. 158:5484-91.

Hayashi Y, Haneji N, and Hamano H 1996. Cytokine gene expression and autoantibody production in Sjogren's syndrome of MRL/lpr mice. *Autoimmunol*. 23:269-277.

Ansar Ahmed, S., 2000. The Immune system as a potential target for environmental estrogens (endocrine disruptors): A new emerging field. *Toxicology,*150: 191-2006

Roubinian JR., Talal N., Greenspan JS, Goodman JR., Sitteri PC. Effect of castration and sex hormone treatment on survival, anti nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J. Exp. Med.* 147: 1508-1583, 1978.

Ansar Ahmed S. Dauphinee M.J., Montoya I.A., Talal N. 1989. Estrogen induces normal murine CD5+ B cells to produce antibodies. J.Immunol. 142:2647-2653.

Verthelyi D. and Ansar Ahmed S. 1994. 17β-estradiol but not 5-α-dihydrotestosterone induces anti-dsDNA in normal C57BL/6 mice. Endocrinology 135: 2615-2622.

Ansar Ahmed S, and Verthelyi D 1993. Anticardiolipin autoantibodies in normal C57BL/6J mice: Induction by estrogen but not dihydrotestosterone. *J Autoimmun*. 6: 265-279.

Ansar Ahmed S., Gogal R.M. Jr, Walsh J.E. 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to 3H-thymidine incorporation assay. *Journal of Immunological Methods* 170: 211-224.

Donner K.J., Becker K.M., Hissong B.D., Ansar Ahmed S. 1999. Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbestrol. Cytometry 35: 80-90.

Karpuzoglu-Sahin, E., Zhi-Jun, Yin., Sriranganathan, N., Ansar Ahmed,S. 2001. Effects of long-term estrogen treatment on IFN-γ, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. Cytokine, accepted pending revision.

Yin Z-J., Vaught T., Arastu S., Sriranganathan N., Ansar Ahmed S. A new non-radioactive assay that permits determination of proliferation, analysis of mRNA, chromosomal aberrations and flow cytometry of lymphocytes. Journal of Immunological Methods 210:25-39, 1997.

Verthelyi D.,and Ansar Ahmed S. Estrogen increases the number and activity of plasma cells and enhances their autoantibody production in non-autoimmune C57BL/6 mice. Cellular Immunology, 189: 125-134, 1998.

McMurray , R.W,. Hoffman , R.W., Nelson, W., Walker, S.E. 1997Cytokine mRNA expression in the B/W mouse model of systemic lupus erythematosus--analyses of strain, gender, and age effects.Clin Immunol Immunopathol 84: 260-8

Curiel, R.E., Mason, K.M., Dryden, T.D., Maurer, M.J., Bigley, N.J. 1998. Cytokines produced early in picornavirus infection reflect resistance or susceptibility to disease J Interferon Cytokine Res. 18: 587-96

Sarvetnick N and Fox H.S. 1990. Interferon-gamma and the Sexual dimorphism of autoimmunity. *Mol.Bio. Med.* 7; 321-331.

Wilcoxen, S.C., Kirkman, E., Dowdell, K.C., Stohlman, S.A., 2000. Gender-dependent IL-12 secretion by APC is regulated by IL-10. J. Immunol. 164: 6237-6243

Hasbold J., Hong J.S., Kehry M.R., Hodgkin P.D. Integrating signals from IFN-gamma and IL-4 by B cells: positive and negative effects on CD40 ligand-induced proliferation, survival, and division-linked isotype switching to IgG1, IgE, and IgG2a. J Immunol; 163(8):4175-81,

1999.

Johnson-Leger C., Hasbold J., Holman M., Klaus G.G. The effects of IFN-gamma on CD40-mediated activation of B cells from X-linked immunodeficient or normal mice. J Immunol 159(3):1150-9, 1997.

Golden R.J., Noller K.L., Titus-Ernstoff L., Kaufman R.H., Mittendorf R., Stillman R., Reese E.A. 1998. Environmental endocrine modulators and human health: an assessment of the biological evidence. *Crit Rev Toxicol* 28(2):109-227.

Herbst A.L. , Ulfelder H., Poskanzer D.C. 1971. Adenocarcinoma of the vagina. Association of maternal stilbesterol therapy with tumor appearance in young women. *New. Engl. J. Med.* 284:878-881,.

Noller K.L., Blair P.B., O'Brien P.C. Melton III L.J., Offord J.R., Kauffman R.H., Colton T. 1988. Increased occurrence of autoimmune disease among women exposed in utero to diethylstilbestrol. *Fertility & Sterility* 49: 1080-1082.

Wingard D.H., and Turiel J. 1988. Long-term effects of exposure to diethylstilbestrol. *West J Med* 149: 551-554.

Frosberg J.G. 2000. Neonatal estrogen treatment and its consequences for thymus development, serum level autoantibodies to cardiolipin, and the delayed-type hypersensitivity response. *J Tox Env Health.* 29.

Kamiya K., Sato T., Nishimura N., Goto Y., Kano K. Iguchi T. 1996. Expression of estrogen receptor and proto-oncogene messenger ribonucleic acids in reproductive tracts of neonatally diethylstilbestrol-exposed female mice with or without post-pubertal estrogen administration. *Exp Clin Endocrinol Diabetes*;104(2):111-22.

Hendry W.J. 3rd, Zheng X., Leavitt W.W., Branham W.S., Sheehan D.M. 1997. Endometrial

hyperplasia and apoptosis following neonatal diethylstilbestrol exposure and subsequent estrogen stimulation in both host and transplanted hamster uteri. *Cancer Res;* 57(10):1903-8.

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g). FIGURES

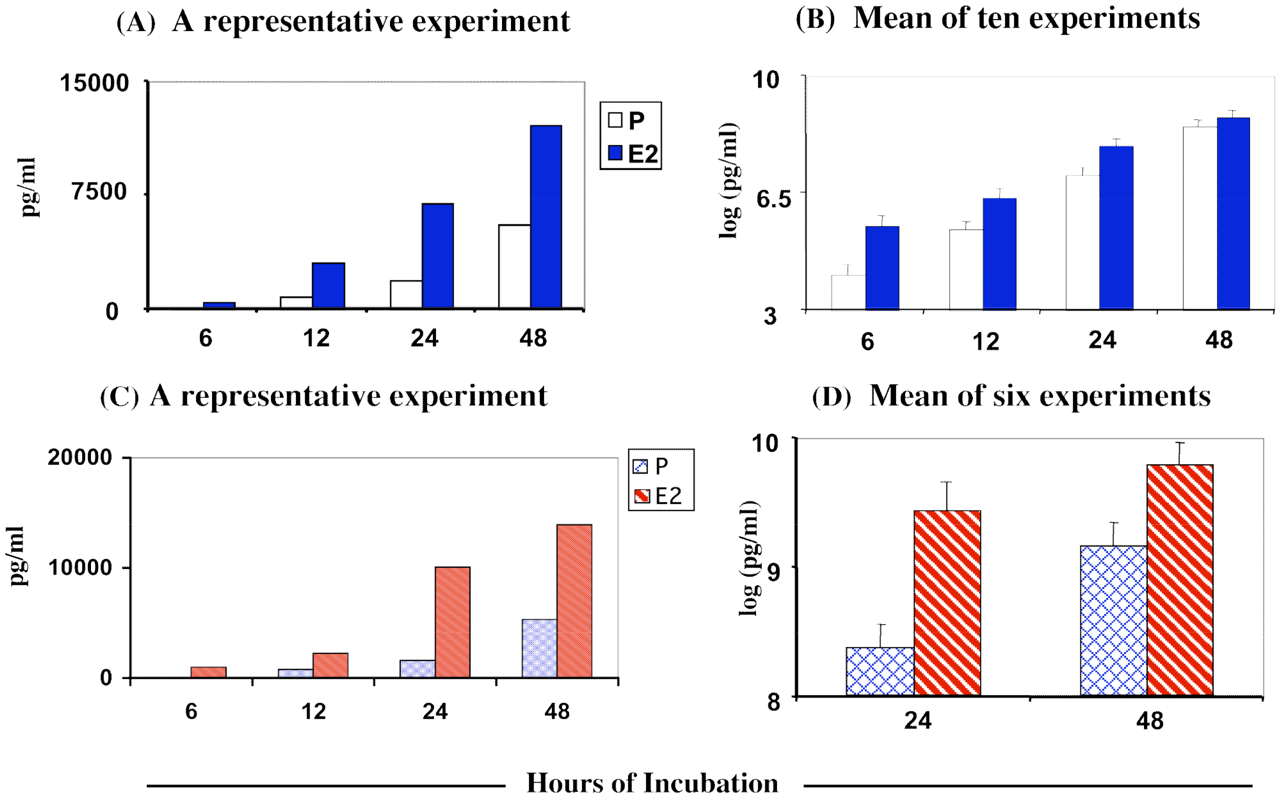

Figure 1. Normal male C57BL/6 mice were orchiectomized during prepubertal life and given either a placebo (P) or 17-β-estradiol (E2) implants as described in the materials and methods section. After 3–5 months of treatment, mice were terminated, splenic lymphocytes isolated and stimulated with an optimal concentration of concanavalin-A (Con-A; 10 μg/ml). The supernatants were collected at 6, 12, 24, and 48 h of culture and analyzed for IFN-γ levels by ELISA. Panel A is a representative experiment. Note the supernatants of splenic lymphocytes from estrogen-treated mice had an overall increased IFN-γ at the above time points studied. Panel B: Analysis of kinetics data from 10 independent experiments revealed that estrogen significantly increased the levels of IFN-γ ($P<0.05$). Panel C depicts an average of two representative experiments from normal female C57BL/6 mice were ovariectomized during prepubertal life and given either a placebo (P) or 17-β-estradiol (E2) implants for 3–5 months. Panel D represents data combined from 6 independent experiments from estrogen/placebo-treated ovariectomized mice, which showed that estrogen significantly increased the levels of IFN-γ ($P<0.05$).

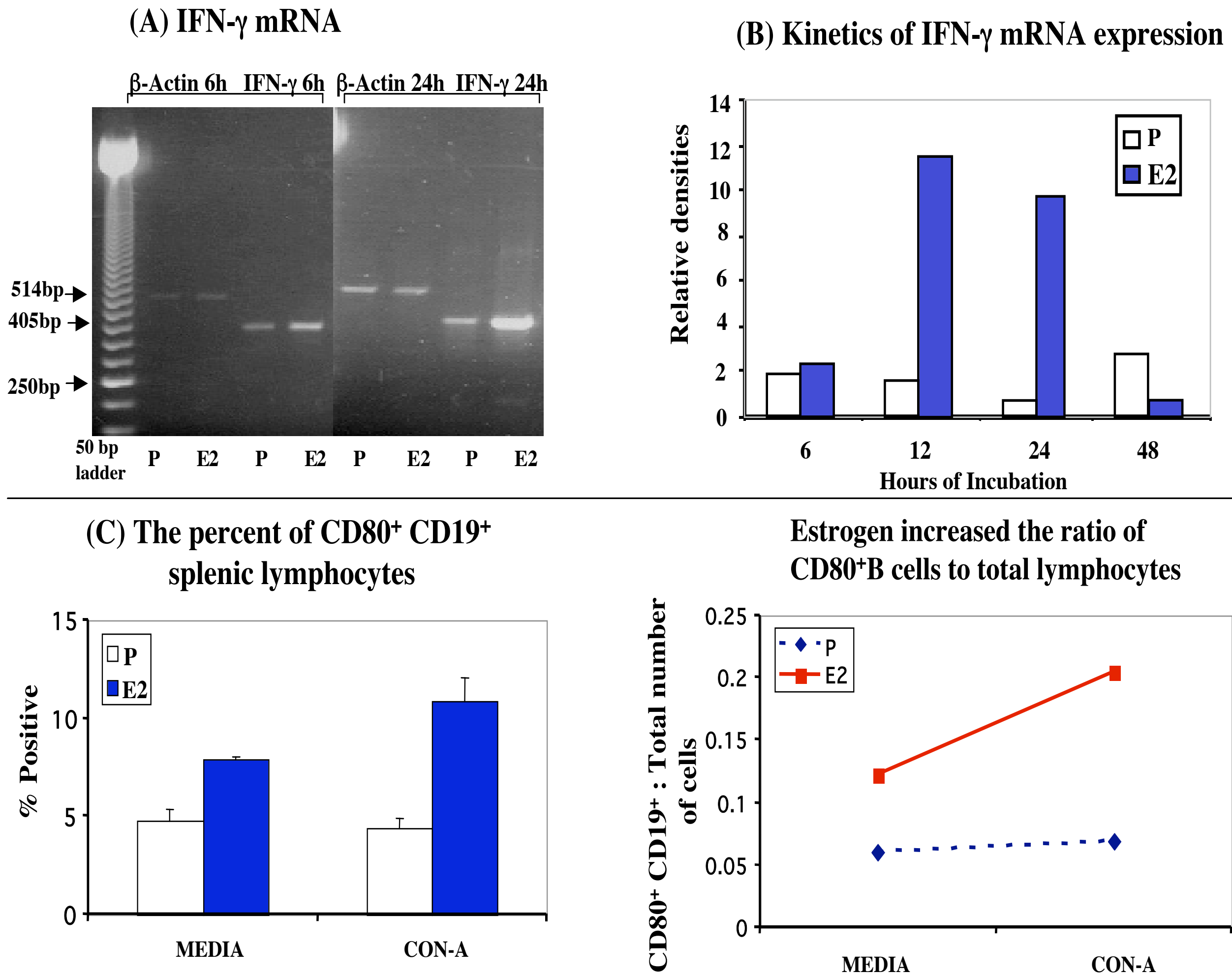

Figure 2. Splenic lymphocytes from mice prepubertally treated with estrogen (E2) or placebo (P) for 4 months were unstimulated or stimulated with Con-A (10 μg/ml) at the above indicated times. IFN-γ mRNA expression was determined using RT-PCR. Panel A shows RT-PCR samples separated on a 2% agarose gel and stained with ethidium bromide (10 μl/ml). Panel B shows relative mRNA values determined after densitometry scanning and normalized to β-actin. Panel C shows Con-A-stimulated splenic lymphocytes were stained with FITC-anti-CD80 plus PE-anti-CD19 monoclonals, or isotype-matched control antibodies, and analyzed by flow cytometry. Data represent 4 independent experiments from estrogen and placebo treated mice (*P*<0.05 by ANOVA). Panel D shows ratios of CD80+CD19+ *B* cells to total cell numbers Data represents four independent experiments (*P*<0.05 by ANOVA).

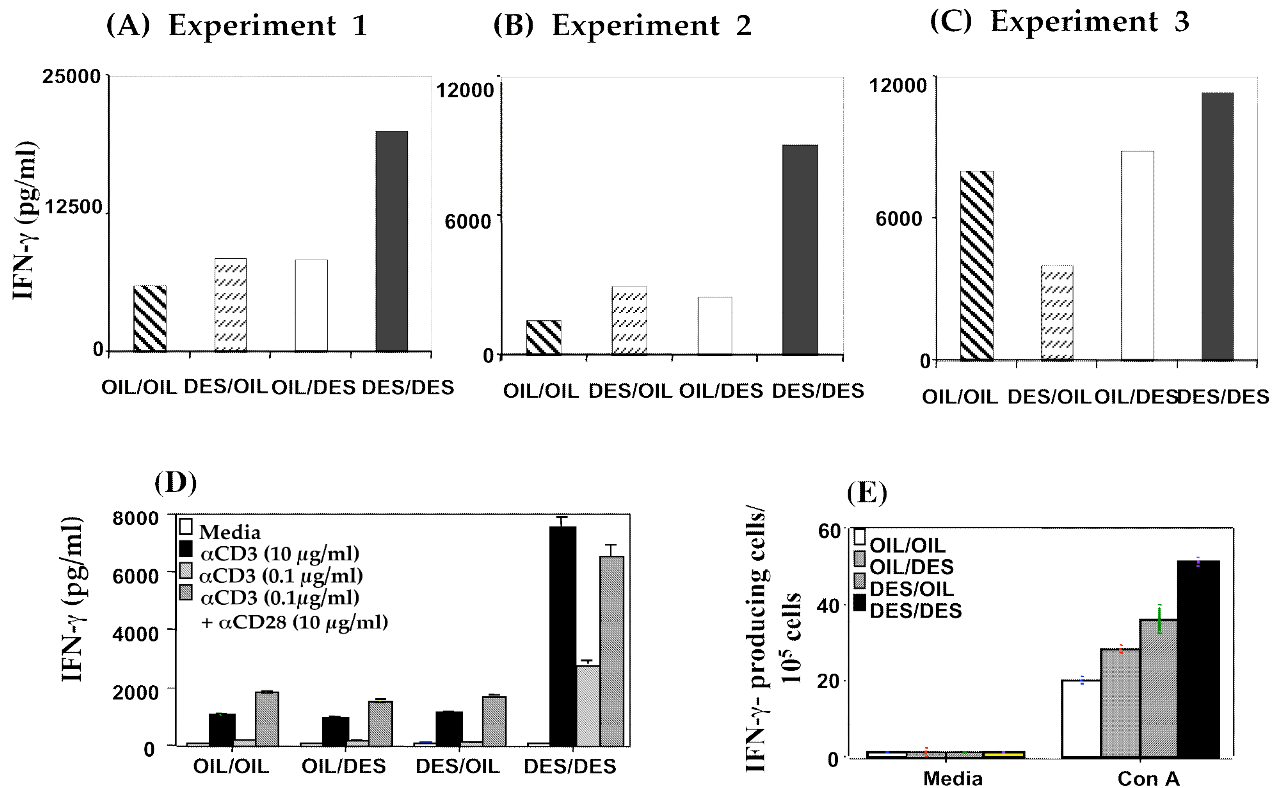

Figure 3. Late-life DES exposure increases IFN-γ secretion in prenatally DES exposed mice. One year-old C57BL6/J male mice which had been prenatally exposed to oil or DES were treated with a second dose of either oil or DES as described in the material and methods. (A) One week after the second exposure, splenic lymphocytes were isolated from these mice and stimulated with Con-A and IFN-γ secretion was assessed by ELISA after 72 h of culture. *n*=6 to 7 mice per group. Three separate experiments are shown. ANOVA analysis revealed that DES treatment significantly increased IFN-γ levels ($P<0.05$). Panel B shows a representative experiment pertaining to IFN-γ levels in the supernatants of splenic lymphocytes from mice which had been prenatally exposed to oil or DES and then received late-life oil or DES stimulated with anti-CD3 mAb (0.1 or 10 μg/ml) or with combined anti-CD3 (0.1 μg/ml) plus anti-CD28 mAb (10 μg/ml) for 72 h (representative of two mice per group). Panel C shows ELIspot data from a representative experiment on IFN-γ-secreting splenic lymphocytes from prenatal oil or DES-treated mice, which were given a second adult dose of oil or DES. The lymphocytes were stimulated with Con-A for 6 h.

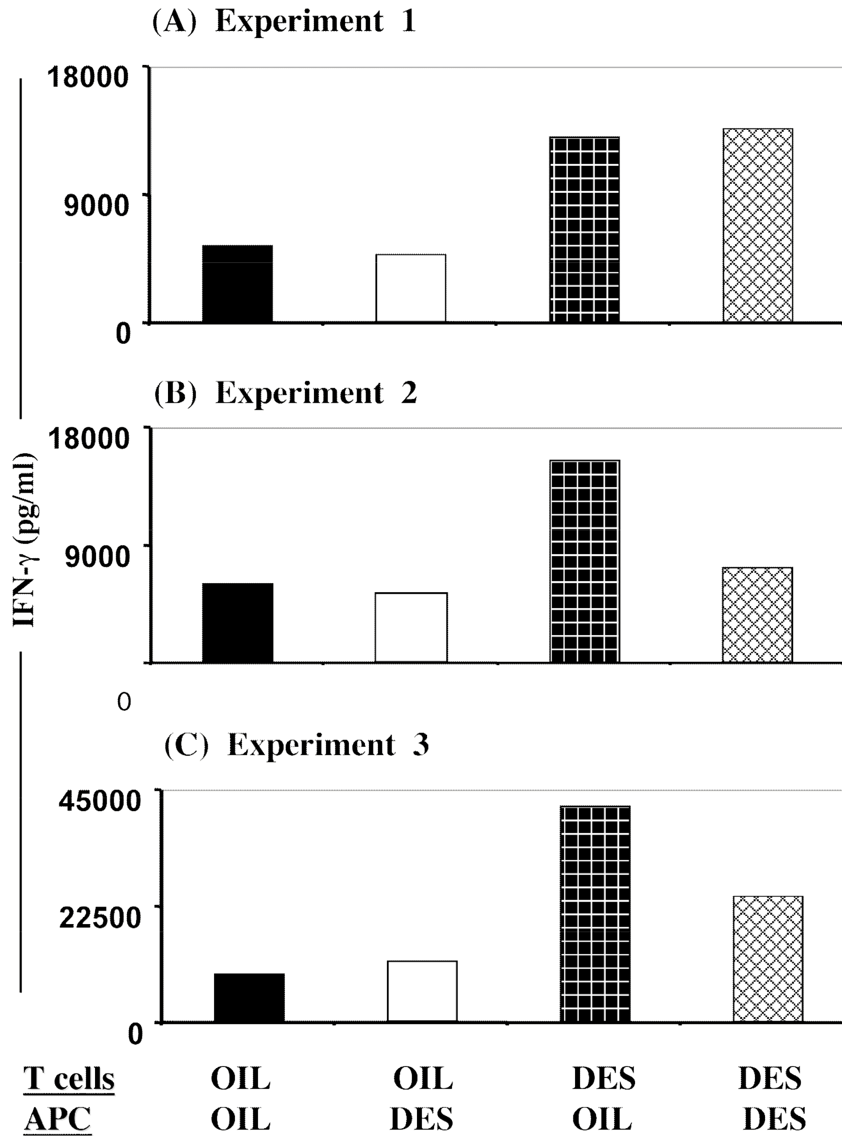

Figure 4. **Adult DES exposure affects T cells but not antigen presenting cells in prenatal DES-exposed mice**. Splenic lymphocytes from OIL/OIL and DES/DES mice were separated into *T* cell and APC fractions using magnetically-labeled anti-Thy1.2 mAb according to the manufacturer's instructions. The fractions were mixed in equal proportions ($5x10^5$ total cells) and stimulated with Con-A. After 72 h incubation, IFN-γ levels in the supernatants were determined by ELISA as described previously. The results are presented as pg/ml of IFN-γ. Three separate experiments are shown as A, B and C.

2.3. A Simpler Method to Quantify Gene Expression by Real-Time PCR: Analysis of Estrogen-Induced IFN-γ Expression (Manuscript to be submitted)

A SIMPLER METHOD TO QUANTIFY GENE EXPRESSION BY REAL-TIME PCR: ANALYSIS OF ESTROGEN-INDUCED IFN-γ EXPRESSION

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a.) ABSTRACT

The immune system is regulated by cytokines, which regulate various processes such as communictation between cells, activation of lymphocytes, proliferation, differentiation, and cell death. Estrogen intensely alters the immune system. We have shown that estrogen treatment results in increased IFN-γ secretion from splenocytes. The elevation of IFN-γ, a key immunomodulatory cytokine, can lead to increased inflammatory and autoimmune responses, which are prevalent in females. Therefore, reliable quantification and detection of IFN-γ mRNA expression becomes crucial in analyzing the response of immune system. We demonstrate with RT-PCR a qualitative increase of IFN-γ mRNA expression in Con-A activated splenocytes from estrogen-treated mice. The Real-Time PCR is used to allow a direct and sensitive quantification of IFN-γ mRNA expression with standards that were developed for each target cDNA (IFN-γ and β-actin). In this study, we describe a simpler method to quantify gene expression and compared this method with the Delta Delta CT method.

b.) INTRODUCTION

Cytokines are immunomodulatory proteins, which regulate vital lymphocyte functions including gene expression, signaling, activation, proliferation, differentiation, and programmed cell death (1-3). Alterations in cytokine levels are often noticed in inflammatory, allergic and infectious diseases. It is, thus, becoming increasingly important to determine the levels of cytokines to clarify immune and pathological pathways (4, 5). Cytokine protein levels are determined by ELISA, ELIspot, and intracellular cytokine staining via flow cytometry, while the levels of cytokine gene expression are assessed by such assays as Northern blotting, *in situ* hybridization, RNase protection assays, and RT-PCR (Reverse Transcription-Polymerase Chain Reaction). After the discovery of PCR (6), which is the most sensitive and accurate quantification method (7), various ways have been used to quantify the results of PCR. In RT-PCR, the product is measured after interruption at a determined cycle number of the exponential phase of PCR. The products are then run on an agarose gel stained with ethidium bromide and the bands are scanned with a densitometer. The intensities from the bands of target genes, such as cytokines, (8, 9) are normalized to the bands from housekeeping genes. A major limitation of RT-PCR is that it is not a quantitative assay and is, perhaps erroneously, referred to as a semi-quantitative gene expression assay. Real-Time RT-PCR, on the other hand, circumvents this limitation because it offers method of absolute quantification of gene expression. This assay measures PCR product accumulation during the exponential phase of the reaction. There are many different detection methods for the quantification of the products of Real Time PCR such as the use of TaqMan Probes (10, 11), Molecular beacons (12, 13), and SYBR Green I (14). SYBR Green I, a DNA-binding dye that intercalates into only double stranded DNA and emits fluorescence upon binding, is now increasingly employed. The advantage of this dye is its flexibility to be used with any primer for any target gene. One disadvantage of this method is decreased specificity due to the risk of amplifying non-specific PCR products (15) and primer dimers (16, 17). Therefore, the RNA that is used during the reverse transcriptase (RT) reaction should be cleaned of genomic DNA and the primers should be designed carefully to avoid primer dimers. Non-specific PCR products and primer dimers can be assessed by using the melting curve at the end of the PCR

reaction (18, 19). SYBR green I has been successfully utilized for the detection and quantification of low levels of cytokine mRNA (10, 20, 21).

Data from Real Time PCR are commonly analyzed by two methods: "the comparative threshold method" and "the standard curve method". Comparative threshold method is used for relative quantification by comparing the relative amount of the target gene to the reference value chosen such as an untreated control sample. The change in target gene expression, which is normalized to a housekeeping gene and related to the control sample, is calculated with the formula: $2^{-\Delta\Delta CT}$. In this equation, ΔΔCT (delta delta threshold cycle)= ΔCT (sample)- ΔCT (control sample). The ΔCT is the CT of the target gene subtracted from the CT of a housekeeping gene (22). In the standard curve method, a sample of known concentration is used to construct the standard curve. The standard curve can be constructed from plasmid ds (double stranded) DNA, *in vitro* transcribed RNA, *in vitro* synthesized ss (single stranded) DNA or any cDNA from the sample expressing the target gene (23, 24). The concentration of the DNA or RNA sample is determined with a spectrophotometer at 260 nm or by using Picogreen, a more sensitive method to determine dsDNA. The standard curve is included in each run of PCR which is important in correction of PCR efficiency and interassay comparison (16).

Both in comparative CT and the standard curve method, normalization with a housekeeping gene is a central issue for accurate quantification and to correct minor variations between samples due to the differences in input RNA or efficiencies of RT reactions. Theoretically, ideal relative gene expressions work best when an endogenous housekeeping gene is expressed at a constant level at different times and remains constant over different experimental treatments (16). A housekeeping gene with all these properties has thus far not been convincingly identified. The most commonly used housekeeping genes are β-actin, GAPDH (glyceraldehyde-3-phosphate-dehydrogenase), 18S RNA, and HPRT (hypoxanthine guanine phosphoribosyl transferase). GAPDH, due to increased expression in proliferating cells, and 18S RNA, due to the absence of a poly-A tail, cannot be used in cDNA synthesis with oligo-dT and are not highly recommended as housekeeping genes (24, 25). β-actin,

although not ideal, is recommended as a better housekeeping gene in many circumstances (25). β-actin mRNA encodes a cytoskeleton protein and is expressed in almost all cells types.

In this part of our studies, we describe RT-PCR and the use of Real Time RT-PCR for quantification of various murine cytokines, such as IFN-γ. To permit direct quantification in Real Time RT-PCR, a set of primers, which amplify target cDNA with known amounts to be used for specific standard curves were developed for target β-actin or IFN-γ cDNA, without co-amplifying the potentially contaminating genomic DNA. In addition, to minimize variation due to amounts of input RNA and reverse transcriptase reaction efficiency, normalization of the assay with the housekeeping gene. β-actin was used both in RT-PCR and Real Time RT-PCR to ensure the credibility of the results.

c.) MATERIALS AND METHODS

Mice: Three-to-four week old C57BL/6 wild-type mice were obtained from Charles River Laboratories and housed 3-5 animals per cage. All mice were maintained at the Center for Molecular Medicine and Infectious Diseases (CMMID) Animal Laboratory facility. Mice were fed on a diet devoid of phyto-estrogens and maintained with a 14/10 of light/dark cycle. Mice were housed in standard cages and terminated by cervical dislocation in accordance with the Virginia Polytechnic Institute and State University Institutional Animal Care guidelines.

Estrogen treatment: Four-to-five week old mice were orchiectomized and given silicone implants which were either a placebo (empty implant as control) or contained 17-β estradiol (estrogen; Sigma-Aldrich Inc., MO St. Louis, MO) by standard procedures as in our previously reported procedures (26, 27). The implants were placed under the skin on the dorsal side approximately near the neck. Mice were maintained 1.5 to 3 months after implantation.

Isolation and culture of splenic lymphocytes: Spleens were collected under sterile conditions and lymphocytes were isolated according to our previously published methods (28 , 29, 30). Briefly, spleens were gently dissociated by teasing on a sterile 60-

mesh steel screen (Sigma-Aldrich Inc., St. Louis, MO). The cell suspensions were washed in incomplete RPMI-1640 media (CellGro, Mediatech, Herndon, VA). Lymphocytes were enriched via removal of red blood cells after treating with ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) (31). Cells were washed and suspended in phenol red free RPMI-1640 media (Mediatech, Herndon, VA) supplemented with 10% charcoal-stripped heat inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 200 mM L-glutamine (Mediatech, Herndon, VA), 5000 IU/mL penicillin, 5000 mg/mL streptomycin (Mediatech, Herndon, VA), and 100X non essential amino acids (Mediatech, Herndon, VA). The isolated lymphocytes were then counted and viability was assessed by the trypan blue exclusion method as described in our earlier studies (30). Five hundred microliters of cells of $5x10^6$ cells/ml were added to 24-well round flat plates containing 500 μl per well of complete phenol red-free RPMI-1640 media with or without the T cell mitogen, Concanavalin-A (Con-A, 10 μg/ml; Sigma-Aldrich Inc., MO). The cell cultures were incubated for 24 hours at 37 C with 5% CO_2. Supernatants were collected in microcentrifuge tubes and centrifuged at 5000 rpm for 5 minutes. The cells and supernatants were frozen at -70^0C.

Isolation of RNA and cDNA synthesis: Cells were subjected to RNA isolation using RNase easy mini columns (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. Briefly, 350 μl of RLT buffer with β-mercaptoethanol were added to splenic lymphocytes cultured for 24 hours with Con-A and homogenized by pipetting. An equal volume of 70% molecular grade ethanol was added to the sample and the sample was applied on a RNeasy mini column. The tube was centrifuged for 15 sec at 9100 x g and the flow through was discarded. DNase digestion was then performed to increase the quality of RNA isolation. Eighty μl of DNase I mix were added on the silica gel membrane of the column and incubated at room temperature for 15 minutes. Three hundred fifty μl of RW1 buffer were added to the column and centrifuged for 15 seconds at 9100 x g. The column was washed twice with RPE buffer. The column was transferred to a new tube and RNA was eluted by the addition of RNase-DNase free sterile water. The cDNA was synthesized using the SuperScript™ First-strand synthesis system following manufacturer's instructions (Invitrogen

Corp., Carlsbad, CA). Briefly, 5 μl of random hexamers (50 ng/ml) and 1 μl of 10 mM dNTP mix were added to isolated total RNA and incubated at 65°C for 5 minutes and then chilled on ice. Nine μl of the reaction mixture, which consisted of 10X RT buffer, 25 mM $MgCl_2$, 0.1 mM DTT, and RnaseOUT recombinant ribonuclease inhibitor were applied to each RNA mixture. Fifty units of Superscript™ II RT were added to each mix and incubated for 10 min at room temperature. The mixtures were incubated at 42°C for 50 min to synthesize cDNA. The reaction was terminated at 70°C for 15 min and chilled on ice. The Rnase H was added to degrade any remaining RNA to enhance the quality of the cDNA.

Primer design: Primers were designed to span a large segment of the target genes using an online program, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primers that were designed were as follows: β-actin (965 base pairs) forward 5'-ATTGTTACCAACTGGGACGA-3', reverse 5'-CTGCGCAAGTTAGGTTTTGT-3'; IFN-γ (966 base pairs) forward 5'-CAATGAACGCTACACACTGC-3', reverse 5'-CTGCTTTCTTTCAGGGACAG-3'; (One Trick Pony Oligos, Ransom Hill Bioscience, Ramona, CA).

RT-PCR assay: The reverse transcriptase-polymerase chain reaction was used to detect β-actin and IFN-γ mRNA expression for the generation of standards and detection of cytokine gene expression in the samples. After cDNA synthesis, the PCR reactions were performed using Platinium® PCR SuperMix (Invitrogen Corp., Carlsbad, CA) following manufacturer's instructions. For the construction of standards, the primers for β-actin and IFN-γ that were described in "The primer design" of the methodology were used. β-actin (yielding a product size of 474 bp) and IFN-γ (yielding a product size of 284 bp) primers were purchased from Maxim Biotech Inc. (San Francisco, CA) and used for RT-PCR. The PCR reaction for both the construction of standards and samples was performed under the following conditions: 35 cycles of 94°C for 1 min, annealing at 58°C for 1 min, and amplification at 72°C for 1 min. Twelve microliters of PCR products from the samples were run in a 2% agarose gel containing ethidium bromide (10 μl per 100 ml of gel). Polaroid pictures of these gels were taken and PCR products were quantified with a scanning densitometer (Kodak Image Station,

Perkin Elmer Life Sciences Inc., Boston MA), normalized using β-actin (housekeeping gene), and expressed as relative densities. PCR products for the standard curve were run on a 0.8 % agarose gel containing ethidium bromide (10 μl per 100 ml of gel) and visualized over UV light. The bands of the target genes were cut out of the gel using a scalpel blade for isolation using a MinElute Gel Extraction kit (QIAGEN Inc., Valencia, CA).

Extraction of target genes: PCR products were excised from the agarose gel and isolated a using MinElute Extraction kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). Briefly, each gel slice was weighed and 3 volumes of Buffer AG were added to 1 volume of gel. The gel was incubated at 50°C for 10 min and vortexed every 2-3 minutes during the incubation. After the gel was dissolved completely, 1 volume of isopropanol was added, mixed via inverting, applied to a MinElute column, and centrifuged for 1 min. After discarding the flow-through, the column was applied with Buffer QG and spun for a minute. The MinElute column was washed with Buffer PE and DNA was eluted using Rnase-Dnase free water.

Measurement of total cDNA after gel extraction, Picogreen: PicoGreen (Molecular Probes Inc., Eugene, OR) reagent, the most sensitive reagent for detecting dsDNA in solution, such as cDNA, was used to detect the amount of target cDNAs after PCR. A two μg/ml stock solution of dsDNA in TE buffer was prepared and samples were diluted 20 fold in Dnase-Rnase free TE buffer. One hundred μl of sample and Picogreen were added to a 96-well plate and incubated in the dark for 5 mins. After incubation, the sample and standards were read using a fluorometer (CytoFlour, PerSeptive Biosystems Inc., MA) (excitation ~480nm, emission ~520nm). Double stranded DNA diluted 2 fold to generate a standard curve of fluorescence versus DNA concentration was used to detect the amount of cDNA in the samples.

Real time PCR: After cDNA synthesis, Real Time PCR was performed using Platinium UDG PCR SuperMix (Invitrogen Corp., Carlsbad, CA) and SybrGreen I (Molecular Probes Inc., Eugene, OR) following the manufacturer's instructions. β-actin and IFN-γ primers were purchased from Maxim Biotech Inc. (San Francisco, CA). The Real Time PCR reaction was

performed under the following conditions: 35 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec, and amplification at 72°C for 30 sec. Upon amplification of the target gene, integration of the double stranded DNA binding dye, SybrGreen I into the amplification product results in increased fluorescence, which is a direct consequence of target amplification during PCR. Threshold cycle (Ct) values are then calculated by determining the point at which the fluorescence exceeds the fluorescence baseline, called the threshold limit. The comparative Delta Delta Ct (ΔΔCt) method was used to determine the fold change of the target gene between the Con-A stimulated cells from placebo versus estrogen treated mice. The direct quantification of the Real Time RT-PCR products was performed by using the standard curve generated using respective standards that had known amounts of cDNA in femtograms (which were 10 fold diluted). The Real Time RT-PCR reaction was performed under the following conditions: 35 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec, and amplification at 72°C for 30 sec for both standards and unknown samples. Normalization of the target gene IFN-γ (284 bp) was done by normalizing to the housekeeping gene, β-actin (474 bp). Standard curves were used when the efficiency of the reaction, which is represented by R-square of the curve, was not higher than 100%. Values were recorded as femtogram ratios of the target gene (IFN-γ) normalized to the housekeeping gene (β-actin).

Statistics: An unpaired Student's T-test was used to assess whether the mean of the treatments differed from each other significantly. *P*-values less than 0.05 were considered as significant. Data are represented as mean ± standard error of the mean.

d.) RESULTS

IFN-γ transcription is induced after estrogen treatment detected via semi-quantitative RT-PCR. Supernatants from Con-A stimulated splenic lymphocytes, incubated for 24 hrs, from both estrogen and placebo treated mice showed a significant increase of secreted IFN-γ protein as shown in previous data (28, 29). We also looked at kinetics of IFN-γ gene expression at 6, 12, 24, and 48 hours, demonstrated by a representative experiment included in Chapter 2 (28). The gene expression of IFN-γ was determined using RT-PCR. The data

showed that IFN-γ gene expression was increased in Con-A (10 μg/ml)-stimulated splenic lymphocytes from estrogen-treated mice after 24 hours of culture (**Figure 1**). It is well established with our data that estrogen treatment does increase IFN-γ both at the protein and gene expression level.

Estrogen treatment does increase IFN-γ transcription quantitatively: Real Time RT-PCR. By RT-PCR analysis it appears that IFN-γ gene expression in splenic lymphocytes may be increased, we then used a quantitative method, Real Time RT-PCR. We used two methods to calculate IFN-γ expression, the Comparative Ct method (ΔΔCt method) and the Standard curve method. In the standard curve method, we amplified large cDNA segments both for β-actin (965 base pairs) and IFN-γ (966 base pairs) using RT-PCR. The target genes were excised out of a 0.8% gel under UV light and purified using a MinElute kit as described in the Materials and Methods. The amount of isolated genes, IFN-γ and β-actin, were calculated using a Picogreen Kit and diluted 1/10 with sterile Dnase-Rnase free water to construct the standard curve. Standard curves were used when the efficiency of the reaction which is represented by R-square of the curve, was not higher than 100% (**Figure 2**). The Real Time PCR iCycler program uses the standard curve formula to calculate the amount of β-actin and IFN-γ from the samples (**Figure 2**). The values obtained from the standard curve method were recorded as femtogram ratios of the target gene (IFN-γ) normalized to the housekeeping gene (β-actin) (**Figure 3**). The comparative Delta Delta Ct (ΔΔCT) method was used to determine the fold change of the target gene (IFN-γ) between the cells from placebo (control sample) versus estrogen-treated (target sample) that were stimulated with Con-A for 24 hours (**Figure 4**). Fold change demonstrated the increase in expression of IFN-γ cells from estrogen-treated mice over cells from placebo-treated mice.

e.) DISCUSSION

Much attention has up until now been focused on the physiological and general effects of estrogen and estrogenic compounds on reproductive functions. Detailed studies addressing immunological consequences such as cytokines associated with *in vivo* estrogen exposure are

lacking. Cytokines are immunomodulatory proteins, which regulate immune responses to infections and other pathologies such as autoimmune diseases.

The need for more-sensitive methods for the detection of cytokines is increasing due to immunological pathologies and immune responses to various stimulants and chemicals. In this study, we explored the IFN-γ expression in estrogen-treated splenic lymphocytes using SYBR Green I Real Time PCR. We showed that the Real Time PCR assay correlates with the RT-PCR assay with great sensitivity for IFN-γ detection.

The RT-PCR assay is used for detecting the low expression of target gene transcription. It is more qualitative than quantitative (or even semi-quantitative) providing a base to further explore target genes in a quantitative fashion using different techniques like Northern Blotting or Real Time RT-PCR analysis. It requires the detection of PCR products by assays such as agarose gel eletrophoresis with ethidium bromide staining (17) or chemilluminecent or radioactive labeling and Southern blotting (32), where it enables the researcher to see the actual end-product. The disadvantage of RT-PCR is that it is not able to absolutely quantify the levels of expression of the target gene. Unlike conventional RT-PCR, Real Time RT-PCR allows quantitation of even the lowest levels of expression of target genes via monitoring the product accumulation during the exponential phase (33). This technique is rapid, accurate, and sensitive. For both RT and Real Time PCR to be reliable, they both need to be optimized carefully and a relevant housekeeping gene should be used for normalization. Our previous studies showed that estrogen upregulates IFN-γ protein and expression (28, 29). We again demonstrated that estrogen treatment increased IFN-γ expression in Con-A stimulated lymphocytes as detected with RT-PCR demonstrating the reproducibility of the data. Although we showed the expression of IFN-γ mRNA, we quantitated the IFN-γ expression using Real Time PCR with SYBR Green I. SYBR Green I is much cheaper than Taqman Probes or other possible quenchers and molecular probes. Since it is not gene specific and it incorporates into double stranded PCR products during the reaction, SYBR Green I is a more flexible and cheaper reagent to use in the quantification of several genes. On the other hand, one must be careful in cleaning the total RNA of genomic DNA to avoid co-amplification of genomic DNA along with cDNA. To prevent the co-amplification a Dnase should be used

during RNA isolation and the primers should be designed to be RNA-specific as much as possible. Primers could be tested against genomic DNA or a cDNA prepared from non Dnase-treated RNA to see whether there is non-specific amplification. The non-specific amplification could be observed on an agarose gel, which is generally larger than the PCR product obtained from a clean cDNA preparation. Our RNA isolates were treated with Dnase before the cDNA reaction and there was no non-specific amplification observed both on gels from RT-PCR and Real Time RT-PCR products. The melting curve obtained at the end of Real Time PCR reaction also indicates whether there is non-specific amplification or primer-dimer formations. In addition, it can be used to detect allelic dissociations using SYBR Green I (34, 35).

To quantify the Real Time RT-PCR products, we constructed standards from the RNA of Con-A-stimulated murine splenic lymphocytes. A standard curve was contructed for IFN-γ and β-actin to calculate the amount of the genes expressed based on the formula provided by the program. By including a standard curve with known serial dilutions in the Real Time RT-PCR assay, unknown amounts of expression of IFN-γ is quantified both in samples from placebo and estrogen-treated mice. In the absence of a standard curve, a ΔΔCt method can be used to demonstrate the fold change of the IFN-γ gene in estrogen-treated samples over placebo –treated (control) samples after being normalized to β-actin. In this study, we showed that estrogen-treatment significantly and quantitatively increases IFN-γ expression in Con-A stimulated murine splenocytes as calculated with Standard Curve and ΔΔCt methods.

RT-PCR is a good tool to explore the expression of several genes as a primary line of detection. Real Time PCR assay is important in the easy, sensitive, and quick determination of expression of target genes and can be used to further quantify mRNA expression that is observed by RT-PCR. Our lab currently uses these methods to detect various cytokines and transcription factors in unstimulated and stimulated murine splenocytes from placebo and estrogen-treated mice. In conclusion, IFN-γ and β-actin standards to establish Standard Curve and SYBR Green I Real Time RT-PCR assay were confirmed to be a flexible, economical, and sensitive way for detecting IFN-γ expression in estrogen treated samples. The advantages

our methodology and analysis are the use of SYBR green I, which allows many reactions to be performed with different primers, the analysis and calculation are less time consuming than the plasmid approach, and it is relatively inexpensive.

f.) REFERENCES

1. Agnello, D., C. S. Lankford, J. Bream, A. Morinobu, M. Gadina, J. J. O'Shea, and D. M. Frucht. 2003. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol 23:147*.
2. Shuai, K., and B. Liu. 2003. Regulation of JAK-STAT signalling in the immune system. *Nat Rev Immunol 3:900*.
3. Thomson, A. W. 1991. Immunomodulation: suppression, enhancement and autoimmunity. *Curr Opin Biotechnol 2:247*.
4. Rabinovitch, A. 1998. Animal models of type 1 diabetes are relevant to human IDDM--use caution. *Diabetes Metab Rev 14:189*.
5. Dai, Z., and F. G. Lakkis. 1999. The role of cytokines, CTLA-4 and costimulation in transplant tolerance and rejection. *Curr Opin Immunol 11:504*.
6. Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol 51 Pt 1:263*.
7. Wang, T., and M. J. Brown. 1999. mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal Biochem 269:198*.
8. O'Garra, A., and P. Vieira. 1992. Polymerase chain reaction for detection of cytokine gene expression. *Curr Opin Immunol 4:211*.
9. Montgomery, R. A., and M. J. Dallman. 1997. Semi-quantitative polymerase chain reaction analysis of cytokine and cytokine receptor gene expression during thymic ontogeny. *Cytokine 9:717*.
10. Overbergh, L., D. Valckx, M. Waer, and C. Mathieu. 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine 11:305*.
11. Killgore, G. E., B. Holloway, and F. C. Tenover. 2000. A 5' nuclease PCR (TaqMan) high-throughput assay for detection of the mecA gene in staphylococci. *J Clin Microbiol 38:2516*.

12. Tyagi, S., and F. R. Kramer. 1996. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol 14:303*.

13. Tyagi, S., D. P. Bratu, and F. R. Kramer. 1998. Multicolor molecular beacons for allele discrimination. *Nat Biotechnol 16:49*.

14. Yin, J. L., N. A. Shackel, A. Zekry, P. H. McGuinness, C. Richards, K. V. Putten, G. W. McCaughan, J. M. Eris, and G. A. Bishop. 2001. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. *Immunol Cell Biol 79:213*.

15. Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. *Genome Res 6:986*.

16. Giulietti, A., L. Overbergh, D. Valckx, B. Decallonne, R. Bouillon, and C. Mathieu. 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods 25:386*.

17. Kuschnaroff, L. M., D. Valckx, J. Goebels, O. Rutgeerts, H. Heremans, G. Froyent, and M. Waer. 1997. Effect of treatments with cyclosporin A and anti-interferon-gamma antibodies on the mechanisms of immune tolerance in staphylococcal enterotoxin B primed mice. *Scand J Immunol 46:459*.

18. Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem 245:154*.

19. Elenitoba-Johnson, K. S., S. D. Bohling, C. T. Wittwer, and T. C. King. 2001. Multiplex PCR by multicolor fluorimetry and fluorescence melting curve analysis. *Nat Med 7:249*.

20. Hein, J., U. Schellenberg, G. Bein, and H. Hackstein. 2001. Quantification of murine IFN-gamma mRNA and protein expression: impact of real-time kinetic RT-PCR using SYBR green I dye. *Scand J Immunol 54:285*.

21. Ramos-Payan, R., M. Aguilar-Medina, S. Estrada-Parra, Y. M. J. A. Gonzalez, L. Favila-Castillo, A. Monroy-Ostria, and I. C. Estrada-Garcia. 2003. Quantification of cytokine gene expression using an economical real-time polymerase chain reaction method based on SYBR Green I. *Scand J Immunol 57:439*.

22. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods 25:402*.
23. Bustin, S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol 29:23*.
24. Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol 25:169*.
25. Suzuki, T., P. J. Higgins, and D. R. Crawford. 2000. Control selection for RNA quantitation. *Biotechniques 29:332*.
26. Ansar Ahmed, S., T. B. Aufdemorte, J. R. Chen, A. I. Montoya, D. Olive, and N. Talal. 1989. Estrogen induces the development of autoantibodies and promotes salivary gland lymphoid infiltrates in normal mice. *J Autoimmun 2:543*.
27. Ansar Ahmed, S., and D. Verthelyi. 1993. Antibodies to cardiolipin in normal C57BL/6J mice: induction by estrogen but not dihydrotestosterone. *J Autoimmun 6:265*.
28. Karpuzoglu-Sahin, E., B. D. Hissong, and S. Ansar Ahmed. 2001. Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. *J Reprod Immunol 52:113*.
29. Karpuzoglu-Sahin, E., Y. Zhi-Jun, A. Lengi, N. Sriranganathan, and S. Ansar Ahmed. 2001. Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. *Cytokine 14:208*.
30. Donner, K. J., K. M. Becker, B. D. Hissong, and S. A. Ahmed. 1999. Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. *Cytometry 35:80*.
31. Ansar Ahmed, S., R. M. Gogal, Jr., and J. E. Walsh. 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J Immunol Methods 170:211*.
32. Hanson, M. S., M. Cetkovic-Cvrlje, V. K. Ramiya, M. A. Atkinson, N. K. Maclaren, B. Singh, J. F. Elliott, D. V. Serreze, and E. H. Leiter. 1996. Quantitative thresholds of MHC class II I-E expressed on hemopoietically derived antigen-presenting cells in

transgenic NOD/Lt mice determine level of diabetes resistance and indicate mechanism of protection. *J Immunol 157:1279*.

33. Freeman, W. M., S. J. Walker, and K. E. Vrana. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques 26:112*.

34. Donohoe, G. G., M. Laaksonen, K. Pulkki, T. Ronnemaa, and V. Kairisto. 2000. Rapid single-tube screening of the C282Y hemochromatosis mutation by real-time multiplex allele-specific PCR without fluorescent probes. *Clin Chem 46:1540*.

35. Marziliano, N., E. Bevilacqua, D. Pirulli, A. Span, A. Amoroso, and S. Crovella. 2000. Single tube melting temperature assay for rapid and sensitive detection of the most frequent hemocromatosis mutations, C282Y and H63D. *Haematologica 85:990*.

g.) Figures

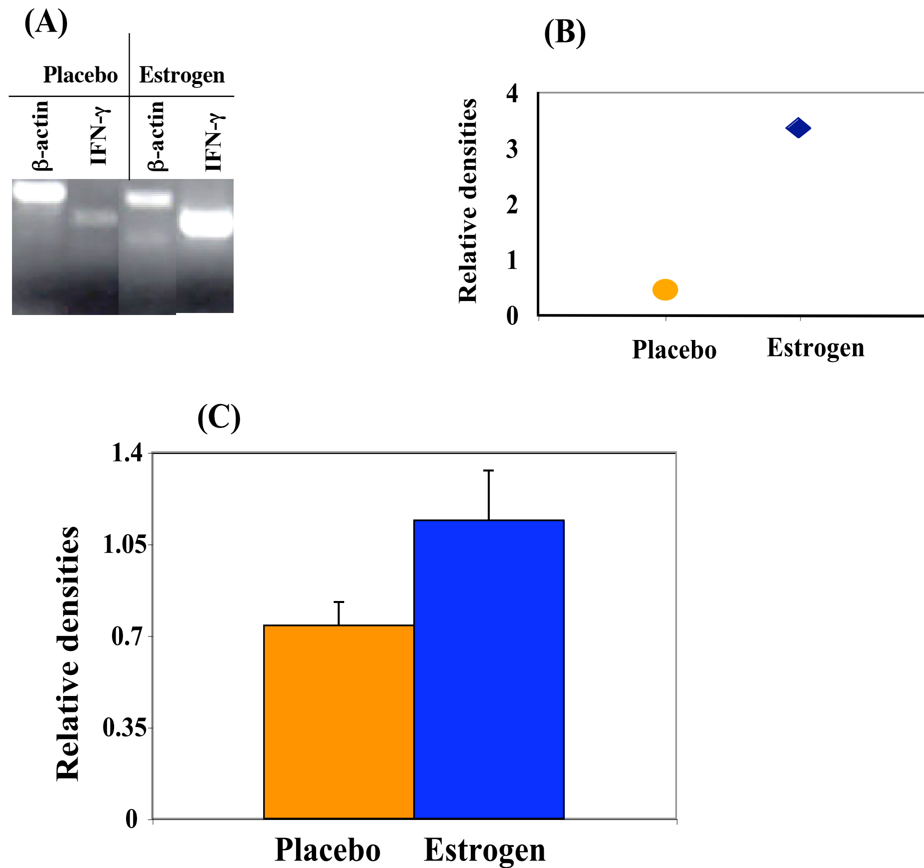
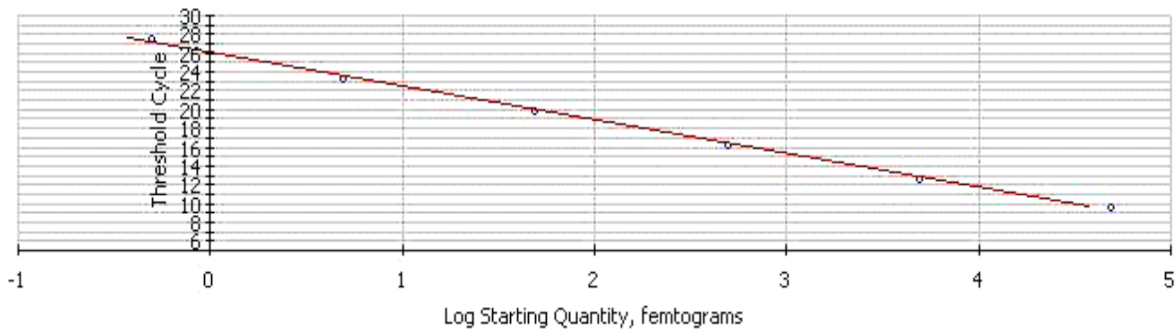


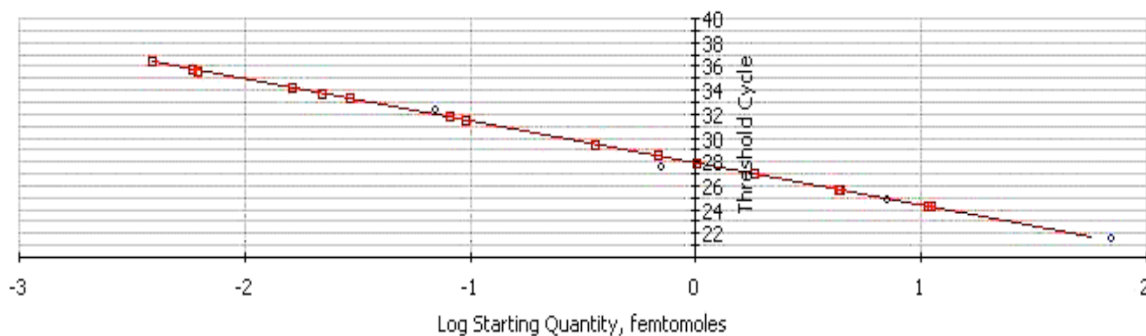
Figure 1. Estrogen tended to increase IFN-γ mRNA expression as detected by RT-PCR. Splenic lymphocytes from mice treated with estrogen or placebo were stimulated with Con-A (10 μg/ml) for 24 hours. The IFN-γ mRNA expression was determined using RT-PCR. Panel A shows RT-PCR samples separated on a 2% agarose gel and stained with ethidium bromide (10 μl/ml). Panel B shows the relative mRNA values of RT-PCR samples in Panel A determined after densitometry scanning and normalized to β-actin. Panel C shows the mean relative mRNA values of IFN-γ from Con-A-stimulated splenic lymphocytes. Data represents 14 independent experiments from estrogen and placebo-treated mice (*p=0.0818*).

(A) β-actin Standard Curve



Correlation coefficient: 0.999, Slope -3.596, Intercept: 26.140, Y=-3.596X+26.140, PCR Efficiency: 89.7%.

(B) IFN-γ Standard Curve



Correlation coefficient: 0.992, Slope –3.496, Intercept: 27.895, Y=-3.496X+27.895, PCR Efficiency: 93.21%.

Figure 2. The standard curve method for quantification of the target gene, IFN-γ and the housekeeping gene, β-actin. The amplified large cDNA segments required for both β-actin (965 bp) and IFN-γ (966 bp), using RT-PCR, were isolated using a MinElute kit as described in Materials and Methods. The amount of isolated genes was calculated and diluted 1/10 with sterile Dnase-Rnase free water to construct the standard curve. The standard curves were used when the efficiency of the reaction, which is represented by R-square of the curve, was not higher than 100%. Panel A shows the standard curve for housekeeping gene, β-actin and Panel B shows the standard curve for IFN-γ. These graphs are representative of one experiment each.

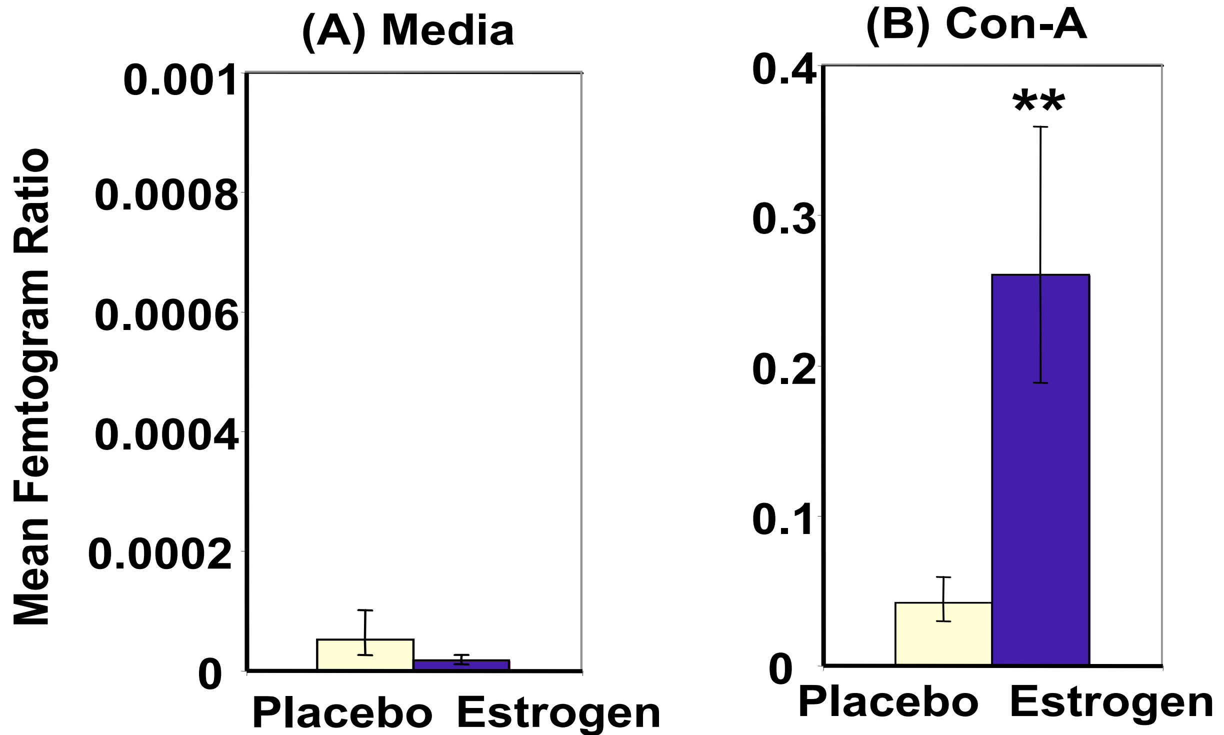
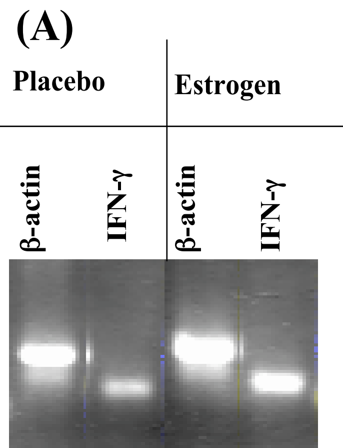


Figure 3. Estrogen increased IFN-γ mRNA expression, quantitative detection with Real Time RT-PCR. IFN-γ mRNA that was amplified with Real Time RT-PCR and normalized to β-actin was represented as mean femtogram ratio from splenic lymphocytes stimulated with Con-A from placebo and estrogen-treated mice. Panel A shows IFN-γ expression in unstimulated splenic lymphocytes left in media for 24 hrs (n=3, $p=0.3181$). Panel B shows IFN-γ mRNA expression in splenic lymphocytes stimulated for 24 hrs with Con-A (n=10 mice per placebo or estrogen treatment; $p<0.01$). The values were recorded as femtogram ratios of the target gene (IFN-γ) normalized to the housekeeping gene (β-actin). Data are represented as mean ± standard error of mean.



(B)

	ΔCT Placebo		ΔCT Estrogen	ΔΔCT (Estrogen-Placebo)	FOLD CHANGE
IFN-γ Placebo	7.9	**IFN-γ Estrogen**	6	-1.9	3.73

Figure 4. The comparative CT method (ΔΔCT), relative quantification of the target gene: β-actin and IFN-γ were amplified with Real Time RT-PCR and relative fold change of IFN-γ expression in cells from estrogen treated mice in relation to cells from placebo treated mice was relatively quantitated using the ΔΔCT method. Panel A shows Real Time RT-PCR products after the reaction. Panel B shows the calculation of the ΔΔCT method (Comparative CT method) and the fold change of IFN-γ, the target gene.

CHAPTER 3. Estrogen regulation of IFN-γ dependent iNOS expression

This chapter deals with addressing the central question " *Does estrogen (17-β estradiol) alter interferon-γ (IFN-γ) mediated downstream events, iNOS, nitric oxide, and iNOS-inducible COX-2?*" This chapter will present evidence to show the activated splenic lymphocytes from estrogen-treated mice upregulate IFN-γ-dependent iNOS, nitric oxide, and iNOS-inducible COX-2.

This chapter is divided into 2 subsections, which are likely to result in two significant publications.

(1) **Subsection 1:** "**Estrogen upregulates IFN-γ-dependent iNOS gene expression, nitric oxide and COX-2 in splenocytes activated with T cell stimulants**. Ebru Karpuzoglu-Sahin, Jillian B. Fenaux, Rebecca Phillips, Andrea Lengi, François Elvinger, S. Ansar Ahmed. To be submitted to The Journal Of Immunology". *This study shows that estrogen upregulates the expression of IFN-γ-dependent iNOS, nitric oxide and COX-2. Studies in orchiectomized males demonstrated the importance of costimulatory interaction between CD28 and B7.1/B7. for estrogen induced increase of IFN-γ, iNOS, nitric oxide and COX-2 expression by splenocytes.*

(2) **Subsection 2:** "**Estrogen Regulation Of The Inducible Nitric Oxide Synthase Gene And Nitric Oxide In Immune Cells: Implications For Immunity And Autoimmunity**. Ebru Karpuzoglu-Sahin and S. Ansar Ahmed. To be submitted to Endocrine Reviews". *This section is a brief review about the duplicitous nature of iNOS-derived nitric oxide in immune system different tissues in relation to cytokines, cell survival, apoptosis, and the effects of estrogen on nitric oxide were covered in this part. The data in this section also demonstrated that estrogen upregulated nitric oxide secretion and iNOS mRNA expression not only in males, but also in splenocytes from estrogen-treated ovariectomized female mice.*

3.1. Estrogen upregulates IFN-γ-dependent iNOS gene expression, nitric oxide, and COX-2 in splenocytes activated with T cell stimulants.

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a.) ABSTRACT:

Estrogen is implicated in many autoimmune diseases and is a robust immunomodulator, including enhancing interferon-γ (IFN-γ), a cytokine believed to upregulate inducible nitric oxide synthase (iNOS). A notable gap in the literature is the lack of information on the regulation of nitric oxide in immune tissues by estrogen. We now show that activation of splenocytes with T cell stimulants (Concanavalin-A or anti-CD3 antibodies) results in copious release of nitric oxide in cultures from estrogen, but not placebo-treated mice. Moreover, even submitogenic doses of T cell stimulants induced nitric oxide in splenocytes from estrogen-treated, but not from placebo-treated, mice. Con-A-activated splenocytes from estrogen-treated mice also have upregulated iNOS mRNA, iNOS protein and COX-2 (a nitric oxide-regulated downstream pro-inflammatory protein) when compared to controls. Our studies suggest that the induction of nitric oxide by activated splenocytes from estrogen-treated mice is mediated through IFN-γ. First, blocking co-stimulatory signals mediated through interactions of CD28 and B7 molecules by CTLA-4Ig markedly decreased not only IFN-γ, but also nitric oxide. Second, estrogen-treatment of IFN-γ-knockout (IFN-γ(-/-)) mice or interferon regulatory factor-1 knock out (IRF-1(-/-)) mice did neither induce iNOS protein nor nitric oxide. Finally, *in vitro* addition of recombinant IFN-γ to Con-A-activated splenocytes from wild-type mice induced nitric oxide or iNOS protein. Overall, this is a first report to show that estrogen treatment upregulates iNOS gene expression, iNOS protein, nitric oxide, and COX-2 as an indirect consequence of activation of T cells via IFN-γ. These findings may have wide implications to inflammatory disorders and immunity to infections.

b.) INTRODUCTION

It is now well appreciated that nitric oxide has broad physiological and pathological effects on many tissues including the immune system (1, 2). Nitric oxide, a short-lived ($t_{1/2}$ seconds) but highly reactive biomolecule, rapidly diffuses through membranes and readily reacts with diverse substances in a wide-range of cells. Nitric oxide is known to interact with: aqueous oxygen (to produce relatively unreactive nitrate and nitrite), transition metals (to form metal-nitric oxide adducts), superoxide (to generate highly toxic peroxynitrite), heme, Fe-S clusters, Zn-S clusters and sulfadyryl clusters (to exert distinct biological effects) (3-6). It is therefore not surprising that nitric oxide plays a central role in the physiology and pathology in various tissues. Nitric oxide is produced by specific enzymes referred to as nitric oxide synthases (NOS). These enzymes catalyze the production of nitric oxide and *L*-citrulline from *L*-arginine in the presence of NADPH-derived electrons, and O_2. There are three well-known isoforms of nitric oxide synthases, (1) neuronal nitric oxide synthase (nNOS), (2) endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) (2). All three main isoforms of NOS share common structural motifs, have nearly alike catalytic mechanisms, and require cofactors for activity including flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopetrin calmodulin, heme, and thiol donors. Three distinct domains appear to be necessary for catalytic activity including, a reductase domain at the C-terminus, a calmodulin-binding domain, and an oxygenase domain. The reductase domain, which has many similarities to cytochrome P-450 reductase, transfers electrons from NADPH to the oxygenase domain. The oxygenase domain then catalyzes the conversion of arginine into citrulline. Despite several common features among the three isoforms of NOS, there are a number of key differences with respect to individual functions. For example, each NOS isoform has distinct transcriptional regulation and post-transcriptional regulation of catalytic activity, anatomic location, differential ability to release the amount of nitric oxide, and physiological functions. nNOS is constitutively present in the brain, the peripheral nervous system, and skeletal tissues. nNOS-derived nitric oxide is generated at low levels (in the picomolar range) in short bursts and is

believed to play an important role in brain-neuromodulation (7, 8). eNOS is also constitutively present, but its principal location is in vascular endothelial cells and cardiomyocytes (9). Nitric oxide–derived from eNOS is also present in low picomolar amounts and is thought to be involved in vasodilation to regulate blood flow and pressure. In contrast to these two NOS isoforms, iNOS is not constitutively present, but rather is induced by cytokines (IFN-γ, TNF-α) or other immunological stimuli (LPS). Although iNOS can be induced in various immune cells, macrophages appear to be the principal source of iNOS (1, 2, 10). Relatively high levels of nitric oxide (in the nanomolar range) are generated by iNOS in macrophages.

The role of nitric oxide in immunity is well appreciated (2, 11). For example, the healing of excisional wounds is significantly delayed in iNOS-deficient mice when compared to wild-type mice (2). Nitric oxide-derived from iNOS is critical for controlling infections (1, 12, 13). Infected or activated macrophages produce nitric oxide derived from iNOS, which assists in killing the invading microbes by increasing dsDNA breaks and oxidation of lipids and DNA. Moreover, nitric oxide will interact with oxygen free radicals such as superoxide to result in highly toxic peroxynitrite ($ONOO^-$). Nitric oxide may also play a pivotal role in inflammation by increasing the production and/or increasing the enzymatic activity of cyclooxygenase-2 (COX-2), (14, 15). COX-2 is inducible directly or indirectly by nitric oxide. It is expressed in many cells especially in macrophages and monocytes (16-20).

While an appropriate increase in nitric oxide is critical for effective immunity, aberrant levels of nitric oxide have been associated with many pathological conditions such as neurodegenerative disorders, apoptosis, hypertension, hypercholestrolemia, diabetes, heart failure, infections, autoimmune diseases, asthma, and septic shock (1, 21-23). Autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), and glomerulonepthritis associated with increased IFN-γ levels demonstrate high expression of iNOS and nitric oxide (1, 22). This implies that the level of nitric oxide and the duration of its presence must be regulated to maintain health. Thus understanding of regulation of NOS is of biomedical importance. One of

the important regulators of NOS is estrogen. In several non-immune tissues estrogen has been shown to be powerful regulator of nitric oxide that is presumably generated by nNOS and eNOS (24, 25). While estrogen has been shown to regulate nitric oxide in non-immune (reproductive and nervous) tissues, there is little information on the regulation of nitric oxide by estrogen in immune tissues, particularly in relation to T-macrophage interactions. Therefore, this study addresses this important aspect, especially considering that estrogen is implicated in many inflammatory and autoimmune diseases (26-32). Further, we have recently reported that splenocytes from estrogen-treated mice, when exposed to T cell stimulants (such as Concanavalin-A (Con-A)), release high levels of interferon-gamma (IFN-γ) (33, 34). To date, there have been no studies that have addressed the relationship between IFN-γ, iNOS/nitric oxide and COX-2 in T cell activated splenocytes. This is the first study that establishes such a link and demonstrates the effects of *in vivo* estrogen treatment on IFN-γ-inducible iNOS expression, NO production, and COX-2 expression in mouse splenic lymphocytes.

c.) MATERIALS AND METHODS

Mice: Three-to-four week old C57BL/6 wild-type male mice were obtained from Charles River Laboratories (Wilmington, MA) and housed 3-5 animals per cage. IFN-γ knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME) and IRF-1 knockout mice were obtained from Amgen (Thousand Oaks, CA; Georgio Senaldi). Both IFN-γ(-/-) and IRF-1(-/-) were on a C57BL/6 background. Mice were housed in standard cages, fed on a diet that is devoid of phytoestrogens, and maintained in a 14/10 light/dark cycle at the Center for Molecular Medicine and Infectious Diseases (CMMID) Animal Laboratory facility. Two, four, or eight mice were introduced into experiments at any given experiment date. Mice were terminated by cervical dislocation in accordance with the Virginia Polytechnic Institute and State University Institutional Animal Care guidelines.

Estrogen treatment: Four-to-five week old mice were orchiectomized and given silastic implants that were either placebo (empty implant as a control) or estrogen implants

containing 17 β-estradiol (Sigma-Aldrich Inc., MO) by standard procedures that were extensively reported previously (33-36).

Isolation of splenic lymphocytes: Spleens were collected under sterile conditions and lymphocytes were isolated according to our previously published methods (33, 34, 37). Briefly, spleens were gently dissociated by teasing on a sterile 60-mesh steel screen (Sigma-Aldrich Inc., MO). The cell suspensions were washed in incomplete RPMI-1640 media (CellGro, Mediatech, Herndon, VA). Lymphocytes were enriched by removing red blood cells from splenocytes after treating with ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) (38). Care was taken to culture cells in phenol-red deficient media (since phenol-red is estrogenic) and charcoal-stripped FBS (to remove estrogens and estrogen-binding proteins) to ensure that estrogen exposure was restricted to *in vivo* period only. Cells were thus washed and suspended in phenol red-free RPMI-1640 media (Mediatech, Herndon, VA) that was supplemented with 10% charcoal-stripped, heat inactivated-fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 200 μM L-glutamine (Mediatech, Herndon, VA), 5000 IU/ml penicillin, 5000 μg/ml streptomycin (Mediatech, Herndon, VA), and 5 ml of 100X nonessential amino acids (Mediatech, Herndon, VA). Isolated lymphocytes were then counted and cell viability was assessed by the trypan blue exclusion method as described in our earlier studies (37). Cell numbers were assessed with a CASY-1 Cell Counter and Analyzer System (Scharfe System GmbH, Reutingen, Germany) and adjusted to 5 x 10^6 cells/ml as described in our previous studies (37). In our experience, cells ranging from 5 to 10 microns predominantly constitute the lymphocyte population (37, 39).

Culture of lymphocytes: Five-hundred microliters of cells at $5x10^6$ cells/ml were added to 24-well round flat-bottomed plates containing 500 μl per well of complete phenol red-free RPMI-1640 media with or without the T cell mitogen, Concanavalin-A (Con-A, 10 μg/ml or submitogenic dose of Con-A, 1 μg/ml; Sigma-Aldrich Inc., MO) or anti-CD3 antibodies (0.1, 1, and 10 μg/ml; eBioscience Inc., CA). In selected cultures, cells were exposed to Con-A in the presence or absence of an optimal concentration of CTLA-4Ig fusion protein (15 μg/ml) (Alexis Biochemicals San Diego, CA) or cultured with

recombinant IFN-γ (1000 pg/ml or 10 ng/ml; BDPharmingen, San Diego, CA). The CTLA-4Ig fusion protein blocks the interaction of B7.1/B7.2 to CD28 co-stimulatory molecules. In selected cultures, cells were exposed to Con-A for 3 hours, washed, and then cultured with recombinant IFN-γ (10,000, 1000,and 100 pg/ml; BDPharmingen, San Diego, CA) for 24 hrs. Cell cultures were incubated for 24 or 48 hrs at 37 C with a 5% CO_2 environment. Supernatants were collected in microcentrifuge tubes and centrifuged at 5000 RPM for 5 minutes to collect the cellular fractions and supernatants, which were immediately frozen at -70^0C until use.

Determination of IFN-γ protein: Levels of IFN-γ in the supernatants of activated lymphocytes in culture were analyzed with an IFN-γ specific ELISA that has been extensively described previously (33, 34). Briefly, ninety-six well Maxisorp high binding immunoassay plates were coated overnight with anti-IFN-γ antibodies (anti-IFN-γ clone R4-6A2, ATCC, HB-170; BDPharmingen, San Diego, CA) in PBS and then blocked with 2% BSA for 1 hr. Plates were incubated for 2 hrs with either supernatants (diluted as necessary) or serial dilutions of known concentrations of IFN-γ to generate a standard curve. Next, biotin conjugated anti-IFN-γ antibodies (BDPharmingen, San Diego, CA) were applied to the plates for 1 hr and then overlaid with avidin-horseradish peroxidase solution (Vector Labs, Burlingame, CA). The color was developed using TMB as the substrate (3,3',5,5'-tetra-methylbenzidine) (KPL, Gaithersburg, MD) and 0.18 M sulfuric acid to stop the reaction. Plates were read at 450 nm with an ELISA reader (Molecular Devices Sunnyvale, CA). The IFN-γ protein levels were calculated with the formula obtained from the standards using SoftMax Pro software from Molecular Devices Inc.

Isolation of RNA and cDNA synthesis, and Primer Design: RNA was isolated from splenic lymphocytes that were cultured for 24 hrs with Con-A with RNase easy mini columns (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. Following the elution of RNA with sterile water from RNase easy columns, RNA was used to synthesize cDNA using the SuperScript™ First-stand synthesis system following the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Primers were designed to span a large segment of the target genes using an online program, Primer 3

(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Primers designed were as follows: β-actin (965 base pairs) forward 5'-ATTGTTACCAACTGGGACGA-3', reverse 5'-CTGCGCAAGTTAGGTTTTGT-3'; and iNOS (854 base pairs) forward 5'-TGTGTTCCACCAGGAGATGT-3', reverse 5'-AGGTGAGCTGAACGAGGAG-3' (One Trick Pony Oligos, Ransom Hill Bioscience, Ramona, CA).

The RT-PCR assay: The reverse transcriptase-polymerase chain reaction was used to detect β-actin and iNOS mRNA expression for the generation of standards. After cDNA synthesis, the PCR reactions were performed using Platinium® PCR SuperMix (Invitrogen Corp., Carlsbad, CA) per manufacturer's instructions. The β-actin and iNOS primers were purchased from Maxim Biotech Inc. (San Francisco, CA). The PCR reaction was performed under the following conditions: 35 cycles of 94°C for 1 min, annealing at 58°C for 1 min, and amplification at 72°C for 1 min. At the end of the RT-PCR reaction 12 μl of PCR products were run on a 2% agarose gel containing ethidium bromide (10 μl per 100 ml of gel). Photographs of the PCR products were taken and the density of bands determined by scanning densitometry and a Kodak Image Station (Perkin Elmer Life Sciences Inc., MA). The data were normalized to β-actin, a house-keeping gene, and expressed as relative densities. The PCR products that were used for the generation of the standard curve for Real Time-PCR were visualized over UV light.

Extraction of target genes: The PCR products were excised from the agarose gel and isolated using a MinElute Extraction kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). Briefly, the gel slice was weighed and 3 volumes of Buffer AG were added to 1 volume of gel. The gel was incubated at 50°C for 10 min and vortexed every 2-3 minutes during the incubation. After the gel was dissolved completely, 1 volume of isopropanol was added, mixed via inverting, applied to the MinElute column, and centrifuged for 1 min. After discarding the flow-through, the column was applied with Buffer QG and spun for one minute. The MinElute column was washed with Buffer PE and DNA was eluted using Rnase-Dnase free water.

Measurement of total cDNA after gel extraction: PicoGreen (Molecular Probes Inc., Eugene, OR) is considered to be a sensitive reagent for detecting dsDNA, such as cDNA, in solution. Following the manufacturer's instructions, a 2 μg/ml stock solution of dsDNA in TE buffer was prepared and samples were diluted 20-fold in DNase-RNase-free TE buffer. One-hundred μl of sample and Picogreen were added to a 96-well plate and incubated in the dark for 5 min. After incubation, the sample and standards were read using a fluorometer (CytoFlour, PerSeptive Biosystems Inc., MA) (excitation ~480nm, emission ~520nm). Double stranded DNA, diluted 2-fold to generate a standard curve of fluorescence versus DNA concentration, was used to detect the amount of cDNA in the samples.

Real time PCR: After cDNA synthesis, Real Time PCR was performed using Platinium UDG PCR SuperMix (Invitrogen Corp., Carlsbad, CA) and SybrGreen I (Molecular Probes Inc., Eugene, OR) following the manufacturer's instructions. The β-actin and iNOS primers were purchased from Maxim Biotech Inc. (San Francisco, CA). The Real Time PCR reaction was performed under the following conditions: 35 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec, and amplification at 72°C for 30 sec. β-actin mRNA encodes a cytoskeleton protein, which is expressed among almost all cell types. It is widely used to normalize results in Real-Time RT-PCR assays. Upon amplification of the target gene, integration of the double stranded DNA binding dye, SybrGreen I, into the amplification product results in an increase in fluorescence, which is a direct consequence of target amplification during PCR. Direct quantification of the PCR products was performed using the standard curve generated from appropriate standards that had known amounts of cDNA in femtograms. The target gene expression was normalized to β-actin. The fold change in iNOS expression in Con-A or Con-A and recombinant IFN-γ (rIFN-γ) stimulated lymphocytes to iNOS expression from placebo treated (control) IFN-γ knockout (IFN-γ(-/-)) mice was calculated using the ΔΔCT method: $2^{-\Delta\Delta CT}$. In this equation, ΔΔCT (delta delta threshold cycle)= ΔCT (estrogen)-ΔCT (placebo, control). ΔCT is the CT (threshold cycle) of the target gene, iNOS, subtracted from the CT of β-actin.

Detection of nitric oxide: A Greiss Assay was used to determine the presence of nitric oxide. In this assay, nitrite, an end product of nitric oxide oxidation is measured in tissue culture fluid. Greiss Assay reagents included 1% (w/v) sulfanilamide (Sigma-Aldrich Inc., MO) and 0.1% (w/v) naphthylenediamine dihydrochloride (Sigma-Aldrich Inc., MO). Both reagents were dissolved separately in 2.5% phosphoric acid. Immediately after collecting the tissue culture, 50 μl of both reagents were added to an equal volume of the culture supernatant in a 96-well round bottom plate. A serial dilution of sodium nitrite beginning at 1000 μM to 0.5 μM was used as a standard. Five to ten minutes after adding the reagents, the plates were read on an optical density plate reader set at 550 nm (Molecular Devices, Sunnyvale, CA). The levels of nitric oxide were calculated using the formula obtained from the standards using SoftMax Pro software from Molecular Devices Inc.

Determination of iNOS and COX-2 protein: Western blot assays were used to detect the iNOS and COX-2 protein levels in whole cell lysates of placebo and estrogen-treated splenic lymphocytes that were cultured with Con-A (10 μg/ml) or Con-A (10 μg/ml) plus CTLA-4Ig (15 μg/ml). At the end of the incubation period, cells were harvested and resuspended in 15μl of lysis buffer (50mM Tris pH: 7.4, 300mM NaCl, 2mM EDTA pH: 8, 0.5% Triton X-100, and 30 μl of freshly added mammalian protease inhibitor cocktail (10 μg/ml, Sigma-Aldrich Inc., MO), vortexed and incubated on ice for 15 minutes. Fifteen μl of 2x sample buffer (Laemmli Sample Buffer; Sigma-Aldrich Inc., St Louis MO) were added to the 15 μl of lysate and heated at 95^0C for 5 minutes. Samples were electrophoresed on a 12.5% gel at 25 mA constant current until the dye ran off bottom. The proteins were transferred to PDVF transfer membranes (Amersham Biosciences, CA) by blotting for 1hr 15 mins at 240 mA constant current. After transferring the proteins to a PDVF membrane, it was blocked in 5% milk in TBS-T for 1 hr at room temperature. The blot was incubated with anti-iNOS antibody (rabbit polyclonal IgG (sc-650), Santa Cruz Inc., CA) and COX-2 primary antibody (rabbit polyclonal IgG (sc-1745), Santa Cruz Inc., CA) diluted in blocking buffer (1:1000) overnight on a rocking platform. The blots were rinsed briefly in TBS-T, then washed 3 times for 10 minutes with TBS-T. The secondary HRP-conjugated antibody (anti-rabbit IgG, 1:2000, Santa

Cruz Inc., CA) was applied in blocking buffer to each blot and incubated for 1 hr. After blots were washed, they were labeled using an ECL protocol (Amersham Pharmacia Biotech, NJ) and the bands were visualized using a Kodak Image Station (Perkin Elmer Life Sciences Inc., MA).

Statistics: Data were analyzed using SAS software (SAS Institute Inc., Cary, NC) at the Laboratory for Study Design and Statistical Service, VMRCVM by co-author Dr. F. Elvinger. Data distributions and variances were evaluated using the UNIVARIATE procedure and data were logarithmically transformed when warranted. Transformed data were evaluated by analysis of variance (ANOVA) using the MIXED and GLM procedures. Model for the split-plot design included treatment, date of experiment, and their interactions tested by the main plot error term (treatment x date of experiment). Stimulant, time of measurement and interactions with treatment were tested by the overall error term. Statistically significant interactions were further investigated using the SLICE option to test the simple main effects. p-values less than 0.05 were considered to be significant. The data are logarithmically transformed and geometric means and standard errors were then back transformed for presentation in figures. Therefore, in some cases the standard errors are non-symmetric.

d.) RESULTS

Estrogen induces IFN-γ:

Consistent with our recently reported studies (33, 34), Con-A-activated splenocytes from estrogen-treated mice tended to have increased levels of IFN-γ when compared to similar cultures from placebo-treated mice ($p<0.05$) (**Figure 1, Panel A**). Since estrogen treatment increased IFN-γ levels, we next investigated whether IFN-γ-inducible iNOS and its products were also altered in Con-A-activated splenocytes of estrogen-treated mice.

Activation of splenocytes with T cell stimulation upregulates the levels of nitric oxide in estrogen-treated mice:

Activation of splenocytes with a T cell stimulant, Con-A, led to a marked increase in the levels of nitric oxide in cultures from estrogen-treated mice when compared to placebo-treated mice both at 24 hrs ($p<0.05$) and 48 hrs ($p<0.005$) of culture (**Figure 1, Panel B & C**). The level of nitric oxide in the supernatants of Con-A-activated splenocytes from estrogen-treated mice was higher at 48 hrs of culture compared to 24 hrs of culture. Interestingly, nitric oxide was not detectable in the supernatants of unstimulated cells of even estrogen-treated mice, implying that T cell activation is necessary for nitric oxide release.

Anti-CD3-activated splenocytes from estrogen-treated mice also demonstrated increased levels of nitric oxide:

We next determined whether activation of splenocytes with an optimal concentration of anti-CD3 antibodies (10 μg/ml), also induces the release of nitric oxide. As is apparent in **Figure 2, Panel A,** anti-CD3-activated splenocytes from estrogen-treated mice released significantly higher levels of nitric oxide when compared to cultures from placebo-treated mice ($p<0.05$). Similar results were also evident in cultures exposed to a 10x lower dose of anti-CD3 antibodies (1 μg/ml) (**Panel B**, $p<0.05$). Impressively, detectable levels of nitric oxide were evident in the supernatants of splenocytes from estrogen-treated mice when exposed to even a submitogenic dose of anti-CD3 antibodies (0.1 μg/ml) **Panel C,** $p<0.05$). Nitric oxide was not detectable in cultures that were left unstimulated (media only) or exposed to control antibodies (data not shown).

Estrogen treatment increases iNOS mRNA expression in Con-A-activated Splenocytes:

Since estrogen increased nitric oxide production in the supernatants of Con-A or anti-CD3-activated splenic lymphocytes from estrogen-treated mice, we next investigated

whether this increase in nitric oxide is also reflected at the level of iNOS gene transcription. Con-A-activated splenocytes from estrogen-treated mice tended to have an apparent relative increased levels of iNOS gene expression as demonstrated by RT-PCR analysis when compared to cultures from placebo-treated mice (**Figure 3,** $p<0.05$). Figure 3A shows the mean relative densities of PCR products, while Figure 3B shows a representative gel. Since RT-PCR is not a quantitative assay and merely provides a relative rough estimate of the gene expression, we next performed Real-Time PCR analysis for quantitative expression of iNOS gene expression. By Real-Time PCR analysis, Con-A-activated splenocytes from estrogen-treated mice also demonstrated significantly increased levels of expression of the iNOS gene when compared to similar cultures from placebo-treated mice ($p<0.0001$, **Figure 3C)**. Panel D of Figure 3 shows a representative gel of end-product of Real Time PCR assay as an additional confirmation of real Time PCR reaction and to demonstrate that PCR products of Real Time and RT-PCR assay are comparable.

Estrogen treatment increases iNOS protein in Con-A or Anti-CD3-activated splenocytes:

Given that Con-A or anti-CD3 activated splenocytes from estrogen-treated mice had increased levels of nitric oxide and increased expression of the iNOS gene, we next determined whether iNOS protein is also expressed at a higher level in these mice. Con-A-activated splenocytes from estrogen-treated mice had increased expression of iNOS protein compared to similar cultures from placebo-treated mice (**Figure 4A,** $p<0.005$**)**. Similarly, splenocytes from estrogen-treated mice, when exposed to various doses of anti-CD3 antibodies, also demonstrated increased levels of iNOS protein (**Figure 4B-D**, $p<0.05$). Interestingly, exposure of splenocytes to submitogenic dose of anti-CD induced detectable expression of iNOS protein bands only in cells from estrogen-treated mice but not in cells from placebo-treated mice (**Figure 4D**, $p<0.05$).

Activated splenocytes from estrogen-treated mice had increased nitric oxide regulated Cox-2 protein expression:

Several studies have shown that nitric oxide upregulates levels of COX-2 (40, 41). Given our observation of increased levels of nitric oxide in activated splenocytes from estrogen-treated mice, we then determined whether the expression of COX-2 protein levels in these cells is also altered. The relative expression of COX-2 protein was upregulated in Con-A-activated splenocytes from estrogen-treated mice when compared to controls **(Figure 5,** $p<0.05$**)**. Similarly, splenocytes from estrogen-treated mice, when activated with anti-CD3, also had increased expression of COX-2 compared to controls (n=4 per treatment, data not shown).

Blocking CD28 and B7 molecular interactions inhibits the secretion of IFN-γ and nitric oxide:

Since T cells require at least 2 signals for activation, the importance of molecular interactions of the co-stimulatory molecules CD28 on T cells and B7.1 (CD80)/B7.2 (CD86) on antigen presenting cells was investigated. Remarkably, blocking the interaction of CD28 with B7 molecules with a CTLA-4Ig fusion protein dramatically decreased the level of IFN-γ in the supernatants (**Figure 6A,** $p<0.05$). The decrease in IFN-γ was evident in both estrogen as well as placebo-treated samples, indicating the importance of this pathway and interaction of T cells with antigen presenting cells. Stimulation of enriched T cells only or enriched macrophages only with Con-A did not induce detectable levels of nitric oxide thereby indicating that physical interactions of T cells with macrophages are necessary for nitric oxide induction (data not shown). Blockade of CD28 and B7 interactions by CTLA-4Ig also significantly decreased the levels of nitric oxide in the supernatants of Con-A-activated splenocytes. The ability of CTLA-4Ig to downregulate nitric oxide was particularly evident in samples from estrogen-treated mice. With RT-PCR analysis, it was observed that the addition of CTLA-4Ig also resulted in an apparent relative decrease in iNOS gene expression (**Figure 7,** $p<0.005$). The decrease in IFN-γ and nitric oxide levels due to co-culturing with CTLA-4Ig was not due to cytotoxic effects of this fusion protein, since the viability of cells that were cultured with and without CTLA-4Ig were comparable (Media: Placebo=$9.8 \times 10^5 \pm 3.9 \times 10^5$, Estrogen= $1.7 \times 10^6 \pm 9.3 \times 10^5$; Con-A: Placebo=$5.9 \times 10^5$ ±

$2.8x10^5$, Estrogen= $6.2x10^5 \pm 2.2x10^5$; Con-A and CTLA-4Ig: Placebo=$8.4x10^5 \pm 1.9x10^5$, Estrogen= $6.9x10^5 \pm 2.4x10^5$).

Blockade of CD28 and B7 interactions also decreases COX-2 levels:

Splenocytes from estrogen and placebo-treated mice were cultured with Con-A in the presence or absence of CTLA-4Ig to determine the level of COX-2. As expected, Con-A activation of splenocytes from estrogen-treated mice, but not from placebo-treated mice, resulted in increased expression of COX-2 protein (**Figure 8A & B)**. Co-culturing with CTLA-4Ig tended to decrease the level of expression of COX-2 (**Figure 8A & B**; $p<0.05$).

The estrogen-induced increase in nitric oxide is IFN-γ-dependent: To demonstrate the importance of IFN-γ in the induction of iNOS and nitric oxide IFN-γ(-/-) mice (n=5 per treatment) were utilized. In none of the five IFN-γ(-/-) mice that were treated with estrogen (n=5) or placebo (n=5) iNOS mRNA was detectable by Real Time PCR in Con-A activated splenocytes. Further, there were neither detectable levels of iNOS protein in Con-A activated splenocytes from estrogen or placebo-treated IFN-γ(-/-) mice nor detectable levels of nitric oxide in the supernatants of these cultures.

Moreover, we also utilized estrogen (n=10) and placebo (n=10) treatment to Interferon Regulatory Factor-1 (IRF-1)-deficient mice. These mice had very low levels of IFN-γ (Media: not detectable; Con-A: Placebo: 210.97±42.2 pg/ml, Estrogen: 413.33±108.3 pg/ml after 24 hrs of culture; Media: not detectable; Con-A: Placebo: 670.02 ± 116.6 pg/ml, Estrogen: 1432.8 ± 240.5 pg/ml after 48 hrs of culture; n=10 per treatment) and undetectable levels of nitric oxide or iNOS. Overall, these data imply that estrogen-induced expression of iNOS is dependent upon IFN-γ.

Since the above data implied the importance of IFN-γ in induction of iNOS/nitric oxide (rather than direct effect of estrogen induction of iNOS/nitric oxide), we next utilized wild-type estrogen-treated mice and splenocytes from these mice were stimulated with a

submitogenic dose of Con-A (1 μg/ml) in the presence or absence of rIFN-γ (10 ng/ml). Interestingly, stimulation of splenocytes with a submitogenic dose of Con-A (1 μg/ml) induced detectable levels of nitric oxide in the supernatants of estrogen-treated wild-type mice, when compared to placebo-treated mice (**Figure 9**; $p<0.05$). No nitric oxide was detectable in unstimulated cultures from estrogen or placebo-treated mice. Deliberate addition of IFN-γ to aliquots of these cultures induced nitric oxide (**Figure 9**) indicating that nitric oxide generation is responsive to and dependent on IFN-γ.

e.) DISCUSSION:

It is now recognized that human exposure to estrogens occurs through multiple means including endogenous, pharmaceutical, and environmental sources (27, 42). Many studies have clearly shown that the immune system is highly responsive to estrogens (27, 28, 43-46). All the major cells of the immune system (T, B, macrophages, and dendritic cells) are potential targets for estrogens. Furthermore, estrogens have been incriminated in many inflammatory and autoimmune conditions (27, 28, 32, 44). Therefore, it is not surprising that studies aimed at understanding the effects of estrogen on the immune system are recognized as being of increasing biomedical importance.

A significant novel observation in this study is that splenocytes from estrogen-treated mice, when exposed to T cell stimulants, release abundant amounts of nitric oxide in supernatants. The levels of nitric oxide in the supernatants of Con-A-activated splenocytes from estrogen-treated mice were particularly high after 48 hrs of culture. Estrogen appears to sensitize splenocytes to release nitric oxide since splenocytes from estrogen-treated mice, but not from placebo-treated mice, when exposed to even sub-mitogenic doses of anti-CD3 antibodies or Con-A induced detectable levels of nitric oxide. The increase in nitric oxide in Con-A or anti-CD3-activated splenocytes from estrogen-treated mice is due to estrogen-induced up-regulation of iNOS mRNA (as demonstrated by Real-Time PCR) and a concomitant increase in iNOS protein (as demonstrated by Western blot assays). Our studies show that one indirect consequence of T cell activation is the release of nitric oxide.

Recent studies have also shown that nitric oxide can induce the expression of cyclooxygenase-2 (COX-2) or prostaglandin H synthase, an enzyme that catalyzes arachidonic acid for the formation of prostaglandin in tissues (47-49). For example, studies in mouse skin cells have shown that the suppression of iNOS by specific inhibitors also inhibited COX-2, while the addition of nitric oxide donors induced the expression of COX-2 (47). Similarly, studies in three colon cancer cell lines have shown that nitric oxide also upregulated the expression of COX-2 (48). Further, nitric oxide is necessary for maintaining prolonged COX-2 mRNA expression in IFN-γ and LPS-stimulated macrophage cell lines (18, 40) as well as peritoneal macrophages (19), and iNOS and nitric oxide upregulated COX-2 in myocardial cells (49). To our knowledge, the relationship of nitric oxide and COX-2 in immune cells such as splenocytes has thus far not been investigated. Our studies clearly show that Con-A activation of splenocytes not only upregulated nitric oxide, but also COX-2 expression.

It is noteworthy that T cell stimulation was necessary to induce the release of nitric oxide, since nitric oxide was not released in unstimulated cultures. It is also of note that whole splenocytes (a mixture of T, B, and macrophages) were necessary to demonstrate the increased release of nitric oxide, since nitric oxide was not detectable in either Con-A-activated enriched T cells alone or Con-A-activated non-T cells (mostly macrophages) alone from estrogen-treated mice (data not shown). We further show that the up-regulation of iNOS/nitric oxide by estrogen is mediated by IFN-γ. Blocking the molecular interactions of CD28 and B7 molecules with a CTLA-4Ig fusion protein markedly decreased not only IFN-γ secretion, but also iNOS mRNA and nitric oxide. Blocking CD28 and B7 interactions also significantly decreased COX-2 levels. The dependence of nitric oxide on IFN-γ is also evident by the observations that estrogen administration to either IFN-γ deficient mice or IRF-1 deficient mice (which have very low levels of IFN-γ) did not upregulate the levels of nitric oxide or iNOS mRNA. Moreover, direct addition of recombinant IFN-γ to Con-A-activated splenocytes from wild-type tended to upregulate nitric oxide or iNOS protein, respectively. Furthermore, the kinetics of induction of IFN-γ and nitric oxide also suggests that estrogen induces

IFN-γ first, which is then followed by nitric oxide. For example, intracellular IFN-γ is readily measured in freshly-isolated splenocytes from estrogen-treated mice and detectable levels of IFN-γ in the supernatants of Con-A-activated splenocytes from estrogen-treated mice become evident as early as 6 hours of culture (33). High levels of IFN-γ in cultures of Con-A-activated splenocytes from estrogen-treated mice are evident at 24 hours of culture (which continue to rise at 48 hours of culture). In contrast to the kinetics of estrogen-induced IFN-γ, nitric oxide is not detectable in either fresh cultures or after 6 hours of culture. At 24 hours of culture, the levels of nitric oxide are detectable and high levels become evident only after 48 hours of culture.

Several *in vitro* studies have also shown the effect of estrogen on iNOS or nitric oxide in non-immune cells such as isolated rat aortic rings, ovine coronary artery cells, rat myocytes, rat uterus and endothelial cells, and macrophage-like cell lines (J774, RAW-264.7) and peritoneal macrophages (50-55). Depending upon the cell type and the stimulus, direct exposure of these cells to estrogen either increased or suppressed iNOS or nitric oxide. For example, estrogen increased iNOS expression in RAW-267.7 cells and peritoneal macrophages (50, 55) or suppressed IL-1 induced iNOS expression in isolated rat aortic cells (52). Our studies differed from these studies in several important respects. First, to date no studies have been conducted on the *in vivo* effects of estrogen. Second, our studies examined the consequence of T cell activation in splenocytes by studying the interactions of T cells and macrophages. The use of splenocytes simulates as natural state as possible, where lymphocytes (T, B) and antigen presenting cells (e.g. macrophages, dendritic cells) physically co-exist and interact to influence one another. This later point is highlighted by the fact that blocking the interactions of T and antigen presenting cells through CTLA-4Ig markedly decreased IFN-γ and nitric oxide. In this experimental setting, *in vivo* estrogen treatment did not directly induce iNOS or nitric oxide rather these effects were mediated through induction of cytokines such as IFN-γ.

These studies provide a new understanding of estrogen modulation of the immune system via release of nitric oxide, especially considering that nitric oxide has potent effects on the immune system including defense against microbial agents, cellular proliferation,

apoptosis, and cell survival (2, 56, 57). Further, one of the downstream nitric oxide-induced events is the induction of COX-2 (47-49), which suggests that estrogen also modulates eicosanoid production that is involved in inflammation. Thus, estrogen promotion of inflammation and certain autoimmune diseases may be mediated by upregulation of pro-inflammatory IFN-γ, which in turn can induce iNOS and nitric oxide (**Figure 10**). Nitric oxide in turn can induce COX-2, which is largely responsible for prostaglandin-E2 secretion (58). These are new observations that may provide insights into mechanisms of estrogen-induced promotion of inflammatory diseases and aid design of new therapeutic interventions to manipulate levels of nitric oxide (Non-steroidal anti-inflammatory drugs (NSAID-NO)).

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f.) REFERENCES

1. Bogdan, C. 2001. Nitric oxide and the immune response. *Nat Immunol 2:907*.
2. MacMicking, J., Q. W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu Rev Immunol 15:323*.
3. Wink, D. A., I. Hanbauer, M. B. Grisham, F. Laval, R. W. Nims, J. Laval, J. Cook, R. Pacelli, J. Liebmann, M. Krishna, P. C. Ford, and J. B. Mitchell. 1996. Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. *Curr Top Cell Regul 34:159*.
4. Lipton, S. A. 1996. Distinctive chemistries of no-related species. *Neurochem Int 29:111*.
5. Kolb, H., and V. Kolb-Bachofen. 1998. Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? *Immunol Today 19:556*.
6. Ludwig, M. L., and M. A. Marletta. 1999. A new decoration for nitric oxide synthase - a Zn(Cys)4 site. *Structure Fold Des 7:R73*.
7. Huang, P. L., and E. H. Lo. 1998. Genetic analysis of NOS isoforms using nNOS and eNOS knockout animals. *Prog Brain Res 118:13*.
8. Mungrue, I. N., and D. S. Bredt. 2004. nNOS at a glance: implications for brain and brawn. *J Cell Sci 117:2627*.
9. Sessa, W. C. 2004. eNOS at a glance. *J Cell Sci 117:2427*.
10. Liew, F. Y., and F. E. Cox. 1991. Nonspecific defence mechanism: the role of nitric oxide. *Immunol Today 12:A17*.
11. Sicher, S. C., M. A. Vazquez, and C. Y. Lu. 1994. Inhibition of macrophage Ia expression by nitric oxide. *J Immunol 153:1293*.
12. Bogdan, C. 1997. Of microbes, macrophages and nitric oxide. *Behring Inst Mitt:58*.
13. Bogdan, C., M. Rollinghoff, and A. Diefenbach. 2000. The role of nitric oxide in innate immunity. *Immunol Rev 173:17*.

14. Fu, J. Y., J. L. Masferrer, K. Seibert, A. Raz, and P. Needleman. 1990. The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *J Biol Chem 265:16737*.

15. Xie, W. L., J. G. Chipman, D. L. Robertson, R. L. Erikson, and D. L. Simmons. 1991. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci U S A 88:2692*.

16. Hinz, B., K. Brune, and A. Pahl. 2000. Prostaglandin E(2) upregulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochem Biophys Res Commun 272:744*.

17. Hinz, B., K. Brune, and A. Pahl. 2000. Cyclooxygenase-2 expression in lipopolysaccharide-stimulated human monocytes is modulated by cyclic AMP, prostaglandin E(2), and nonsteroidal anti-inflammatory drugs. *Biochem Biophys Res Commun 278:790*.

18. Salvemini, D., T. P. Misko, J. L. Masferrer, K. Seibert, M. G. Currie, and P. Needleman. 1993. Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A 90:7240*.

19. Habib, A., C. Bernard, M. Lebret, C. Creminon, B. Esposito, A. Tedgui, and J. Maclouf. 1997. Regulation of the expression of cyclooxygenase-2 by nitric oxide in rat peritoneal macrophages. *J Immunol 158:3845*.

20. Chun, K. S., H. H. Cha, J. W. Shin, H. K. Na, K. K. Park, W. Y. Chung, and Y. J. Surh. 2003. Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-{kappa}B. *Carcinogenesis*.

21. Vladutiu, A. O. 1995. Role of nitric oxide in autoimmunity. *Clin Immunol Immunopathol 76:1*.

22. Singh, V. K., S. Mehrotra, P. Narayan, C. M. Pandey, and S. S. Agarwal. 2000. Modulation of autoimmune diseases by nitric oxide. *Immunol Res 22:1*.

23. Kroncke, K. D., K. Fehsel, and V. Kolb-Bachofen. 1998. Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol 113:147*.

24. Rosenfeld, C. R., C. Chen, T. Roy, and X. Liu. 2003. Estrogen selectively up-regulates eNOS and nNOS in reproductive arteries by transcriptional mechanisms. *J Soc Gynecol Investig 10:205*.

25. Grohe, C., S. Kann, L. Fink, P. C. Djoufack, M. Paehr, M. van Eickels, H. Vetter, R. Meyer, and K. B. Fink. 2004. 17 Beta-estradiol regulates nNOS and eNOS activity in the hippocampus. *Neuroreport 15:89*.

26. Olsen, N. J., and W. J. Kovacs. 1996. Gonadal steroids and immunity. *Endocr Rev 17:369*.

27. Ansar Ahmed, S., B. D. Hissong, D. Verthelyi, K. Donner, K. Becker, and E. Karpuzoglu-Sahin. 1999. Gender and risk of autoimmune diseases: possible role of estrogenic compounds. *Environ Health Perspect 107 Suppl 5:681*.

28. Ansar Ahmed, S., W. J. Penhale, and N. Talal. 1985. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol 121:531*.

29. Homo-Delarche, F., F. Fitzpatrick, N. Christeff, E. A. Nunez, J. F. Bach, and M. Dardenne. 1991. Sex steroids, glucocorticoids, stress and autoimmunity. *J Steroid Biochem Mol Biol 40:619*.

30. Cutolo, M., B. Seriolo, B. Villaggio, C. Pizzorni, C. Craviotto, and A. Sulli. 2002. Androgens and estrogens modulate the immune and inflammatory responses in rheumatoid arthritis. *Ann N Y Acad Sci 966:131*.

31. Friedman, A., and Y. Waksman. 1997. Sex hormones and autoimmunity. *Isr J Med Sci 33:254*.

32. Lahita, R. G. 1999. The role of sex hormones in systemic lupus erythematosus. *Curr Opin Rheumatol 11:352*.

33. Karpuzoglu-Sahin, E., B. D. Hissong, and S. Ansar Ahmed. 2001. Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. *J Reprod Immunol 52:113*.

34. Karpuzoglu-Sahin, E., Y. Zhi-Jun, A. Lengi, N. Sriranganathan, and S. Ansar Ahmed. 2001. Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. *Cytokine 14:208*.

35. Ansar Ahmed, S., M. J. Dauphinee, A. I. Montoya, and N. Talal. 1989. Estrogen induces normal murine CD5+ B cells to produce autoantibodies. *J Immunol 142:2647*.

36. Ansar Ahmed, S., and D. Verthelyi. 1993. Antibodies to cardiolipin in normal C57BL/6J mice: induction by estrogen but not dihydrotestosterone. *J Autoimmun 6:265*.

37. Donner, K. J., K. M. Becker, B. D. Hissong, and S. A. Ahmed. 1999. Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. *Cytometry 35:80*.

38. Ansar Ahmed, S., R. M. Gogal, Jr., and J. E. Walsh. 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J Immunol Methods 170:211*.

39. Calemine, J. B., R. M. Gogal, Jr., A. Lengi, P. Sponenberg, and S. A. Ahmed. 2002. Immunomodulation by diethylstilbestrol is dose and gender related: effects on thymocyte apoptosis and mitogen-induced proliferation. *Toxicology 178:101*.

40. Perkins, D. J., and D. A. Kniss. 1999. Blockade of nitric oxide formation down-regulates cyclooxygenase-2 and decreases PGE2 biosynthesis in macrophages. *J Leukoc Biol 65:792*.

41. Ishimura, N., S. F. Bronk, and G. J. Gores. 2004. Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. *Am J Physiol Gastrointest Liver Physiol 287:G88*.

42. Ansar Ahmed, S. 2000. The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. *Toxicology 150:191*.

43. Puzanov, I. J., M. Bennett, and V. Kumar. 1996. IL-15 can substitute for the marrow microenvironment in the differentiation of natural killer cells. *J Immunol 157:4282*.

44. Ansar Ahmed, S., and N. Talal. 1989. Sex hormones and autoimmune rheumatic disorders. *Scand J Rheumatol 18:69*.

45. Castagnetta, L., O. M. Granata, A. Traina, L. Cocciadiferro, A. Saetta, R. Stefano, M. Cutolo, and G. Carruba. 2002. A role for sex steroids in autoimmune diseases: a working hypothesis and supporting data. *Ann N Y Acad Sci 966:193*.

46. Verthelyi, D. 2001. Sex hormones as immunomodulators in health and disease. *Int Immunopharmacol 1:983*.

47. Chun, K. S., H. H. Cha, J. W. Shin, H. K. Na, K. K. Park, W. Y. Chung, and Y. J. Surh. 2004. Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-kappaB. *Carcinogenesis 25:445*.

48. Liu, Q., S. T. Chan, and R. Mahendran. 2003. Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines. *Carcinogenesis 24:637*.

49. Xuan, Y. T., Y. Guo, Y. Zhu, H. Han, R. Langenbach, B. Dawn, and R. Bolli. 2003. Mechanism of cyclooxygenase-2 upregulation in late preconditioning. *J Mol Cell Cardiol 35:525*.

50. Azenabor, A. A., S. Yang, G. Job, and O. O. Adedokun. 2004. Expression of iNOS gene in macrophages stimulated with 17beta-estradiol is regulated by free intracellular Ca2+. *Biochem Cell Biol 82:381*.

51. Tamura, K., K. Yamaguchi, and H. Kogo. 2000. 17Beta-estradiol inhibits ovariectomy-induced expression of inducible nitric oxide synthase in rat aorta in vivo. *Life Sci 66:PL 259*.

52. Kauser, K., D. Sonnenberg, P. Diel, and G. M. Rubanyi. 1998. Effect of 17beta-oestradiol on cytokine-induced nitric oxide production in rat isolated aorta. *Br J Pharmacol 123:1089*.

53. Baker, A. E., V. M. Brautigam, and J. J. Watters. 2004. Estrogen Modulates Microglial Inflammatory Mediator Production via Interactions with Estrogen Receptor {beta}. *Endocrinology*.

54. Nuedling, S., R. H. Karas, M. E. Mendelsohn, J. A. Katzenellenbogen, B. S. Katzenellenbogen, R. Meyer, H. Vetter, and C. Grohe. 2001. Activation of estrogen receptor beta is a prerequisite for estrogen-dependent upregulation of nitric oxide synthases in neonatal rat cardiac myocytes. *FEBS Lett 502:103*.

55. Hong, M., and Q. Zhu. 2004. Macrophages are activated by 17beta-estradiol: possible permission role in endometriosis. *Exp Toxicol Pathol 55:385*.

56. Kroncke, K. D., K. Fehsel, C. Suschek, and V. Kolb-Bachofen. 2001. Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *Int Immunopharmacol 1:1407*.

57. Dimmeler, S., and A. M. Zeiher. 1997. Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. *Nitric Oxide 1:275*.

58. Steer, S. A., and J. A. Corbett. 2003. The role and regulation of COX-2 during viral infection. *Viral Immunol 16:447*.

g.) FIGURES

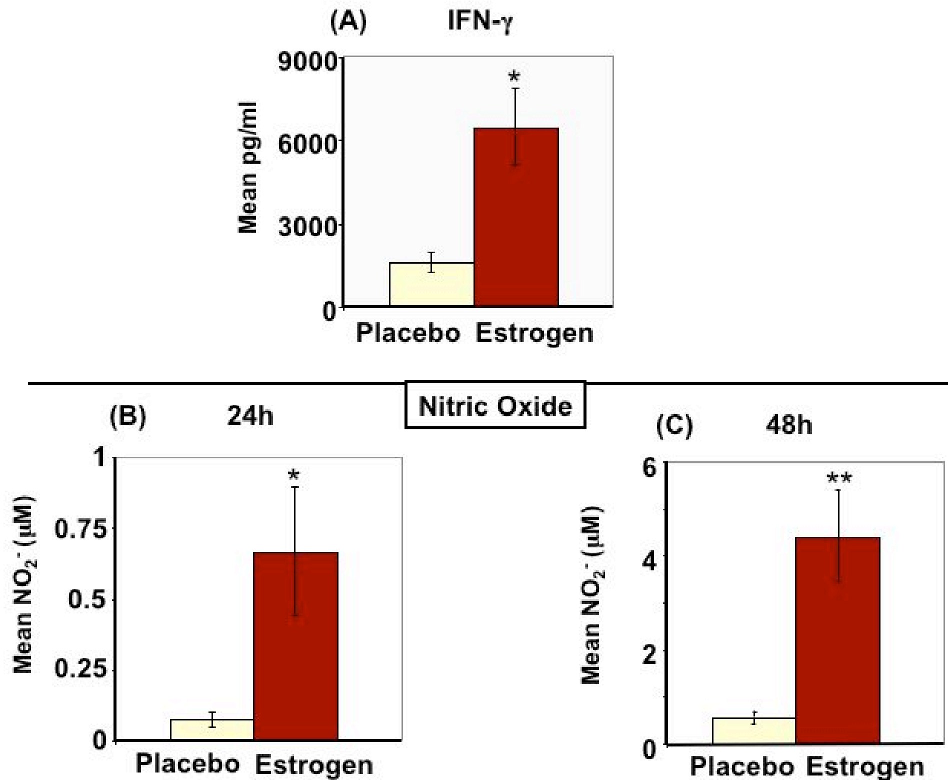

Figure 1. Estrogen induces IFN-γ and augments the levels of IFN-γ-inducible nitric oxide: Splenocytes were isolated from estrogen or placebo-treated orchiectomized mice. Cells were stimulated with an optimal concentration of Concanavalin-A (Con-A; 10 μg/ml) or left unstimulated (media only) and the levels of IFN-γ and nitric oxide in the supernatants were determined by a specific ELISA and a Griess Assay, respectively. Supernatants of Con-A-activated splenic lymphocytes cultured for 24 hrs from estrogen-treated mice had significantly higher levels of IFN-γ compared to placebo controls (Panel A; n=15 mice per placebo or estrogen-treatment, $p<0.05$). Panel B shows the levels of nitric oxide in the supernatants from splenocytes cultured with Con-A for 24 hrs (n=14 mice per placebo or estrogen-treatment; $p<0.05$). Panel C shows the levels of nitric oxide in the supernatants from splenocytes cultured with Con-A for 48 hrs (n=13 mice per placebo or estrogen-treatment; $p<0.005$). In the supernatants of splenic lymphocytes left unstimulated in media IFN-γ or nitric oxide were not detectable. Data are presented as geometric means with standard errors.

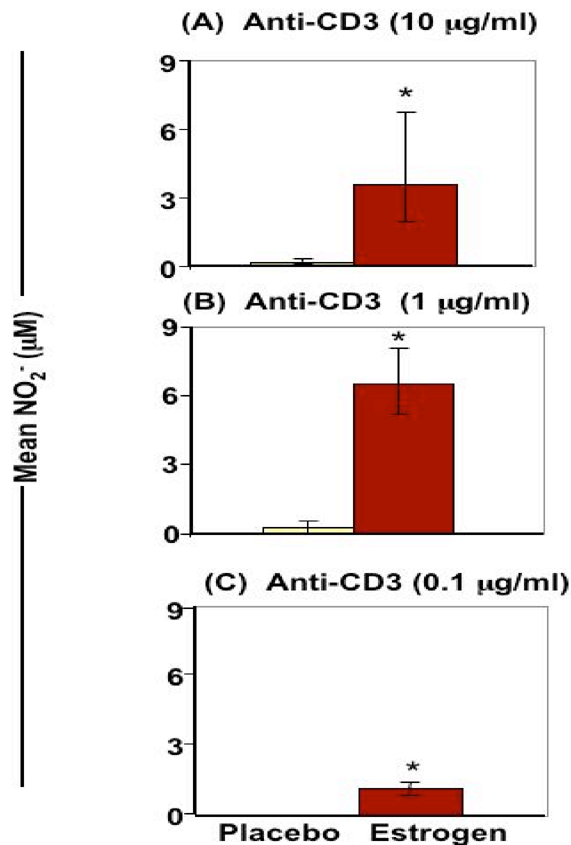

Figure 2. Anti-CD3 activation of splenocytes from estrogen-treated mice induces increased levels of nitric oxide: Splenocytes from estrogen or placebo-treated orchiectomized mice were stimulated *in vitro* with anti-CD3 antibodies (10, 1, or 0.1 μg/ml) or left unstimulated (media only) for 48 hrs. The level of nitric oxide was measured with Griess Assay. Panel A shows levels of nitric oxide in the supernatants from splenic lymphocytes cultured with 10 μg/ml of anti-CD3 antibody (Placebo: n=7 mice, estrogen: n=7 mice; $p<0.05$). Panel B shows the levels of nitric oxide in the supernatants from splenic lymphocytes cultured with 1 μg/ml of anti-CD3 antibody (Placebo: n=13 mice, estrogen: n=13 mice; $p<0.05$). Panel C shows the levels of nitric oxide in the supernatants from splenic lymphocytes cultured with 0.1 μg/ml of anti-CD3 antibody (Placebo: n=11 mice, estrogen: n=11 mice; $p<0.05$). Nitric oxide was not detectable in the supernatants of splenocyte cultures that were not stimulated (i.e. in media only). Nitric oxide was also not detectable in selected cultures, which were exposed to control antibodies. Data are presented as geometric means with standard errors.

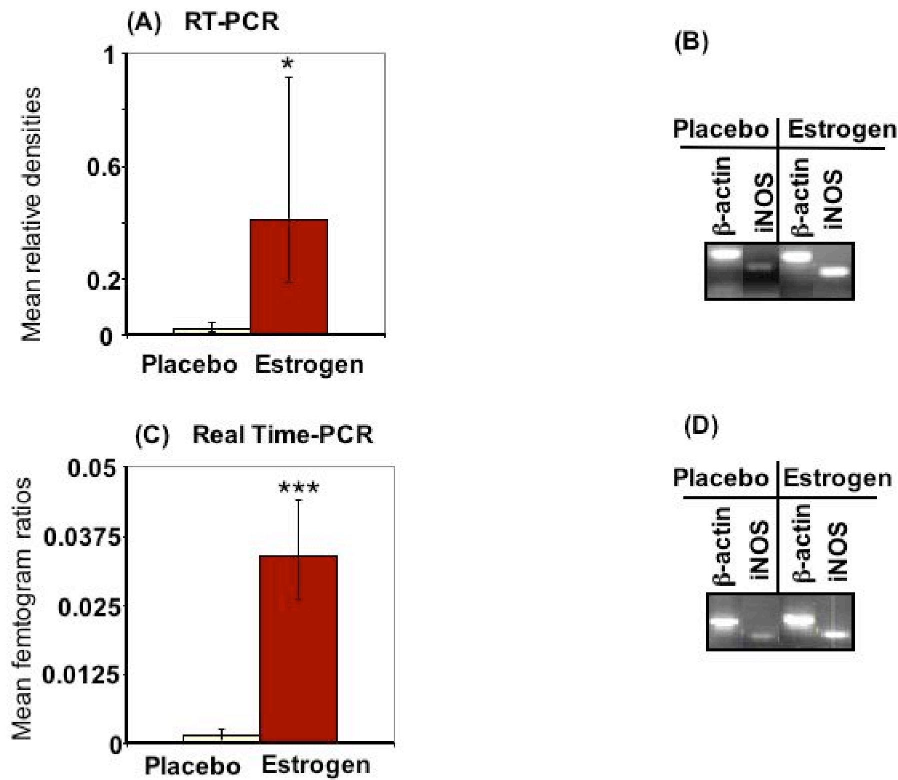

Figure 3. Estrogen upregulates iNOS mRNA expression: Splenic lymphocytes from estrogen or placebo-treated orchietomized mice were stimulated with Con-A (10 µg/ml) for 24 hrs and the relative expression of iNOS mRNA was determined by RT-PCR (Placebo: n=22 mice, estrogen: n=22 mice; $p<0.05$) (Panel A). RT-PCR products were scanned with a scanning densitometer and normalized to their own β-actin, a house-keeping gene. Panel B shows a representative result for of iNOS mRNA expression after RT-PCR. Panel C shows the quantitative detection of iNOS mRNA expression by the Real Time PCR assay. The Real Time PCR products were calculated in femtograms using the standard curve derived from cDNA standards of the respective genes, iNOS and β-actin. The expression of iNOS mRNA was normalized to β-actin. The data are presented as geometric means of femtogram ratios with standard error bars (Placebo: n=8 mice, estrogen: n=8 mice; $p<0.0001$). Panel D shows representative end product of Real Time PCR. Data are presented as geometric means of relative densities with standard error bars.

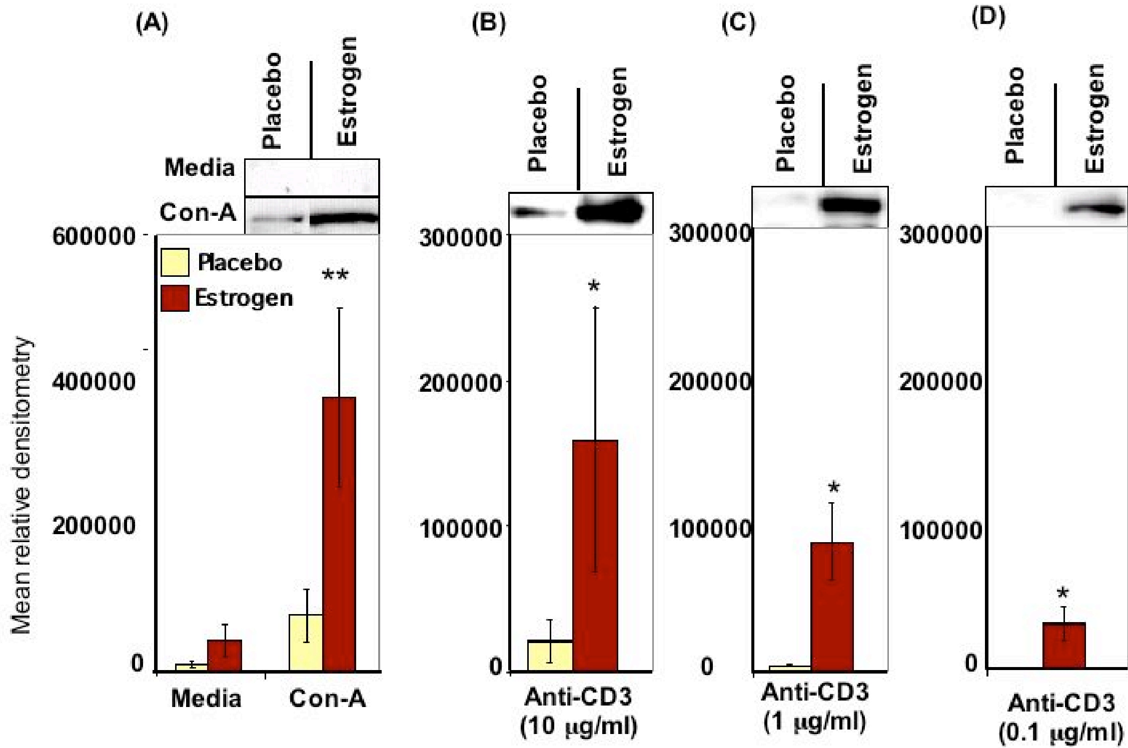

Figure 4. **The expression of iNOS protein is increased in estrogen-treated splenic lymphocytes**: Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml), anti-CD3 antibodies (10, 1, or 0.1 μg/ml), or left unstimulated (media) for 24 hrs. The expression of iNOS protein was determined by the Western blot analysis. The top portion of Panel A shows a representative experiment with unstimulated (media) or Con-A-stimulated cells from estrogen or placebo-treated orchiectomized male mice. The bottom portion of Panel A shows mean relative densitometry data for iNOS protein expression (Media: Placebo: n=6 mice, estrogen: n=6 mice; Con-A: Placebo: n=6 mice, estrogen: n=6 mice; $p<0.005$). The top portion of Panel B shows a representative experiment for iNOS protein from splenocytes from estrogen or placebo-treated orchiectomized mice, which were stimulated with 10 μg/ml of anti-CD3 antibodies. The bottom portion of Panel B shows mean relative densitometry data for iNOS protein expression (Placebo: n=3 mice, estrogen: n=3 mice; $p<0.05$). Panel C: the bottom portion shows mean relative densitometry data for iNOS protein expression (Placebo: n=4 mice, estrogen: n=4 mice; $p<0.05$). The top portion shows a representative experiment of 1 μg/ml of anti-CD3 antibody-stimulated cells from estrogen or placebo-treated orchiectomized mice. The bottom portion of Panel D shows mean relative densitometry data for iNOS protein expression (Placebo: n=5 mice, estrogen: n=5 mice; $p<0.05$). The top portion of Panel D shows a representative experiment of 0.1 μg/ml of anti-CD3 antibody-stimulated cells from estrogen or placebo-treated orchiectomized mice. All data are presented as geometric means with standard error bars.

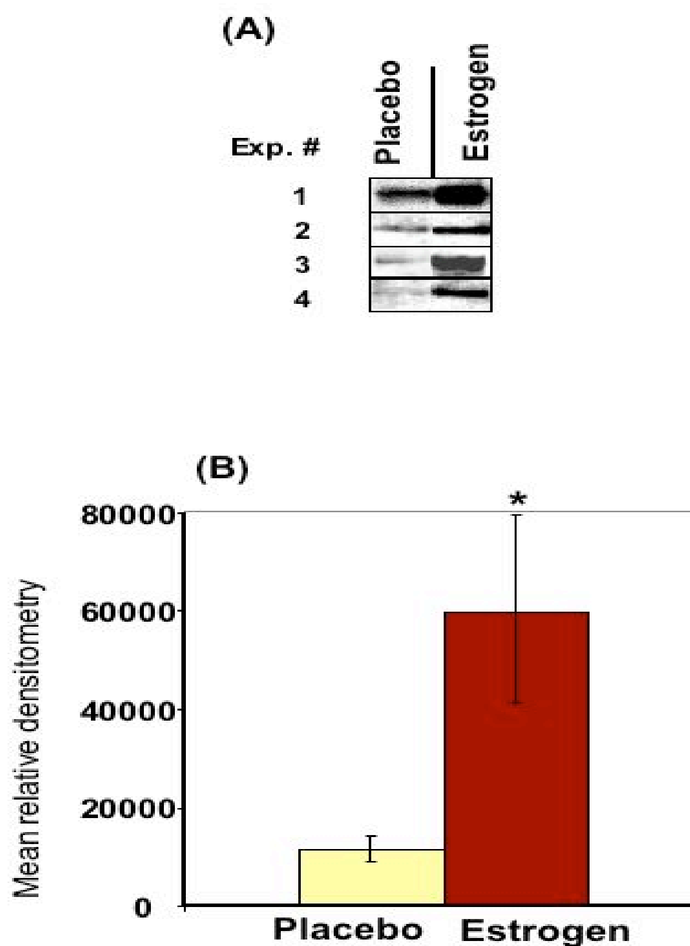

Figure 5 Cox-2 expression is increased in estrogen-treated splenic lymphocytes: Splenic lymphocytes from estrogen (n=4) or placebo (n=5)-treated mice were stimulated with Con-A (10 μg/ml) and cultured for 48 hrs. The expression of Cox-2 protein was determined by Western blot analysis. Panel A shows Cox-2 protein expression from 4 different experiments. Panel B shows the relative densitometry data of Cox-2 expression presented [geometric means with standard error bars (Placebo: n=5 mice, estrogen: n=5 mice; $p<0.05$)] for Con-A stimulated cells from estrogen or placebo-treated orchiectomized mice.

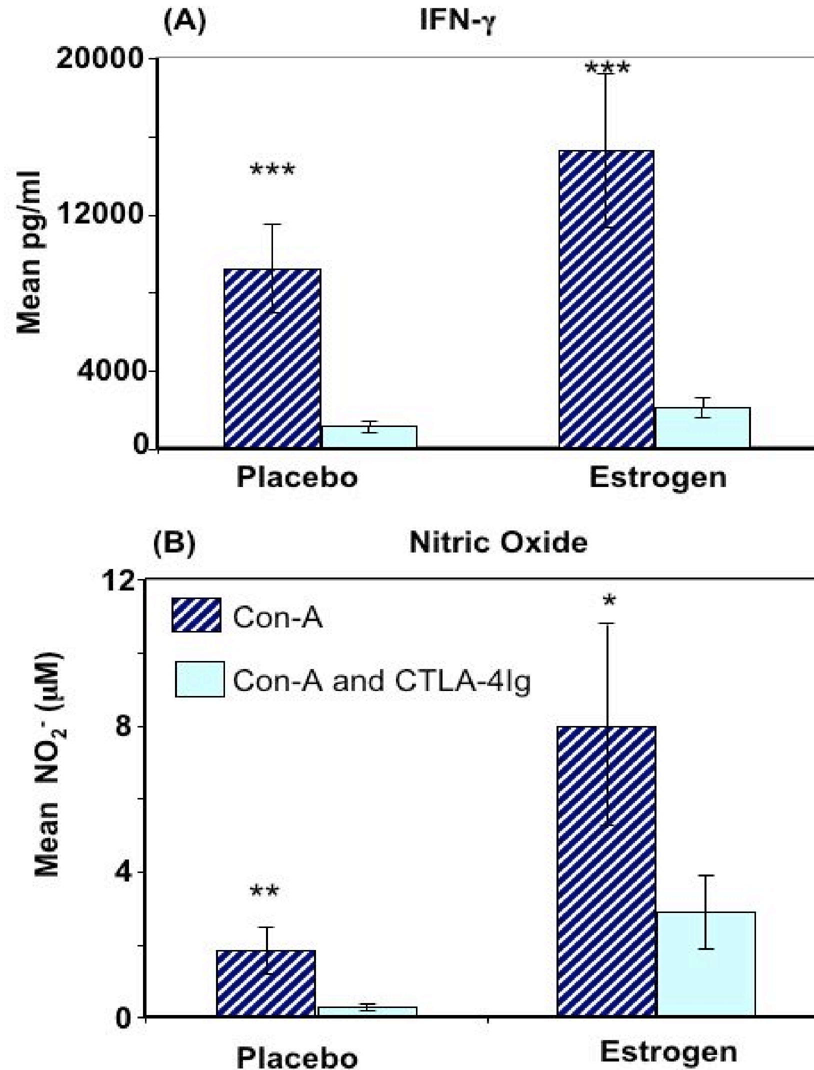

Figure 6. Blocking CD28-B7 molecular interactions inhibits IFN-γ and IFN-γ-inducible nitric oxide: Splenic lymphocytes isolated from estrogen or placebo-treated gonadectomized male mice were cultured with Con-A (10 μg/ml) in the presence or absence of CTLA-4Ig fusion protein (15 μg/ml) for 24 or 48 hrs. The levels of IFN-γ in the supernatants of Con-A stimulated splenic lymphocytes were significantly decreased in cultures with CTLA-4Ig. This was particularly evident in cultures from estrogen-treated mice as shown in Panel A (Con-A: Placebo n=15 mice, estrogen n=20 mice; Con-A and CTLA-4Ig: Placebo n=15 mice, estrogen n=15 mice; $p<0.05$). Similarly, blocking CD28 and B7 interactions with CTLA-4Ig also abrogated nitric oxide release as shown in Panel B (Con-A: Placebo n=15 mice, estrogen n=20 mice; Con-A and CTLA-4Ig: Placebo n=15 mice, estrogen n=15 mice; $p<0.0001$). Data are presented as geometric means with standard errors. IFN-γ and nitric oxide were only detected in Con-A or Con-A and CTLA-4Ig activated cells but not in unstimulated (media only) cells.

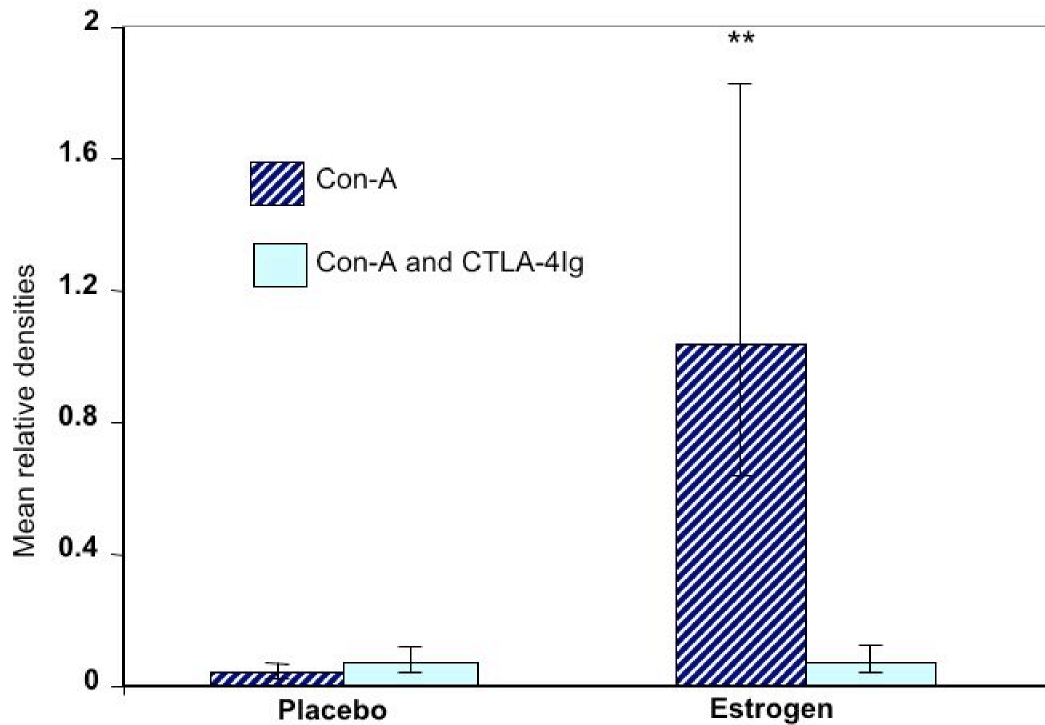

Figure 7. iNOS mRNA expression is drastically reduced in the absence of costimulation: Splenic lymphocytes from estrogen or placebo-treated orchietomized male mice were stimulated with Con-A (10 μg/ml) or Con-A and CTLA-4Ig (15 μg/ml) for 24 hrs. The iNOS RT-PCR products were scanned with a scanning densitometer and normalized to β-actin, a house-keeping gene. The expression of iNOS mRNA is presented as geometric means of relative densities with standard error bars (Placebo: n=5 mice, estrogen: n=5 mice; $p<0.005$).

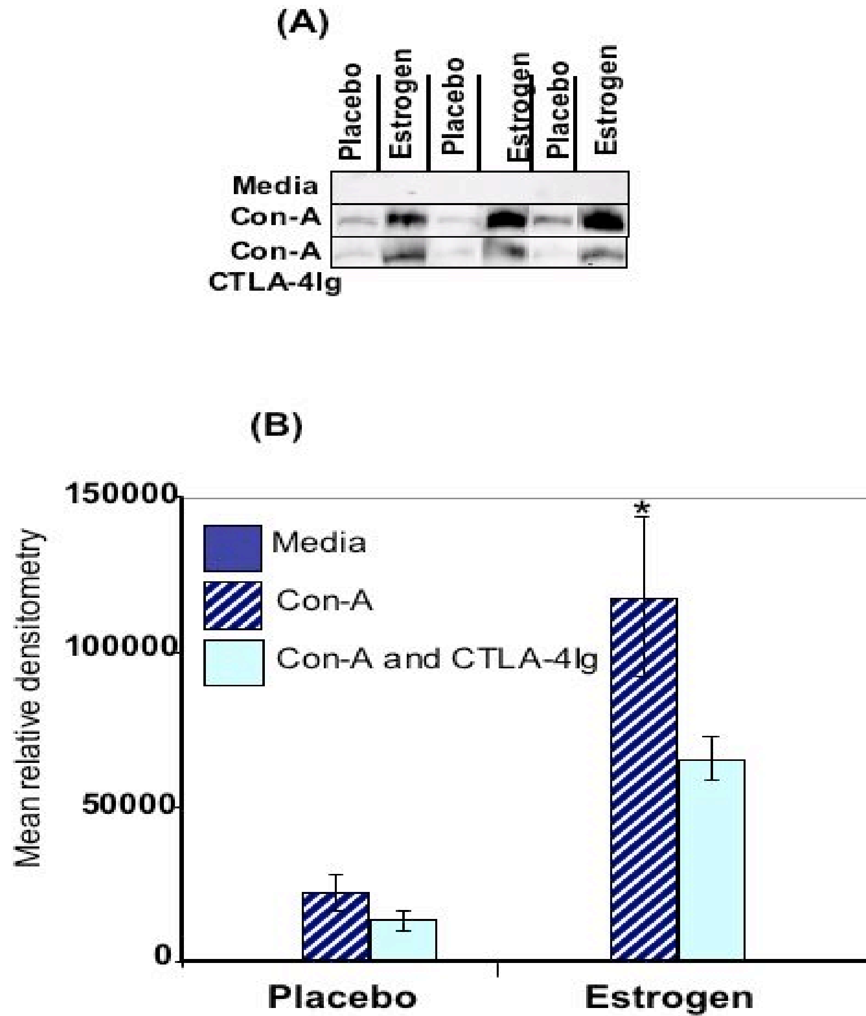

Figure 8 Cox-2 expression is increased in estrogen-treated splenic lymphocytes: Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml) or Con-A (10 μg/ml) and CTLA-4Ig (15 μg/ml) for 48 hours of incubation. The expression of Cox-2 protein was determined by Western blot analysis. Panel A shows three representative experiments of Cox-2 protein expression in cells stimulated with Con-A or Con-A and CTLA-4Ig or left unstimulated in media for 48 hrs. Panel B shows the relative densitometry data for Cox-2 protein expression (Placebo: n=3 mice, estrogen: n=3 mice; $p<0.05$). Data are presented as geometric means with standard error bars.

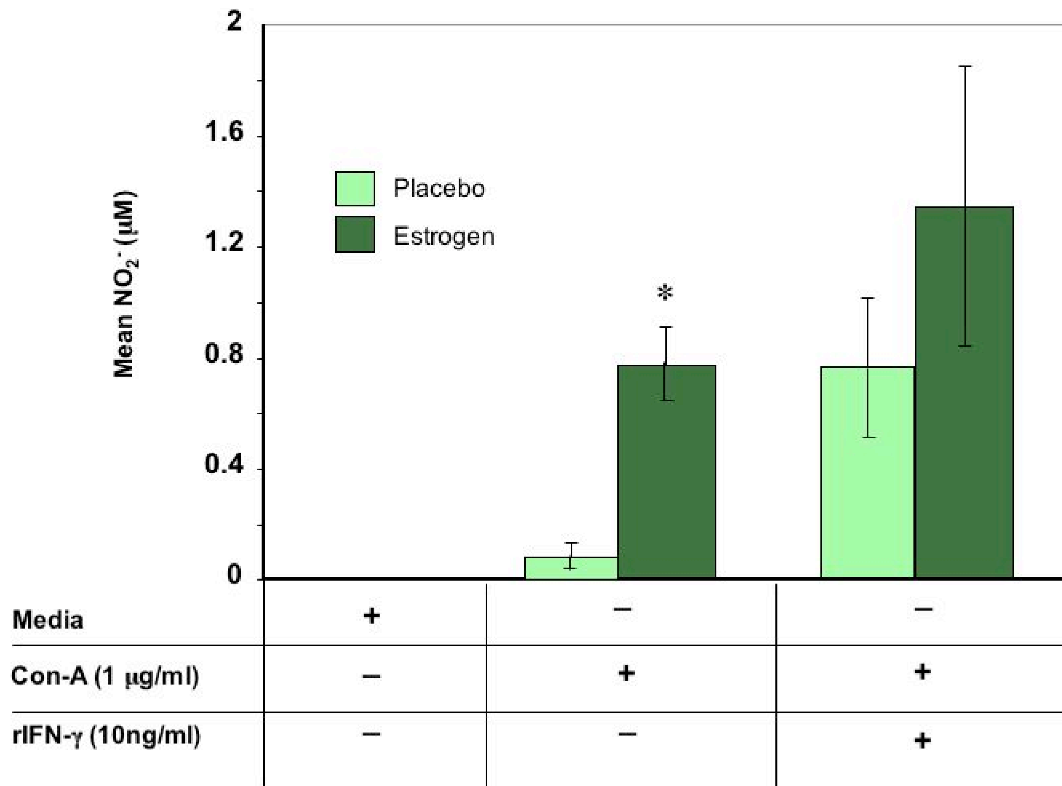

Figure 9. **Low doses of Con-A and rIFN-γ stimulation increase nitric oxide**: Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with submitogenic doses of Con-A (1 μg/ml) with or without recombinant IFN-γ (rIFN-γ, 10 ng/ml) for 24 hours of incubation. Levels of nitric oxide in the supernatants of Con-A and rIFN-γ-stimulated splenic lymphocytes were significantly increased (Placebo: n=8 mice, estrogen: n=8 mice; $p<0.05$). Data are presented as geometric means with standard error bars.

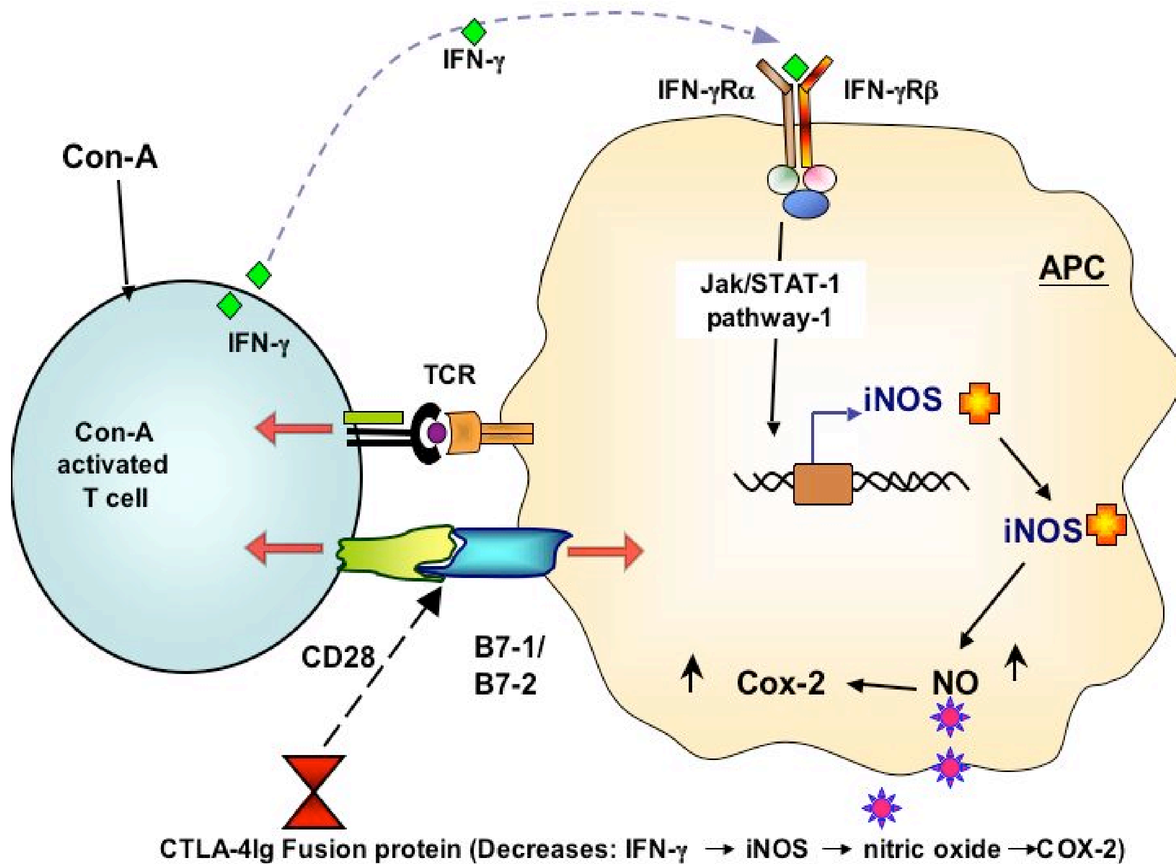

Figure 10. The effect of estrogen activated splenocytes IFN-γ, IFN-γ-inducible iNOS, nitric oxide, and nitric oxide-stimulated Cox-2: Panel A: Signaling through T cell receptor (TCR) and co-stimulatory molecules such as CD28 and B7.1/B7.2, is critical to the activation of lymphocytes. Concanavalin–A (Con-A), a strong pan-T cell stimulant, results in increased levels of IFN-γ protein in the supernatants of splenic lymphocytes from estrogen-treated mice. IFN-γ has been shown to: activate macrophages, increase inducible nitric oxide synthase (iNOS) activity, induce Cox-2 via IFN-γ inducible nitric oxide, and confer immunity against intracellular pathogens. Antigen presenting cells (mostly macrophages) are important target cells for IFN-γ. IFN-γ binds to specific IFN-γ receptors, which often exist as heterodimers of IFN-γ-Rα and IFN-γ-Rβ chains. The binding of IFN-γ to its receptors results in the activation of members of the Janus Kinase family, Jak-1 by IFN-γ-Rα and Jak-2 by IFN-γ-Rβ, which in turn leads to activation of STAT-1 (signal transducers and activators of transcription-1) pathway. Activation of cells by IFN-γ results in activation of IFN-γ-inducible genes, such as iNOS. Under the influence of iNOS, there is conversion of L-arginine to L-citrulline resulting in the generation of nitric oxide (NO). The increase in Nitric Oxide (NO) could also result in increased Cox-2 protein expression. Blocking CD28-B7.1/B7.2 interactions of Con-A activated splenocytes with CTLA-4Ig results in decreased IFN-γ secretion, which in turn decreases the activation of iNOS genes and nitric oxide release. Diminished nitric oxide levels also decrease Cox-2 protein.

3.2 Estrogen Regulation of The Inducible Nitric Oxide Synthase Gene and Nitric Oxide in Immune Cells: Implications for Immunity and Autoimmunity

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a.) INTRODUCTION

Intense research in many biological disciplines have revealed that nitric oxide has a pleiotropic effects on diverse tissues, implying a fascinating broad biological role (1-3). A wide-range of tissues, including the immune system, are targets of nitric oxide. The levels of nitric oxide must be carefully regulated to maintain homeostasis. Appropriate levels of nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS) assists in mounting an effective defense against invading microbes. Conversely, inability to generate nitric oxide results in serious, even fatal, susceptibility to infections (4). Further, dysregulation or overproduction of nitric oxide has been implicated in the pathogenesis of many disorders, including atherosclerosis, neurodegenerative diseases, inflammatory and autoimmune diseases, and cancer. Therefore, depending upon the levels of nitric oxide generated, a potential exists for nitric oxide to behave like a "double-edged" biological sword. Thus, it is pivotal to understand the regulation of nitric oxide. In this review, we focus on the regulation of nitric oxide levels in lymphoid cells by estrogen, a subject of burgeoning interest and broad ramifications. Prior to dwelling on this subject, it is pertinent to discuss the physiology of nitric oxide and the role of nitric oxide in the immune system.

Biochemistry of Nitric Oxide Synthases:

Nitric oxide, a small molecule with a very short life span (t=1/2 second), diffuses through cell membranes and interacts with various targets. It exerts its biological functions by interacting with heme groups, Iron-Sulfur (Fe-S) or Zinc-Sulfur (Zn-S) clusters, and sulfhydryl moieties directly or indirectly via its intermediates (5-7). Nitric oxide can S-nitrosylate and regulate the activity of various proteins such as transcription factors by binding to zinc fingers or cysteine residues in these molecules. Nitric oxide forms intermediates such as nitrite (NO_2^-) and nitrate (NO_3^-), as well as more reactive products such as peroxynitrite ($ONOO^-$) when reacted with O_2 in aqueous solutions.

Nitric oxide is synthesized by three different Nitric Oxide Synthases (NOS), which catalyze the formation of nitric oxide and L-citrulline by oxidation of L-arginine in the

presence of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH4), and thiol donor (**Figure 1**).

There are three known isoforms of Nitric Oxide Synthases: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). Their molecular weights are 160, 135, and 130 kDa, respectively. These enzymes have similar structures but different catalytical properties (8-11). The catalytic activity of NOS is provided by 3 different domains: reductase, calmodulin-binding, and oxygenase. The calmodulin domain is flanked by a reductase domain at the C-terminal end and an oxygenase domain at the amino-terminal end. The reductase domain at the C-terminal region, interacts with the cofactors FMD and FAD which are important in the transfer of electrons from NADPH to the oxygenase domain. The second region, called the calmodulin-binding domain, is important in the activity of all isoforms of NOS by sensing the intracellular concentration of Ca_2^+, although it acts differently depending on the type of NOS isoforms. The oxygenase domain has a key role in catalyzing the NOS substrate, L-arginine that results in the generation of nitric oxide (**Figure 2**).

Figure 1.

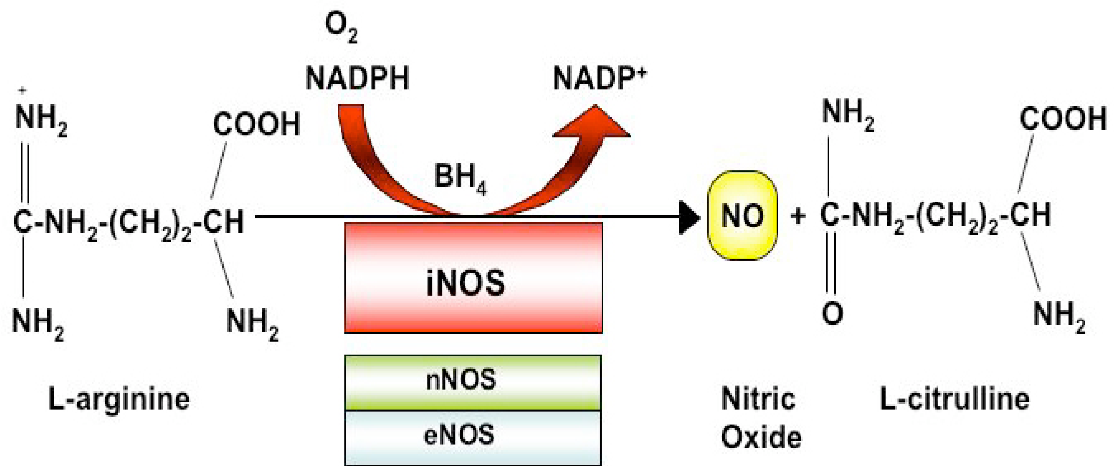
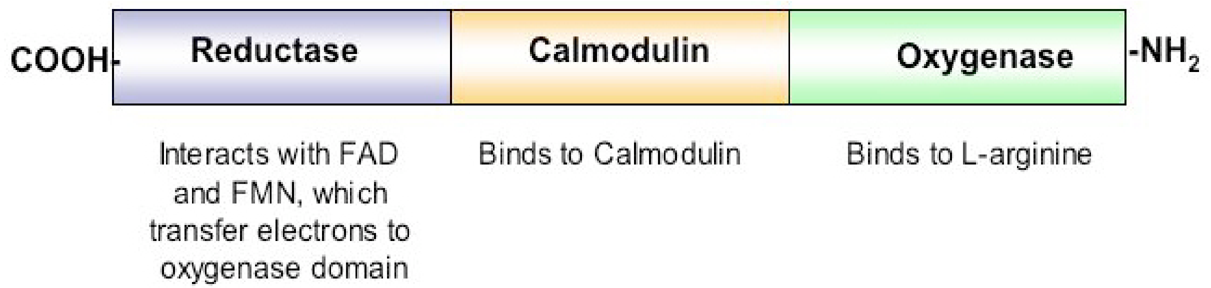


Figure 2. General Structure of NOS



Key similarities and dissimilarities among the three NOS isoforms, nNOS, eNOS, and iNOS are summarized in Table 1. Neuronal NOS is expressed in the nervous system and in skeletal muscles. Depending on where nNOS is expressed, it can act as a neuromodulator in the central nervous system, as a neurotransmitter in the peripheral nervous system, and as a key molecule in the regulation of neuroendrocrine functions and control of smooth muscles (12-14). Although it is constitutively expressed at low levels (in the picomolar range) in these tissues, nNOS expression can alter depending on pathological and physiological stimulation (15, 16) (**Table 1**). Dysregulation of nitric oxide production by nNOS results in neurodegenerative disorders in peripheral and central nervous system.

Endothelial NOS is found primarily in cardiomyocytes and endothelial cells. Similar to nNOS, a low output of nitric oxide is sustained by constitutive expression of endothelial NOS (eNOS). Basal levels of nitric oxide generation by eNOS are required for normal homeostasis of the vasculature system, such as maintaining blood flow and pressure (13, 17), inhibiting platelet aggregation, and regulation of vascular smooth muscle proliferation (17, 18) (**Table 1**). The distruption in eNOS derived nitric oxide levels causes several disorders such as hypertension, hypercholesterolemia, diabetes, and heart failure.

The third isoform is inducible NOS. This differs from nNOS and eNOS in that it is not constitutively present rather it is induced in activated antigen presenting cells (mostly macrophages) and various other types of cells in response to inflammatory cytokines (19, 20). Further, unlike nNOS and eNOS, very low concentrations of intracellular Ca_2^+ bind to calmodulin (3, 8) and is often referred to as not dependent on Ca_2^+ for its enzymatic activity. Another distinguishing feature of iNOS from the other two NOS (nNOS and eNOS) is that it is capable of inducing high levels of nitric oxide (in the nanomolar range) (3). High levels of nitric oxide produced by iNOS exert potent anti-microbial and cytotoxic effects on the immune system (**Table 1**). The dysregulation of iNOS derived nitric oxide production takes place in a wide range of cases such as infections, Rheumatoid Arthritis (RA), Crohn's Disease, asthma, and septic shock.

Table 1. Key Features of Three Nitric Oxide Synthases

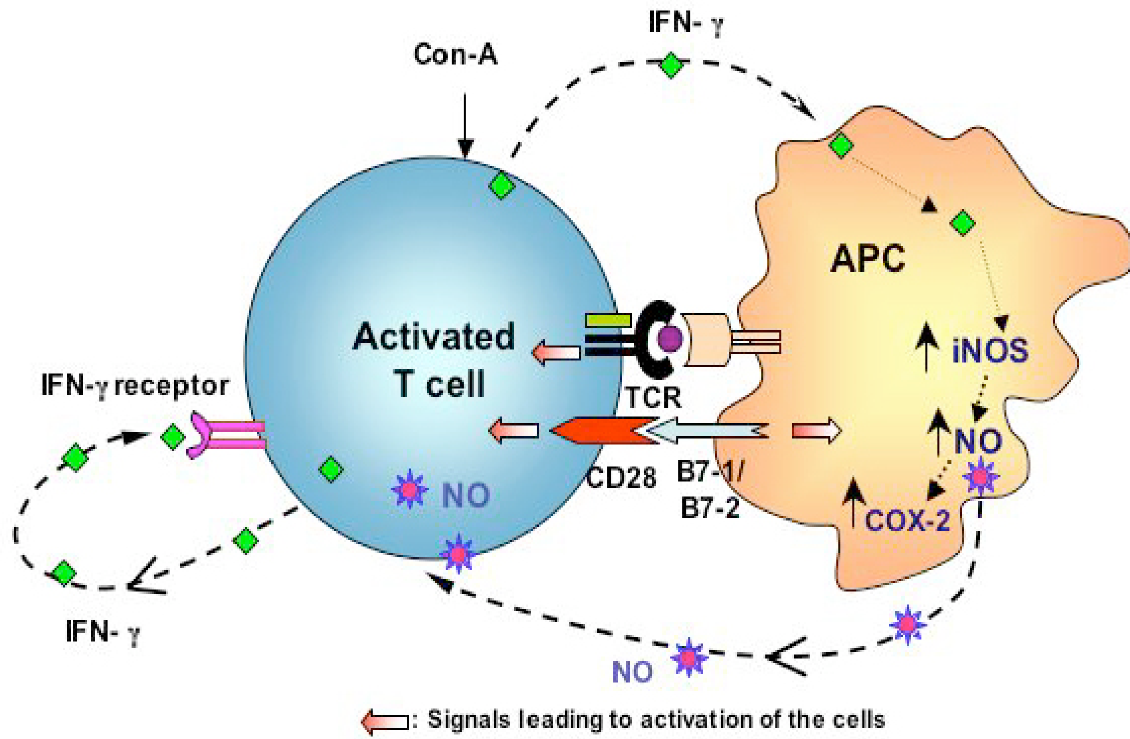
	nNOS	eNOS	iNOS
Distribution	Brain, Peripheral (PNS) and Central Nervous System (CNS), and skeletal muscles	Vascular endothelial cells, Cardiomyocytes	Macrophages
Known functions	Brain- Neuromodulator PNS- Neurotransmittor Skeletal muscles- Regulation of smooth muscle control and neuroendocrine functions	Vasodilator Regulation of blood flow and pressure	Activation of APCs against infections and microbes Cell death
Expression	Constitutively present	Constitutively present	Inducible
Concentration	In picomolar range	In picomolar range	In nanomolar range
Calcium dependence	Ca_2^+ dependent	Ca_2^+ dependent	Small quantities of Ca_2^+ are sufficient. Mostly considered as Ca_2^+ independent
Cellular location	Cytosol (?)	Found in Caveolae of membrane bound to Calveolin-1	Cytoplasm
Associated disorders	Dysregulation associated with neurodegenerative disorders	Hypertension, hypercholesterolemia, diabetes, heart failure. At low concentrations prevents apoptosis of endothelial cells, inhibits platelet aggregation, and smooth muscle proliferation	Infections, Rheumatoid Arthritis, Crohn's disease, asthma, septic shock

c.) iNOS, NITRIC OXIDE, and IMMUNITY

The iNOS-derived nitric oxide is principally generated from the cells of the macrophage-monocyte lineage. This includes: monocytes, macrophages (21), peritoneal macrophages (22, 23), microglia, and Kupffer cells. In addition, certain T (24) and B cell lines (25, 26), optic nerve astrocytes (27), hepatocytes, neutrophils, vascular smooth muscle cells, and endothelial cells (28) can also release nitric oxide derived from iNOS. Nitric oxide has potent effects on the immune system including the following.

The iNOS gene is activated by selected immunological stimuli especially cytokines such as IFN-γ. Studies show that IFN-γ, secreted by T cells, is required for the production of nitric oxide and the expression of iNOS (3, 29). Macrophages are notable target cells for IFN-γ. This cytokine has been shown to activate macrophages, increase iNOS activity (3), induce MHC class I and II expression (30), and confer immunity against intracellular pathogens (31). IFN-γ binds to specific IFN-γ receptors (heterodimers of IFN-γR1 or IFN-γRα and IFN-γR2 or IFN-γRβ chains) (32-34) and activate members of the Janus Kinase family. Jak-1 binds IFN-γRα and Jak-2 binds IFN-γRβ. Activated Jaks in turn, phosphorylate tyrosine residues of signal transducers and activators of transcription-1 (STAT-1) (35). Activated STAT-1 dimerizes and binds to specific DNA response elements to regulate STAT-1-controlled genes including the iNOS gene culminating in the induction of the enzyme iNOS protein (**Figure 3**). iNOS subsequently catalyzes the generation of nitric oxide, which upregulates proinflamatory Cyclooxygenase-2 (COX-2) and diffuses out of macrophages to affect various other cells in the cellular level.

Figure 3. Induction of iNOS and Nitric Oxide (NO) in Con-A stimulated cells



Nitric oxide functions as a cytotoxic agent against invading microbes (4, 36), it regulates the functions of diverse cells of the immune system (1, 2, 4, 37, 38), and it modulates apoptosis and cell viability (39, 40). The level of nitric oxide is important in the enhancement or retardation of inflammation. Healing of excisional wounds takes longer in iNOS deficient mice compared to wild type mice (3). There is substantial evidence suggesting that increased nitric oxide is involved in human and rodent septic shock (41), which results in decreased blood pressure, vascular hyporeactivity, platelet aggregation, and neutrophil adhesion (42). Enhanced levels of nitric oxide has been incriminated in tissue injury in several autoimmune diseases including rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), and glomerulonepthritis (4, 43). Interestingly, these autoimmune diseases, which demonstrate upregulation of iNOS/nitric oxide also exert higher levels of production of Th1 type cytokines: IFN-γ, IL-1β, and TNF-α (2, 44).

One of the most important roles of nitric oxide is to control infection by inhibiting the growth of bacteria, viruses, and parasites. Increased activity of iNOS is observed in monocytes or macrophages from hosts with infectious or inflammatory diseases (3). Some of the infections that are controlled by iNOS include Herpes simplex virus type 1 (45), Coxsackie virus B3 (46), *Listeria monocytogenes* (47), *Mycobacterium tuberculosis* (48), *Mycoplasma pulmonis* (49), *Leishmania donovani* (50), *Leishmania major* (51-53), *Tryposonoma cruzi* (54), and *Toxoplasma gondii* (55).

iNOS has an important role in the regulation of the immune system because it influences antigen-presenting cells. Infected or activated macrophages can produce iNOS derived nitric oxide, which results in Reactive Oxygen Intermediates (ROI): O_2^-, H_2O_2, and $OH^.$. ROI kill infectious agents such as viruses, bacteria, protozoa, fungi, by decreasing glutathione, increased double-stranded DNA breaks, and oxidation of lipids and DNA (56). Superoxide produced by macrophages or neutrophils in inflamed tissues will react with nitric oxide to form reactive nitrogen intermediates (RNO) such as peroxynitrite ($ONOO^-$), these molecules will directly affect invading pathogens and confine them in the infected area to be eliminated with minimal tissue damage to the host. These ROI

may further enhance nitric oxide synthesis by these macrophages (57). An elevation in NO level is not always beneficial to the host. It may become detrimental to cells and tissues. Reaction initiated by ROI can result in tissue destruction by necrosis or apoptosis (1, 4). Nitric oxide can also have an inhibitory effect on protein synthesis, phagocytosis, and MHC class II expression on peritoneal macrophages (58). Nitric oxide also plays an important role in controlling tumor growth. For example, nitric oxide has also been shown to have a cytotoxic effect on some tumor cells (3, 19, 59). Further, excessive production of nitric oxide may result in the suppression of solid tumor growth (60) preventing metastasis of neoplastic cells.

At a cellular level, nitric oxide can have selective effects on different T cell subsets. For example, nitric oxide inhibits the expansion of Th1 but not Th2 clones (3, 24). Further, nitric oxide inhibits the secretion of IFN-γ by Th1 cells, but has no appreciable effects on IL-4 secretion by Th2 cells (24). The observations that nitric oxide inhibits IFN-γ is interesting (and has potentially significant implications) considering that IFN-γ potently activates nitric oxide. The ability of nitric oxide in turn to switch off IFN-γ, provides a means of regulation of pro-inflammatory IFN-γ. This "turning off" of IFN-γ may be an effective way of preventing generation of dangerous levels of IFN-γ. The effect of nitric oxide on T cells is dependent upon the level of exposure of nitric oxide as well as the timing of exposure (i.e. naïve versus activated T lymphocytes). For example, addition of high concentrations of the nitric oxide donor S-nitroso-N-acetylpenicillamine (500μM; SNAP) to Th1 cells inhibits IFN-γ production, while exposure of these Th1 cells to a lower concentration of SNAP (10μM) leads to an increased secretion of IFN-γ (52). In support of the latter, it has been shown that in the absence of nitric oxide (as noticed in iNOS knockout mice), enhanced IFN-γ and IL-12 secretion were evident (51, 61) and higher levels of IFN-γ production were observed in *Leishmania major* infected iNOS mice compared to wild-type (52). It also appears that the effect of nitric oxide on T cells is dependent upon when exposure of nitric oxide occurs. For example, exposure of human T cells first to various nitric oxide donors, SNAP, Dipropylenetriamine NONOate (DPTA-NONOate), (Z)-1-[-2-(aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-nonoate), subsequently followed by activation with anti-CD3 and

anti-CD28 antibodies resulted in a significant decrease in IFN-γ secretion, without affecting Th2 cytokines, IL-4 and IL-5 (62). However, coincubation of nitric oxide donors (i.e. SNAP, SIN-1) (addition of nitric oxide) with anti-CD3 or PMA/ionomycin-activated human T cells has no effect on the levels of IFN-γ or IL-12 (63). Although the precise mechanisms underlying these observations are not clearly evident, these studies suggest that exposure of cells to nitric oxide, prior to T cell activation may blunt key signaling events (possibly S-nitrosylation) that are involved in transcription and/or synthesis of IFN-γ.

While IFN-γ is known to upregulate iNOS, other cytokines such as TGF-β, IL-4, IL-13, and IL-10 act to prevent excessive production of nitric oxide by iNOS (64-66).

c.) iNOS, NITRIC OXIDE, and APOPTOSIS

In addition to the central role of nitric oxide in immunoregulation, immunity against infections, and cell differentiation, nitric oxide also influences apoptosis. Intense research in apoptosis has revealed that tightly regulated apoptosis is essential for viability in both unicellular and multicellular organisims. Physiologically, apoptosis is critical in the regulation of developmental tissues, and removal of damaged cell populations in many organ systems such as neuronal, cardiac, and immune cells. Further, apoptosis is essential for maintanence of immune homeostasis. This includes death of dangerous autoreactive and defective lymphocytes during development (also regarded as negative selection), elimination of clonally-expanded immune cells after effectively countering infection, and death of infected or abnormal cells by cytotoxic lymphocytes. Any defects or dysregulation of apoptosis of cells in the immune system may result serious health consequences, including the development of autoimmunity and/or cancer. Excessive apoptosis of lymphocytes can result in immunological and developmental defects, and immunodeficiency disorders. Given that apoptosis is central to homeostasis, the ability of nitric oxide to regulate apoptosis adds another dimension of physiological and/or pathological role of nitric oxide. iNOS-derived nitric oxide is capable of inducing or preventing cell death depending on the cell type, the stimulation, and the level of nitric

oxide production. Therefore, the effects of nitric oxide can be divided into two types, pro-apoptotic and anti-apoptotic.

The Pro-Apoptotic Effects of Nitric Oxide, The Product of inducible NOS

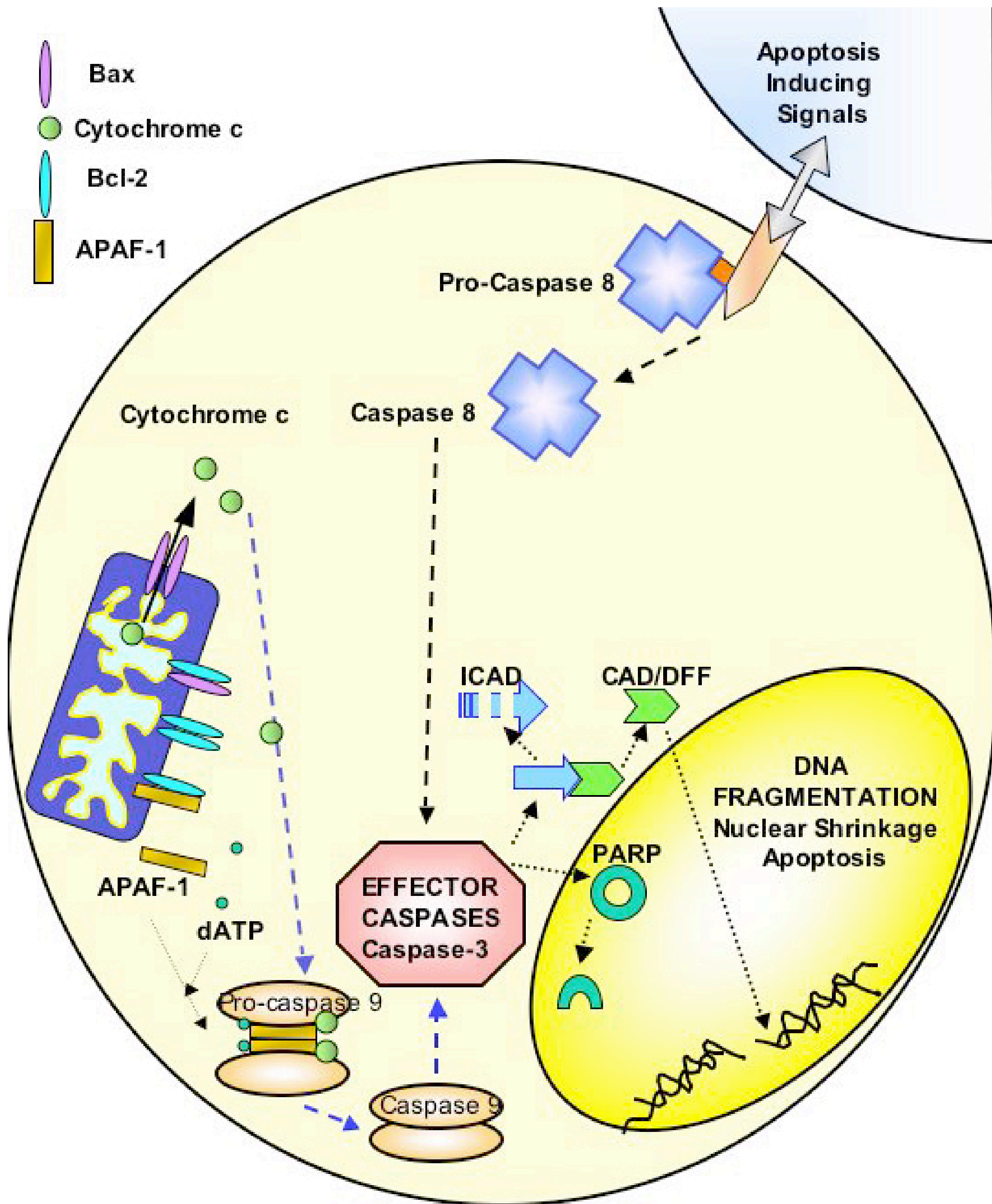
iNOS-derived nitric oxide has been shown to induce apoptosis in several cell types (67-74). Augmented levels of nitric oxide can initiate apoptotic pathway by membrane permeabilization, altering the balance in expression of pro and anti-apoptotic genes, leading to caspase activation, which results in nuclear condensation and fragmentation. Increased concentrations of either exogenous or endogenous nitric oxide, compared to physiological levels of nitric oxide present in the cell, result in decreased cell viability. *In vitro* addition of with increasing concentrations of nitric oxide donors (0.6-1mM) such as sodium nitroprusside (SNP), S-nitroso-N-acetyl-DL-penicillamine (SNAP), S-nitrosogluthatione (GSNO), and S-nitroso-N-acetyl-cysteine (SNAC) to various cell types such as embryonic chick cardiomyocytes (75), human neuroblastoma cells (SHSY-5Y) (76), human myeloid leukemia cells (77), and serum-starved primary cultures of neonatal and adult rat ventricular myocytes (38) has been shown to enhance the incidence of apoptosis.

Nitric oxide alters the balance between anti- and pro- apoptotic genes: The rate and extent of apoptosis appears to depend upon alterations in the cellular balance of the pro-apoptotic proteins (e.g. Bax and Bak) with the anti-apoptotic proteins (e.g. Bcl-2 and Bcl-x_L). Selective expression of these genes is also regulated by nitric oxide alone (either added exogenously via nitric oxide donors or physiologically induced by cytokines) or in combination with the oxidant species produced as a result of increased nitric oxide. In many (but not all) cells undergoing apoptosis, there is an increase in the expression of pro-apoptotic genes, Bax or Bak, while there is a concomitant decrease in Bcl-2 and Bcl-x_L genes. Expression of the pro-apoptotic genes, such as Bak and Bax, is increased after treatment with increased concentrations of nitric oxide donors in cardiac myocyte cells (38), enterochromaffin-like (ECL) cells (neuroendocrine cells) (78), Meg-01 and HEL megakaryocytoid cell lines (79), and bone marrow macrophages (80). This

increase is detected at both mRNA and protein levels of these genes using RT-PCR and Western blot assays. Escalation of endogenous nitric oxide levels by the induction of iNOS protein in cardiac myocytes by cytokines such as recombinant IL-1β and recombinant IFN-γ, is associated with increased expression of Bax (81). Nitric oxide-mediated cell death in myocytes is attenuated by the use of non-specific or specific iNOS inhibitors, which demonstrates the direct effect of nitric oxide in these cells (81). The rebalance of Bcl-2 to Bax is critical to the recovery of cells from programmed cell death. Neurons co-cultured with astrocytes, which undergo cell death, experience increased iNOS production from astrocytes and upregulation of Bax protein levels (82). The same profile of increased nitric oxide in relation to decreased levels of anti-apoptotic Bcl-2 or Bcl-x_L expression is observed in many cells such as megakaryocytoid cell lines (79), U937 cells (77), neurons (82), and RAW 264.7 macrophages (83). Inhibition of nitric oxide production with iNOS inhibitors such as N(G)-monomethyl-L-arginine (L-NMMA) suppresses neuronal cell death (82) in enterochromaffin-like cells (78) and increases the Bcl-2 to Bax ratio (82). Nitric oxide donors promote apoptosis via cytotoxic products formed when nitric oxide reacts with superoxide.

Nitric oxide causes release of Cytochrome-c and activation of caspases: This review is not intended to provide detailed molecular events involved in apoptosis. Rather, only key events that are crucial to the understanding of the regulation of apoptosis by nitric oxide are discussed. Downregulation of anti-apoptotic Bcl-2 and/or upregulation of Bax homodimers generally result in mitochondrial changes and the release of Cytochrome-c (84-86). The release of cytochrome-c regulates the downstream events that culminate in apoptotsis. Free cytochrome-c in the cytoplasm binds to ATP-dependent activation of the death regulator apoptotic protease-activating factor-1 (APAF-1), Caspase-9, and an ATP-forming complex called an apoptosome, which activates caspases (**Figure 4**) (86).

Figure 4. Molecular Mechanisms of Apoptosis



Caspases are found as inactive pro-caspases (zymogens) in viable cells (87) and are activated in response to many pathways such as Fas-FasL, TNFR1-TNF-α, which activate precursor Caspases such as Caspase-8 or Caspase-10 (84, 85, 87-89). A receptor free caspase activation pathway occurs by release of cytochrome-c from mitochondria to the cytoplasm. Cytochrome-c released into the cytoplasm triggers the activation of initiator caspases such as Caspase-9, which in turn stimulates downstream pro-effector caspases (90, 91). Effector caspases that are activated by initiator caspases and their own active forms are Caspase-3, Caspase-7, and Caspase-6. Activation of Caspase 3 (CPP32, apopain) causes the activation of a nucleosomal DNase that results in DNA laddering and cell death.

Studies performed in relation to elevated levels of nitric oxide and a disturbance in the balance of Bcl-2 to Bax proteins suggest the presence of increased cytochrome-c in the cytosol and elevated Caspase-3 activity. Treatment of RAW 264.7 macrophages with the nitric oxide donors, S-nitrosoglutathione or spermine-NO, causes cytochrome-c to be released into the cytosol (83). When the rat pheochromocytoma cell line (PC12) is cultured with the nitric oxide donors, 1 mM S-nitroso-N-acetyl-DL-penicillamine (SNAP) or 1 mM diethylenetriamine-NO adduct (NOC-18), cytochrome-c is released from mitochondria into the cytosol, accompanied by activation of caspases (92).

Various cells and cell lines such as the rat PC12 cell line (92), neonatal rat cardiac myocyte cells (38), rat neurons (82), RAW 264.7 (macrophage cell line) (83), and HL-60 cell line (human promyelocytic leukemia cell line) (93) cultured with exogenous nitric oxide, exhibited an increase in the enzymatic activity of Caspase-3. Cleavage of inactive pro-Caspase-3 (32 kDa) to active Caspase-3 (17 kDa) upon exposure to nitric oxide and inhibition of the activation of pro-Caspase-3 by the nitric oxide inhibitor, L-NAME in AK-5 tumor cell cultures confirmed the action of nitric oxide on caspases (94).

Once the effector caspases (Caspase-3, Caspase-7, or Caspase-6) are activated, they start to inactivate the proteins that protect viable cells from apoptosis, such as inhibitor of

caspase-activated DNase (ICAD) and Poly-ADP-Ribose polymerase (PARP). ICAD (DFF45) is an inhibitor of nuclease Caspase-activated deoxyribonuclease (CAD), which is responsible for DNA fragmentation (85, 89). Caspase-3 releases CAD from the inactive ICAD/CAD complex and therefore initiates the formation of DNA breaks (85, 86, 89). Another substrate of Caspase-3, PARP, synthesizes homopolymers of ADP-ribose in response to DNA damage and is responsible for the maintenance of genomic integrity. The activation of Caspase-3 results in cleavage of PARP and therefore increases DNA fragmentation. Proteolytic cleavage of PARP (from 116 kDa to a 85 kDa product) is associated with the presence of iNOS expression demonstrating the effect of endogenous nitric oxide production on cells. Cardiac rat myocytes, treated with IL-1β, and AK-5 macrophage tumor cells cultured with natural killer cells have increased endogenous nitric oxide production and increased PARP cleavage (38, 94).

Changes in DNA fragmentation and nuclei in response to nitric oxide: Apoptosis has been noticed in cells exposed to relatively high levels of nitric oxide, either exogenously (delivered through increased concentrations of nitric oxide donors) or endogenously (after stimulation of cells with inflammatory cytokines such as TNF-α, IL-1β, IFN-γ). Nitric oxide-mediated cell death that is primarily due to apoptosis can be confirmed by DNA fragmentation. The characteristic cleavage of chromatin DNA during apoptosis into nucleosomes of 180 base pairs can be assayed by DNA gel electrophoresis, enzymatic labeling of DNA strands [terminal deoxynucleotidyl transferase biotin-mediated dUTP nick-end labeling (TUNEL)], or fluorescent microscopic analysis with nuclei staining.

Exposure of several cell lines to high levels of exogenous nitric oxide, nitric oxide donors induced apoptosis, characterized by increased levels of nuclei showing apoptotic bodies, chromatin condensation, and DNA fragmentation. These include adult rat and human islet cells (95), human neuroblastoma cells (SHSY-5Y) (76), embryonic chick cardiomyocytes (75), bone marrow macrophages (80), neonatal rat cardiac myocyte cells (38), megakaryocytoid cell lines (Meg-01 and HEL) (79), the human myeloid leukemia U9 cell line (77, 89), AK-5 tumor cells (94), the human leukemia cell line, HL-60 cell

line(93), and $CD4^+CD8^+$ thymocytes, (96). The elevation in endogenous nitric oxide levels by inflammatory cytokines (recombinant IL-1β, TNF-α, and IFN-γ), in serum-starved neonatal rat ventricular myocytes (81), megakaryocytoid cell lines (79), and RAW 264.7 cells results in augmented DNA fragmentation analogous to that observed in the presence of nitric oxide donors. Data show that apoptosis is caused by increased hydrogen peroxide formation in the J774 macrophage cell line cultured with the nitric oxide donors, diethylenetriamine/NO adduct (DETA/NO) and S-nitrosoglutathione (GSNO) (77, 97).

The effect of endogenous or exogenous nitric oxide treatment on the cell viability is also demonstrated by the addition of nitric oxide scavengers like hemoglobin or nitric oxide inhibitors (L-NAME, L-nitroarginine) to neonatal or adult rat ventricular myocytes (81, 98), which results in decreased DNA laddering and chromatin condensation.

High levels of nitric oxide derived from iNOS switches programmed cell death to necrosis: In healthy cells, the mitochondrial membrane potential is a good indicator of the energy status of mitochondria, cellular homeostasis, and cell viability. A collapse in membrane potential has been associated with apoptosis. Maintenance of membrane potential is important for cell survival since disruption results in the release of cytochrome-c to the cytosol and the activation of caspases. Apoptosis is a process that requires ATP and depletion of ATP in addition to collapse in the membrane potential of mitochondria can switch the process into necrosis. The amount of nitric oxide and status of the cells are both extremely important in determining the fate of cells, whether they enter into apoptosis or necrosis. Stimulation of RAW 264.7 cells with LPS/IFN-γ results in production of endogenous nitric oxide up to 100 mM. At these concentrations of nitric oxide, apoptosis is detected. Lactate dehydrogenase (LDH) leakage from cells, an indicator of necrosis, is not observed at these concentrations probably demonstrating the need for a higher concentration of nitric oxide to drive cells into necrosis (99). In the absence of glucose, PC12 cells (rat pheochromocytoma cell line) (92) and the HL-60 cell line (93), as well as RAW264.7 (83) and J774 macrophage cell lines (77, 97), which are depleted of ATP, show a shift from apoptosis to necrosis, with LDH release and lack of

caspase activation upon addition of high concentrations of nitric oxide donors (>1mM). In the presence of glucose, the number of apoptotic cells is much higher in nitric oxide donor treated PC12 cells. Overall, it appears that nitric oxide-induced necrosis is mediated by energy failure in these cells.

The Effect of Nitric Oxide on the Survival of Cells from Apoptosis

Remarkably, in relation to apoptosis and cell survival, nitric oxide behaves like a "double-edge" sword. In contrast to the pro-apoptotic effects of nitric oxide discussed above, nitric oxide has also been reported to inhibit apoptosis in eosinophils, murine and human lymphocytes, certain tumor cell lines, human endothelial cells, and rat cardiac muscle cells subjected to mechanical stress (100-107). The mechanisms responsible for these responses to nitric oxide have not been defined in detail.

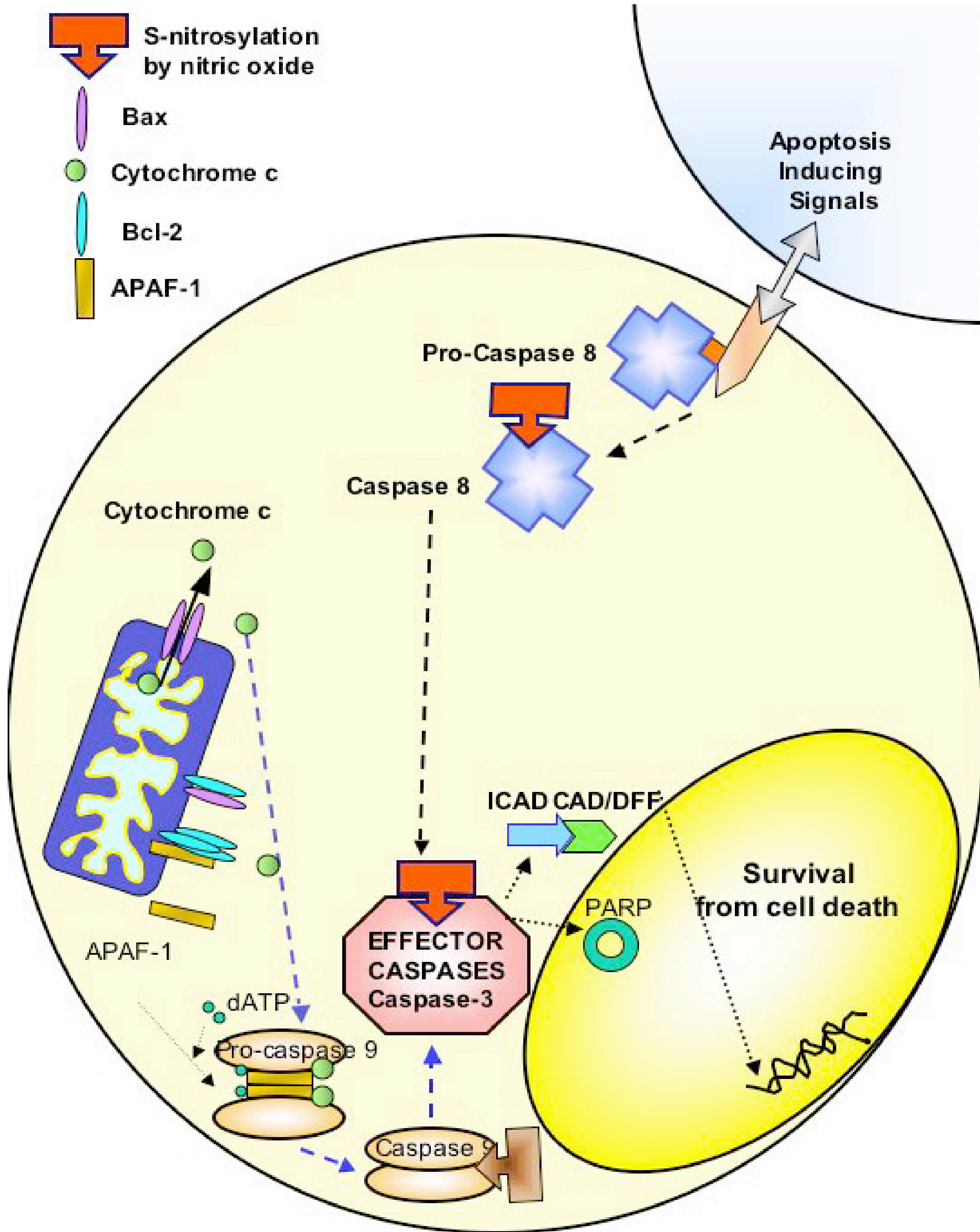
Increased expression of anti-apoptotic genes in the presence of nitric oxide: There is substantial evidence for the effect of nitric oxide on the expression of anti-apoptotic genes and proteins in various cell types. Both endogenously produced nitric oxide by the cytokine challenge and exogenously applied nitric oxide result in an increase of anti-apoptotic Bcl-2 mRNA in UVA treated endothelial cells (104), murine peritoneal macrophages (PEC), and endothelial cells (105). Nitric oxide-treated cells do not have an increase in Bax mRNA demonstrating the importance of the Bcl-2/Bax ratio to the control of the onset of apoptosis. Immortalized porcine endothelial cells cultured in the presence of iNOS derived nitric oxide are resistant to TNF-α-induced apoptosis and there is a sustained expression of Bcl-2 and Bcl-x_L (76, 108).

Addition of inhibitors of nitric oxide production to cultures of PEC, RAW 264.7, or UVA-treated endothelial cells protects these cells from nitric oxide induced cell death in the presence of high levels of the nitric oxide donor, SNAP (2mM for 8 hrs or 24 hrs) (104, 109). These cells have normal levels of *bcl-x_L* and *bcl-2* expression. Inhibition of endogenous nitric oxide by a specific iNOS inhibitor in melanoma cell lines and by 6-

mercaptopurine (6-MP) in LPS-activated enriched B cells downregulates the level of Bcl-2 proteins and increases the expression of Bax (101, 110).

The Inactivation of Caspases by S-nitrosylation: Although increased levels of endogenous or exogenous nitric oxide can lead to increased enzyme activity of caspases resulting in apoptosis in different cell types, physiological or low doses of iNOS-derived nitric oxide or nitric oxide donors can result in rescue of cells from programmed cell death. Nitric oxide is capable of modifying proteins such as Caspases that contain cysteine residues via S-nitrosylation of the thiol group (**Figure 5**). The decrease in Caspase-3 enzyme activity can be credited to this modification seen after *in vitro* incubation with nitric oxide donors. Dithiothreitol (DTT) is able to reverse the nitric oxide inhibition of apoptosis, indicating direct S-nitrosylation of the caspase catalytic cysteine residue, Cys163, a functionally essential amino acid conserved among ICE/CPP-32-like proteases by nitric oxide (111).

Figure 5. S-nitrosylation by Nitric Oxide and Apoptosis



Activity of Caspase-3 in the cell lysates of human umbilical vein endothelial cells (112), melanoma cell lines (110), and primary rat hepatocytes (113) is significantly decreased when exposed to low concentrations of nitric oxide donors, which resembles native endogenous nitric oxide production. The decrease in Caspase-3 activity is detected by checking for the active cleavage product (17 kDa) and enzymatic activity. Physiological levels of nitric oxide prevent apoptosis by interfering with the activation of the caspase cascade in human endothelial cells (111). Caspase-3 activity is increased in melanoma cell line lysates in the presence of increasing concentrations of iNOS inhibitors. Expression of Caspase-3 mRNA is also increased after iNOS inhibitor treatment (110).

Cell lysates from rat hepatocytes, which are stimulated to express iNOS by IFN-γ, IL-1β, and TNF-α, or exposed to nitric oxide donors and then incubated with DTT, demonstrate increased Caspase-3 activity compared to control cells (110). The increase in Caspase activity in response to DTT, which removes thiol bound nitric oxide groups from proteins and causes "de-S-nitrosylation" of the proteins, specifically shows the S-nitrosylation effect of NO on these cysteine proteases (110).

While high doses of nitric oxide lead to direct DNA damage or exhibit cytotoxic effects, the posttranslational modification of Caspase-3 by S-nitrosylation in the presence of low concentrations of nitric oxide causes the inactivation of caspase and therefore, prevents cells from apoptosis (113).

The Inhibitory Effect of Nitric Oxide on DNA Fragmentation Factors: The inhibitory effect of nitric oxide on Caspase-3 activity by S-nitrosylation of the enzyme is observed in two substrates of caspase-3, PARP, and ICAD. The cleavage of intact PARP (85 kDa) by Caspase-3 to an inactive fragment (31 kDa) is suppressed by the pre-incubation of recombinant Caspase-3 with the nitric oxide donor, SNAP (26). The activity of Caspase-3 is restored after incubation with DTT, which disturbs S-nitrosylation of the enzyme (26). In primary rat hepatocytes, PARP is cleaved in response to TNF-α/Act-D treatment (113). The exogenous and endogenous nitric oxide produced

by iNOS suppresses Caspase-3 and Caspase-8 activity and PARP cleavage (113). The basal NOS activity in human leukocytes inhibits Fas-induced cleavage of poly (ADP-ribose) polymerase (114). Inhibition of Fas induced apoptosis by basal levels of nitric oxide in human cell lines takes place by S-nitrosylation on the cysteine catalytic site (114). S-nitrosylated inactive caspases in unstimulated human cell lines could be stimulated via the Fas pathway resulting in denitrosylation and activation of cell death (115). Therefore, it appears that the level of nitric oxide and the apoptotic signals determine cell viability.

Cleavage of ICAD (DFF-45), another indicator of Caspase-3 activity, is decreased in MCF-7 cells incubated with TNF-α/Act-D and nitric oxide donors as detected with Western blot assays (116).

Nitric Oxide Decreases DNA Laddering and Other Morphological Characteristics of Apoptosis: Several studies have shown that the presence of nitric oxide in these cells leads to decreased DNA laddering and increased cell viability. For example, protection against TNF-α/Act-D, anti-Fas monoclonal antibodies, 6-mercaptopurine or UVA (UV light) induced cell death is achieved after the incubation of γd T cells (117), LPS-stimulated mature splenic B cells (101), eosinophils from the human nasal polyps (100), human umbilical venous endothelial cells (HUVEC) (107), and primary rat hepatocytes (113) with nitric oxide donors or increased endogenous iNOS-derived nitric oxide levels.

Spontaneous apoptosis can occur *in vitro* in some cell types as a result of nutrient inadequacy and withdrawal of growth factors (118). The spontaneous apoptosis can be countered in several cell types such as splenic enriched B cells from BALB/c mice (102), B-cell chronic lymphocytic leukemia (B-CLL) cells (119), rat hepatocytes (26), rat preovulatory follicles (23), melanoma cell lines and adult human melanocytes (110) by increased endogenous nitric oxide levels or after incubation with nitric oxide donors such as SNAP. Further, anti-apoptotic effect of nitric oxide on B-cell chronic lymphocytic

leukemia cells (119), melanoma cell lines, and adult human melanocytes (110) are counteracted by NOS inhibitors.

To date, the data on the effect of estrogen on apoptosis in relation to iNOS/ nitric oxide is inconclusive. For example, anterior pituitary cells from ovariectomized rats cultured in the presence of estrogen (10^{-9} M) with TNF-α and nitric oxide inhibitor, NAME (0.5 mM), have increased apoptosis and enhanced the pro-apoptotic action of TNF-α in the absence of nitric oxide (120). This particular study does not specify which NOS (nNOS, eNOS, or iNOS) is responsible for this action. On the other hand, subcutaneous one-time estrogen treatment (100 μg/ml) of ovariectomized female rats with ischemic damage demonstrates a protective effect on neuronal cells from apoptosis (121). In this case, estrogen treatment results in diminished NF-κB, IκB, and iNOS expressions, which are rapidly activated during ischemia/reperfusion. In summary, the discrepancy between these studies can be due to the effect of estrogen and/or the nature of nitric oxide (either cytotoxic or cytoprotective) on different cell types or experimental conditions, a subject that needs further investigation. We are currently investigating the effects of estrogen on apoptosis of splenocytes in relation to iNOS and nitric oxide.

d.) iNOS, NITRIC OXIDE, and ESTROGEN

Nitric oxide is regulated by many endogenous factors including hormones such as estrogens. The effects of estrogen on iNOS and nitric oxide have been investigated in non-immune tissues such as aortic cells (122, 123), cardiac myocytes (124), and kidney epithelial cells (125). Whereas estrogen has a suppressive effect on iNOS expression from rat isolated aortic endothelial cells (85, 122, 123), microglial cells (44, 126), and vascular endothelial cells (89, 127), while in other tissues such as rat uterus (*in vivo* E2) (128), ovine coronary artery cells (single dose of E2 *in vivo*) (129), and rat cardiac myocytes (*in vitro* E2) (124, 130) estrogen increased the levels of iNOS expression and/or nitric oxide. Divergent effects of estrogen on nitric oxide could be due to differences in cell types, the dose of estrogen, and the difference in the time of administration and culture.

A notable observation in endocrinology is the recognition that estrogen is not merely a reproductive hormone. Rather, it has potent effects (physiological and pathological) on diverse non-reproductive tissues including the immune system. While the effects of estrogen on reproductive tissues are well established, the influence of estrogens on the immune system is increasingly being appreciated over the years. While the effects of estrogen on non-immune tissues is reported, there are only a limited number of studies, that have demonstrated the effect of estrogen on iNOS/NO by immune cells. The regulation of nitric oxide by estrogen is clearly important considering that estrogen is a potent immunomodulator including modulating the activity of macrophages, that are important producers of iNOS-derived nitric oxide. Estrogen treatment activates phagocytic activity (131) and enhances Fc-γR expression on splenic-macrophage (132). *In vitro* exposure of peritoneal macrophages (133, 134) or macrophage-monocyte-like cell lines (RAW 264.7) (135) to 17-β estradiol has been shown to increase iNOS expression and release nitric oxide.

Since there were no studies on *in vivo* effects of estrogen on iNOS expression and nitric oxide release by splenocytes, we addressed this gap in our recent studies. We showed that Con-A-activated splenic lymphocytes from outbred CD-1 mice exposed to short-term, relatively low doses of subcutaneous injections of estrogen (2 and 4 μg/ 100 g body weight) treatment increased nitric oxide in the supernatants (In press). This increase is especially apparent with higher doses of 17-β estradiol (4 μg/ 100 g body weight). Further, in our recent studies we have shown that splenocytes from orchiectomized C57BL/6 mice given estrogen implants (1-3 months) when activated in vitro with T cell mitogens [Concanavalin-A (Con-A) or anti-CD3 antibodies] released copious amounts of nitric oxide in the supernatants (To be submitted) This increase in nitric oxide in splenocytes from estrogen treated (but not placebo-treated) mice was in part due to increased transcription of iNOS mRNA as determined by Real Time PCR and increased levels of iNOS protein as determined by Western blot assay (To be submitted). This is the first study to show that *in vivo* estrogen treatment leads to activated splenocytes to secrete proinflammatory nitric oxide and iNOS gene upregulation. To further determine whether

estrogen also enhances iNOS/NO in female mice, we next prepubertally ovarietomized C57BL/6 mice and gave either placebo and estrogen implants. After 2-3 months of treatment, splenic lymphocytes from these mice were cultured in the presence or absence of Con-A. Similar to our findings in gonadectomized male mice given estrogen, we find that the level of nitric oxide was significantly increased in the supernatants from Con-A activated splenocytes from estrogen-treated ovariectomized female C57BL/6 mice when compared to similar cultures from placebo-treated mice ($p<0.001$; **Figure 6**). It is important to note that high levels of nitric oxide was detectable only in splenocytes from estrogen-treated mice that were activated with a T cell mitogen (e.g. Con-A) and not in unstimulated (media only) cells. This implies that estrogen is not directly stimulating splenocytes to induce iNOS, rather it is mediated through activated cells. In our recent elaborate studies, we find that estrogen-induced upregulation of iNOS /nitric oxide in activated splenocytes is likely to be mediated through IFN-γ. This is based on the following findings: (1) Estrogen treatment of IFN-γ knockout mice did not induce iNOS or nitric oxide. (2) Estrogen treatment of Interferon Regulatory Factor-1 (IRF-1) knockout mice (in collabration with G. Senaldi), which also have low levels of IFN-γ did not induce detectable levels of nitric oxide. (3) Direct addition of recombinant IFN-γ to splenocytes (that were briefly activated with Con-A) from estrogen-treated but not placebo-treated mice resulted in upregulation of iNOS protein. (4) Blocking of CD28-B7 interactions with CTLA-4Ig markedly diminishes IFN-γ levels as well as nitric oxide. Similarly in this study, we find that splenocytes from ovariectomized estrogen-treated mice, the increase in nitric oxide levels in Con-A activated splenocytes from estrogen-treated mice can be abrogated by blocking CD28 and B7 interactions (**Figure 6**). Thus, implying the importance of this pathway in regulating nitric oxide levels. There was an apparent increase in the relative levels of iNOS mRNA in Con-A activated splenocytes from ovariectomized estrogen-treated mice (**Figure 7**). In our previous studies, using Con-A activated splenocytes from estrogen-treated gonadectomized male mice, we had shown by the Real Time PCR assay that iNOS mRNA was significantly increased compared to the placebo-treated mice (in press). In the present studies, the RT-PCR data needs to be confirmed with Real Time PCR assay. These results demonstrate the *in vivo* effect of estrogen on iNOS expression and iNOS derived nitric oxide from splenic

lymphocyte cultures and support our previous data observed in estrogen-treated gonadectomized male inbred C57BL/6 mice as well as short-term estrogen treatment of intact out-bred CD1 male mice.

Overall, these studies demonstrate that estrogen regulates iNOS expression and/or release of nitric oxide in many non-immune and immune (splenocytes) cells. The precise effects of estrogen on nitric oxide are dependent upon several factors including different cell types, the dose of estrogen, the duration of cell culture period, and the response to IFN-γ.

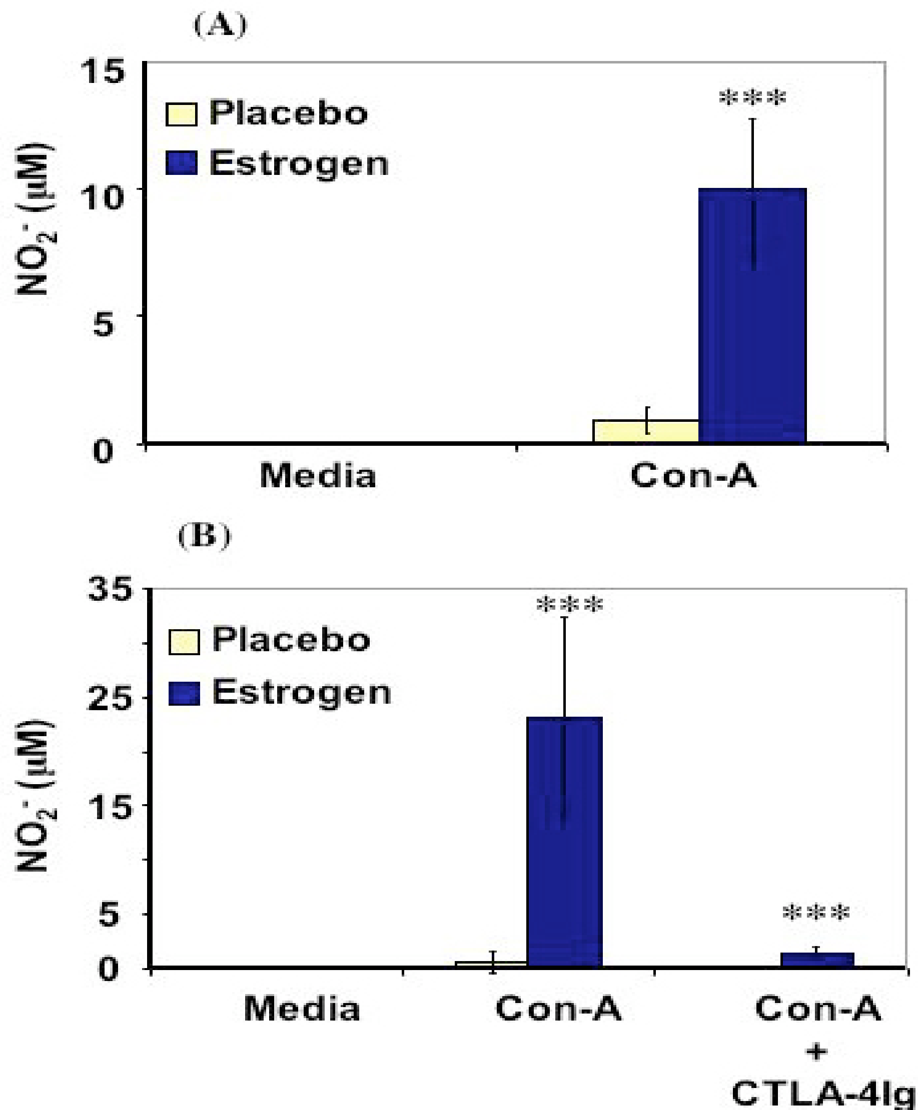


Figure 6. Estrogen-treated female mice have increased nitric oxide levels Splenic lymphocytes isolated from gonadectomized female mice given placebo or estrogen implants were stimulated with: Con-A (10 μg/ml) or Con-A + CTLA-4Ig fusion protein (15 μg/ml; Alexis Biochemicals San Diego, CA), or left unstimulated (media) for 48 hrs. Supernatants were collected and measured indirectly to determine the levels of nitric oxide with Griess Assay. Amount of nitric oxide was determined using the formula obtained from the standard curve. Data are presented as means with standard error bars. Estrogen treatment increases nitric oxide levels in the supernatants from Con-A stimulated splenocytes when compared to that from placebo-treated mice (**Panel A**; Media n=8 mice per placebo or estrogen treatment, Con-A n=8 mice per placebo or estrogen treatment; ***$p<0.001$). Blocking with Con-A+CTLA-4Ig fusion protein leads to a decrease in nitric oxide levels when compared to that from Con-A stimulated splenocytes from estrogen-treated mice (**Panel B**; Media n=6 mice per placebo or estrogen treatment, Con-A n=6 mice per placebo or estrogen treatment; ***$p<0.001$, Con-A+CTLA-4Ig n=6 mice per placebo or estrogen treatment;***$p<0.001$).

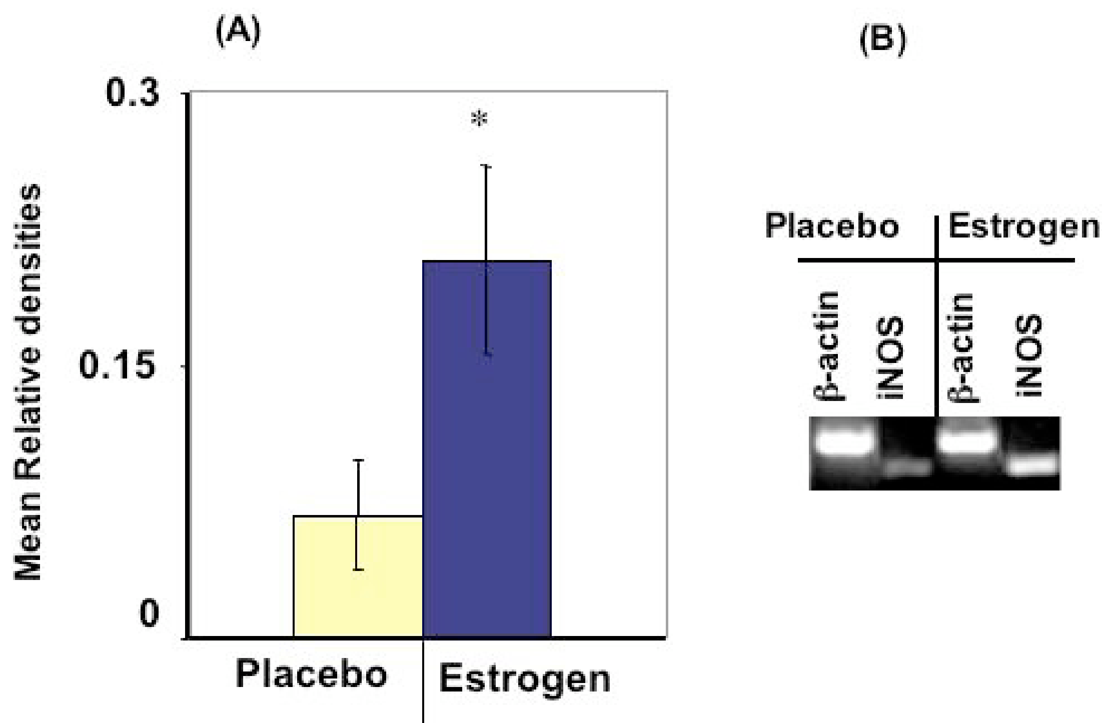


Figure 7. Estrogen increases iNOS mRNA expression in ovariectomized female mice Splenic lymphocytes from ovariectomized female mice that were given estrogen or placebo implants were stimulated with Con-A (10 μg/ml). The expression of iNOS mRNA was detected using RT-PCR after 24 hours of culture. RT-PCR products were scanned with a scanning densitometer and normalized to β-actin. The expression of iNOS is expressed as means of relative densities with standard error bars. Panel A represents the data for iNOS expression from Con-A stimulated splenic lymphocytes after 24 hours of culture (n=7 mice per placebo treatment, n=8 mice per estrogen treatment *$p<0.05$). Panel B shows a representative scan.

e.) NITRIC OXIDE, COX-2, and ESTROGEN

iNOS derived-nitric oxide can induce several genes and their products in various cell types. One of these iNOS-inducible genes is Cyclooxygenase-2 (COX-2). COX-2, a member of cyclooxygenases family, is also known as prostaglandin H synthase. It catalyzes for the formation of prostaglandin PGE_2 from arachidonic acid in tissues (136). COX-2 is expressed in activated cells and as a result of pathologic stimuli. COX-2 is also upregulated in several pathological conditions including cancer and autoimmune diseases. COX-2 mRNA and protein are induced in a time and dose-dependent manner in inflammatory cells, such as human macrophages and polymorphonuclear leukocytes in response to IL-1β, IL-8, TNF-α, and IFN-γ (137, 138), murine and rat macrophages stimulated with LPS and IFN-γ(139-141). Studies show that iNOS and COX-2 are frequently expressed together in tumor cells (142) and various cells such as keratinocytes, macrophage cell lines and endothelial cells (140, 143). Prolonged COX-2 mRNA expression in IFN-γ and LPS activated murine macrophages is maintained by endogenous nitric oxide (144). In addition to the increased COX-2 enzyme activity, due to iNOS-derived nitric oxide in macrophages, elevated COX-2 expression is observed in murine cholangiocytes (145), mouse keratinocytes (143), mouse myocardiocytes (146), and colon cancer cell lines (142). The relationship between IFN-γ, iNOS, and COX-2 can be deciphered in studies using iNOS knockout and IFN-γ knockout mice. Peritoneal macrophages from iNOS knockout mice that are stimulated with IFN-γ and LPS have no nitric oxide production (147). Moreover, IFN-γ knockout mice have not only decreased nitric oxide levels but also the formation of the COX-2 product PGE_2 (Prostaglandin E2) is significantly decreased (80%) (147). Interestingly, although COX-2 product PGE_2 is decreased in IFN-γ knockout mice, the levels of COX-2 itself are not different compared to control mice (147). These results indicate that absence of iNOS-derived nitric oxide affects the formation of PGE_2 from the COX-2 enzyme, rather than protein expression of COX-2 (148). Other studies have shown that the suppression of LPS and IFN-γ mediated nitric oxide and iNOS via capsaicin treatment of the murine macrophage cell line (RAW264.7) decrease COX-2 mRNA expression and PGE_2 secretion (148).

The effect of estrogen on COX-2 in various tissues is demonstrated by several studies. Estrogen treatment of vascular endothelial cells (149), as well as human umbilical vein endothelial cells (150) increases COX-2 expression. Egan et al (151) have shown that estrogen upregulates COX-2 activity and PGE_2 production via Estrogen Receptor subunit alpha (ERα) providing atheroprotection. Although it was shown that macrophage PGE_2 production was increased in burn-traumatized pro-estrus female mice compared to traumatized males or control pro-estrus female mice (152, 153), there is no relevant data that demonstrates the effect of estrogen on COX-2 expression in non-autoimmune and non-traumatized immune system. We have previously shown that estrogen upregulates IFN-γ, IFN-γ-inducible iNOS and its end-product nitric oxide from splenocytes compared to controls. Therefore, we evaluated the effect of estrogen treatment on iNOS-inducible COX-2 expression in the immune system. Our studies demonstrated that estrogen treatment increased iNOS-inducible COX-2 protein expression in Con-A stimulated splenocytes that correlates with the increased iNOS expression (in press). These data imply that estrogen upregulates iNOS and nitric oxide which may have a vital impact in inflammatory disorders.

f.) CONCLUSIONS

It is now recognized that estrogen has profound effects on the immune system of normal and autoimmune individuals that are exposed to this hormone through various sources. Multiple sources of exposure to estrogenic compounds are recognized. These include, natural endogenous estrogens, intentional exposure to pharmaceutical estrogen (such as oral contraceptives and estrogen replacement therapy), and inadvertent human exposure to estrogenic compounds including environmental estrogens. Environmental estrogens are a large part of endocrine-disrupting chemicals, such as pesticides, insecticides, and industrial chemicals, are capable of mimicking or blocking the effects of estrogen (154). These estrogenic compounds include methoxychlor, dioxin, DDT, and bisphenol A. Estrogenic exposure also occurs through consumption of phytoestrogens that are found in soybeans, cabbage, and other edible plants (154, 155). The precise impact of estrogenic

compounds on health is therefore of significant health concern. One mechanism by which estrogens could modulate the immune system is through affecting cytokines and/or altering the response to cytokines. For example, 17-β estradiol markedly increases the promoter activity of IFN-γ as well as IFN-γ secretion from splenic lymphocytes (156). We showed that estrogen treatment of gonadectomized male C57BL/6 mice results in a significant increase of the IFN-γ, in Con-A stimulated splenic lymphocytes (157, 158). Increased levels of IFN-γ protein are also observed in the supernatants of splenic lymphocytes from outbred CD-1 mice that are treated with relatively low doses of 17-β estradiol for short durations followed by an increase in nitric oxide in supernatants from Con-A-activated splenocytes from estrogen-treated mice as well (in press). Our studies show that estrogen upregulates IFN-γ and IFN-γ-inducible Nitric Oxide Synthase (iNOS) and nitric oxide which are both key molecules in physiological processes such as immunity against intracellular infections and pathological processes such as autoimmune diseases and cancer. Although nitric oxide is synthesized by many cell types that are involved in inflammation and innate immunity, nitric oxide demonstrates a dual character depending on the levels produced. At moderately high levels, nitric oxide can be constructive for the immune system with a cytoprotective character as being anti-bacterial, anti-parasitic and anti-viral and anti-apoptotic for immune cells, whereas in the presence of prolonged or severe pathological conditions, very high levels of iNOS-derived nitric oxide can demonstrate a destructive character leading to apoptosis or even necrosis of immune cells. There is also evidence that high concentrations of nitric oxide can suppress the IL-2 and IFN-γ secretion and macrophage activity resulting in the upregulation of IL-4 and driving the T cell differentiation into a Th2 profile (43). This double nature of nitric oxide helps to maintain the homeostasis of the immune system.

Our studies are the first to demonstrate profound effects of estrogen and the link between IFN-γ, iNOS, and nitric oxide in splenic lymphocytes. These results are significant due to the fact that increased IFN-γ and IFN-γ-induced nitric oxide have been associated with autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (159-162), Rheumatoid Arthritis (RA) (163-167), where estrogen has detrimental effects in the progress of these particular autoimmune diseases accompanied with a higher incidence in

females than males. Therefore, mechanistic studies that decipher the molecular and cellular pathways of estrogen-induced IFN-γ, iNOS, and the cellular responses due to exposure to these molecules, are critically important in understanding how estrogens influence healthy individuals and the induction or progression of the disease.

The complexity and intricate nature of immunomodulatory effects of estrogen requires detailed and through investigation of several molecular and cellular pathways to explore key questions such as: what kind of cells are overstimulated to produce IFN-γ and iNOS-derived nitric oxide, what are the consequences of increased nitric oxide on different cells of the immune system after estrogen treatment. These data will provide new information to focus on molecular mechanisms of estrogen treatment and nitric oxide in the immune system. It is hoped that these new findings of linking estrogen with IFN-γ and IFN-γ induced iNOS/nitric oxide and iNOS-induced COX-2 may have important implications to certain inflammatory, autoimmune diseases, and neoplastic disorders. It is conceivable that new therapeutic approaches can be designed to disrupt or regulate the sequence of events.

g.) REFERENCES

1. **Bogdan C, Rollinghoff M, Diefenbach A** 2000 The role of nitric oxide in innate immunity. Immunol Rev 173:17-26
2. **van der Veen RC** 2001 Nitric oxide and T helper cell immunity. Int Immunopharmacol 1:1491-500
3. **MacMicking J, Xie QW, Nathan C** 1997 Nitric oxide and macrophage function. Annu Rev Immunol 15:323-50
4. **Bogdan C** 2001 Nitric oxide and the immune response. Nat Immunol 2:907-16
5. **Wink DA, Hanbauer I, Grisham MB, et al.** 1996 Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. Curr Top Cell Regul 34:159-87
6. **Lipton SA** 1996 Distinctive chemistries of no-related species. Neurochem Int 29:111-4
7. **Kolb H, Kolb-Bachofen V** 1998 Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator? Immunol Today 19:556-61
8. **Gorren AC, Mayer B** 1998 The versatile and complex enzymology of nitric oxide synthase. Biochemistry (Mosc) 63:734-43
9. **Stuehr DJ** 1999 Mammalian nitric oxide synthases. Biochim Biophys Acta 1411:217-30
10. **Ludwig ML, Marletta MA** 1999 A new decoration for nitric oxide synthase - a Zn(Cys)4 site. Structure Fold Des 7:R73-9
11. **Werner ER, Werner-Felmayer G, Mayer B** 1998 Tetrahydrobiopterin, cytokines, and nitric oxide synthesis. Proc Soc Exp Biol Med 219:171-82
12. **Christopherson KS, Bredt DS** 1997 Nitric oxide in excitable tissues: physiological roles and disease. J Clin Invest 100:2424-9
13. **Huang PL, Lo EH** 1998 Genetic analysis of NOS isoforms using nNOS and eNOS knockout animals. Prog Brain Res 118:13-25
14. **Bolanos JP, Almeida A** 1999 Roles of nitric oxide in brain hypoxia-ischemia. Biochim Biophys Acta 1411:415-36

15. **Forstermann U, Boissel JP, Kleinert H** 1998 Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). Faseb J 12:773-90
16. **Kone BC** 1999 Localization and regulation of nitric oxide synthase isoforms in the kidney. Semin Nephrol 19:230-41
17. **Moncada S** 1999 Nitric oxide: discovery and impact on clinical medicine. J R Soc Med 92:164-9
18. **Lloyd-Jones DM, Bloch KD** 1996 The vascular biology of nitric oxide and its role in atherogenesis. Annu Rev Med 47:365-75
19. **Taylor BS, Alarcon LH, Billiar TR** 1998 Inducible nitric oxide synthase in the liver: regulation and function. Biochemistry (Mosc) 63:766-81
20. **Taylor AW, Yee DG, Streilein JW** 1998 Suppression of nitric oxide generated by inflammatory macrophages by calcitonin gene-related peptide in aqueous humor. Invest Ophthalmol Vis Sci 39:1372-8
21. **Assreuy J, Cunha FQ, Epperlein M, et al.** 1994 Production of nitric oxide and superoxide by activated macrophages and killing of Leishmania major. Eur J Immunol 24:672-6
22. **Matsuura M, Saito S, Hirai Y, Okamura H** 2003 A pathway through interferon-gamma is the main pathway for induction of nitric oxide upon stimulation with bacterial lipopolysaccharide in mouse peritoneal cells. Eur J Biochem 270:4016-25
23. **Chun SY, Eisenhauer KM, Kubo M, Hsueh AJ** 1995 Interleukin-1 beta suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. Endocrinology 136:3120-7
24. **Taylor-Robinson AW** 1997 Counter-regulation of T helper 1 cell proliferation by nitric oxide and interleukin-2. Biochem Biophys Res Commun 233:14-9
25. **Koide N, Sugiyama T, Mu MM, et al.** 2003 Gamma interferon-induced nitric oxide production in mouse CD5+ B1-like cell line and its association with apoptotic cell death. Microbiol Immunol 47:669-79

26. **Kim YM, Talanian RV, Billiar TR** 1997 Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. J Biol Chem 272:31138-48
27. **Neufeld AH, Liu B** 2003 Comparison of the signal transduction pathways for the induction of gene expression of nitric oxide synthase-2 in response to two different stimuli. Nitric Oxide 8:95-102
28. **Bogdan C** 2000 The function of nitric oxide in immune system. In: Handbook of experimental Pharmacology. Springer, Berlin, New York
29. **van der Veen RC, Dietlin TA, Hofman FM** 2003 Tissue expression of inducible nitric oxide synthase requires IFN-gamma production by infiltrating splenic T cells: more evidence for immunosuppression by nitric oxide. J Neuroimmunol 145:86-90
30. **Burney S, Tamir S, Gal A, Tannenbaum SR** 1997 A mechanistic analysis of nitric oxide-induced cellular toxicity. Nitric Oxide 1:130-44
31. **Boehm U, Klamp T, Groot M, Howard JC** 1997 Cellular responses to interferon-gamma. Annu Rev Immunol 15:749-95
32. **Bach EA, Szabo SJ, Dighe AS, et al.** 1995 Ligand-induced autoregulation of IFN-gamma receptor beta chain expression in T helper cell subsets. Science 270:1215-8
33. **Rigamonti L, Ariotti S, Losana G, et al.** 2000 Surface expression of the IFN-gamma R2 chain is regulated by intracellular trafficking in human T lymphocytes. J Immunol 164:201-7
34. **Larkin J, 3rd, Johnson HM, Subramaniam PS** 2000 Differential nuclear localization of the IFNGR-1 and IFNGR-2 subunits of the IFN-gamma receptor complex following activation by IFN-gamma. J Interferon Cytokine Res 20:565-76
35. **Leonard WJ, O'Shea JJ** 1998 Jaks and STATs: biological implications. Annu Rev Immunol 16:293-322
36. **Liew FY, Li Y, Moss D, Parkinson C, Rogers MV, Moncada S** 1991 Resistance to Leishmania major infection correlates with the induction of nitric oxide synthase in murine macrophages. Eur J Immunol 21:3009-14

37. **Bogdan C** 1997 Of microbes, macrophages and nitric oxide. Behring Inst Mitt:58-72

38. **Ing DJ, Zang J, Dzau VJ, Webster KA, Bishopric NH** 1999 Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. Circ Res 84:21-33

39. **Brune B, von Knethen A, Sandau KB** 1999 Nitric oxide (NO): an effector of apoptosis. Cell Death Differ 6:969-75

40. **Kroncke KD, Fehsel K, Suschek C, Kolb-Bachofen V** 2001 Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. Int Immunopharmacol 1:1407-20

41. **Vallance P, Moncada S** 1993 Role of endogenous nitric oxide in septic shock. New Horiz 1:77-86

42. **Ruetten HaT, C.** 2000 Chapter 47. In: Ignarro L (ed) Nitric Oxide: Biology and Pathology. Academic Press

43. **Singh VK, Mehrotra S, Narayan P, Pandey CM, Agarwal SS** 2000 Modulation of autoimmune diseases by nitric oxide. Immunol Res 22:1-19

44. **Lieb K, Engels S, Fiebich BL** 2003 Inhibition of LPS-induced iNOS and NO synthesis in primary rat microglial cells. Neurochem Int 42:131-7

45. **MacLean A, Wei XQ, Huang FP, Al-Alem UA, Chan WL, Liew FY** 1998 Mice lacking inducible nitric-oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses. J Gen Virol 79 (Pt 4):825-30

46. **Zaragoza C, Ocampo C, Saura M, et al.** 1998 The role of inducible nitric oxide synthase in the host response to Coxsackievirus myocarditis. Proc Natl Acad Sci U S A 95:2469-74

47. **MacMicking JD, Nathan C, Hom G, et al.** 1995 Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 81:641-50

48. **MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF** 1997 Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci U S A 94:5243-8

49. **Hickman-Davis J, Gibbs-Erwin J, Lindsey JR, Matalon S** 1999 Surfactant protein A mediates mycoplasmacidal activity of alveolar macrophages by production of peroxynitrite. Proc Natl Acad Sci U S A 96:4953-8
50. **Murray HW, Nathan CF** 1999 Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani. J Exp Med 189:741-6
51. **Wei XQ, Charles IG, Smith A, et al.** 1995 Altered immune responses in mice lacking inducible nitric oxide synthase. Nature 375:408-11
52. **Niedbala W, Wei XQ, Piedrafita D, Xu D, Liew FY** 1999 Effects of nitric oxide on the induction and differentiation of Th1 cells. Eur J Immunol 29:2498-505
53. **Diefenbach A, Schindler H, Donhauser N, et al.** 1998 Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity 8:77-87
54. **Yoneto T, Yoshimoto T, Wang CR, et al.** 1999 Gamma interferon production is critical for protective immunity to infection with blood-stage Plasmodium berghei XAT but neither NO production nor NK cell activation is critical. Infect Immun 67:2349-56
55. **Scharton-Kersten TM, Yap G, Magram J, Sher A** 1997 Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. J Exp Med 185:1261-73
56. **Pacelli R, Wink DA, Cook JA, et al.** 1995 Nitric oxide potentiates hydrogen peroxide-induced killing of Escherichia coli. J Exp Med 182:1469-79
57. **Nathan CF, Root RK** 1977 Hydrogen peroxide release from mouse peritoneal macrophages: dependence on sequential activation and triggering. J Exp Med 146:1648-62
58. **Sicher SC, Vazquez MA, Lu CY** 1994 Inhibition of macrophage Ia expression by nitric oxide. J Immunol 153:1293-300
59. **Kleinert H, Schwarz PM, Forstermann U** 2003 Regulation of the expression of inducible nitric oxide synthase. Biol Chem 384:1343-64
60. **Akaike T, and Maeda, H.** 2000 Chapter 46. In: Ignarro L (ed) Nitric oxide: Biology and Pathobiology. Academic Press

61. **Huang FP, Niedbala W, Wei XQ, et al.** 1998 Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. Eur J Immunol 28:4062-70
62. **Roozendaal R, Vellenga E, Postma DS, De Monchy JG, Kauffman HF** 1999 Nitric oxide selectively decreases interferon-gamma expression by activated human T lymphocytes via a cGMP-independent mechanism. Immunology 98:393-9
63. **Bauer H, Jung T, Tsikas D, Stichtenoth DO, Frolich JC, Neumann C** 1997 Nitric oxide inhibits the secretion of T-helper 1- and T-helper 2-associated cytokines in activated human T cells. Immunology 90:205-11
64. **Bogdan C, Vodovotz Y, Nathan C** 1991 Macrophage deactivation by interleukin 10. J Exp Med 174:1549-55
65. **Vodovotz Y, Bogdan C, Paik J, Xie QW, Nathan C** 1993 Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. J Exp Med 178:605-13
66. **Bogdan C, Vodovotz Y, Paik J, Xie QW, Nathan C** 1994 Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. J Leukoc Biol 55:227-33
67. **Zhuang JC, Wogan GN** 1997 Growth and viability of macrophages continuously stimulated to produce nitric oxide. Proc Natl Acad Sci U S A 94:11875-80
68. **Messmer UK, Brune B** 1994 Modulation of inducible nitric oxide synthase in RINm5F cells. Cell Signal 6:17-24
69. **Messmer UK, Brune B** 1996 Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. Biochem J 319 (Pt 1):299-305
70. **Messmer UK, Brune B** 1996 Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD+ content, and p53 accumulation. Arch Biochem Biophys 327:1-10
71. **Messmer UK, Lapetina EG, Brune B** 1995 Nitric oxide-induced apoptosis in RAW 264.7 macrophages is antagonized by protein kinase C- and protein kinase A-activating compounds. Mol Pharmacol 47:757-65

72. **Messmer UK, Reed UK, Brune B** 1996 Bcl-2 protects macrophages from nitric oxide-induced apoptosis. J Biol Chem 271:20192-7
73. **Messmer UK, Reimer DM, Reed JC, Brune B** 1996 Nitric oxide induced poly(ADP-ribose) polymerase cleavage in RAW 264.7 macrophage apoptosis is blocked by Bcl-2. FEBS Lett 384:162-6
74. **Sarih M, Souvannavong V, Adam A** 1993 Nitric oxide synthase induces macrophage death by apoptosis. Biochem Biophys Res Commun 191:503-8
75. **Rabkin SW, Kong JY** 2000 Nitroprusside induces cardiomyocyte death: interaction with hydrogen peroxide. Am J Physiol Heart Circ Physiol 279:H3089-100
76. **Wagle A, Singh JP** 2000 Fibroblast growth factor protects nitric oxide-induced apoptosis in neuronal SHSY-5Y cells. J Pharmacol Exp Ther 295:889-95
77. **Brockhaus F, Brune B** 1998 U937 apoptotic cell death by nitric oxide: Bcl-2 downregulation and caspase activation. Exp Cell Res 238:33-41
78. **Mahr S, Neumayer N, Gerhard M, Classen M, Prinz C** 2000 IL-1beta-induced apoptosis in rat gastric enterochromaffin-like cells is mediated by iNOS, NF-kappaB, and Bax protein. Gastroenterology 118:515-24
79. **Battinelli E, Loscalzo J** 2000 Nitric oxide induces apoptosis in megakaryocytic cell lines. Blood 95:3451-9
80. **Xaus J, Comalada M, Valledor AF, et al.** 2000 LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-alpha. Blood 95:3823-31
81. **Arstall MA, Sawyer DB, Fukazawa R, Kelly RA** 1999 Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ Res 85:829-40
82. **Tamatani M, Ogawa S, Niitsu Y, Tohyama M** 1998 Involvement of Bcl-2 family and caspase-3-like protease in NO-mediated neuronal apoptosis. J Neurochem 71:1588-96
83. **Brockhaus F, Brune B** 1999 p53 accumulation in apoptotic macrophages is an energy demanding process that precedes cytochrome c release in response to nitric oxide. Oncogene 18:6403-10

84. **Earnshaw WC, Martins LM, Kaufmann SH** 1999 Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383-424

85. **Thornberry NA, Lazebnik Y** 1998 Caspases: enemies within. Science 281:1312-6

86. **Gajewski TF, Thompson CB** 1996 Apoptosis meets signal transduction: elimination of a BAD influence. Cell 87:589-92

87. **Rathmell JC, Thompson CB** 1999 The central effectors of cell death in the immune system. Annu Rev Immunol 17:781-828

88. **Cryns V, Yuan J** 1998 Proteases to die for. Genes Dev 12:1551-70

89. **Sakahira H, Enari M, Nagata S** 1998 Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96-9

90. **Liu X, Kim CN, Yang J, Jemmerson R, Wang X** 1996 Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86:147-57

91. **Li P, Nijhawan D, Budihardjo I, et al.** 1997 Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-89

92. **Bal-Price A, Brown GC** 2000 Nitric-oxide-induced necrosis and apoptosis in PC12 cells mediated by mitochondria. J Neurochem 75:1455-64

93. **Takeda Y, Tashima M, Takahashi A, Uchiyama T, Okazaki T** 1999 Ceramide generation in nitric oxide-induced apoptosis. Activation of magnesium-dependent neutral sphingomyelinase via caspase-3. J Biol Chem 274:10654-60

94. **Jyothi MD, Khar A** 1999 Induction of nitric oxide production by natural killer cells: its role in tumor cell death. Nitric Oxide 3:409-18

95. **Hadjivassiliou V, Green MH, James RF, Swift SM, Clayton HA, Green IC** 1998 Insulin secretion, DNA damage, and apoptosis in human and rat islets of Langerhans following exposure to nitric oxide, peroxynitrite, and cytokines. Nitric Oxide 2:429-41

96. **Fehsel K, Kroncke KD, Meyer KL, Huber H, Wahn V, Kolb-Bachofen V** 1995 Nitric oxide induces apoptosis in mouse thymocytes. J Immunol 155:2858-65
97. **Borutaite V, Brown GC** 2003 Nitric oxide induces apoptosis via hydrogen peroxide, but necrosis via energy and thiol depletion. Free Radic Biol Med 35:1457-68
98. **Pinsky DJ, Aji W, Szabolcs M, et al.** 1999 Nitric oxide triggers programmed cell death (apoptosis) of adult rat ventricular myocytes in culture. Am J Physiol 277:H1189-99
99. **Eu JP, Liu L, Zeng M, Stamler JS** 2000 An apoptotic model for nitrosative stress. Biochemistry 39:1040-7
100. **Hebestreit H, Dibbert B, Balatti I, et al.** 1998 Disruption of fas receptor signaling by nitric oxide in eosinophils. J Exp Med 187:415-25
101. **Hortelano S, Bosca L** 1997 6-Mercaptopurine decreases the Bcl-2/Bax ratio and induces apoptosis in activated splenic B lymphocytes. Mol Pharmacol 51:414-21
102. **Genaro AM, Hortelano S, Alvarez A, Martinez C, Bosca L** 1995 Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. J Clin Invest 95:1884-90
103. **van der Veen RC, Dietlin TA, Pen L, Gray JD** 1999 Nitric oxide inhibits the proliferation of T-helper 1 and 2 lymphocytes without reduction in cytokine secretion. Cell Immunol 193:194-201
104. **Suschek CV, Krischel V, Bruch-Gerharz D, et al.** 1999 Nitric oxide fully protects against UVA-induced apoptosis in tight correlation with Bcl-2 up-regulation. J Biol Chem 274:6130-7
105. **Hemmrich K, Suschek CV, Lerzynski G, Kolb-Bachofen V** 2003 iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage. J Appl Physiol 95:1937-46
106. **Dimmeler S, Zeiher AM** 1997 Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. Nitric Oxide 1:275-81

107. **Dimmeler S, Haendeler J, Nehls M, Zeiher AM** 1997 Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. J Exp Med 185:601-7

108. **Delikouras A, Hayes M, Malde P, Lechler RI, Dorling A** 2001 Nitric oxide-mediated expression of Bcl-2 and Bcl-xl and protection from tumor necrosis factor-alpha-mediated apoptosis in porcine endothelial cells after exposure to low concentrations of xenoreactive natural antibody. Transplantation 71:599-605

109. **Okada S, Zhang H, Hatano M, Tokuhisa T** 1998 A physiologic role of Bcl-xL induced in activated macrophages. J Immunol 160:2590-6

110. **Salvucci O, Carsana M, Bersani I, Tragni G, Anichini A** 2001 Antiapoptotic role of endogenous nitric oxide in human melanoma cells. Cancer Res 61:318-26

111. **Li J, Billiar TR, Talanian RV, Kim YM** 1997 Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. Biochem Biophys Res Commun 240:419-24

112. **Rossig L, Fichtlscherer B, Breitschopf K, et al.** 1999 Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. J Biol Chem 274:6823-6

113. **Li J, Bombeck CA, Yang S, Kim YM, Billiar TR** 1999 Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes. J Biol Chem 274:17325-33

114. **Mannick JB, Miao XQ, Stamler JS** 1997 Nitric oxide inhibits Fas-induced apoptosis. J Biol Chem 272:24125-8

115. **Mannick JB, Hausladen A, Liu L, et al.** 1999 Fas-induced caspase denitrosylation. Science 284:651-4

116. **Kim YM, Kim TH, Seol DW, Talanian RV, Billiar TR** 1998 Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome c release. J Biol Chem 273:31437-41

117. **Sciorati C, Rovere P, Ferrarini M, Heltai S, Manfredi AA, Clementi E** 1997 Autocrine nitric oxide modulates CD95-induced apoptosis in gammadelta T lymphocytes. J Biol Chem 272:23211-5

118. **Thompson CB** 1995 Apoptosis in the pathogenesis and treatment of disease. Science 267:1456-62

119. **Zhao H, Dugas N, Mathiot C, et al.** 1998 B-cell chronic lymphocytic leukemia cells express a functional inducible nitric oxide synthase displaying anti-apoptotic activity. Blood 92:1031-43
120. **Candolfi M, Jaita G, Zaldivar V, Zarate S, Pisera D, Seilicovich A** 2004 Tumor necrosis factor-alpha-induced nitric oxide restrains the apoptotic response of anterior pituitary cells. Neuroendocrinology 80:83-91
121. **Wen Y, Yang S, Liu R, et al.** 2004 Estrogen attenuates nuclear factor-kappa B activation induced by transient cerebral ischemia. Brain Res 1008:147-54
122. **Kauser K, Sonnenberg D, Diel P, Rubanyi GM** 1998 Effect of 17beta-oestradiol on cytokine-induced nitric oxide production in rat isolated aorta. Br J Pharmacol 123:1089-96
123. **Tamura K, Yamaguchi K, Kogo H** 2000 17Beta-estradiol inhibits ovariectomy-induced expression of inducible nitric oxide synthase in rat aorta in vivo. Life Sci 66:PL 259-64
124. **Nuedling S, Karas RH, Mendelsohn ME, et al.** 2001 Activation of estrogen receptor beta is a prerequisite for estrogen-dependent upregulation of nitric oxide synthases in neonatal rat cardiac myocytes. FEBS Lett 502:103-8
125. **Neugarten J, Ding Q, Friedman A, Lei J, Silbiger S** 1997 Sex hormones and renal nitric oxide synthases. J Am Soc Nephrol 8:1240-6
126. **Baker AE, Brautigam VM, Watters JJ** 2004 Estrogen Modulates Microglial Inflammatory Mediator Production via Interactions with Estrogen Receptor {beta}. Endocrinology
127. **Noris M, Todeschini M, Zappella S, et al.** 2000 17beta-estradiol corrects hemostasis in uremic rats by limiting vascular expression of nitric oxide synthases. Am J Physiol Renal Physiol 279:F626-35
128. **Ogando D, Farina M, Ribeiro ML, et al.** 2003 Steroid hormones augment nitric oxide synthase activity and expression in rat uterus. Reprod Fertil Dev 15:269-74
129. **Mershon JL, Baker RS, Clark KE** 2002 Estrogen increases iNOS expression in the ovine coronary artery. Am J Physiol Heart Circ Physiol 283:H1169-80

130. **Nuedling S, Kahlert S, Loebbert K, et al.** 1999 17 Beta-estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo. Cardiovasc Res 43:666-74

131. **Chao TC, Phuangsab A, Van Alten PJ, Walter RJ** 1996 Steroid sex hormones and macrophage function: regulation of chemiluminescence and phagocytosis. Am J Reprod Immunol 35:106-113

132. **Gomez F, Ruiz P, Bernal JA, Escobar M, Garcia-Egido A, Lopez-Saez JJ** 2001 Enhancement of splenic-macrophage Fcgamma receptor expression by treatment with estrogens. Clin Diagn Lab Immunol 8:806-10

133. **Hong M, Zhu Q** 2004 Macrophages are activated by 17beta-estradiol: possible permission role in endometriosis. Exp Toxicol Pathol 55:385-91

134. **You HJ, Kim JY, Jeong HG** 2003 17 beta-estradiol increases inducible nitric oxide synthase expression in macrophages. Biochem Biophys Res Commun 303:1129-34

135. **Azenabor AA, Yang S, Job G, Adedokun OO** 2004 Expression of iNOS gene in macrophages stimulated with 17beta-estradiol is regulated by free intracellular Ca2+. Biochem Cell Biol 82:381-90

136. **Steer SA, Corbett JA** 2003 The role and regulation of COX-2 during viral infection. Viral Immunol 16:447-60

137. **Barrios-Rodiles M, Chadee K** 1998 Novel regulation of cyclooxygenase-2 expression and prostaglandin E2 production by IFN-gamma in human macrophages. J Immunol 161:2441-8

138. **Maloney CG, Kutchera WA, Albertine KH, McIntyre TM, Prescott SM, Zimmerman GA** 1998 Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. J Immunol 160:1402-10

139. **Lee SH, Soyoola E, Chanmugam P, et al.** 1992 Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J Biol Chem 267:25934-8

140. **Akarasereenont P, Mitchell JA, Appleton I, Thiemermann C, Vane JR** 1994 Involvement of tyrosine kinase in the induction of cyclo-oxygenase and nitric oxide synthase by endotoxin in cultured cells. Br J Pharmacol 113:1522-8

141. **Misko TP, Trotter JL, Cross AH** 1995 Mediation of inflammation by encephalitogenic cells: interferon gamma induction of nitric oxide synthase and cyclooxygenase 2. J Neuroimmunol 61:195-204

142. **Liu Q, Chan ST, Mahendran R** 2003 Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines. Carcinogenesis 24:637-42

143. **Chun KS, Cha HH, Shin JW, et al.** 2004 Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-kappaB. Carcinogenesis 25:445-54

144. **Perkins DJ, Kniss DA** 1999 Blockade of nitric oxide formation down-regulates cyclooxygenase-2 and decreases PGE2 biosynthesis in macrophages. J Leukoc Biol 65:792-9

145. **Ishimura N, Bronk SF, Gores GJ** 2004 Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. Am J Physiol Gastrointest Liver Physiol 287:G88-95

146. **Xuan YT, Guo Y, Zhu Y, et al.** 2003 Mechanism of cyclooxygenase-2 upregulation in late preconditioning. J Mol Cell Cardiol 35:525-37

147. **Marnett LJ, Wright TL, Crews BC, Tannenbaum SR, Morrow JD** 2000 Regulation of prostaglandin biosynthesis by nitric oxide is revealed by targeted deletion of inducible nitric-oxide synthase. J Biol Chem 275:13427-30

148. **Chen CW, Lee ST, Wu WT, Fu WM, Ho FM, Lin WW** 2003 Signal transduction for inhibition of inducible nitric oxide synthase and cyclooxygenase-2 induction by capsaicin and related analogs in macrophages. Br J Pharmacol 140:1077-87

149. **Pedram A, Razandi M, Aitkenhead M, Hughes CC, Levin ER** 2002 Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J Biol Chem 277:50768-75

150. **Akarasereenont P, Techatraisak K, Thaworn A, Chotewuttakorn S** 2000 The induction of cyclooxygenase-2 by 17beta-estradiol in endothelial cells is mediated through protein kinase C. Inflamm Res 49:460-5

151. **Egan KM, Lawson JA, Fries S, et al.** 2004 COX-2-derived prostacyclin confers atheroprotection on female mice. Science 306:1954-7

152. **Stapleton PP, Strong VE, Freeman TA, Winter J, Yan Z, Daly JM** 2004 Gender affects macrophage cytokine and prostaglandin E2 production and PGE2 receptor expression after trauma. J Surg Res 122:1-7

153. **Gregory MS, Duffner LA, Hahn EL, Tai HH, Faunce DE, Kovacs EJ** 2000 Differential production of prostaglandin E(2) in male and female mice subjected to thermal injury contributes to the gender difference in immune function: possible role for 15-hydroxyprostaglandin dehydrogenase. Cell Immunol 205:94-102

154. **Colborn T, vom Saal FS, Soto AM** 1993 Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378-84

155. **Whitten PL, Lewis C, Russell E, Naftolin F** 1995 Potential adverse effects of phytoestrogens. J Nutr 125:771S-776S

156. **Fox HS, Bond BL, Parslow TG** 1991 Estrogen regulates the IFN-gamma promoter. J Immunol 146:4362-7

157. **Karpuzoglu-Sahin E, Zhi-Jun Y, Lengi A, Sriranganathan N, Ansar Ahmed S** 2001 Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. Cytokine 14:208-17

158. **Karpuzoglu-Sahin E, Hissong BD, Ansar Ahmed S** 2001 Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. J Reprod Immunol 52:113-27

159. **Weinberg JB** 1998 Nitric oxide as an inflammatory mediator in autoimmune MRL-lpr/lpr mice. Environ Health Perspect 106 Suppl 5:1131-7

160. **Gilkeson GS, Mudgett JS, Seldin MF, et al.** 1997 Clinical and serologic manifestations of autoimmune disease in MRL-lpr/lpr mice lacking nitric oxide synthase type 2. J Exp Med 186:365-73

161. **Weinberg JB, Granger DL, Pisetsky DS, et al.** 1994 The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide synthase expression in MRL-lpr/lpr mice, and

reduction of spontaneous glomerulonephritis and arthritis by orally administered NG-monomethyl-L-arginine. J Exp Med 179:651-60

162. **Belmont HM, Levartovsky D, Goel A, et al.** 1997 Increased nitric oxide production accompanied by the up-regulation of inducible nitric oxide synthase in vascular endothelium from patients with systemic lupus erythematosus. Arthritis Rheum 40:1810-6

163. **Choi JW** 2003 Nitric oxide production is increased in patients with rheumatoid arthritis but does not correlate with laboratory parameters of disease activity. Clin Chim Acta 336:83-7

164. **Grant DD, Goldstein R, Karsh J, Birnboim HC** 2001 Nitric oxide donors induce large-scale deletion mutations in human lymphoblastoid cells: implications for mutations in T-lymphocytes from arthritis patients. Environ Mol Mutagen 38:261-7

165. **Hilliquin P, Borderie D, Hernvann A, Menkes CJ, Ekindjian OG** 1997 Nitric oxide as S-nitrosoproteins in rheumatoid arthritis. Arthritis Rheum 40:1512-7

166. **Ueki Y, Miyake S, Tominaga Y, Eguchi K** 1996 Increased nitric oxide levels in patients with rheumatoid arthritis. J Rheumatol 23:230-6

167. **Sakurai H, Kohsaka H, Liu MF, et al.** 1995 Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 96:2357-63

CHAPTER 4. Effects of Estrogen on IFN-γ-Inducing Cytokines, Transcription Factors, and Costimulatory Molecules.

a.) INTRODUCTION

The differentiation of naïve T helper cells into T helper 1 (Th1) or T helper 2 (Th2) cells is critical to mount an effective and appropriate immune response. A disturbance in the balance of Th1 and Th2 cells and their products may lead to several disorders such as autoimmune diseases, susceptibility to infections, fetal loss, and even cancer. T helper subsets (Th1 and Th2) are distinguished by the patterns of cytokines they secrete, the expression of unique surface markers, and their expression of specific transcription factors (**Table 1**).

Table 1. Molecular Markers for Th1 and Th2 Cells

Receptor markers	**Th1**	**Th2**
IL-12 Receptorβ2	+	—
IL-18 Receptorβ	+	—
IFNγ Receptor	Only α receptor	Both α & β
IL-1 Receptor	—	+
Chemokine Receptors	CCR-5, CXCR-3, CCR1	CCR3, CCR4, CCR-8, CR
Transcription factors	**Th1**	**Th2**
T-bet	+	—
Hlx	+	—
Eomes	+	—
ERM	+	—
CIITA	+	—
GATA-3	—	+
c-maf	—	+
JunB	—	+

The defining key cytokines for Th1 and Th2 cells are IFN-γ and IL-4, respectively. The development or promotion of naïve T cells into effector Th1 cells occurs in relation to several factors. It is now apparent that induction of IFN-γ is dependent upon several factors including activation, IFN-γ-inducing cytokines, costimulatory signals, and IFN-γ-

inducing transcription factors. Given that presented data in previous chapters demonstrates that estrogen treatment tends to result in increased IFN-γ (protein and mRNA) as well as increased levels of IFN-γ-inducible iNOS and nitric oxide production, this part of the dissertation focuses on how estrogen regulates IFN-γ production. Specifically, this chapter will focus on the effects of estrogen on IFN-γ-inducing cytokines, IFN-γ-inducing transcription factors, and selected costimulatory molecules.

Therefore, this chapter is divided into three subsections:

Subsection 1. IFN-γ-inducing cytokines and receptors

Subsection 2. Induction of IFN-γ-inducing transcription factors

Subsection 3. Costimulatory molecules

4.1. Effects of Estrogen Treatment on IFN-γ-Inducing Cytokines and Their Receptors on Murine Splenocytes

a.) ABSTRACT

There are several cytokines that are known to modulate the secretion of IFN-γ by T cells (IFN-γ-inducing cytokines), including IL-12, IL-18, IL-15, and the newly discovered IL-27. Sex hormones, such as 17-β-estradiol, are believed to play a major role in the dichotomy of immune responses between sexes. Previously, we have shown that estrogen treatment tends to induce IFN-γ and upregulate IFN-γ end-products, such as iNOS. The effect of estrogen on IL-12p70, IL-18, IL-15, IL-27, and their respective receptors is unknown. In this study, we demonstrate that Con-A-stimulated splenocytes from estrogen-treated mice have increased levels of IL-18 and IL-15 proteins, whereas IL-12p70 protein levels are decreased. The mRNA expression levels of IL-18, IL-15, and IL-27 are increased in Con-A-stimulated splenocytes from estrogen-treated mice as detected by RT and/or Real Time PCR. Interestingly, estrogen treatment increases expression of the signaling subunits: IL-12Rβ2 and IL-18Rβ from Con-A-stimulated splenocytes. Deliberate addition of IFN-γ to low dose Con-A-stimulated (1μg/ml) splenocytes results in increased IL-12Rβ2 from estrogen treated mice. The possibility of increased response due to estrogen treatment was explored with the addition of rIL-12p70 addition to splenocytes. We found that such cultures from estrogen-treated mice that were stimulated with submitogenic dose of Con-A have increased levels of IFN-γ, iNOS mRNA, and nitric oxide in comparison to controls. This is the first report to show that estrogen increases IFN-γ-inducing cytokines (IL-18, IL-15, IL-27) as well as enhanced IL-18Rβ, and IL-12Rβ2 in response to IFN-γ. We conclude that in this model estrogen alters the expression of IFN-γ inducing cytokines and their receptors.

b.) INTRODUCTION

To date, there are several cytokines (principally secreted by antigen presenting cells) that are known to modulate the secretion of IFN-γ by T cells. These IFN-γ-inducing cytokines include IL-12, IL-18, IL-15, and the recently discovered IL-27. Although IL-12, IL-18, IL-27, and IL-15 are all involved in the induction of IFN-γ they do not seem to act independently. Rather, they appear to work in concert or in a cascade fashion. For example, in mouse and human cells, IL-12 by itself does not induce IFN-γ production even at very high doses (1), emphasizing the need for other cytokines such as IL-18 or IL-27 for the induction of IFN-γ.

Interleukin-27 (IL-27)

Recently, a new member of the heterodimeric family of cytokines, called Interleukin-27 (IL-27) was described (2). IL-27 consists of a p40 protein that is called Epstein-Barr virus (EBV)-induced gene 3 (EIB3) and p28, which is related to IL-12p35. It is produced early by activated antigen presenting cells and works synergistically with IL-12 to induce the proliferation of naive T cells and IFN-γ (2). IL-27 binds to an orphan receptor called TCCR (WSX-1), which is mainly expressed on naive T cells, but is also expressed at a low level on differentiated Th1 cells (3). TCCR (WSX-1) knockout mice show decreased IFN-γ levels and increased susceptibility to intracellular pathogens and gram-negative bacteria (3-5). TCCR together with another subunit called gp130 is responsible for the binding and signaling of IL-27 (6). TCCR-IL-27 signaling induces the expression of IL-12Rβ2 and the Th1 transcription factor T-bet in naïve T and NK cells regardless of IFN-γ preparing them to be differentiated into Th1 lymphocytes for IFN-γ secretion (7-9). Induction of T-bet by IL-27 results in increased IL-12Rβ2 expression (8). The upregulation of IL-12Rβ2 expression on these naïve T cells suggests that IL-27 acts earlier than IL-12. Signaling through TCCR induces phosphorylation of STAT-1 and STAT-3 (7, 8). The activation of STAT-1 by IL-27 results in the suppression of GATA-3, a Th2 type transcription factor, which facilitates the effects of T-bet and IFN-γ-inducing

transcription factor. On the other hand, IL-27 can induce T-bet in a STAT-1 independent fashion as well (7). Although IL-27 can stimulate T-bet expression, it cannot increase IFN-γ secretion from these cells by itself (7). IL-27 does not directly induce IFN-γ production from $CD4^+$ T naïve cells but it does do so in the presence of IL-12 (9).

In addition to directing naïve T cells toward a Th1 type response, IL-27 also regulates Ig class switching in B cells. The IL-27 receptor, TCCR, is expressed on naïve mouse splenic B lymphocytes (10). Activation of B cells with anti-CD40 or LPS in combination with IL-27 causes STAT-1 phosphorylation, increased T-bet expression, and IgG2a class switching. T-bet, but not IFN-γ, is necessary for IgG2a class switching in LPS-activated B cells (10). The primary roles of IL-27 are to prepare T cells for early Th1 commitment, to activate B lymphocytes for class switching, and to increase responsiveness to IL-12.

Interleukin-12 (IL-12) and IL-12 Receptors

Interleukin-12 is an immunomodulatory cytokine, which is important for protective immunity against intracellular pathogens and for the differentiation of naive $CD4^+$ cells into Th1 cells. This heterodimeric cytokine is composed of two subunits, p40 and p35, which form the active p70 dimers (11). The p40 subunit is constitutively produced by macrophages. This subunit can dimerize with itself to form a IL-12p40 homodimer, which in turn binds to the IL-12 receptors and appears to block the IL-12 signaling pathway. On the other hand, the p35 subunit is expressed ubiquitously and constitutively at very low levels. It is also upregulated upon stimulation or infection (12, 13). The p35 subunit is biologically inactive and cannot homodimerize with itself (14). IL-12p70 heterodimers are produced by activated macrophages, neutrophils, and dendritic cells. IL-12p70 induces IFN-γ production from T (15-17), NK (18) and other cells such as B cells (19). Natural killer cells respond to IL-12 and IL-18 by secreting large amounts of IFN-γ to be used during early immune defense. IFN-γ, in turn, upregulates the secretion of IL-12p70 resulting in a positive feedback loop (20). IL-12 also induces the expression of the IL-18 receptor on T cells, which is considered to be a Th1-specific marker (18) (**Figure**

1). IL-12 acts together with IL-18 to induce the secretion of IFN-γ in antigen presenting cells and T cells (21, 22).

IL-12 binds to its receptors, IL-12Rβ1 and IL-12Rβ2, to induce signaling in T cells. IL-12Rβ2 is strictly expressed on Th1 cells (**Figure 1**), which is necessary for lineage commitment and maintaining IL-12 responsiveness (23). IL-12Rβ1 is more important for ligand binding (24), while IL-12Rβ2 serves a critical role as the signal transducing subunit of the receptor heterodimer. Both subunits are required for the signal transduction in the presence of active IL-12p70 (25). The IL-12p40 subunit interacts with IL-12Rβ1 while the IL-12p35 subunit of the IL-12p70 heterodimer specifically interacts with IL-12Rβ2 (24). Resting T cells do not express IL-12Rβ1 or β2 on the cell surface. During Th2 type cell differentiation, the expression of IL-12Rβ2 is significantly decreased and this loss can be reversed by addition of IFN-γ to mouse cell cultures (26). Further, IL-12 itself appears to upregulate IL-12 receptors since the addition of recombinant IL-12 to mouse lymph node cultures resulted in increased mRNA of both IL-12 receptor subunits. IFN-γ secretion is also elevated after treatment with recombinant IL-12 (rIL-12) in these cultures (26). Splenic lymphocytes from IL-12Rβ2 knockout mice, when stimulated *in vitro* with Con-A, fail to produce IFN-γ, implying that the IL-12Rβ2 subunit is critical for IL-12 induction of IFN-γ (24). In contrast, the Th2 cytokine, IL-4 inhibits expression of IL-12Rβ2 (23, 27).

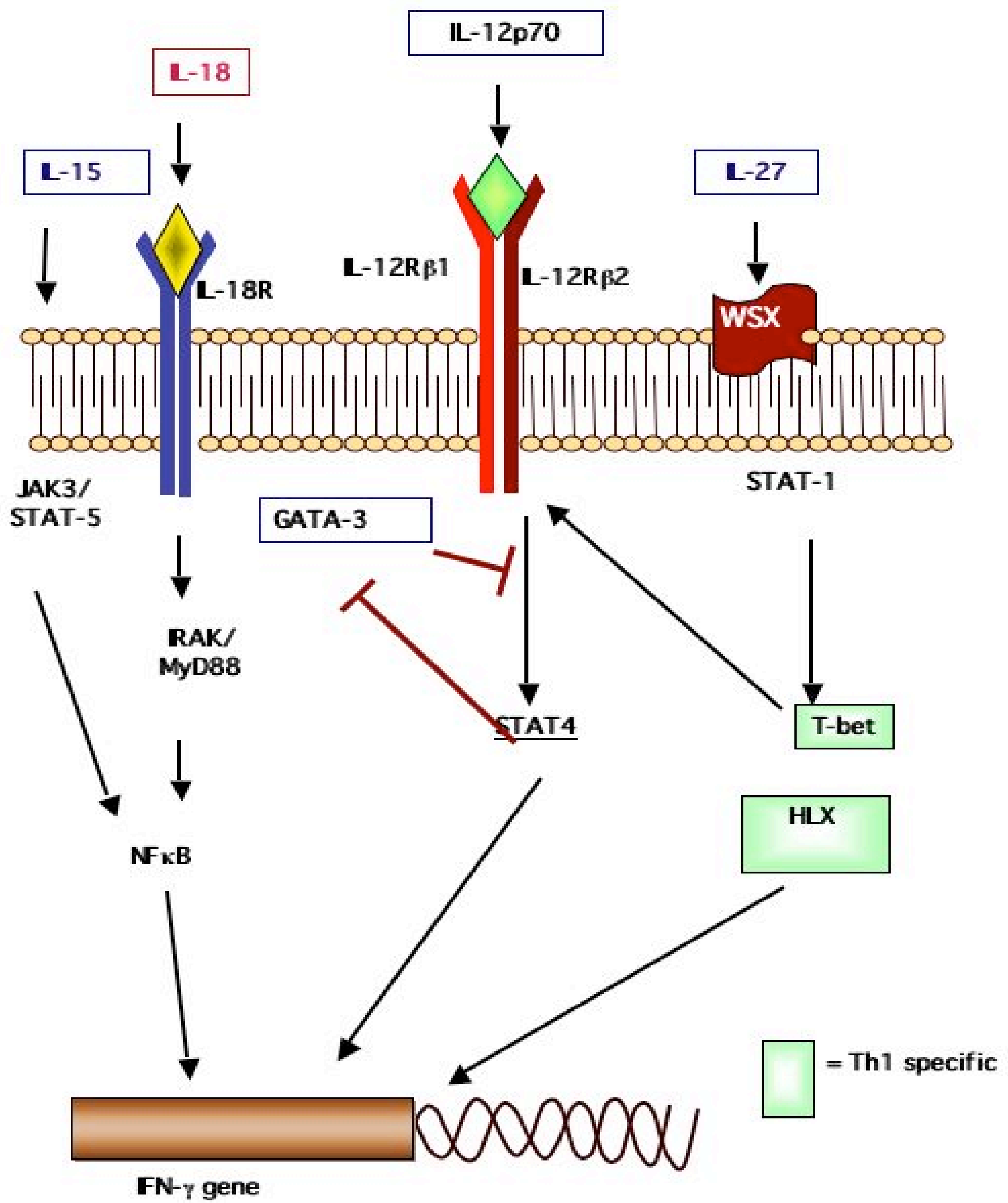


Figure 1. A model for IFN-γ inducing cytokines and their respective receptors

The binding of IL-12 to IL-12Rβ2 results in phosphorylation and activation of STAT-4, a transcription factor that links IL-12 receptors to IL-12 induced gene transcription (23). Following binding of IL-12p70 to the IL-12 receptor complex, the receptor bound proteins Tyk2 and Jak1 are phosphorylated, which in turn phosphorylate STAT-4. After activation, STAT-4 subunits dimerize and translocate to the nucleus where they bind to the murine IFN-γ gene and modulate its expression (28). IFN-γ secretion, in the presence of IL-12 and STAT-4 activation, is enhanced by increased promoter binding activity of c-Jun/AP-1 in T cells (29). The significance of STAT-4 in IFN-γ induction is highlighted by the fact that STAT-4 deficient mice have minimal levels of IFN-γ secretion upon stimulation (18, 30, 31) and impaired Th1 cell development (32, 33).

Interleukin-18 (IL-18) and IL-18 Receptors

Interleukin-18 (interferon gamma (IFN-γ)-inducing factor) is produced in an inactive form, pro-IL18 precursor, which is cleaved by caspase-1 to form the active form of IL-18. Since it is capable of inducing IFN-γ, it is defined as one of the IFN-γ inducing cytokines. Active IL-18 is produced by murine splenic macrophages, peritoneal exudate cells, keratinocytes, and alveolar macrophages after stimulation (34-36). Various stimulants of IL-18 include numerous macrophage stimulants such as LPS, endotoxins from gram-negative bacteria, and cytokines, which include IL-1, TNF, and IL-6. IL-18 directly regulates the development and activity of cytotoxic T and NK cells by upregulating perforin dependent cytotoxic activity and FasL expression (37-40). IL-18 plays an important role in the clearance of intracellular bacteria by activated antigen presenting cells and polarization of naïve T cells to Th1 lymphocytes (41, 42).

Although IL-18 is considered to be an IFN-γ-inducing cytokine like IL-12, individually, neither IL-18 nor IL-12 alone are effective in inducing optimal levels of IFN-γ. However, IL-18 in combination with IL-12 does induce IFN-γ secretion from Th1 cells. The addition of exogenous IL-18 to naïve T cells in the absence of IL-12 cannot induce IFN-γ, while the combination of IL-12 and IL-18 increase the level of IFN-γ protein, which is

accompanied by an increase in IL-12Rβ2 and differentiation of naïve T cells into IL-18R expressing Th1 cells (43-45). Pretreatment of B or T cells with IL-12 makes them responsive to IL-18 resulting in increased proliferation and IFN-γ secretion, presumably due to increased expression of IL-18 receptors (46, 47). The IL-18 receptor complex is a heterodimer consisting of IL-18Rα and IL-18Rβ. The IL-18 binding subunit IL-18Rα is constitutively expressed on $CD4^+$ T cells and freshly isolated thymocytes (48) and its expression is increased in the presence of IL-12 and IFN-γ (49). The non-binding stimulatory receptor subunit IL-18Rβ has increased expression upon stimulation of fresh thymocytes with IL-12 and Con-A (48). After the binding of IL-18 to IL-18Rα, the signaling subunit, IL-18Rβ binds and forms a heterodimer. IL-18 receptor proteins and mRNA are specifically expressed in murine Th1 cells after stimulation, especially in the presence of IL-12 (50). IL-12-driven upregulation of IL-18Rα for the binding of IL-18 and IL-18Rβ for signaling results in activation of the IL-18 signaling pathway. This activation augments IL-12Rβ2 expression on Th1 cells and results in more upregulation of IL-12 secretion leading to a positive feedback loop, which is critical to the development of IFN-γ secreting cells (51). Th2 cells did not express IL-18 receptors. While stimulation of Th1 cells with anti-CD3, IL-12, and IL-18 results in IFN-γ production, Th2 cells do not show a response (46). Although IL-18Rα and β are constitutively expressed in the human natural killer cell line, NKO, in the absence of IL-12, IL-18 does not induce IFN-γ implying the cooperation of IL-18 and IL-12 in inducing IFN-γ(52). An increase in IL-18Rs, due to IL-12, leads to enhanced IL-18-induced activation and sensitizes the cells to respond to lower concentrations of IL-18. Although studies have shown that IL-18, in combination with IL-12, induces IFN-γ and Th1 responses, IL-18 can also induce Th2 responses in the absence of IL-12p70 (41, 42).

Unlike IL-12/IL-12 Receptor binding, binding of IL-18 to the IL-18 Receptor heterodimer does not induce the JAK/STAT pathway in Th1 cells (15). Rather, it results in the binding of MyD88, which in turn results in the autophosphorylation and dissociation of IL-1R-associated kinase (IRAK) from its receptor complex (53-55). The interaction of IRAK with TNFR-associated factor-6 (TRAF-6) leads to the formation of

active NF-κB, which is composed of either a p65 homodimer or a p50/p65 heterodimer. Activated NF-κB is translocated into the nucleus where it binds to specific regulatory DNA sequences in the promoter of the IFN-γ gene (53-56). Although IL-18 treatment can induce NF-κB activation in Th1 cells, it was not enough to induce IFN-γ (57). These results suggest that IL-18 activation of NF-κB and IL-12 activation of the STAT-4 pathway work in harmony for the enhancement of IFN-γ expression and for the development of Th1 cells.

Interestingly, IL-18 and IL-12 can also induce IFN-γ in B cells and bone marrow macrophages. IL-18 in combination with IL-12 induces IFN-γ and IgG2a production from anti-CD40 stimulated B cells (19). In addition to B cells, IL-18, and IL-12 stimulate bone marrow macrophages to produce enhanced levels of IFN-γ (58, 59).

Interleukin-15 (IL-15)

Interleukin 15, another IFN-γ inducing cytokine, is a 14-15 kDa polypeptide. The mRNA of IL-15 is barely detectable in T cells and it is mainly expressed in the placenta, skeletal muscle, kidney, lung, heart, fibroblasts, epithelial cells, monocytes, macrophages and dendritic cells (DCs) (60-62). Secretion of biologically active IL-15 by monocytes and macrophages is regulated post-transcriptionally (61, 63). IL-15 is an important cytokine for: memory and activated $CD8^+$ T cells as a growth factor (62), for the development and survival of NK cells (64), NKT cells, $TCR\gamma\delta^+$ intestinal intraepithelial lymphocytes, and for the functional maturation of dendritic cells and macrophages (60). Although T cells, NK cells, and antigen presenting cells have IL-15 Receptors, the role of IL-15/IL-15 Receptor signaling is not clear in the development of these cells (65).

IL-15 upregulates IFN-γ secretion by $CD4^+$ T blast cells but not naïve T cells (66). Upregulation of IFN-γ secretion by $CD4^+$ cells in the presence of IL-15 producing monocytes may be due to increased IL-12Rβ1 and IL-12 expression where it synergizes with IL-12 (67). Expression of IL-15 may be regulated by IFN-γ since IL-15 expression is increased in anti-CD3 stimulated splenic lymphocytes from wild-type mice, while there

is no detectable IL-15 mRNA expression from the stimulated lymphocytes from IFN-γ Receptor knockout mice (68). IFN-γ mRNA expression is increased in human $CD4^+$ and $CD8^+$ T cells after exposure to Con-A and IL-15 (69). The combination of IL-15 and IL-12 is found to be less potent than IL-18 and IL-12 treatment of resting NK cells for increased IFN-γ production (70). Signaling through IL-15 receptors results in the translocation of STAT-1, STAT-3, STAT-4, and STAT-5 to the regulatory sites of the IFN-γ gene (71). The mRNA expression of T-bet, IFN-γ, IL-12Rβ, and IL-18Rβ are rapidly upregulated after stimulation with IL-15 in the human natural killer cell line, NK-92, and in peripheral blood T cells (71, 72). Pretreatment of the NK-92 cell line with IL-15 augments IL-12-stimulated STAT-4 binding to the IFN-γ-activated sequence (GAS) of the IL-2 Receptor (71). Although treatment of human T and NK cells with IL-15 leads to increased IFN-γ protein secretion, the addition of IL-18 and IL-21 enhances IL-15-induced IFN-γ gene expression even more, further supporting the hypothesis that multiple cytokines work in concert for upregulation of IFN-γ (72).

c.) MATERIALS AND METHODS

Mice: Three-to-four week old C57BL/6 wild-type mice were obtained from Charles River Laboratories and housed 3-5 animals per cage. All mice were maintained at the Center for Molecular Medicine and Infectious Diseases (CMMID) Animal Laboratory facility. Mice were fed on a diet that is devoid of synthetic or phyto-estrogens and maintained in 14/10 hrs of a light/dark cycle. Mice were housed in standard cages and terminated by cervical dislocation in accordance with Virginia Polytechnic Institute and State University Institutional Animal Care guidelines.

Estrogen Treatment Four-to-five week old mice were orchiectomized and given silicone implants prepared as either a placebo (empty implant as a control) or as estrogen implants containing 17-β estradiol (Sigma-Aldrich Inc., MO St. Louis, MO) by standard procedures described previously (73, 74) as described in Chapter 3.

Isolation and Culture of Splenic Lymphocytes: Spleens were collected under sterile conditions and lymphocytes were isolated as described in Chapter 3. Five hundred microliters of cells ($5x10^6$ cells/ml) were added to 24-well round flat plates containing 500 μl per well of complete phenol red free RPMI-1640 with or without the T cell mitogen, Concanavalin-A (Con-A, 10 μg/ml; Sigma-Aldrich Inc., MO). Splenic lymphocytes were also cultured with Con-A (1 μg/ml), Con-A (1 μg/ml) and recombinant IL-12p70 (20 ng/ml, 419-ml/cf, R&D Systems Inc., Minneapolis, MN.), or Con-A (1μg/ml) and recombinant IFN-γ (10 ng/ml, BDPharmingen San Diego, CA) for 24 hrs. Cell cultures were incubated for 24 hrs at 37 C with 5% CO_2. They were collected in microcentrifuge tubes and centrifuged at 5000 rpm for 5 minutes. The cells and supernatants were frozen at -70^0C until use.

Isolation of RNA and cDNA Synthesis: Isolation of total RNA and cDNA synthesis were performed as described in Chapter 3.

Primer Design: Primers were designed to span a large segment of the target genes to be used during the construction of standard curves for Real Time PCR was described in Chapter 3. The primers that were designed were as follows: β-actin (product: 965 bp) forward 5'-ATTGTTACCAACTGGGACGA-3', reverse 5'-CTGCGCAAGTTAGGTTTTGT-3'; IL-18 (product: 320 bp) forward 5'-CTTTGGCCGACTTCACTGTA-3', reverse 5'-GTCCTGGAACACGTTTCTGA-3'; IL-15 (535 bp) forward 5'-GCATCATGAAAAGGGACATT-3', reverse 5'-CTGTAAGAAGACACAAAACCAAG -3' (One Trick Pony Oligos, Ransom Hill Bioscience, Ramona, CA). The primers for IFN-γ and iNOS were explained in previous sections.

RT-PCR Assay: The reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect mRNA expression of IFN-γ inducing cytokines (IL-18, IL-15, IL-27) and the housekeeping gene, β-actin, for the generation of standards or/and detection of cytokine gene expression in the samples were described in detail in Chapter 3. For the construction of standards, the primers for β-actin, IL-18, and IL-15 that were described in

"The primer design" section of the methodology were used. β-actin (product: 349 bp), IL-18 (product: 325 bp), and IL-15 (product: 138 bp) primers were purchased from Maxim Biotech Inc. (San Francisco, CA). The IL-27 primer (product: 204 bp) was purchased from R and D Systems Inc, and used according to the PCR program as instructed by the manufacturer: 35 cycles of 94°C for 45 sec, annealing at 55°C for 45 sec, and amplification at 72°C for 45 sec. The same PCR program was used for the primers designed and purchased from Maxim Biotech Inc.. The PCR reaction for both the construction of standards and samples was performed under the following conditions: 35 cycles of 94°C for 1 min, annealing at 58°C for 1 min, and amplification at 72°C for 1 min. Twelve microliters of PCR products from the samples were run in a 2% agarose gel containing ethidium bromide (10 μl per 100 ml of gel). The PCR products were quantified with a scanning densitometer (Kodak Image Station, Perkin Elmer Life Sciences Inc., Boston MA), normalized using β-actin, and expressed as relative densities as described in Chapter 3.

Extraction of Target Genes and Measurement of Total cDNA after Gel Extraction, Picogreen: The extraction and quantification (with Picogreen) of the PCR products used for the standard curve was explained in detail in Chapter 3.

Real Time PCR: The Real Time PCR using SybrGreen I was performed as explained in detail in Chapter 3. β-actin, IFN-γ, iNOS, IL-18, and IL-15 primers were purchased from Maxim Biotech Inc. (San Francisco, CA). The Real Time PCR reaction was performed under the following conditions: 35 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec, and amplification at 72°C for 30 sec for both standards and unknown samples. Direct quantification of the Real Time RT-PCR products was performed by using the standard curve generated using respective standards that had known amounts of cDNA in femtograms (which were 10 fold diluted). Normalization of the target genes was done by normalizing to the housekeeping gene, β-actin. Values were recorded as femtogram ratios of the target gene (IFN-γ, iNOS, IL-18, or IL-15) normalized to β-actin.

Detection of IL-12Rβ1 and IL-12Rβ2 Expression: For flow cytometric analysis, splenic lymphocytes from estrogen or placebo-treated gonadectomized male C57BL/6 mice were cultured with or without mitogenic Con-A (10 μg/ml), or submitogenic Con-A (1 μg/ml) with or without recombinant IFN-γ (rIFN-γ, 10 ng/ml, BDPharmingen San Diego, CA) and/or recombinant IL-12 p70 (rIL-12p70, 20 ng/ml, R and D Systems Inc.) for 24 hrs. One-hundred microliters of 5 x10^6 cells/ml were plated and cultured in 96-well round bottom tissue culture plates (Corning, NY). The cultured cells were stained with appropriate monoclonal antibodies and analyzed by flow cytometry according to previously reported procedures (73, 75). The following monoclonal antibodies (mAbs) were used in this study: fluorescein isothyocynate (FITC)- or phycoerythrin (PE)-conjugated anti-Thy1.2 (CD90.2, clone 53-21), anti-CD45RB (B220) (clone RA3-6B2) (eBioscience Inc. San Diego, CA), and anti-IL12-Rβ1 (CD212, clone 114) (BDPharmingen, San Diego, CA). After the addition of purified anti-IL-12Rβ2 antibody (clone HAM10B9), PE-conjugated anti-hamster IgG (clone G94-905, G70-204; BDPharmingen, San Diego, CA) was added to IL-12Rβ2. For the detection of cell surface markers on cultured lymphocytes, 100 μl of each fluorochrome-conjugated monoclonal antibody in PBS were added to each well followed by incubation at 4 C in the dark for 30 minutes. Samples were washed with PBS followed by analysis on an EPICS XL-MXL flow cytometer (Coulter, Hialeah, FL). Forward scatter (FS) and side scatter (SS) analysis was also included for both splenic lymphocytes in order to visualize various lymphocyte subsets. Data were represented as mean relative percent expression of surface marker.

Detection of cytokines with ELISA: IL-12p70 and IL-18: Protein levels of active IL-12p70 in the supernatants of Con-A (10 μg/ml)-stimulated splenic lymphocytes from placebo or estrogen-treated gonadectomized male mice were determined using a Mouse IL-12p70 ELISA Readt-SET-go! kit following the manufacturer's instructions (eBioscience Inc. San Diego, CA). Concisely, 96-well flat-bottom high-binding NUNC Maxisorp plates were coated with Capture IL-12 antibody (clone C18.2) overnight in coating buffer and then blocked with Assay Diluent for 1 hr at room temperature subsequently washing with commercial Wash Buffer (Mouse IL-12p70 ELISA Readt-

SET-go! Kit). Plates were overlaid with 100 μl of supernatants or 100 μl of recombinant IL-12p70 as a standard with serial dilutions for the development of a standard curve. Plates were incubated at $4^{o}C$ overnight, washed with Wash Buffer, and biotin-conjugated IL-12 antibody (clone C17.8) was applied to the plates and incubated for 1 hr. Following the incubation, plates were thoroughly washed and overlaid with avidin-horseradish peroxidase solution for 30 mins. After a final wash, the wells were incubated with 100 μl of Substrate Solution for 15 mins and 50 μl of Stop Solution were added to stop the reaction. Plates were read at 450 nm on an ELISA reader and the values of 570 nm were subtracted from values obtained at 450 nm (Molecular Devices, Sunnyvale, CA). The levels of free IL-12p70 in the supernatants were calculated by the formula obtained from standards using SoftMax Pro software from Molecular Devices Inc.

Levels of IL-18 protein in the supernatants of splenic lymphocytes stimulated with or without Con-A (10 μg/ml) from placebo or estrogen-treated gonadectomized male mice were detected using a Mouse IL-18 ELISA kit following the manufacturer's instructions (MBL LTD, Nagoya, Japan). Briefly, 100 μl of samples or standards were added to mouse anti-IL-18 antibody coated wells and incubated for 1hr at room temperature. After the incubation, wells were washed and incubated with conjugated anti-IL-18 antibody for 1 hr. One-hundred μl of substrate TMB (3,3',5,5'-tetra-methylbenzidine) were added to each well for 30 mins and the reaction was terminated by final addition of stop solution (0.5 mol/L sulfuric acid) to the wells. Plates were read at 450 nm with an ELISA reader (Molecular Devices Sunnyvale, CA). IL-18 protein levels were calculated by the formula obtained from the standards using SoftMax Pro software from Molecular Devices Inc.

Determination of Intracellular IL-15 protein: Western blot assays were used to detect the IL-15 protein levels in whole cell lysates of placebo and estrogen-treated unseparated splenic lymphocytes at 5x 10^{6} cells/ml, which were cultured with Con-A (10 μg/ml) for 24 hrs. At the end of the incubation period, cells were harvested and resuspended in 15μl of lysis buffer (50mM Tris pH: 7.4, 300 mM NaCl, 2mM EDTA pH: 8, 0.5% Triton X-100, 30 μl of freshly added mammalian protease inhibitor cocktail (10 μg/ml, Sigma-Aldrich Inc., MO), vortexed and incubated on ice for 15 minutes. Fifteen μl of 2x sample

buffer (Laemmli Sample Buffer, Sigma-Aldrich Inc., St Louis MO) were added to the 15 μl of lysate and heated at 95^0C for 5 minutes. Samples were electrophoresed on a 15.5% gel at 25 mA constant current until the dye ran off bottom. The proteins were transferred to PDVF transfer membranes (Amersham Biosciences, CA) by blotting for 1hr at 240 mA constant current. After transferring the proteins to a PDVF membrane, it was blocked in 5% milk in TBST for 1 hr at room temperature. The blot was incubated with anti-IL-15 antibody (rabbit polyclonal IgG, eBioscience Inc. San Diego, CA) diluted in blocking buffer (1:1000) overnight on a rocking platform. The blots were rinsed briefly in TBST, then washed 3 times for 10 mins with TBST. The secondary HRP-conjugated antibody (anti-rabbit IgG, 1:2000, Santa Cruz Inc., CA) was applied in blocking buffer to each blot and incubated for 1 hr. After blots were washed, they were labeled using the ECL protocol (Amersham Pharmacia Biotech, NJ) and the bands were visualized using a Kodak Image Station (Perkin Elmer Life Sciences Inc., MA).

Detection of nitric oxide and IFN-γ protein: Levels of nitric oxide and IFN-γ in the supernatants of splenic lymphocytes cultured with Con-A (1 μg/ml) or Con-A (1 μg/ml) and recombinant IL-12p70 (20 ng/ml, 419-ml/cf, R&D Systems Inc., Minneapolis, MN.) for 24 hrs were detected using a Griess Assay and an IFN-γ specific ELISA, as described in Chapter 3 and previous sections.

Statistics: Data were analyzed using SAS software (SAS Institute Inc., Cary, NC). Data distributions and variances were evaluated using the UNIVARIATE procedure and data were logarithmically transformed when warranted. Transformed data were evaluated by analysis of variance (ANOVA) using the MIXED and GLM procedures. The model for the split-plot design included treatment, date of experiment, and their interactions tested by the main plot error term mouse ID (treatment x date of experiment). Stimulant, time of measurement and interactions with treatment were tested by the overall error term. Statistically significant interactions were further investigated using the SLICE option to test the simple main effects. *P*-values less than 0.05 were considered to be significant. Logarithmically transformed data means and standard errors were back transformed for

presentation in graphs; representation of standard errors therefore is non-symmetric about the means.

d.) RESULTS

The mRNA Expression of IL-27 is Elevated in Con-A Activated Splenocytes from Estrogen-Treated Mice

In addition to IL-12, IL-18, and IL-15, a newly discovered cytokine, IL-27, has been found to be important in the initiation of Th1 responses by induction of IFN-γ, T-bet, and IL-12Rβ2 expression (2, 7, 8, 76). Since estrogen increased the expression of IFN-γ inducing cytokines and their respective receptors, we investigated whether estrogen treatment affected the expression of IL-27 in Con-A stimulated splenic lymphocytes. Con-A stimulated splenic lymphocytes from estrogen-treated mice appeared to express increased IL-27 mRNA when compared to placebo-treated mice ($p<0.05$) (**Figure 2, Panel A**). RT-PCR products for IL-27 also had a visual increase as shown in the gel picture (**Figure 2, Panel B**).

Con-A-Activated Splenocytes from Estrogen-Treated Mice Have Decreased Levels of the IFN-γ Inducing Cytokine IL-12p70

Interestingly, Con-A-activated splenocytes from estrogen-treated mice had decreased levels of IL-12p70 when compared to similar cultures from placebo-treated mice after 24 or 48 hrs of culture as demonstrated by a specific ELISA ($p<0.05$) (**Figure 3, Panel A & B**). Since these results were unexpected, we confirmed the assay with another kit (Quantikine M IL-12p70 Immunoassay, R&D Systems, Minneapolis, MN) and similar trends were also noted (data not shown). There was no detectable IL-12p70 protein in the media after 48 hrs of culture.

Activation of Splenocytes Via T Cell Stimulation Upregulates the Expression of IL-12Rβ2 in Estrogen-Treated Mice

Since estrogen treatment decreased IL-12p70 protein levels, we next investigated whether IL-12 receptors, specifically IL-12Rβ1, the binding subunit, and IL-12Rβ2, the signaling subunit, were also altered in Con-A-activated splenocytes from estrogen-treated mice. The expression of IL-12Rβ2, the signal transducing subunit, on splenic lymphocytes activated with the pan-T cell stimulant, Con-A (10 μg/ml), was increased in cultures from placebo ($p<0.01$) and estrogen-treated ($p<0.001$) mice when compared to the expression of IL-12Rβ on unstimulated (media) cells (**Figure 4, Panel A**). Expression of IL-12Rβ2 was significantly increased on Con-A stimulated lymphocytes from estrogen-treated mice in comparison to placebo-treated mice implying an upsurge in IL-12p70 signal transduction ($p<0.05$) (**Figure 4, Panel A**). The level of IL-12Rβ1 expression, the IL-12 binding subunit, on Con-A-activated splenocytes from estrogen-treated mice was higher than the expression on unstimulated cells suggesting increased binding of IL-12 on the IL-12 receptor complex in estrogen-treated mice upon stimulation ($p<0.05$) (**Figure 4, Panel B**). There was no remarkable difference between the IL-12Rβ1 expression on lymphocytes from both estrogen and placebo-treated mice after stimulation with or without Con-A (**Figure 4, Panel B**).

The Estrogen-Induced Increased Expression of IL12Rβ2, But Not IL-12Rβ1, Is Enhanced By Recombinant IFN-γ and/or Recombinant IL-12p70.

In this part of the study, we chose to use a submitogenic dose of Con-A (1 μg/ml) rather than an optimal dose of Con-A (10 μg/ml) to better dissect the effects of various IFN-γ-inducing cytokines and/or their receptors. The addition of recombinant IL-12 (rIL-12, 20 ng/ml) to splenic lymphocytes, stimulated with a submitogenic dose of Con-A (1 μg/ml), led to detectable expression of IL-12Rβ2 on the cells from estrogen-treated mice, when compared to placebo-treated mice ($p<0.05$) (**Figure 5, Panel B**). IL-12Rβ2 expression on unstimulated splenocytes (media) from estrogen-treated mice was not different when compared to that from placebo-treated mice (data not shown) (**Figure 4, Panel A**). Deliberate addition of recombinant IFN-γ (rIFN-γ, 10 ng/ml) in combination with Con-A (1 μg/ml) elevated IL-12Rβ2 expression on lymphocytes from estrogen-treated mice with

regard to the expression from placebo-treated mice ($p<0.05$) (**Figure 5, Panel C**). There was a significant difference in the expression of IL-12Rβ2 on cells from estrogen-treated mice upon addition of rIFN-γ compared to the expression on cells stimulated with only Con-A ($p<0.001$) (**Figure 5, Panel A**) or Con-A and rIL-12 ($p<0.01$). The expression of IL-12Rβ2 was higher on cells from estrogen-treated mice ($p<0.05$) in Con-A (1 μg/ml) stimulated lymphocytes supplemented with both of the recombinant cytokines, rIFN-γ (10 ng/ml) and rIL-12 (20 ng/ml) (**Figure 5, Panel D**). Representative flow cytometric analysis, as determined by forward and side scatter analysis, are shown in **Figure 6.** Although the IL-12Rβ2 levels were higher on cells stimulated with Con-A, rIFN-γ, and rIL-12 than the level expressed on Con-A or Con-A and rIL-12 activated cells, there was no difference between the estrogen-treated cells stimulated with Con-A and rIFN-γ or Con-A, rIFN-γ, and rIL-12 implying the importance of IFN-γ in the amplification of IL-12Rβ2 and increased IL-12 signaling. The activation of placebo or estrogen treated cells with Con-A, Con-A and rIL-12, Con-A and rIFN-γ, or Con-A, rIL-12, and rIFN-γ for 24 hrs did not affect the expression of IL-12Rβ1 (**Figure 5, Panels E, F, G, and H**). Representative flow cytometric analysis of IL-12Rβ1 expression as determined by forward and side scatter analysis were shown in **Figure 7**.

Addition of rIL-12p70 Alters the Expression of IFN-γ and Its End-Product Nitric Oxide From Estrogen-Treated Mice

Since deliberate addition of rIFN-γ and/or rIL-12 in the presence of a submitogenic dose of Con-A (1 μg/ml) altered IL-12Rβ2 expression on lymphocytes from estrogen-treated mice, we investigated whether addition of rIL-12 will affect the levels of IFN-γ mRNA and IFN-γ protein expression of IFN-γ inducible Nitric Oxide Synthase (iNOS) and IFN-γ inducible nitric oxide (NO) as a result of estrogen treatment. Con-A (1 μg/ml) and rIL-12 (20 ng/ml)-activated splenocytes from estrogen-treated mice tended to have increased levels of IFN-γ gene expression as demonstrated by Real Time-PCR analysis compared to the expression from placebo-treated mice and other stimulations of Con-A (1 μg/ml) or Con-A (1 μg/ml) and rIFN-γ (10 ng/ml) (**Figure 8, Panel A**). As noticeable in Panel B,

the activation of estrogen-treated splenic lymphocytes with Con-A (1 μg/ml) and rIL-12 resulted in augmentation of the iNOS mRNA expression in comparison to placebo treated mice ($p<0.05$). The expression of iNOS was also increased in cells stimulated with Con-A (1 μg/ml) and rIFN-γ (10 ng/ml) demonstrating the effect of IFN-γ on iNOS gene expression ($p<0.05$) (**Figure 8, Panel B**). Protein levels of IFN-γ were significantly increased in the supernatants of Con-A and rIL-12 stimulated splenic lymphocytes both from placebo and estrogen-treated mice ($p<0.001$) (**Figure 9, Panel A**), but there was no apparent difference in IFN-γ protein levels between supernatants from placebo and estrogen-treated mice. This lack of detectable difference upon addition of rIL12 may be due to usage of IFN-γ secreted as bound to receptors and taken inside the cell. On the other hand, the levels of IFN-γ inducible NO were considerably increased in the supernatants of lymphocytes from estrogen-treated mice activated with Con-A (1 μg/ml) ($p<0.05$) or Con-A (1 μg/ml) and rIL-12 ($p<0.05$) for 24 hrs of culture (**Figure 9, Panel B**). Addition of rIL-12 increased nitric oxide secretion in the supernatants of lymphocyte cultures from both placebo and estrogen-treated mice implying the effect of IL-12p70 on IFN-γ inducible nitric oxide.

Estrogen Treatment Increases IL-18 at mRNA and Protein Levels

We next explored whether Con-A (10 μg/ml) activation of splenic lymphocytes from estrogen or placebo-treated lymphocytes altered another IFN-γ inducing cytokine, IL-18. The level of IL-18 gene expression was increased in Con-A-activated splenocytes from estrogen-treated mice as demonstrated by RT-PCR analysis when compared to cultures from placebo-treated mice ($p<0.05$) (**Figure 10, Panel A**). A representative gel picture of RT-PCR products is shown in **Panel B**. In view of the fact that RT-PCR offers an approximate and mostly qualitative estimation of gene expression, we used Real Time PCR analysis for quantitative detection of expression of the IL-18 mRNA expression. Levels of expression of the IL-18 gene in Con-A-activated splenocytes from estrogen-treated mice were also increased when compared to similar cultures from placebo-treated mice as demonstrated by Real-Time PCR analysis ($p\leq0.05$) (**Figure 10, Panel C**). The protein level of IL-18 was also notably increased in the supernatants of Con-A (10

μg/ml)-activated splenic lympocytes from estrogen-treated mice when compared to that of placebo-treated mice after 24 hrs of culture ($p<0.05$) (**Figure 11**). IL-18 protein was not detectable in the supernatants of unstimulated (media) lymphocytes.

IL-18Rβ Gene Expression Is Altered Upon Estrogen Treatment

Since estrogen increased the gene expression and protein levels of IL-18 after Con-A stimulation, we next investigated the expression of IL-18Rβ, an important subunit of the IL-18 Receptor complex and a Th1 marker, which takes part in the formation of a functional IL-18 Receptor heterodimer and regulation of IL-18 signaling. The relative gene expression of IL-18Rβ appeared to be increased in Con-A-activated splenocytes from estrogen-treated mice as demonstrated by RT-PCR analysis in comparison to the expression from placebo-treated mice suggesting increased signaling through the IL-18 Receptor complex ($p<0.005$) (**Figure 12, Panel A**). **Panel B** exhibits a representative gel picture of RT-PCR analysis.

Con-A Stimulated Splenic Lymphocytes From Estrogen-Treated Mice Have Increased IL-15 mRNA and Protein Expression

Besides IL-12 and IL-18, IL-15 is another inducer of IFN-γ mRNA and protein in human and murine lymphocytes alone or upon T cell stimulation (69, 77, 78). Therefore, we subsequently examined the alterations in gene expression and protein levels of IL-15 upon estrogen treatment. The relative IL-15 mRNA expression was noticeably elevated as verified with RT-PCR in Con-A stimulated splenic lymphocytes from estrogen-treated mice after 24 hrs of incubation ($p<0.05$), (**Figure 13, Panel A**). A representative gel picture of RT-PCR analysis was shown in **Panel B**. Similarly, Con-A activated cells from estrogen-treated mice tended to show an increase in IL-15 gene expression as confirmed with Real Time PCR (**Figure 13, Panel C**). Since there is no available commercial ELISA available for the detection of murine IL-15 protein in supernatants, we performed a Western Blot assay to demonstrate whether estrogen treatment altered the intracellular levels of IL-15 protein in unstimulated (media) and Con-A stimulated splenic

lymphocytes. The relative expression of IL-15 protein was upregulated in unstimulated splenocytes from estrogen-treated mice when compared to placebo controls ($p<0.05$; **Figure 14, Panel B**). Panel A shows representative pictures of Western blot assay for IL-15. IL-15 protein expression was noticeably increased in Con-A activated splenocytes from estrogen-treated mice compared to controls ($p<0.05$, **Figure 14, Panel D**) and representative western blot pictures are shown in **Panel C**.

e.) DISCUSSION

In our previous studies, we have shown that estrogen regulates IFN-γ (79, 80) and IFN-γ dependent iNOS and nitric oxide. In this study, we further extend our earlier observations to explore the regulation of IFN-γ-inducing cytokines and their receptors by estrogen.

The recently identified IL-27 can enhance the early production of IFN-γ by naïve $CD4^+$ T cells and NK cells (3, 4). This study is the first to demonstrate that estrogen treatment markedly upregulates the relative IL-27 mRNA expression in Con-A stimulated splenocytes. IL-27 can induce naïve T cells to produce high amounts of IFN-γ and upregulate T-bet and IL-12Rβ2 expression via JAK1/STAT1 activation (7, 8, 81). The studies performed by others, combined with our data discussed above (IL-12Rs), suggest that estrogen regulates Th1 commitment at very early stages affecting IL-27, which acts prior to IL-12 and is important for early Th1 differentiation.

Contrary to our expectations, we found that IL-12p70 protein levels were decreased in the supernatants from Con-A-activated lymphocytes from estrogen-treated mice at 24 hrs and that the level of IL-12p70 protein was drastically reduced by 48 hrs of culture. Given that the IL-12p70 protein detected in the supernatants is free-unbound protein, it is very possible that the decrease in the level of IL-12p70 after estrogen treatment may be due to increased levels of IL-12R and possibly to enhanced binding to the IL-12Receptor complex. Although, it is known that mice that are deficient in IL-12p70 demonstrate reduced Th1 responses and IFN-γ levels (33, 82), in our study, we did not observe decreased levels of IFN-γ or changes in nitric oxide, rather we found the opposite to be

the case. It is also possible that enhanced levels of IL-12p70 protein levels in supernatants of splenocytes from estrogen-treated mice might have been expressed at an earlier time point than 24 hours of culture.

We further explored the IL-12 receptors by investigating its subunits separately. Since binding of IL-12p70 protein to the IL-12Receptor complex is important in signaling through STAT-4, we explored the expression of β1, the binding subunit, and β2, the signaling subunit, on splenocytes from placebo and estrogen-treated mice. The expression of IL-12Rβ2 subunit was noticeably increased on Con-A-activated splenocytes from estrogen-treated mice. The IL-12β2 subunit is expressed on Th1 cells in mice and considered to be a Th1 differentiation marker (27). Increased levels of IL-12Rβ2 on splenocytes from estrogen-treated mice in the presence of optimal dose of Con-A (10 μg/ml) suggest that the responsiveness of lymphocytes to IL-12 signaling can be upregulated, and therefore, increase IFN-γ secretion upon estrogen treatment.

Recent studies demonstrate that the expression of IL-12Rβ2 on cells of the immune system is induced by IFN-γ and IL-12p70, where as the IL-12Rβ1 subunit is constitutively expressed on $CD4^+$ T cells after TCR stimulation (25, 83). In addition, in the absence of IL-4, IL-12 is enough to upregulate IL-12Rβ2, while in the presence of IL-4, IFN-γ is required in combination with IL-12 to induce IL-12Rβ2 expression (51). IL-12Rβ2 expression and responsiveness to IL-12 are both decreased when IFN-γ levels are diminished (51). These data suggest that IFN-γ is required to suppress the negative effect of IL-4 on Th1 differentiation by upregulation of IL-12Rβ2. The role of IFN-γ in the regulation of IL-12Rβ2 expression on splenocytes from estrogen-treated mice was clearly demonstrated by deliberately adding recombinant IFN-γ in the presence of a submitogenic dose of Con-A (1μg/ml). Activation of splenocytes with a submitogenic dose of Con-A (1 μg/ml) from placebo or estrogen-treated mice provides enough signal to achieve primary stimulation of lymphocytes resulting in low levels of IL-12Rβ1 and β2 expression. Addition of recombinant IL-12 and/or recombinant IFN-γ with a submitogenic dose of Con-A results in increased IL-12Rβ2, but did not affect IL-12Rβ1

expression on splenocytes from estrogen-treated mice. Therefore, we believe that IFN-γ, combined with IL-12, is an important factor for regulating IL-12Rβ2 expression and for regulating the responsiveness of lymphocytes to IL-12 in estrogen-treated mice.

It is notable that estrogen treatment upregulates many IFN-γ-inducing cytokines and their receptors on splenic lymphocytes. We further showed that estrogen treatment renders splenic lymphocytes hyperresponsive to IFN-γ and the IFN-γ inducing cytokine, IL-12. Stimulation of splenocytes with a submitogenic dose of Con-A (1μg/ml) and recombinant IL-12 markedly increased the expression of IFN-γ and iNOS mRNA. Addition of recombinant IFN-γ did not affect IFN-γ mRNA, but iNOS mRNA was increased in response to IFN-γ. Levels of IFN-γ and nitric oxide were significantly elevated due to stimulation with recombinant IL-12 and Con-A (1μg/ml). Nitric oxide levels in the supernatants from recombinant IL-12 and Con-A (1μg/ml) activated lymphocytes were noticeably higher from estrogen-treated mice compared to control mice. We showed that IL-12Rβ2 expression was upregulated in estrogen-treated mice. In addition to this, a possible reason for hyperresponsiveness of splenocytes from estrogen-treated mice could be enhanced IL-12 activation of STAT-4. Studies have shown that in TCR activated T cells stimulated with IL-12, STAT-4 complexed with c-Jun interacts with AP-1 and induces a high binding form of AP-1. Since STAT-4 cannot bind to the IFN-γ promoter by itself, the presence of AP-1, a potent transcription factor for IFN-γ, is required to bind and activate the IFN-γ promoter (29, 84, 85). Furthermore, stimulation of naïve T cells ($CD4^+$ and $CD8^+$) from C57BL/6 mice via the TCR and IL-12 increases histone hyperacetylation, which is involved in the function of genomic nuclear matrix attachment regions and other nuclear functions that regulate gene transcription (86). This further upregulates the transcription of the IFN-γ gene by 100 fold under Th1 differentiation conditions (87-90). Therefore, the possibility that estrogen-treated mice may have more histone hyperacetylation of the IFN-γ gene in splenocytes compared to placebo-treated mice cannot be excluded.

IL-18, another important IFN-γ inducing cytokine, is unlike IL-12, in that it cannot drive Th1 responses by itself, but synergizes with IL-12 to upregulate the production of IFN-γ and Th1 differentiation (15). The treatment of EAE mice with estrogen implants for 7 days (2.5 mg E2 (1500–2000 pg/ml serum, 20–50% of pregnancy levels) before the start of disease led to increased expression of IL-18 mRNA in fresh splenocytes as detected with a GeneArray (91). In this study, we have shown that estrogen treatment of non-autoimmune, wild-type mice demonstrated a significant increase in IL-18 mRNA expression as shown with RT and Real Time RT-PCR, as well as IL-18 protein expression from Con-A stimulated splenocytes.

Similar to the IL-12 and IL-12 Receptor complex, binding of IL-18 to its receptor complex, which is composed of IL-18Rα, the binding subunit, and IL-18Rβ, the signaling subunit, results in activation of IFN-γ (46, 92, 93). It has been shown IL-18Rβ is an important subunit in the regulation of cellular responses to IL-18. For example, stimulation of thymocytes with Con-A and recombinant IL-12 upregulates IL-18Rβ expression resulting in cells hyperreactive to IL-18 stimulation in terms of both IFN-γ production and cell proliferation (48). Furthermore, NK cells and T cells from MRL/lpr mice that overexpress the IL-18Receptor are hypersensitive to IL-18 (94). We found that estrogen treatment upregulated IL-18Rβ mRNA expression in Con-A activated splenocytes. Due to the absence of a successful antibody for the detection of IL-18Rβ on splenocytes at the time, we were not able to detect the expression of IL-18Rβ on the cell surface. We expect that the increase in IL-18Rβ mRNA expression due to estrogen treatment will be reflected in the expression of IL-18Rβ on lymphocytes. Induction of IFN-γ by IL-12 and IL-18 is associated with the regulation of GADD45β and GADD45γ protein expression (95-97). The increase in IFN-γ expression and IFN-γ inducing cytokines due to estrogen treatment could be a result of elevation of GADD45β or GADD45γ proteins.

IL-15, an IFN-γ inducing cytokine, modulates many immunological functions such as induction of T cell proliferation, activation of cytotoxic effector cells and monocytes, Ig

synthesis by B cells, and differentiation and activation of NK cells (61, 98-100). The relationship between estrogen treatment and IL-15 has not been throughly investigated. Okada et al demonstrated that *in vitro* incubation of human endometrial stromal cells with progesterone and estrogen results in upregulation of IL-15. This increase was not observed in human endometrial cells cultured only with estrogen (101). Although IL-15 mRNA is widely expressed constitutively, many researchers have difficulties demonstrating IL-15 protein in the supernatants of many cells that express IL-15 mRNA (102, 103). Our study is the first to demonstrate that *in vivo* estrogen treatment upregulated IL-15 mRNA in unstimulated lymphocytes left in media and Con-A activated splenocytes. Since, there is no available commercial IL-15 ELISA for the detection of murine IL-15, we used Western blot analysis to demonstrate intracellular IL-15 protein levels. In addition to IL-15 mRNA, IL-15 protein expression was significantly augmented both in Con-A activated cells and unstimulated lymphocytes from estrogen-treated mice. The level of IL-15 protein in unstimulated cells was more than the IL-15 protein detected in Con-A activated splenocytes from estrogen-treated mice. Although precise reasons for this is not clear, one possibility is in unstimulated (media) samples IL-15 is not used up and retained in the cell, whereas after Con-A stimulation there may be increased secretion of IL-15, which led to decreased levels of IL-15 protein inside the cell. Our results may differ from the results obtained by Okada et al due to estrogen treatment (*in vivo* versus *in vitro*), tissue specific responses to estrogen, and/or the differences in species (human versus mouse).

Overall, our studies provide a new insight into how estrogen modulates immune system by regulating IFN-γ inducing cytokines and as a result, IFN-γ and IFN-γ-inducible nitric oxide. In this study, we report that estrogen treatment enhances IFN-γ inducing cytokines, IL-18, IL-15, IL-27, and upregulates the receptors, IL-12Rβ2 and IL-18Rβ, both quantitatively and qualitatively. Estrogen-treated mice appeared to be more responsive to recombinant IL-12 resulting in increased IFN-γ and iNOS. All in all, this study is the first to report that estrogen can regulate IFN-γ inducing cytokines, notably IL-18, IL-15, and IL-27 in splenic lymphocytes. Bearing in mind that estrogen is a potent immunomodulator, it is critical to decipher the molecular components of Th1

differentiation and to shed light on the estrogen-modulated changes in the immune system which may be linked to a number of pathological conditions, including the development of autoimmune and neoplastic disorders.

g.) FIGURES

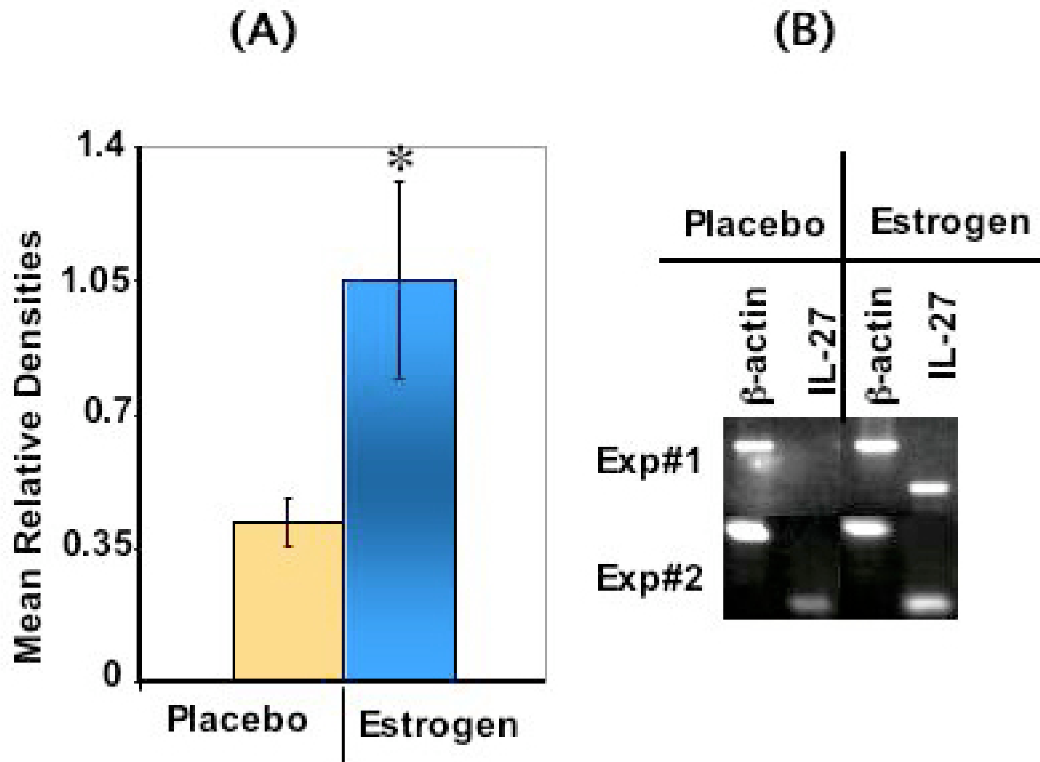


Figure 2. Estrogen treatment increases IL-27 mRNA Expression: Splenocytes stimulated with Con-A (10 μg/ml) from placebo and estrogen-treated mice were cultured for 24 hrs. **Panel A** shows relative densities of IL-27 gene expression was determined with RT-PCR after normalized to β-actin. The IL-27 mRNA expression was increased in splenocytes from estrogen-treated mice (Placebo: n=19 mice, estrogen: n=17 mice; $p<0.05$). Data were presented as means with standard error bars. **Panel B** illustrates two representative experiments for RT-PCR assay.

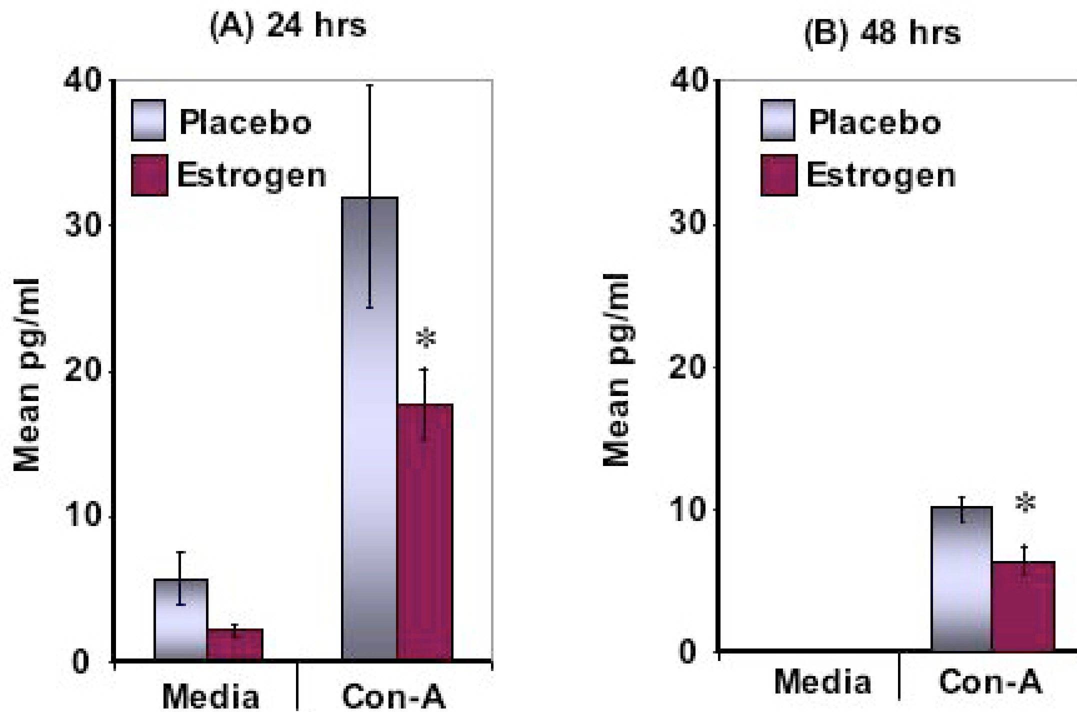


Figure 3. Con-A Activated Splenocytes from Estrogen-Treated Mice Have Decreased Levels of the IFN-γ Inducing Cytokine, IL-12p70: Splenic lymphocytes, which were treated with either estrogen or placebo-treated gonadectomized male mice were stimulated with Concanavalin-A (Con-A; 10 μg/ml) or left unstimulated (media) and the level of IL-12p70 in the supernatants was determined with a specific ELISA. Supernatants of Con-A-activated splenic lymphocytes from estrogen-treated mice had significantly decreased level of IL-12p70 compared to placebo controls (**Panel A**; n=8 mice per placebo or estrogen treatment, $p<0.05$), where as IL-12p70 protein levels in the supernatants from unstimulated splenic lymphocytes showed no difference between placebo and estrogen-treated mice. **Panel B** shows the level of IL-12p70 in the supernatants from splenic lymphocytes cultured with Con-A for 48 hrs (n=6 mice per placebo or estrogen treatment; $p<0.05$). Supernatants of Con-A-activated splenic lymphocytes from estrogen-treated mice had significantly decreased level of IL-12p70 compared to placebo controls after 48 hrs of culture. The level of IL-12p70 was not detectable in the supernatants of splenic lymphocytes left unstimulated in media after 48 hrs. Data are presented as means with standard errors.

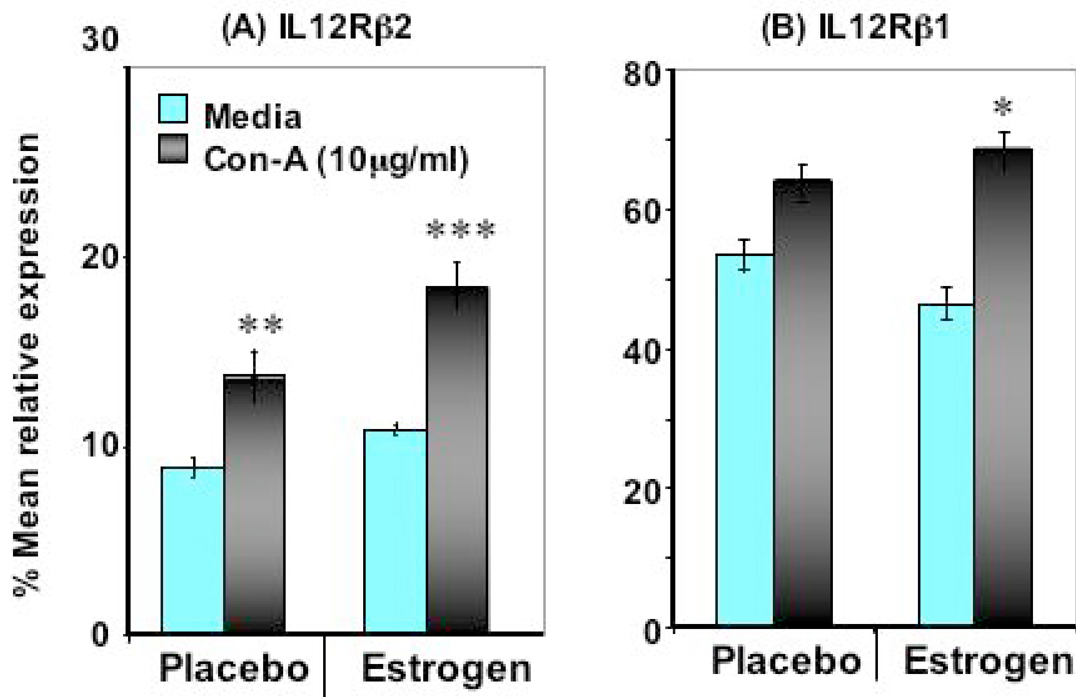


Figure 4. Activation of Splenocytes Via T Cell Stimulation Upregulates the Expression of IL-12Rβ2 in Estrogen-Treated Mice: Splenic lymphocytes from placebo or estrogen-treated mice that were cultured with Con-A (10 μg/ml) or left unstimulated (media) for 24 hrs were stained with PE-anti-IL-12Rβ1, purified anti-IL-12Rβ2 antibody and PE-conjugated anti-hamster IgG monoclonal antibodies, or isotype-matched control antibodies, and analyzed by flow cytometry. The mean percent expression of IL-12Rβ2 was significantly increased on Con-A stimulated splenocytes when compared to unstimulated lymphocytes from placebo-treated ($p<0.01$, n=6 mice per placebo or estrogen treatment; **Panel A**) and estrogen-treated mice ($p<0.001$, n=6 mice per placebo or estrogen treatment; **Panel A**). **Panel B** shows the mean percent expression of IL-12Rβ1 on splenocytes from placebo and estrogen-treated mice. IL-12Rβ1 expression was increased on Con-A stimulated lymphocytes when compared to unstimulated cells from estrogen-treated mice (n=6 mice per placebo or estrogen treatment, $p<0.05$). There was no noticeable difference of IL-12Rβ1 expression on unstimulated and Con-A stimulated lymphocytes from placebo or estrogen-treated mice.

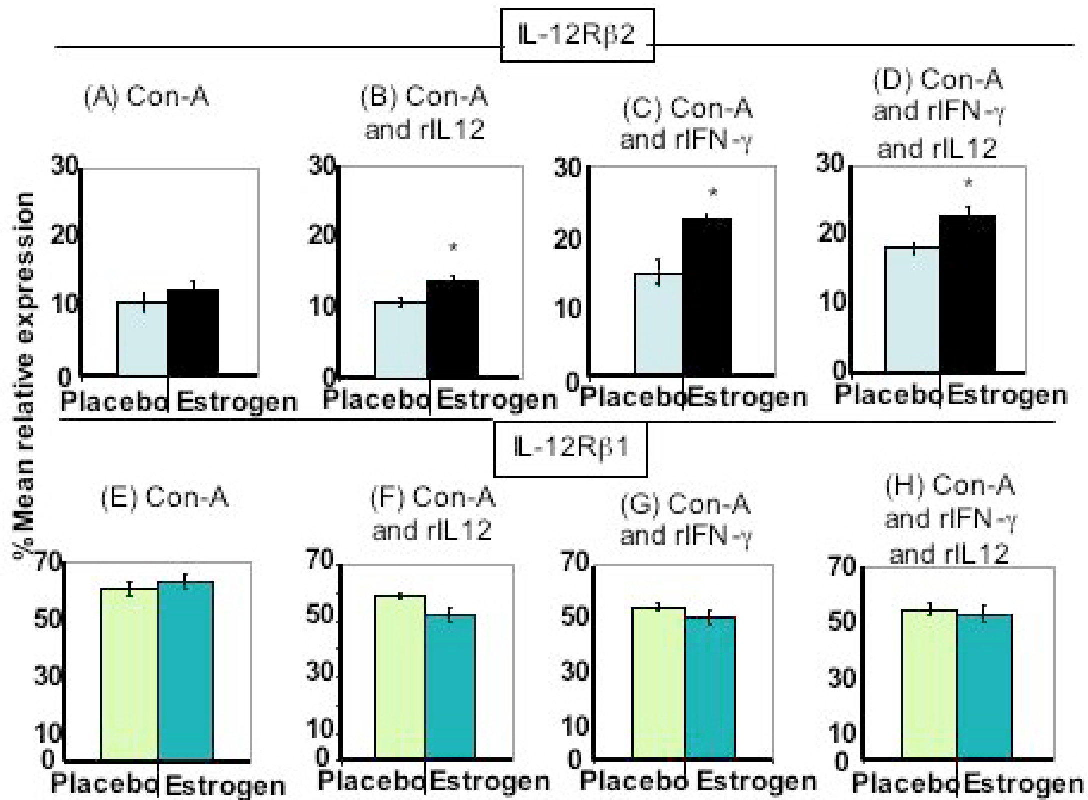


Figure 5. Estrogen-Induced Increase of IL12Rβ2 Expression, but not IL-12Rβ1, Is Enhanced by Recombinant IFN-γ and/or Recombinant IL-12p70: Splenic lymphocytes from placebo or estrogen-treated mice were stimulated with Con-A (1 μg/ml) with or without recombinant IFN-γ (rIFN-γ, 10 ng/ml) and/or recombinant IL-12 p70 (rIL-12p70, 20 ng/ml) for 24 hrs, and then were stained with PE-anti-IL-12Rβ1, purified anti-IL-12Rβ2 antibody and PE-conjugated anti-hamster IgG monoclonals, or isotype-matched control antibodies, and analyzed by flow cytometry. Although the expression of IL-12Rβ2 was not changed on Con-A (1 μg/ml, n=6 mice per placebo or estrogen treatment) stimulated lymphocytes (**Panel A**), IL-12Rβ2 expression was significantly increased on Con-A stimulated lymphocytes after addition of rIL12, rIFN-γ, and rIL-12 and rIFN-γ from estrogen treated mice ($p<0.05$, **Panels B, C, and D**: n=6 mice per placebo or estrogen treatment; n=3 mice per placebo or estrogen treatment, n=3 mice per placebo or estrogen treatment respectively). The mean expression of IL-12Rβ1 did not differ on Con-A (1 μg/ml, **Panel E**) stimulated lymphocytes from placebo or estrogen-treated mice despite the addition of recombinant proteins (rIL-12: **Panel F**; rIFN-γ: **Panel G**; rIL-12 and rIFN-γ: **Panel H**).

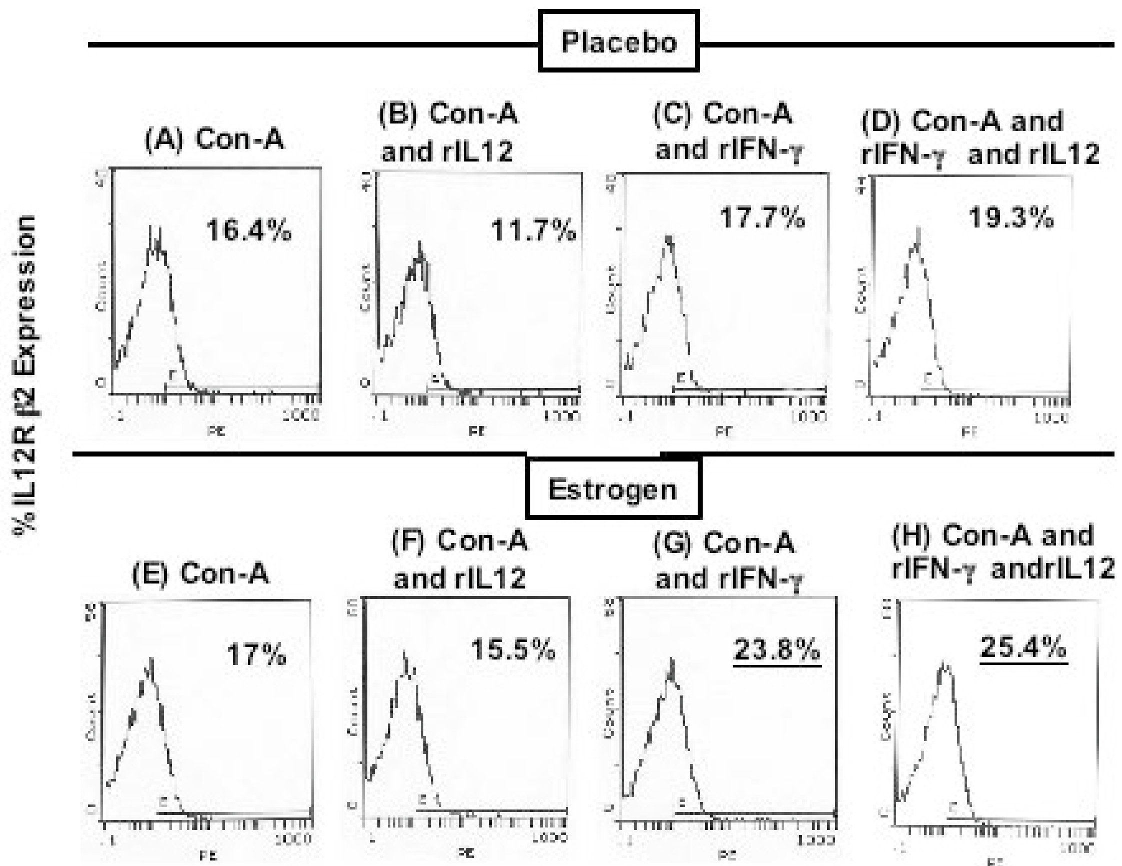


Figure 6. Representative Flow Cytometric Analysis for IL-12Rβ2 Expression Enhanced By Recombinant IFN-γ and/or Recombinant IL-12p70 on Estrogen-Treated Splenocytes: The figure depicts a representative experiment IL-12Rβ2 on lymphocytes from placebo (top row) and estrogen-treated (bottom row) mice stimulated with Con-A (1 μg/ml, Panel **A & E**), and deliberate additions of rIL-12 (20 ng/ml, Panel **B & F**), rIFN-γ (10 ng/ml, Panel **C & G**), or rIL-12 (20 ng/ml) and rIFN-γ (10 ng/ml, Panel **D & H**).

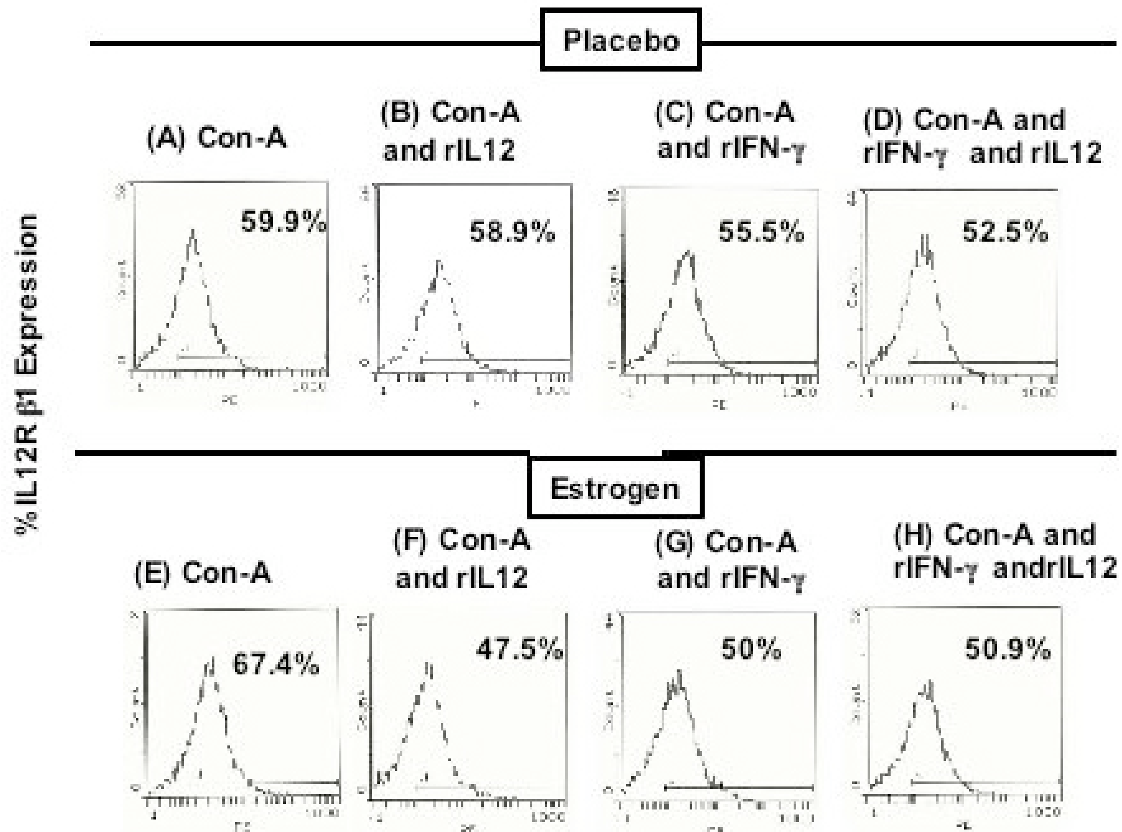


Figure 7. Representative Flow Cytometric Analysis for IL-12Rβ1 Expression Enhanced By Recombinant IFN-γ and/or Recombinant IL-12p70 on Estrogen-Treated Splenocytes: The figure shows a representative experiment for IL-12Rβ1 on lymphocytes from placebo (top row) and estrogen-treated (bottom row) mice stimulated with Con-A (1 μg/ml, **Panel A & E**), and deliberate additions of rIL-12 (20 ng/ml, **Panel B & F**), rIFN-γ (10 ng/ml, **Panel C & G**), or rIL-12 (20 ng/ml) and rIFN-γ (10 ng/ml, Panel **D & H**).

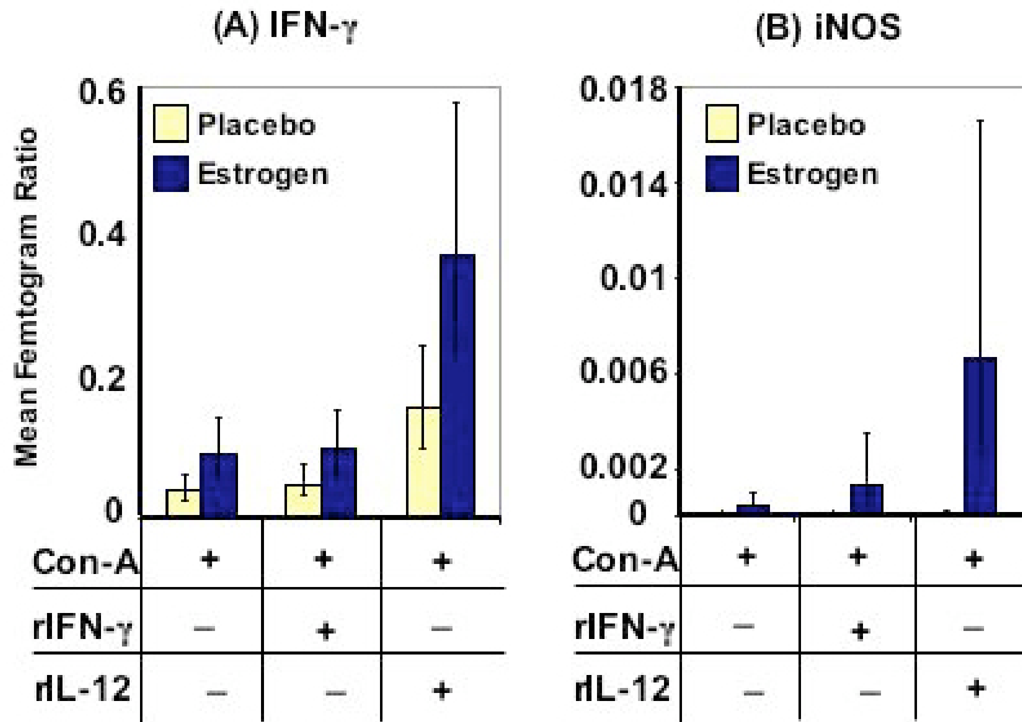


Figure 8. IFN-γ Inducing Cytokine rIL-12p70 Alters Gene Expression of IFN-γ and Its End-Product Nitric Oxide From Estrogen-Treated Mice: Splenic lymphocytes from placebo and estrogen-treated mice were cultured with Con-A (1 μg/ml) with or without rIFN-γ (10 ng/ml) or rIL-12 (20 ng/ml) for 24 hrs. The quantitative detection of gene expressions of IFN-γ and iNOS were performed with Real Time PCR. The expression of IFN-γ and iNOS mRNA were normalized β-actin. As demonstrated in Panel A, the expression of IFN-γ gene showed a tendency to increase in Con-A (n=3 mice per placebo or estrogen treatment) stimulated splenocytes especially after deliberate addition of rIL-12p70 (n=3 mice per placebo or estrogen treatment, $p=0.3$) from after estrogen treatment. The IFN-γ expression did not vary due to addition of rIFN-γ (n=3 mice per placebo or estrogen treatment, $p=0.4$). The iNOS gene expression was significantly increased in consequence of rIFN-γ (n=5 mice per placebo or estrogen treatment, $p<0.05$) or rIL-12 (n=5 mice per placebo or estrogen treatment, $p<0.05$) in Con-A activated lymphocytes from estrogen-treated mice compared to placebo-treated mice (Panel B) Con-A activated lymphocytes did not have significant change in iNOS gene expression between treatments (n=5 mice per placebo or estrogen treatment, $p=0.22$; Panel B). Data are presented as means of femtogram ratios with standard error bars.

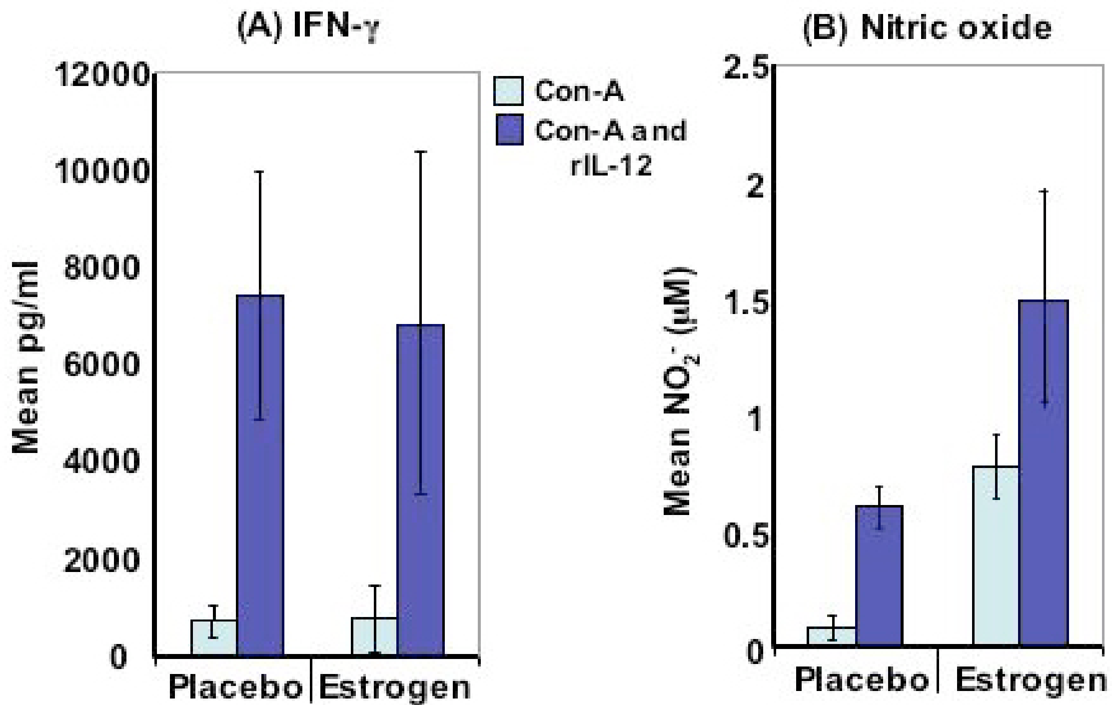


Figure 9. rIL-12p70 induces IFN-γ Protein and Nitric Oxide: Splenic lymphocytes from placebo and estrogen-treated mice were cultured with Con-A (1 µg/ml) with or without rIFN-γ (10 ng/ml) or rIL-12 (20 ng/ml) for 24 hrs. The levels of IFN-γ and nitric oxide in the supernatants were determined with ELISA and Griess Assay, respectively. Addition of rIL-12 to Con-A stimulated splenocytes (n=6 mice per placebo or estrogen treatment, $p<0.05$; Panel A) from placebo and estrogen-treated mice upregulated IFN-γ protein when compared to the supernatants from Con-A stimulated splenocytes. There was no apparent difference in IFN-γ protein secretion due to estrogen treatment (Panel A). Panel B shows the levels of nitric oxide in the supernatants from splenic lymphocytes cultured with Con-A with or without rIL-12 for 24 hr (n=8 mice per placebo or estrogen treatment; $p<0.05$). Estrogen treatment upregulated nitric oxide secretion from Con-A stimulated lymphocytes as compared to controls (n=8 mice per placebo or estrogen treatment, $p<0.05$). Nitric oxide production was significantly increased in the supernatants from Con-A stimulated splenocytes from estrogen-treated mice in response to rIL-12 addition (n=8 mice per placebo or estrogen treatment, $p<0.05$). Levels of IFN-γ protein or nitric oxide were not detectable in the supernatants of splenic lymphocytes left unstimulated in media. Data are presented as means with standard errors.

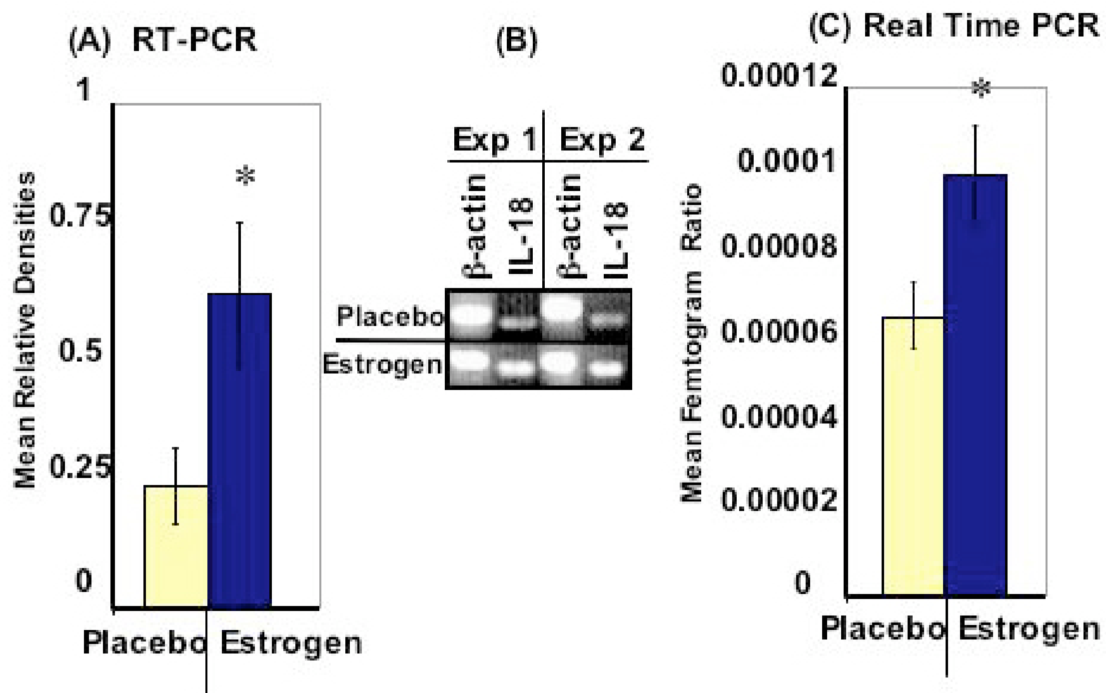


Figure 10. Estrogen Upregulates IL-18 mRNA Expression: Splenic lymphocytes from estrogen or placebo-treated gonadectomized male mice were stimulated with Con-A (10 μg/ml) for 24 hr and relative the expression of iNOS mRNA after normalized to β-actin determined with RT-PCR (n=16 mice per placebo or estrogen treatment, $p<0.05$) (**Panel A**) Data are presented as means of relative densities with standard error bars. **Panel B** shows the representative of IL-18 mRNA expression after RT-PCR. **Panel C** shows the quantitative detection of IL-18 mRNA expression after normalized to β-actin with the Real Time PCR assay. Real Time PCR products were calculated in femtograms using the standard curve derived from cDNA standards of respective genes, IL-18 and β-actin. Data are presented as means of femtogram ratios with standard error bars (n=6 mice per placebo or estrogen treatment, $p\leq0.05$).

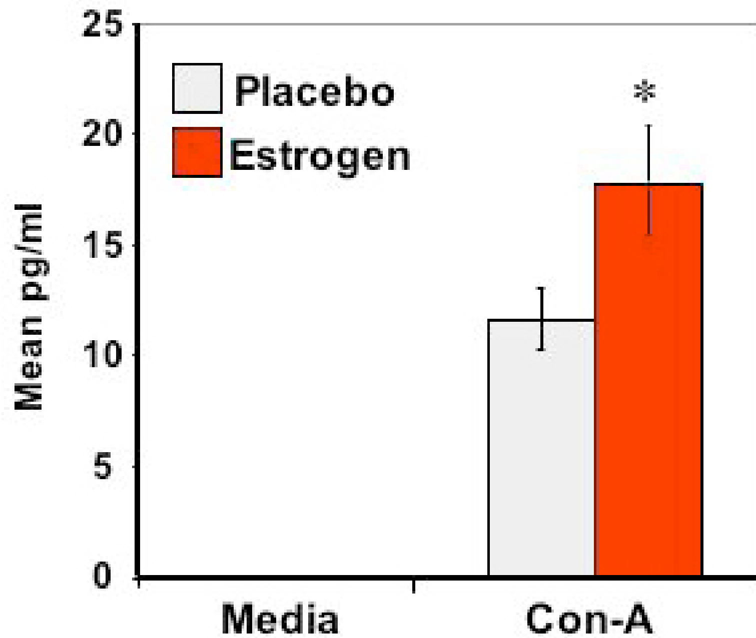


Figure 11. **Expression of IL-18 protein is increased in estrogen-treated splenic lymphocytes**: Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml) or left unstimulated (media) for 24 hrs. The expression of IL-18 protein was determined with ELISA. IL-18 protein level was appreciably increased in the supernatants from Con-A stimulated splenocytes from estrogen-treated mice when compared to placebo-treated mice (n=21 mice per placebo or estrogen treatment; $p<0.05$). IL-18 protein was not detectable in the supernatants from unstimulated lymphocytes. Data were presented as means with standard error bars.

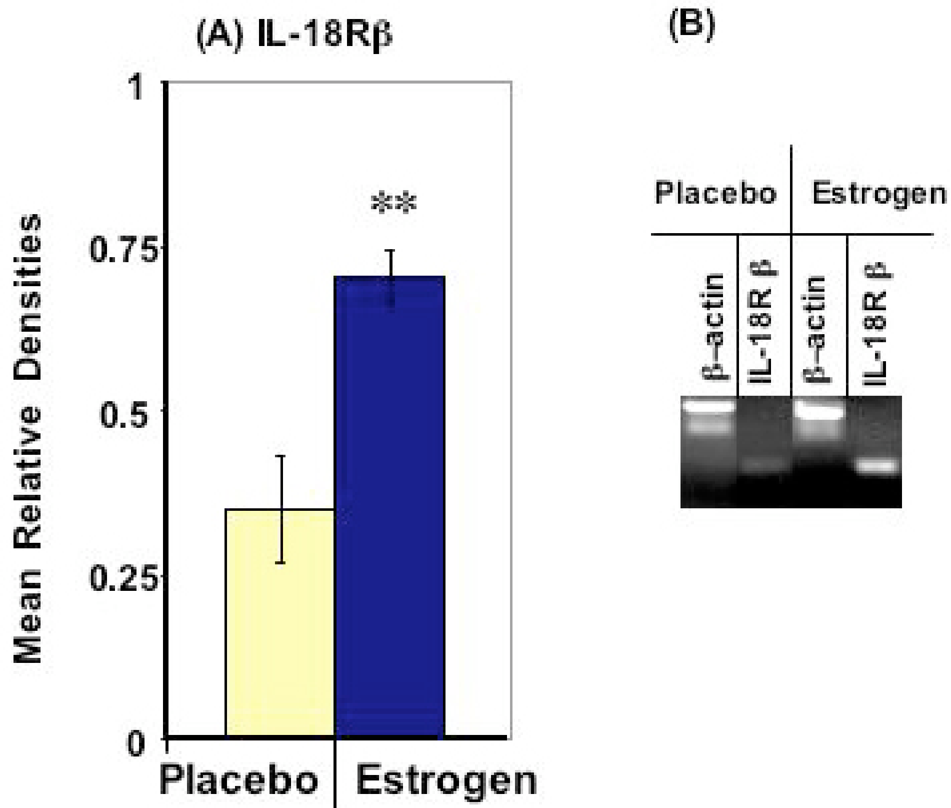


Figure 12. Estrogen Treatment Alters IL-18Rβ Gene Expression: Splenic lymphocytes from estrogen and placebo-treated mice were stimulated with Con-A (10 μg/ml) for 24 hrs. The expression of IL-18R mRNA was determined with RT-PCR and normalized to β-actin. **Panel A** shows mean relative densities of IL-18Rβ expression presented in Con-A stimulated cells from estrogen or placebo-treated orchiectomized male mice (n=7 mice per placebo or estrogen treatment; $p<0.005$). Data were presented as means with standard error bars. **Panel B** depicts a representative gel picture of RT-PCR analysis.

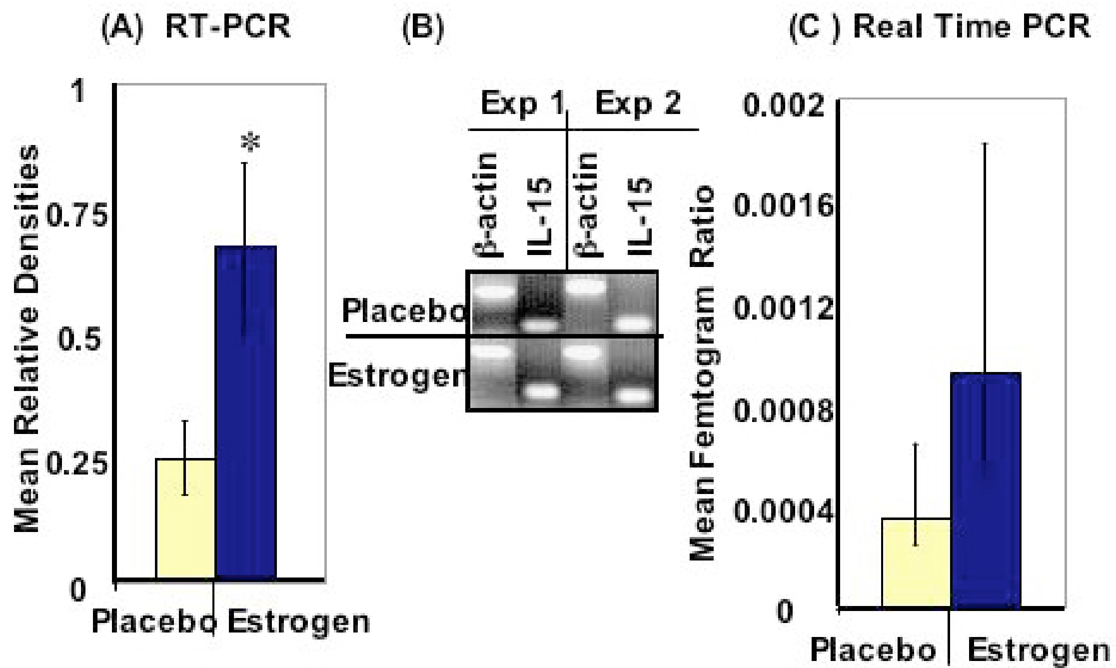


Figure 13. Estrogen Treatment Alters IL-15 Gene Expression: The relative the expression of iNOS mRNA in Con-A (10 μg/ml) stimulated splenic lymphocytes from estrogen-treated mice after 24 hrs of culture was noticeably increased in comparison to placebo-treated mice as determined with RT-PCR (n=10 mice per placebo or estrogen treatment, $p<0.05$; **Panel A**). The RT-PCR product, IL-15 was scanned by scanning densitometer and normalized to β-actin. Data were presented as means with standard error bars. **Panel B** shows two representative experiments of IL-15 mRNA expression after RT-PCR. **Panel C** shows the quantitative detection of IL-15 mRNA expression normalized to β-actin as detected with Real Time PCR assay. Data are presented as means of femtogram ratios with standard error bars (n=7 mice per placebo or estrogen treatment; $p=0.5$).

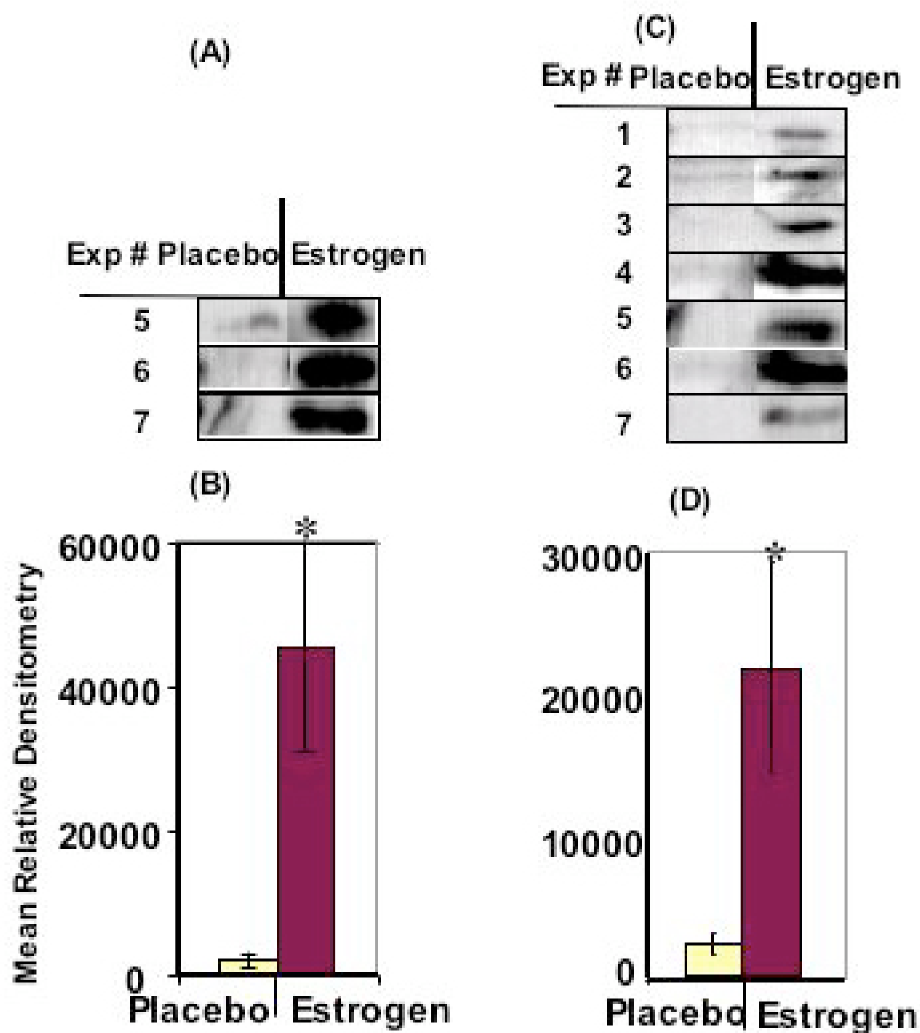


Figure 14. The IL-15 Protein expression is increased in estrogen-treated splenic lymphocytes: Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml) for 48 hours of incubation. The expression of IL-15 protein was determined by the Western Blot assay. Panel A shows mean relative densities of IL-15 protein in unstimulated (media) lymphocytes (n=4 mice per placebo or estrogen treatment; $p<0.05$). Panel B depicts four representative blots from Western blot assay of IL-15 in unstimulated cells. Panel C shows mean relative densitometry results of IL-15 protein in Con-A stimulated splenic lymphocytes (n=8 mice per placebo treatment, n=9 mice per estrogen treatment; $p<0.05$). Seven representative experiments of IL-15 protein expression in cells stimulated with Con-A as western blot pictures are demonstrated in Panel D.

4.2. Effects of Estrogen Treatment on IFN-γ-Inducing Transcription Factors in Splenocytes of Male C57BL/6 Mice.

a.) ABSTRACT

One important mechanism by which 17-βestradiol modulates the immune system is via alteration of expression of IFN-γ and IFN-γ inducing cytokines. Studies have shown that the induction of these cytokines is associated with selective expression or repression of various transcription factors such as T-bet, HLX, Eomes, and IRF-4, which play a critical role in Th1/Th2 differentiation. In addition to these factors, activation of STAT-4, following IL-12 and IL-12 receptor induction, is important in the induction of IFN-γ and differentiation into T helper 1 (Th1) type cells. In our previous studies, we found that estrogen treatment tended to augment in IFN-γ and markedly increase IFN-γ-dependent-iNOS, and IFN-γ inducing cytokines. To further explore the role of estrogen, the potential role of transcription factors involved in IFN-γ synthesis in Con-A stimulated splenocytes from gonadectomized mice treated with placebo or estrogen implants was investigated. We demonstrate that estrogen treatment increases the expression of T-bet mRNA, but not of HLX and Eomes in Con-A stimulated splenocytes. Increased activation of STAT-4 and decreased IRF-4 protein expression in splenocytes from estrogen-treated mice also enables these cells to produce considerable amounts of IFN-γ. These studies confirm the concept that estrogen stimulates Th1 cells to secrete IFN-γ by altering T-bet, STAT-4, and IRF-4 expression.

b.) INTRODUCTION

T-bet

A notable finding in molecular immunology is that the induction of IFN-γ in $CD4^+$ T cells is dependent upon the expression of a specific transcription factor, T-bet. T-bet belongs to the homeobox protein family which is important in the commitment, differentiation, and maintenance of Th1 cells (104). T-bet, a Th1-specific T box transcription factor (Tbx-21), controls the expression of IFN-γ, a hallmark Th1 cytokine (104).

The expression of T-bet is limited to the immune system. T-bet is expressed at very low levels in unstimulated naïve $CD4^+$ T cells and its levels are upregulated following activation through the TCR. While T-bet is essential for the induction of IFN-γ, the levels of T-bet are further increased by IFN-γ demonstrating a positive feedback loop (104). The absence of T-bet in $CD4^+$ T cells results in decreased IFN-γ production, a decrease in the number of IFN-γ producing cells, as well as an increase in Th2 type cytokines (105). Increased expression of T-bet also ensures the progress of Th1 polarization by suppressing GATA-3, a Th2 type transcription factor. The strong role of T-bet in IFN-γ induction is demonstrated by the fact that T-bet transfection of Th2 type murine cells results in decreased IL-4 and IL-5 expression redirecting them to a Th1 type profile (104). Further, human Th2 cells transfected with T-bet, also secrete high levels of IFN-γ and have decreased IL-5 expression. Expression of T-bet also induces IL-12Rβ2 expression on human Th2 cells (106). T-bet knockout mice that exhibit spontaneous reactive airway disease, similar to asthma, (107) with increased Th2 type cytokines show the importance of T-bet in Th1 differentiation (105).

Increased levels of IFN-γ in the cellular environment lead to B cell activation and Ig class switching from IgM to IgG2a and IgG1 (108, 109). The levels of IgG2a, IgG2b, and IgG3 produced by B cells from T-bet knockout mice are diminished and cannot be

recovered in response to IFN-γ (110). Ectopic expression of T-bet in these cells activates IgG2a class switching (110, 111). The induction and regulation of IgG2a class switching takes place in anti-CD40 stimulated B cells from wild-type and IFN-γ knockout mice after the induction of T-bet expression by IL-27 stimulation. There is no activation of Ig class switching from IL-27 and anti-CD40 stimulated B cells from T-bet knockout mice suggesting the importance of T-bet in B cell activities (10). Since T-bet plays an important role in antibody production from B cells from healthy subjects, it is possible that it may have a role in autoimmune diseases. B cell dependent responses such as autoantibody production, hypergammaglobulinemia, and IFN-γ mediated IgG2a production are reduced in lupus prone T-bet knockout mice (110).

Szabo et al. showed that T-bet is critical to the production of IFN-γ from primary splenic NK cells, which had decreased cytotoxic ability in the absence of T-bet (105). Dendritic cells, potent antigen presenting cells with an ability to strongly activate naïve T cells (112, 113), help to initiate primary immune responses and can also produce IFN-γ (21, 114, 115). Expression of IFN-γ in both $CD8a^+$ and $CD8a^-$ murine dendritic cells has been shown to be dependent on T-bet expression. Dendritic cells (DC) from T-bet deficient mice have diminished levels of IFN-γ even after stimulation with IL-12 and IL-18 (116).

Curiously, unlike the critical role of T-bet in IFN-γ induction by $CD4^+$ T cells, NK, and DC cells, there is no change in the levels of IFN-γ or IFN-γ-producing $CD8^+$ T cells in the absence of T-bet (105). This suggests that $CD8^+$ cells may utilize another, possibly yet unidentified, transcription factor.

IFN-γ inducing cytokines such as IL-12, IL-15, IL-27, and IFN-γ itself, contribute to the activation of T-bet by activating various STATs, such as STAT-1 and STAT-4 (71, 72, 104, 117). Recent data show that the activation of T-bet expression is followed by a sequence of events starting with IL-27 (**Figure 1**):

1. IL-27 induces T-bet expression, which then induces chromatin remodeling of the IFN-γ gene (7-9). Interestingly, IL-27 effects take place prior to IL-12 and STAT-4 (118).

2. Elevated T-bet expression increases IL-12Rβ2 expression on lymphocytes, especially T cells, making them more responsive to IL-12 and subsequent STAT-4 activation (118).

3. IL-12/IL-12R signaling results in increased IFN-γ production, which further augments IL-12Rβ2 expression as well as IL-18Rα and β. Further amplification of the chromatin remodeling of the IFN-γ gene happens via IL-12 and IL-18 signaling (27, 118-121).

4. IL-12, in combination with IL-18 and/or IL-15, augments IFN-γ expression from NK, T, and even B cells (71, 72). An increase in IFN-γ may result in a positive feedback loop thus enhancing the expression of T-bet to stabilize the Th1 response (117).

In summary, T-bet influences the generation of Th1 dependent immunity by controlling the commitment of naïve T cells to Th1 polarization, directly affecting potent antigen presenting cells such as dendritic cells (DCs) to produce IFN-γ, increasing the cytotoxicity of NK cells and inducing class switching and/or autoantibody production of B cells.

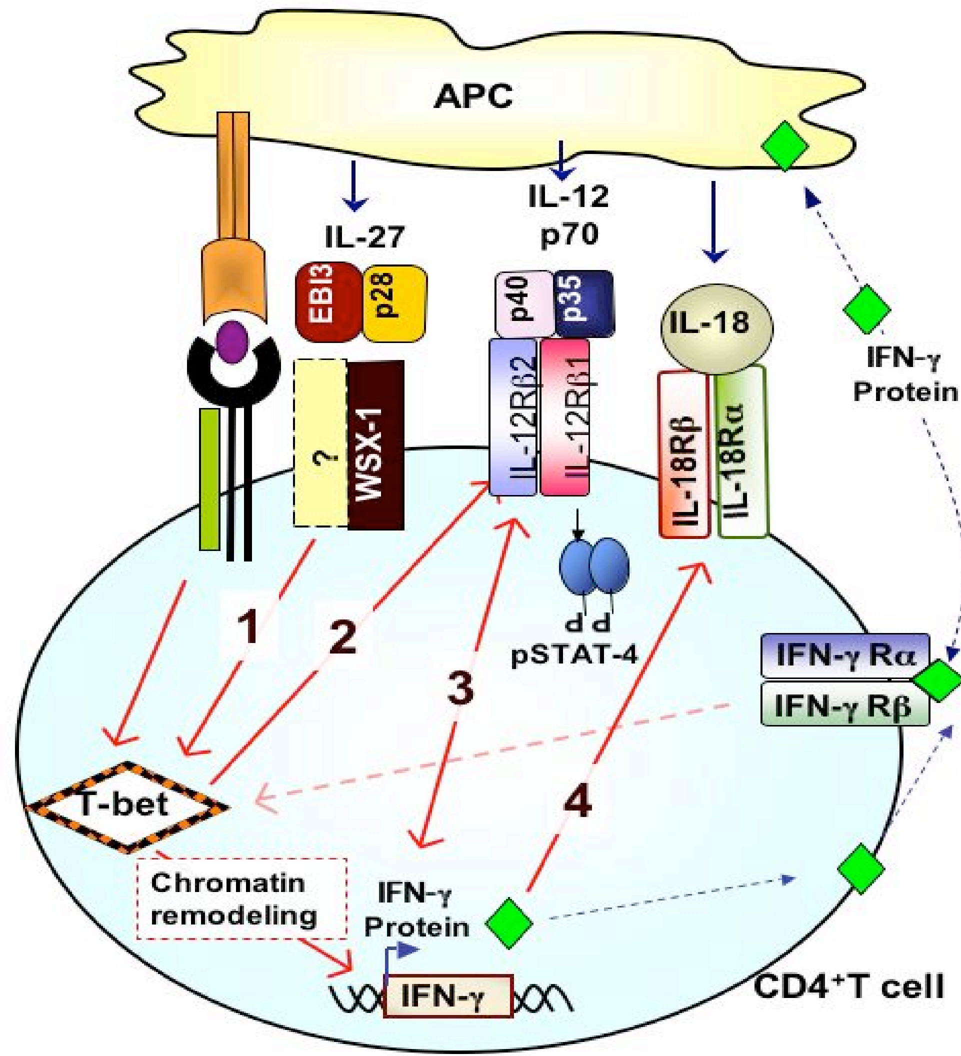


Figure 1. A model for T-bet induction by IL-27 and IL-12 in relation to IFN-γ: Activated Antigen Presenting Cells (APCs) produce IL-27, which binds to its receptor TCCR composed of EBI3, p28, an unknown subunit, and WSX-1, which upregulates T-bet expression in TCR stimultated T cells (#**1**). T-bet induces IFN-γ via chromatin remodeling and upregulates IL-12Rβ2 (#**2**) leading to STAT-4 phosphorylation. Ligation of IL-12p70 to the IL-12R complex results in increased IFN-γ production promoted by STAT-4. IFN-γ positively regulates the expression of IL-12Receptors and IL-12 (#**3**). IFN-γ upregulates IL-18Rβ, which complexes with IL-18Rα bound to IL-18 secreted by APCs. This results in increased IL-18 signaling (#**4**). This results in IRAK dissociation to interact with TRAF-6 leading to activation of NF-kB and up-regulation of IFN-γ. IFN-γ can bind to its receptor complex resulting in further augmentation of T-bet.

HLX

In addition to T-bet, another protein, HLX, from the homeobox family was found to be important in the induction of IFN-γ expression in immature T helper cells and T cell clones (122, 123) (**Figure 2, Panel A**). The inhibition of T-bet expression in primary naïve cells, stimulated under Th1 conditions, diminishes HLX expression suggesting that T-bet induces the homeobox factor, HLX (123). Ectopic expression of HLX cannot induce IFN-γ by itself, while T-bet with or without HLX induces IFN-γ expression. IFN-γ expression is greater in the presence of HLX (123). The biochemical and protein interactions of HLX and T-bet are not yet clear but studies show a genetic interaction between these factors resulting in activation of T-bet target genes such as IFN-γ and endogenous T-bet (123). Chromatin remodeling of the IFN-γ gene is modulated first by T-bet and, in following cell divisions, by the presence of HLX, which also augments IFN-γ expression (123). HLX expression is also found to be upregulated in immature T helper cells (122). Introduction of HLX in T helper cells at a later stage does not result in increased IFN-γ secretion, implying that the ability of HLX to upregulate IFN-γ is restricted to the immature stages of T helper cell development (122). The importance of HLX induction of IFN-γ is further supported by the findings that overexpression of HLX in Th2 cells leads to increased IFN-γ production in $CD4^+$ T cells. Furthermore, HLX transgenic mice have abnormal thymic development and reduced numbers of the $CD4^+CD8^+$ double-positive population (122).

Eomesodermin (Eomes)

The production of IFN-γ in the absence of the transcription factor T-bet in $CD8^+$ T cells from T-bet knockout mice implies a possible T-bet independent mechanism for the regulation of IFN-γ expression. Another T-box-factor, called Eomesodermin (Eomes) that is selectively expressed in $CD8^+$ T cells, but not in $CD4^+$ T cells was recently identified (124) (**Figure 2, Panel B**). The ectopic expression of Eomes in developing Th2 cells with T-bet expression enhances IFN-γ levels without affecting IL-4 protein levels. The introduction of Eomes in T-bet deficient $CD4^+$ T cells induces IFN-γ suggesting that

Eomes is able to induce effector functions in the absence of T-bet (124). Although Eomes is able to induce IL12Rβ2, it was not superior to T-bet in the induction of this receptor subunit (124). In summary, the data suggest that Eomes is an important factor in regulating several functions of $CD8^{+}$T cells, such as IFN-γ production and lytic activity in the absence of T-bet.

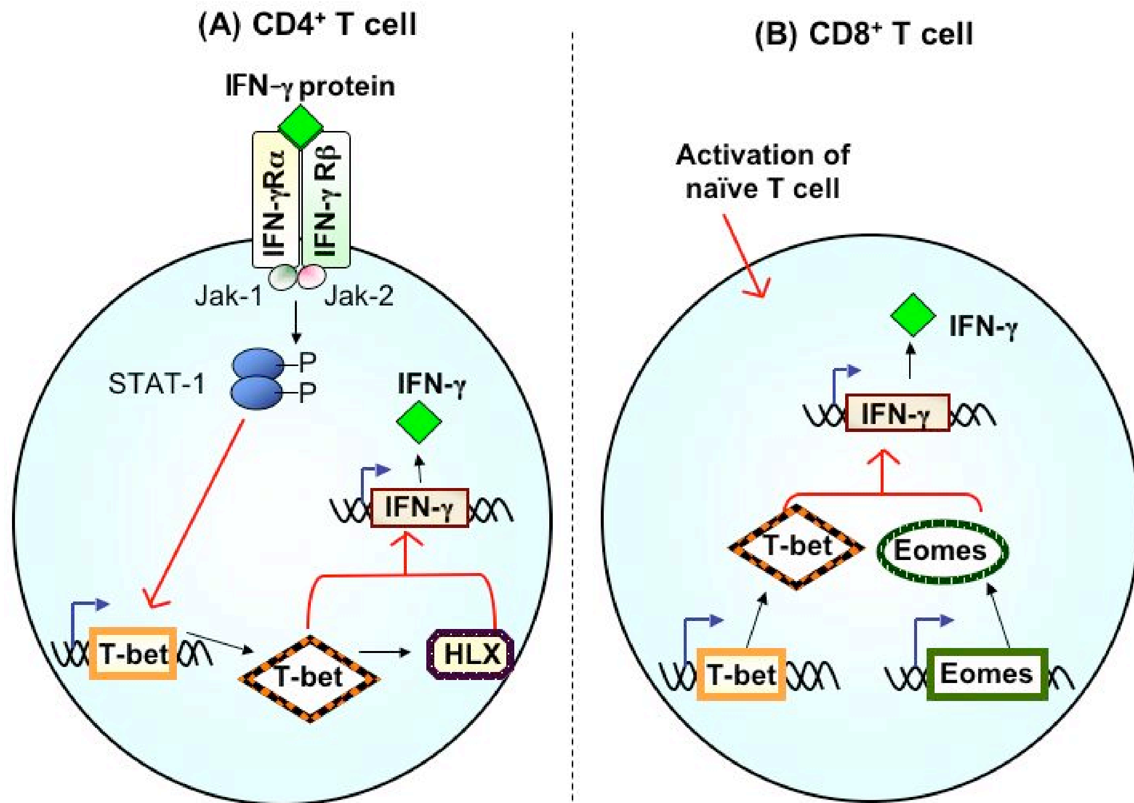


Figure 2. Supplementary IFN-γ-inducing transcription factors: In addition to T-bet, there are two newly discovered transcription factors. **Panel A**: IFN-γ acts on the IFN-γ Receptor complex (IFN-γRα and IFN-γRβ) to activate Jak-1 and Jak-2, which, in turn phosphorylate Stat-1 and induces T-bet expression. The homeobox protein HLX, which is expressed after T-bet induction, upregulates IFN-γ in combination with T-bet in $CD4^{+}$ T cells. **Panel B**: The expression of Eomes in the absence or presence of T-bet leads to IFN-γ production in activated $CD8^{+}$ T cells.

Signal Transducers and Activators of Transcription: STAT-4

Signal Transducers and Activators of Transcription, STATs, constitute another group of transcription factors and they activate the transcription of various genes in response to stimuli such as cytokines (125, 126). One of the most well known of the STATs is STAT-1, which takes part in the signal transduction activities of the IFN-γ-IFN-γ receptor complex and leads to the activation of IFN-γ-inducible genes (127). Briefly, IFN-γRα, the cytokine binding subunit, and IFN-γRβ, the signal transduction subunit, which are coupled with Jak-1 and Jak-2, are activated upon the binding of IFN-γ to its receptors. The IFN-γ-IFN-γ Receptor complex phosphorylates Jak-1 and Jak-2, which in turn leads to the binding of STAT-1. Docking of STAT-1 to the Jaks causes activation of STAT-1 via phosphorylation, which then forms homodimers in the cytosol (128). Homodimers are translocated to the nucleus where they bind to IFN-γ activation sites, called GAS, in the promoters of target genes resulting in alteration of the expression of IFN-γ induced genes (127, 129).

STATs are critical to the development of Th1 or Th2 differentiation of naïve lymphocytes. For example, STAT-4 is strictly expressed in lymphocytes, macrophages (21, 22, 130), dendritic cells (130), natural killer, and T cells (32, 131). IL-12, a cytokine important to the regulation of IFN-γ, phosphorylates STAT-4 through Jak2 and Tyk2 docked to the IL12-Receptor complex (23, 132, 133). Newly discovered IL-27 phosphorylates STAT-4 (7). Jaks phosphorylate STAT-4 and result in activated STAT-4 homodimers that migrate to the nucleus where they bind DNA sequences in the promoters of IL-12-inducible genes (134) such as IFN-γ (85, 135) or IRF-1 (136). There are multiple STAT-4 sites for tyrosine phosphorylation. In addition to tyrosine phosphorylation, STAT-4 can be phosphorylated on serine 721 by IL-12, which is important for maximum activation (137). Phosphorylation of Ser721 mediated through IL-12-IL-12 Receptor interactions, is regulated by p38 mitogen activated kinase (p38-MAPK). Further, disruption of this serine residue results in decreased STAT-4 activation and IFN-γ production (**Figure 3**) (137, 138).

STAT-4 phopshorylation via IL-12 takes place in Th1 cells, but not in Th2 cells, since Th2 cells do not express high levels of IL12-Rβ2 (27), the subunit responsible for effective IL-12 signalling (24). The absence of STAT-4, accompanied with a diminished cell mediated immune response, defective development of Th1 cells, decreased IFN-γ levels (33, 82), and decreased NK cytotoxicity, (32) suggests the importance of STAT-4 in the differentiation of Th1 lymphocytes. The mechanisms that control how estrogen alters STAT-4 signaling to induce Th1 differentiation are inadequately described. Bao et al. show that *in vitro* estrogen treatment of pre-Con-A stimulated $CD4^+$ T cells results in increased phosphorylation of STAT-4 after 20 mins incubation with recombinant IL-12 (20 ng/ml) (139). This suggests increased response to IL-12-induced STAT4 activation by estrogen.

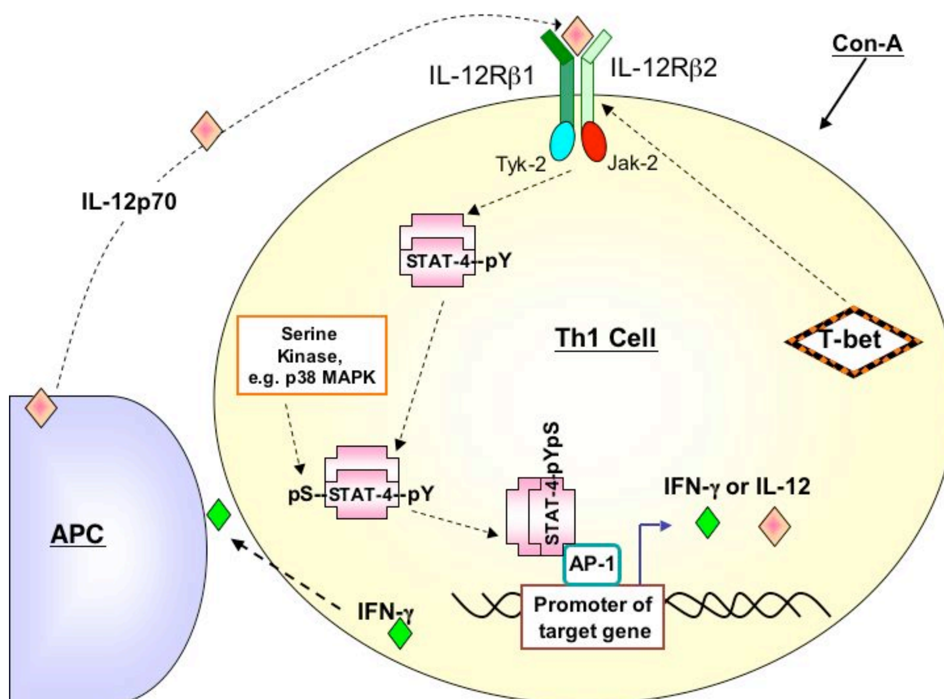


Figure 3. A model for IFN-γ induction via the STAT-4 and IL-12 pathway: IL-12p70 secreted from activated APCs binds to IL-12Rβ1, the binding subunit, and IL-12Rβ2, the signaling subunit to activate Tyk-2 and Jak-1, which in turn recruit and phosphorylate STAT-4 on the tyrosine residue (~700 amino acid, pY). Dimerized and activated STAT-4 is phosphorylated at the serine residue (~727 amino acid, pS) by serine kinases. Activated STAT-4, in turn, alters the expression of target genes by binding to the promoter of target genes or upregulating other transcription factors such as AP-1.

Interferon Regulatory Factors: IRF-4

Interferon regulatory factors are another group of transcription factors involved in the regulation of the immune system through the JAK-STAT pathway, which is activated by IFN-γ and other cytokines leading to transcription of their target genes (140). IRF-1 and IRF-2 are the most well known and characterized members of the IRF family (141-146). Seven other IRFs have been defined to date: IRF-3 (147, 148), IRF-4 (Pip/ LSIRF/ICSAT) (149), IRF-5 (150), IRF-6 (151), IRF-7 (152), IRF-8 (ICSBP) (153, 154), and IRF-9 (ISGF3γ/p48) (155). IRF transcription factors, in general, bind to IFN-stimulated response elements (ISRE), IFN-consensus sequence (ICS), or IFN-regulatory elements (IRF-E) in the promoters of target genes through their DNA binding domains, in which they have homology between different IRF members (156, 157). IRF-1 is expressed by most cell types (141, 144) and is important in IFN-γ signaling and homeostasis of T cells. IRF-1 expression can be induced by IFN-α (158), IFN-γ (159, 160), Con-A (144), and phorbol 12-myristate 13-acetate (PMA) (158) resulting in activation of several genes such as IFN-α (142), iNOS (161), and IRF-2 (162). IRF-4 is expressed mainly in cells of the immune system such as B cells, macrophages, and T cells (163-165). IRF-4 is induced by Con-A, TCR crosslinking via CD3, anti-IgM, PMA, anti-CD40 antibodies, and IL-12 (164, 166-168). IRF-4 can act as an activator or repressor in the development of a Th1 or Th2 profile. The absence of IRF-4 results in severe lymphadenopathy, decreased immunoglobulin levels in sera, impaired T cell cytotoxicty, decreased IFN-γ production, and impaired T and B cell functions (163). IRF-4 is also important in the transition of pre-B cells into the pro-B cell stage as well as the production of light chains of immunoglobulins (169). IRF-4 is also involved in controlling Th2 lymphocyte differentiation. While IRF-4 activates the expression of IL-4 inducible genes (166), it acts as a repressor in the transcription of IFN-γ inducible genes (149). IRF-4 deficient T cells in the presence of IL-12 for Th1 development do not skew towards Th1, similarly IL-4 for Th2 differentiation do not skew towards Th2 profiles suggesting its role in T cell differentiation (170). IRF-4 deficient mice infected with *L. major* produce IFN-γ but the numbers of lymphocytes decline suggesting the need for

IRF-4 to sustain an immune response. T-bet is expressed in $CD4^+$ T cells from IRF-4 deficient mice accompanied with IFN-γ production *in vitro* in response to Th1 polarization (IL-12 and anti-IL4) but not in the presence of Th2 inducing cytokines (IL-4 and anti-IL12) (170, 171). On the other hand, addition of IL-4 to IRF-4 deficient T cell cultures leads to increased IFN-γ production due to decreased GATA-3 protein expression, a key Th2 transcription factor that counters IFN-γ production (170). In conclusion, T lymphocytes from IRF-4 knockout mice can differentiate into Th1 but not Th2 cells. Overall, IRF-4 is essential to the development and differentiation of B cells and is necessary for proper Th2 cell responsiveness to IL-4.

c.) MATERIALS AND METHODS

Mice: Three-to-four week old C57BL/6 wild-type mice were obtained from Charles River Laboratories and maintained as described in first section of this chapter.

Estrogen Treatment: Four-to-five week old mice were orchiectomized and given placebo (control) or 17-β-estradiol implants as described previously in Chapter 3 and previous sections.

Isolation and Cultures of Splenic Lymphocytes: Splenic lymphocytes were collected and cultured with Con-A or left unstimulated in media as previously described in Chapter 3.

Isolation of RNA andcDNA Synthesis: Cells were subjected to RNA isolation and cDNA synthesis was performed as described in the previous sections.

Primer Design: Primers were designed to span a large segment of the target genes to be used during the construction of standard curves for Real Time PCR as explained in Chapter 3. The primers that were designed were as follows: β-actin (product: 965 bp) forward 5'-ATTGTTACCAACTGGGACGA-3', reverse 5'-CTGCGCAAGTTAGGTTTTGT-3'; T-bet (932 base pairs) forward 5'-

CCAACAGCATCGTTTCTTCT-3’, reverse 5’-AGGGGACACTCGTATCAACA-3’(One Trick Pony Oligos, Ransom Hill Bioscience, Ramona, CA).

The RT-PCR Assay: The reverse transcriptase-polymerase chain reaction was used to detect mRNA expression of the IFN-γ inducing transcription factors (T-bet, HLX, Eomes) and the housekeeping gene, β-actin, for the generation of standards and/or for the detection of gene expression in samples were described in detail in Chapter 3. For the construction of standards, the primers for β-actin and T-bet that were described in “The primer design” section of the methodology were used. β-actin (product: 349 bp) and T-bet (product: 254 bp) primers were purchased from Maxim Biotech Inc. (San Francisco, CA). IFN-γ inducing transcription factors HLX and Eomes were designed using the Primer 3 program: HLX (product: 234 bp) forward 5’-ATCTCACATCGCTGCTAACC-3’, reverse 5’-GCTTCCGCTTGTATGTCTGT-3’; Eomes (product: 205 bp) forward 5’-ACACGGATATCACCCAGCTA-3’, reverse 5’-TGAGGCAAAGTGTTGACAAA-3’ (Invitrogen Inc., CA). The same PCR program was used for the primers designed and primers purchased from Maxim Biotech Inc. as described previously. The PCR products of the target genes were quantified with a scanning densitometer and normalized using β-actin. These were expressed as mean relative densities with standard errors in the figures.

Extraction of Target Genes and Measurement of Total cDNA after Gel Extraction, Picogreen: PCR products were isolated and purified as described in the previous section (Chapter 3) of this dissertation. PicoGreen (Molecular Probes Inc., Eugene, OR) was used to detect cDNA in the samples as described in the previous section.

Real Time PCR: Real Time PCR was performed as previously described in Chapter 3. β-actin and T-bet primers were purchased from Maxim Biotech Inc. (San Francisco, CA) and the Real Time PCR reaction was performed under the conditions as previously described in Chapter 3 for both the standards and unknown samples. Normalization of the target genes were performed by normalizing to the housekeeping gene, β-actin. Standard curves were used to calculate the amount of T-bet and β-actin in femtograms. Data were recorded as femtogram ratios of the target gene, T-bet, normalized to β-actin.

Determination of pSTAT-4 and IRF-4 Protein Levels: Western blot assays were used to detect STAT-4, pSTAT-4 and IRF-4 proteins in whole cell lysates of placebo and estrogen-treated unseparated splenic lymphocytes, which were cultured with Con-A (10 μg/ml) or left unstimulated (media) at 37°C with 5% CO_2 for 24 hrs. At the end of the incubation period, cells were harvested and resuspended in 15μl of lysis buffer (50mM Tris pH: 7.4, 300mM NaCl, 2mM EDTA pH: 8, 0.5% Triton X-100, 30 μl freshly added mammalian protease inhibitor cocktail (10 μg/ml, Sigma-Aldrich Inc., MO), vortexed, and incubated on ice for 15 mins. For the detection of phosphorylated STAT-4, cells were lysed with 1 mM sodium vanadate included in the lysis buffer, which is important to inhibit serine and tyrosine phosphatases. Fifteen μl of 2x sample buffer (Laemmli Sample Buffer, Sigma-Aldrich Inc., St Louis MO) were added to 15 μl of lysate and heated at 95^{0}C for 5 mins. Samples were electrophoresed on a 12.5% SDS-PAGE gel at 25 mA constant current until the dye ran off the bottom. Proteins were transferred to PDVF transfer membranes (Amersham Biosciences, CA) by blotting for 1 hr 30 mins at 240 mA constant current. After transfer, the membranes were blocked in 2% BSA in TBST for phosphorylated proteins or 5% milk in TBST (blocking buffer) for 1 hr at room temperature. Blots were incubated with pSTAT-4 primary antibody (rabbit polyclonal IgG; 1:2000; Ser-721, sc-22160R; Santa Cruz Inc., CA), STAT-4 (rabbit polyclonal IgG; 1:1000, sc-486; Santa Cruz Inc., CA), or IRF-4 (goat polyclonal IgG; 1:1000; clone M-17, sc-6059; Santa Cruz Inc., CA) diluted in blocking buffer for overnight on a rocking platform. Blots were rinsed briefly in TBST, then washed 3 times for 10 mins with TBST. The secondary HRP-conjugated antibody (anti-rabbit; 1:4000, or anti-goat IgG; 1:2000; Santa Cruz Inc., CA) were applied in blocking buffer to each blot and incubated for 1 hr. After the blots were washed, the bands were visualized using the ECL protocol (Amersham Pharmacia Biotech, NJ) and quantitated as relative densitometry values using a Kodak Image Station (Perkin Elmer Life Sciences Inc., MA).

Statistics: Data were analyzed using SAS software (SAS Institute Inc., Cary, NC). Data distributions and variances were evaluated using the UNIVARIATE procedure and data were logarithmically transformed when warranted. Transformed data were evaluated by

analysis of variance (ANOVA) using the MIXED and GLM procedures. The model for the split-plot design included treatment, date of experiment, and their interactions tested by the main plot error term mouse ID (treatment x date of experiment). Stimulant, time of measurement and interactions with treatment were tested by the overall error term. Statistically significant interactions were further investigated using the SLICE option to test the simple main effects. p-values less than 0.05 were considered to be significant. The data are logarithmically transformed and geometric means and standard errors were then back transformed for presentation in figures. Therefore, in some cases the standard errors are non-symmetric. Data were represented as means and standard error of means.

d.) RESULTS

Estrogen Upregulates the Key Th1 Transcription Factor T-Bet in Splenocytes

T-bet regulates IFN-γ production and is an important transcription factor in the differentiation of Th1 cells. To date, it is not known whether estrogenic hormones affect the expression of T-bet. Data show that increased T-bet expression leads to effector functions associated with Th1 cells such as enhanced expression of IL-12Rβ2 and IFN-γ (8, 118). In the previous part (**Subsection 4.1**) of this chapter, We have shown that IFN-γ and IFN-γ inducing cytokines such as IL-18 and IL-27, as well as the signaling subunit IL-12Rβ2 are significantly increased upon estrogen treatment in Con-A activated splenocytes. Given its key role in the development of Th1 and IFN-γ production, we therefore, investigated the expression of T-bet after estrogen treatment. Expression of T-bet mRNA in splenic lymphocytes from estrogen and placebo-treated mice was measured after stimulation with Con-A or left unstimulated for 24hrs. Induction of T-bet mRNA expression was observed in Con-A stimulated splenic lymphocytes from estrogen-treated mice as an approximate estimate as detected by RT-PCR. (**Figure 4, Panel B**; $p<0.01$). Figure 4.A depicts a representative gel picture for β-actin and T-bet. The remarkable induction of T-bet gene expression was also observed in Con-A stimulated splenocytes from estrogen-treated mice when compared to placebo-treated mice as detected by Real Time PCR assays (**Figure 5, Panel B**; $p<0.001$). Even in unstimulated cells (media only)

from estrogen-treated mice T-bet mRNA was upregulated, albeit lower than Con-A stimulated cells when compared to placebo controls (**Figure 5, Panel A**).

Expression of the Transcription Factors Eomesodermin and HLX Are Not Altered After Estrogen Treatment

Since estrogen increased T-bet expression in Con-A activated lymphocytes, we next investigated whether expression of HLX, which is another homeobox transcription factor upregulated during early Th1 cell differentiation in combination with T-bet (123), was altered upon estrogen exposure. Estrogen treatment did not appear to alter the expression of HLX in Con-A activated splenic lymphocytes as demonstrated by RT-PCR (**Figure 6, Panel B**). Figure 6.A depicts a representative gel picture of RT-PCR analysis. This aspect needs to be confirmed by Real Time PCR assay.

Since estrogen treatment increased T-bet expression, we explored whether Eomesodermin (Eomes) gene expression was also altered in Con-A activated splenocytes of estrogen-treated mice. As was the case for HLX, the apparent relative Eomes gene expression was not remarkably changed in splenocytes from estrogen-treated mice when stimulated with Con-A (**Figure 6, Panel D**). A representative gel picture of RT-PCR analysis is shown in Figure 6.C.

Estrogen Upregulates STAT-4 Phosphorylation In Splenic Lymphocytes

The activation of STAT-4 through IL-12 and IL-12Rβ2 (120) is important to maintain Th1 differentiation and IFN-γ production. Activation with Con-A caused a noticeable upregulation in the relative expression of serine (S-727) phosphorylation of STAT-4 protein in splenic lymphocytes from estrogen-treated mice compared to placebo treated mice (**Figure 7, Panel A & B**; $p<0.05$). The relative expression of total STAT-4 protein was similar in Con-A stimulated lymphocytes from estrogen and control-treated mice (**Figure 7, Panel C & D**). Although the expression of total STAT-4 was diminished in unstimulated lymphocytes from estrogen-treated mice (**Figure 7, Panel C & D**), estrogen

treatment altered the relative expression of phosphorylated STAT-4 in unstimulated splenic lymphocytes as compared to placebo treated mice (**Figure 7, Panel A & B**).

Expression of IRF-4 Is Diminished In Splenocytes From Estrogen-Treated Mice

In addition to T-box transcription factors such as T-bet, expression of Interferon Regulatory Factors (IRF) such as IRF-4 is important to the development of Th2 cells and IL-4 secretion (172). Since in the absence IRF-4 expression, cells develop into Th1 but not Th2 type cells (171), we examined whether expression of IRF-4 was altered by estrogen treatment. The decrease in the relative expression of IRF-4 protein was evident in Con-A stimulated splenic lymphocytes from estrogen treated mice when compared to placebo treated mice (**Figure 8, Panel A & B**; $p<0.05$). IRF-4 protein expression was also diminished in unstimulated (media only) lymphocytes from estrogen and control treated mice (**Figure 8, Panel A & B)**. In summary, these data suggest that decreased IRF-4 expression results in suppression of Th2 type cells, whereas low levels of IRF-4 may help in maintenance of a Th1 profile in estrogen-treated mice.

e.) DISCUSSION

In our previous studies, we have shown that splenic lymphocytes from estrogen-treated mice tended to have higher levels of IFN-γ, and markedly upregulated IFN-γ dependent iNOS, and iNOS derived nitric oxide when compared to placebo-treated controls. In addition, we have also demonstrated that splenocytes from estrogen-treated mice had elevated levels of IFN-γ inducing cytokines; IL-18, IL-15, IL-27, and the receptors IL-12Rβ2 and IL-18Rβ implying a strong polarization into Th1 type cells. The polarization of naïve T cells into Th1 cells is mediated by the "master" transcription factor T-bet. It is known to regulate the production of IFN-γ and the induction of other key Th1 mediators such as IL-12Rβ2 (27, 104), which were all found to be upregulated due to estrogen treatment. This study is the first to show that estrogen treatment clearly upregulates the mRNA expression of T-bet in Con-A-activated splenocytes as demonstrated qualitatively with RT-PCR as well as quantitatively with Real Time PCR following 24 hours of

culture. T-bet expression in unstimulated lymphocytes was significantly lower than that observed in Con-A stimulated lymphocytes from estrogen-treated mice demonstrating the importance of stimulation and the need for IFN-γ in the cellular environment. Others have demonstrated that T-bet protein by culturing $CD4^+$ T cells for 2 or 3 days with plate-bound anti-CD3 and anti-CD28 antibodies in the presence of recombinant IL-2, recombinant IL-12, and anti-IL-4 antibodies using immunoblot assay (104). Since we preferred to detect the T-bet protein in unseparated splenocyte cultures that are as close to a natural state as possible, rather than artificially drive the culture conditions, we only cultured splenocytes with Con-A for 24 hrs for primary activation. The protein expression of T-bet was not detectable in both the cellular and nuclear extracts from Con-A stimulated splenocytes cultured for 24 hrs (data not shown).

Although it has been suggested that T-bet regulates Th1 development in TCR stimulated cells through the IFN-γ-IFN-R/STAT-1 pathway, but not the IL-12/STAT-4 pathway (117), it has also been shown that T-bet induces the expression of the IL-12Rβ2 chain and other factors, such as GADD45γ, which are involved in IL-12 signaling via STAT-4 activation (106). In the previous part of this chapter, we showed that IL-27, which takes part in the early regulation of Th1 initiation and induction of T-bet and IL-12Rβ2 expression, was also increased in Con-A stimulated lymphocytes from estrogen-treated mice after 24 hours of culture. This could be the initial event in upregulation due to estrogen treatment. Subsequently, the increase in T-bet expression may be due to early induction by IL-27 accompanied by IFN-γ which may resulting in the amplification of Th1 responses in splenocytes from estrogen-treated mice.

A newly discovered transcription factor homeobox protein HLX, is expressed in Th1 cells and is required to enhance T-bet-induced IFN-γ expression (123). Zheng et al demonstrated that the kinetic expression of HLX followed three phases: the constitutive expression of HLX in naive $CD4^+$ T cells, initial reduction of HLX expression through Th1 and Th2 cell differentiation, and up-regulation upon commitment to the Th1 lineage (122). Interestingly, Con-A-activated splenocytes from estrogen-treated mice demonstrated a tendency to have decreased HLX mRNA expression when compared to

placebo-treated mice. Since the result was more qualitative than quantitative due to detection with RT-PCR assays, Real Time PCR should be used to obtain the absolute levels of HLX expression. That being noted, based on the kinetic data from Zheng et al. (122), our results imply that Con-A stimulated splenocytes from estrogen-treated mice may be at the second phase; a decision stage for Th1 vs Th2 response. On the other hand, all the data we have so far indicate that splenocytes from estrogen-treated mice may have already committed to the Th1 type lineage. The discrepancy in this matter could be explained by the use of unseparated splenic lymphocyte cultures from wild-type mice in our study versus the use of T cells, which artificially overexpress HLX, that were differentiated into Th1 or Th2 cells *in vitro*.

Eomesodermin (Eomes) a T-box factor is induced strictly in $CD8^+$ cells and takes part in T-bet–independent IFN-γ induction in $CD8^+$ T cells (124). The inhibition of Eomes results in decreased cytotoxicity and IFN-γ production. Interestingly, Eomes expression was not altered in Con-A stimulated splenocytes from estrogen-treated mice as compared to control mice. This indicates estrogen treatment induces T-bet expression more than it does Eomes. Given that, IFN-γ production may be mostly regulated by $CD4^+$ T cells and T-bet expressing $CD8^+$ T cells, but not by Eomes expressing $CD8^+$ T cells.

In addition to T-bet, Eomes, and HLX, IFN-γ production and Th1 development also depend on other factors such as stimulation through the IL-12/STAT-4 pathway. Although it has been shown that T-bet and IFN-γ expression can take place in the absence of STAT-4 (118, 120), IFN-γ induced T-bet expression in TCR-triggered T cells induce IL-12Rβ2, an important player in Th1 development (117, 120) and increase responsiveness of cells to IL-12 by activating the STAT-4 pathway via phosphorylating the STAT-4 homodimers (106). It has been shown that serine phosphorylation of STAT-4 is important for optimum transcriptional regulation and production of IFN-γ. On the other hand, tyrosine phosphorylated STAT-4 exhibits normal nuclear translocation and DNA binding (138). Serine phosphorylation of STAT-4 is found to be regulated by p38 mitogen-activated protein kinase (MAPK) and can be induced by GADD45β (95) and GADD45γ, regulators of cell cycle progression and differentiation (97). These

intermediates regulate activation of p38 MAPK pathway, effector functions of Th1 cells, cytokine (IL-12 and/or IL-18) and TCR-induced IFN-γ production (95, 97). We have shown that estrogen treatment noticeably increased pSTAT-4 expression (phosphorylated on Serine 727 residue) in Con-A activated splenic lymphocytes when compared to cells from control mice. The expression of total STAT-4 was not altered in Con-A activated splenocytes from estrogen and placebo-treated cells. Although total STAT-4 expression seemed to diminish in unstimulated cells from estrogen-treated mice, the unstimulated cells from estrogen-treated mice demonstrated a higher trend in pSTAT4 expression compared to controls. Based on other studies, estrogen treatment may induce IFN-γ production via the GADD45γ-β/MAP kinase/pSTAT-4 pathway. Overall, our studies suggest that estrogen treatment may induce IFN-γ expression through increased T-bet expression that in turn induces the IL-12Rβ2/pSTAT-4 pathway.

Recent studies have shown that IRF-4 can induce Th2 differentiation (166) and repress IFN-γ inducible genes and Th1 development (149), however in the absence of IRF-4, $CD4^+$ T cells under Th1 conditions produced IFN-γ and expressed T-bet (171). To our knowledge, this is the first study to show that IRF-4 expression was decreased in Con-A stimulated cells from estrogen-treated mice compared to control (placebo-treated mice) implying another mechanism in the elevation of IFN-γ.

In summary, our work demonstrates the multi-faceted molecular regulation of IFN-γ expression by estrogen in splenic lymphocytes. Since the complexity of IFN-γ expression is ever-increasing, our data may help to better explain the affect of estrogen on IFN-γ production by exploring IFN-γ inducing T-bet, STAT-4, and IRF-4 expression and might be beneficial for the treatment of IFN-γ related inflammatory and autoimmune diseases.

g.) FIGURES

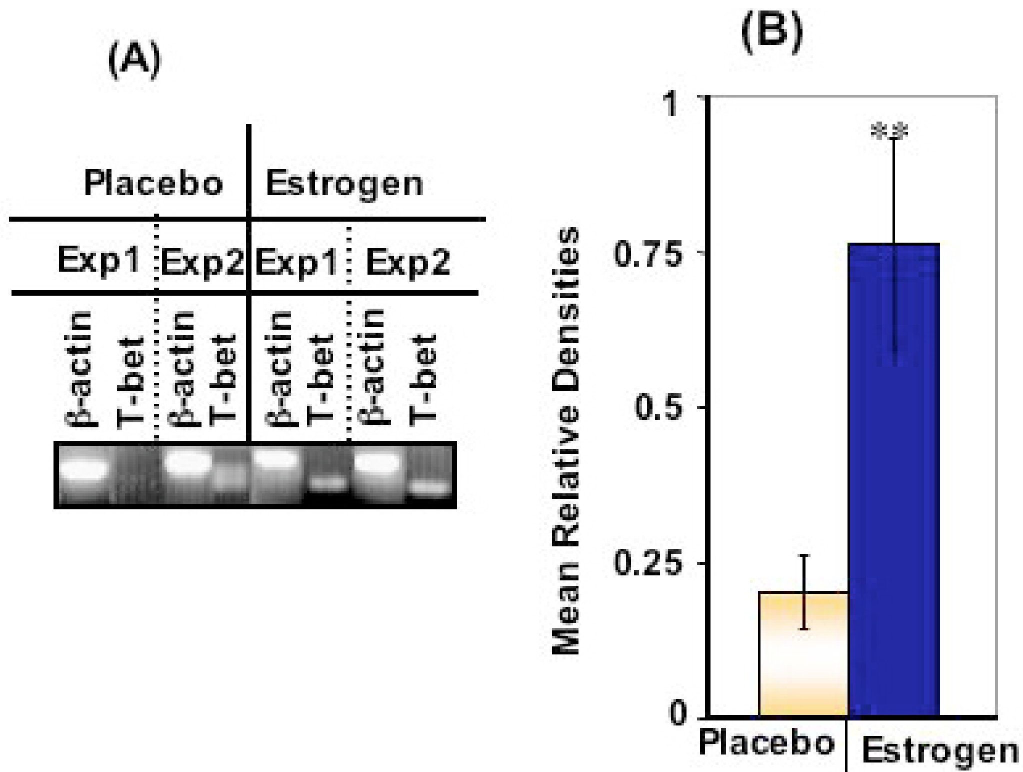


Figure 4. Estrogen upregulates T-bet expression, RT-PCR: Splenic lymphocytes from placebo and estrogen-treated mice were cultured with Con-A (10 μg/ml) for 24hrs. T-bet gene expression was detected with RT-PCR and normalized to the housekeeping gene, β-actin. As demonstrated in **Panel B**, estrogen treatment increased T-bet gene expression in Con-A stimulated splenocytes (n=12 mice per placebo or estrogen treatment, p<0.01). Data are presented as means with standard error bars. **Panel B** illustrates two representative experiments for RT-PCR assay.

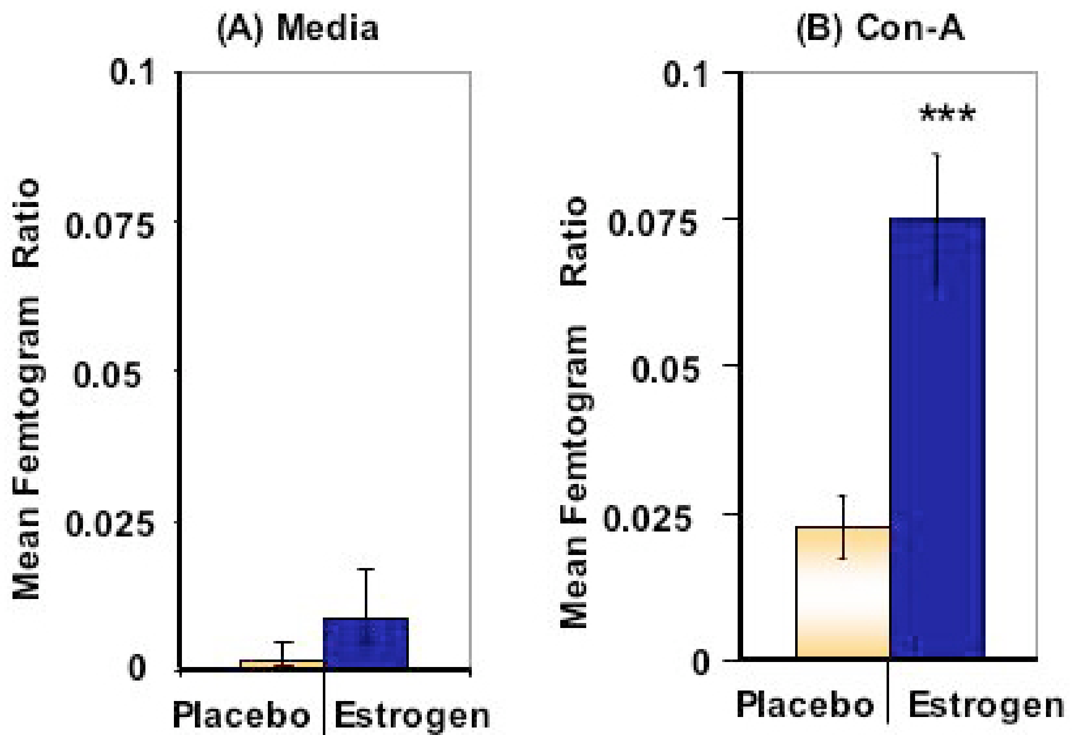


Figure 5. T-bet gene expression is quantitatively increased in estrogen-treated splenocytes, Real Time PCR: Splenic lymphocytes from placebo and estrogen-treated mice were cultured with Con-A (10 μg/ml) or left unstimulated (media) for 24 hrs. The quantitative detection of T-bet gene expression was executed with Real Time PCR and was normalized to β-actin. Data are presented as means of femtogram ratios with standard error bars. Panel A demonstrates the T-bet expression in unstimulated lymphocytes (n=3 mice per placebo or estrogen treatment). T-bet expression was noticeably increased in Con-A stimulated lymphocytes from estrogen-treated mice compared to control mice (Panel B; Placebo: n=8 mice, estrogen: n=9 mice, $p<0.001$).

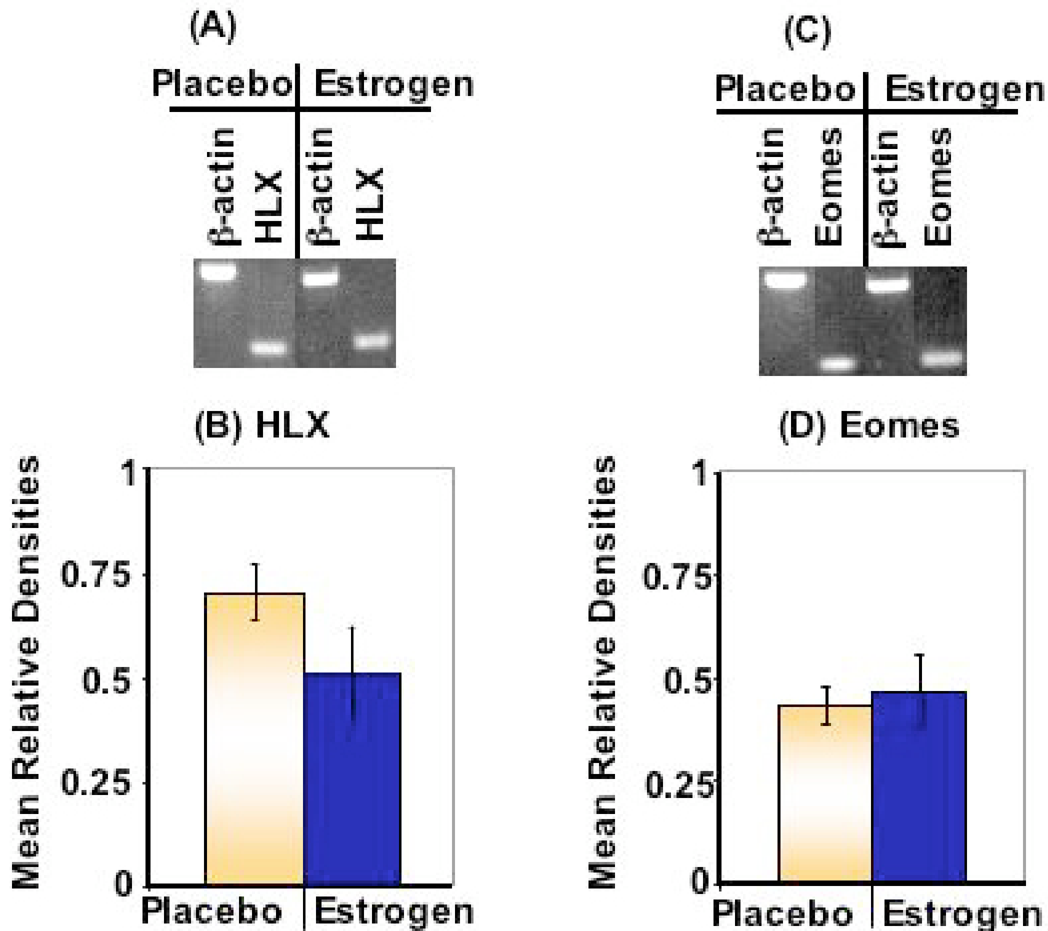


Figure 6. Expression of HLX and Eomes mRNA are not altered in splenocytes from estrogen-treated mice: Splenic lymphocytes from placebo and estrogen-treated mice were cultured with Con-A (10 μg/ml) for 24 hrs. HLX and Eomes gene expression were detected with RT-PCR and normalized to the housekeeping gene, β-actin. HLX mRNA expression was not changed in lymphocytes from estrogen-treated mice (n=9 mice per placebo or estrogen treatment, p=0.17; Panel B). Panel A shows a representative experiment for RT-PCR assay of HLX. As demonstrated in Panel D, estrogen treatment did not affect the expression of Eomes mRNA gene expression in Con-A stimulated splenocytes (n=9 mice per placebo or estrogen treatment, p=0.7). Data are presented as means with standard error bars. Panel C shows a representative experiment for RT-PCR assay of Eomes.

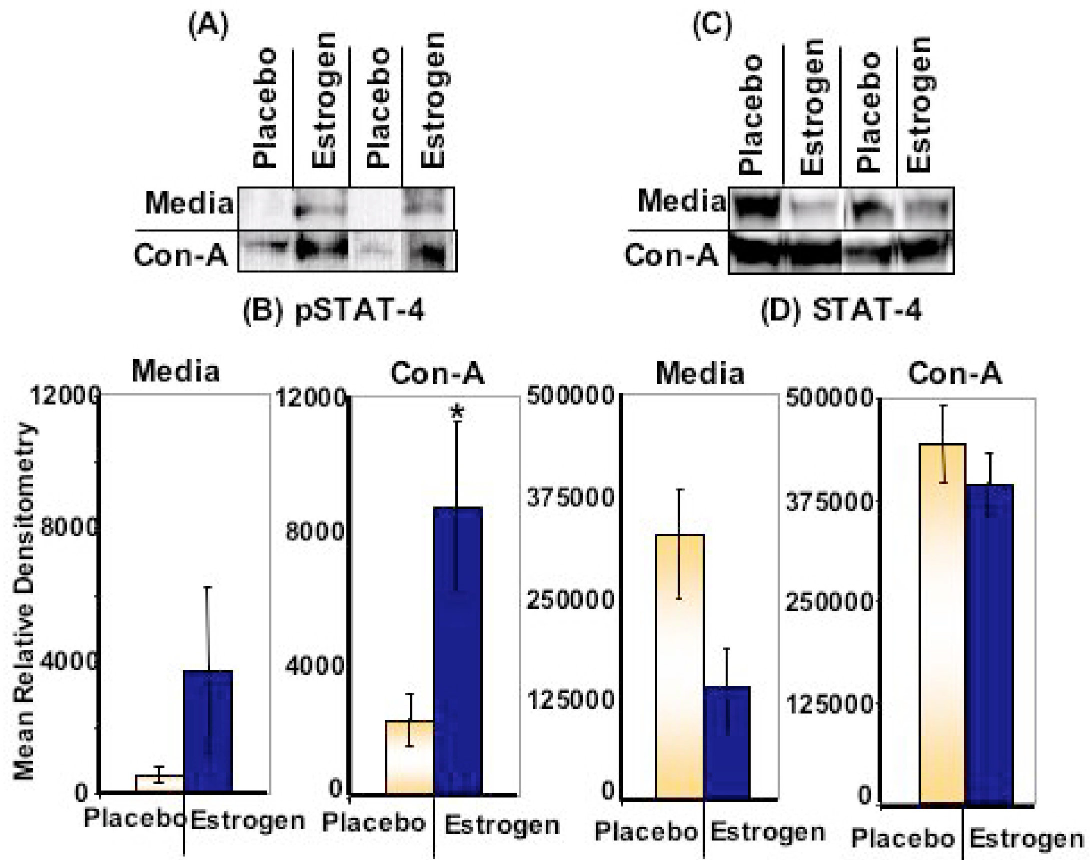


Figure 7. pSTAT-4 protein expression is increased in estrogen-treated splenic lymphocytes whereas STAT-4 is not affected: Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml), Con-A (10 μg/ml) and CTLA-4Ig (15 μg/ml) or left unstimulated in media for 24 hrs. Expression of pSTAT-4 (serine phoshorylated) and STAT-4 protein were determined with Western Blot assays. pSTAT-4 protein expression had a tendency to increase in unstimulated cells (n=5 mice per placebo or estrogen treatment) and demonstrated a significant increase in Con-A-activated splenocytes (n=5 mice per placebo or estrogen treatment, $p<0.05$; **Panel B**) from estrogen-treated mice. Panel A depicts a representative picture of pSTAT-4 protein. As shown in **Panel D**, STAT-4 expression was not altered in Con-A stimulated splenocytes (n=7 mice per placebo or estrogen treatment), whereas it was diminished in unstimulated cells from estrogen treated mice (n=7 mice per placebo or estrogen treatment, not significant). Data are presented as means with standard error bars. A representative experiment for a STAT-4 Western Blot assay is shown in **Panel C**.

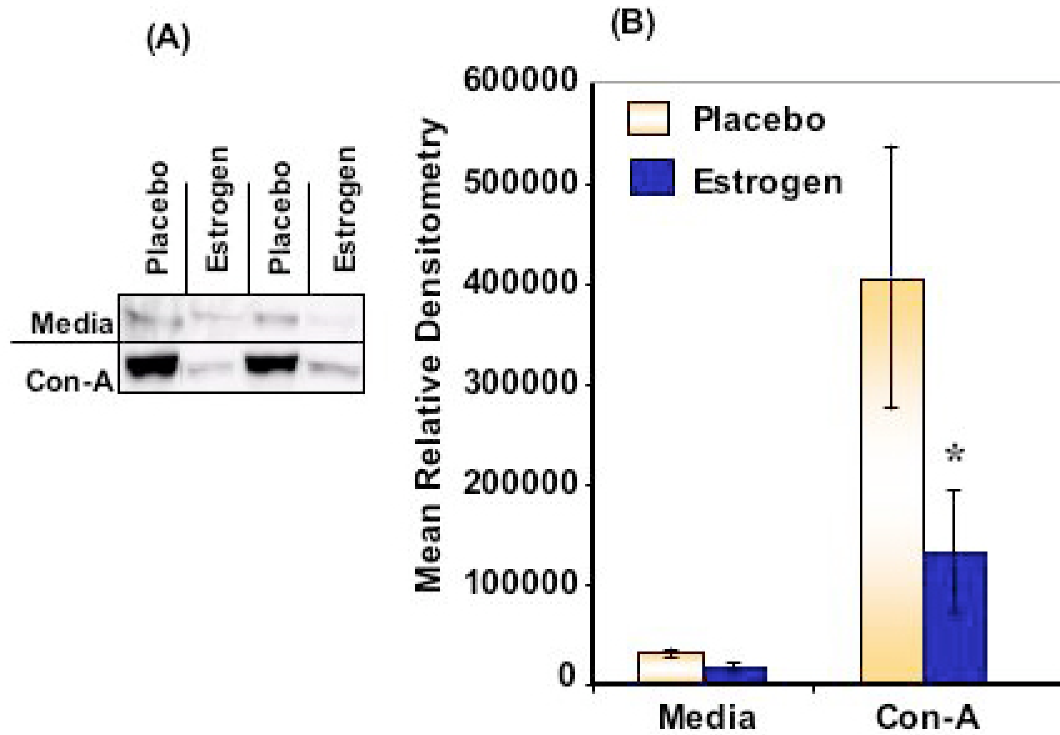


Figure 8. Estrogen treatment downregulates IRF-4 protein in Con-A stimulated splenocytes: Splenic lymphocytes from estrogen or placebo-treated mice were stimulated with Con-A (10 μg/ml) or left unstimulated in media for 24 hrs. IRF-4 protein expression was detected with Western Blot assays. IRF-4 protein was decreased in Con-A stimulated lymphocytes (n=4 mice per placebo or estrogen treatment, $p<0.05$; **Panel B**). The level of IRF-4 protein was lower in unstimulated cells compared to stimulated lymphocytes (n=4 mice per placebo or estrogen treatment; **Panel B**). **Panel A** depicts a representative picture of a Western Blot assay of IRF-4 protein.

4.3. Effects of Estrogen Treatment on IFN-γ-Inducing Costimulatory Molecules: the PD-1/PD-Ligand Pathway.

a.) ABSTRACT

Programmed Death-1 (PD-1) and its respective ligands PD-L1 and PD-L2, members of the B7 family, are important costimulatory molecules expressed on a variety of cells of the immune system. Their main role is to modulate B and T cell responses via regulating the production of cytokines such as IFN-γ. Estrogen, an important sex hormone, has been shown to exert pleiotropic effects on immune system. Our previous data indicate that estrogen upregulates IFN-γ, nitric oxide, IFN-γ inducing cytokines, and antibody production from splenocytes. Optimal activation of T cells involves molecular interactions with other cells, notably antigen-presenting cells (APCs). Costimulatory molecules on APC and cytokines released by these cells play a critical role in regulating the threshold of T cell activation. In this study, we examined the role of recently identified costimulatory molecules, PD-1/PD-L1-PD-L2. So far, there is no available information on whether estrogen alters the expression of PD-1 or its ligands: PD-L1, and PD-L2. We found that estrogen treatment significantly decreased PD-1 expression on B cells, but not on T cells. The overall PD-L1, PD-L2, and PD-1 expression was increased on macrophages from estrogen treated mice. In summary, these results suggest that estrogen treatment alters splenocyte activation through the PD-1/PD-L1-L2 pathway.

b.) INTRODUCTION

CD28 and B7 Costimulation

Activation of naïve T cells depends on at least two signals. The first signal takes place through the TCR (T cell receptor) when ligated with antigenic peptides that are bound to MHC-peptide complex. The second signal is provided by costimulatory molecules that can be divided into two groups depending on their nature as being either positive or negative costimulatory signals. The positive costimulatory signals are important for full activation of T cells after antigen recognition. One type of positive costimulatory molecule is CD28 which is found on T cells, and binds to B7.1 (CD80) or B7.2 (CD86) on antigen presenting cells (173, 174). The interaction between B7s and CD28 increases cytokine production and aids in the survival of T cells (175). Blocking the interaction of CD28 and B7 with a CTLA-4Ig fusion protein, which has a stronger affinity for B7 than does CD28, suppresses auto-aggressive T cells, prolongs graft survival in animal models, and decreases cytokine levels (176-178). CTLA, a member of the CD28/B7 family, binds to B7 molecules and serves as a negative costimulatory receptor, by inhibiting T cell responses and activities such as cytokine production and proliferation (179). Negative co-signaling of lymphocytes drives lymphocytes into anergy or apoptosis by suppressing the necessary signals for survival such as cytokine signaling (180). We showed earlier that blocking CD28 and B7 interactions between splenocytes from estrogen-treated mice with a CTLA-4Ig results in markedly decreased levels of IFN-γ-dependent nitric oxide.

PD-1 and PD-Ligand Costimulation

Recent findings confirm that there is another potentially important negative costimulatory pathway mediated through Programmed Death-1 (PD-1) and its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) (181-184).

Expression and Function of PD-1

Programmed death (PD-1) is an inhibitory receptor expressed mostly on activated T cells (both $CD4^+$ and $CD8^+$ T cells) (185) and on B cells activated by anti-IgM and anti-CD40 antibodies (184, 186). It is constitutively expressed on double negative thymocytes (3-5% of thymocytes, mainly $CD4^-CD8^-$ T cells) (187). PD-1 expression is detected primarily in the T cell zone of the spleen and is co-localized with CD3 expression. PD-1 is also expressed on regulatory $CD4^+CD25^+$ T cells but their function is not yet clearly known (188).

Expression and Function of PD-L1 and PD-L2

PD-L1 (B7-H1) and PD-L2 (B7-DC) are ligands for PD-1 (182, 189-191). The PD-L1 protein is broadly expressed on antigen presenting cells such as dendritic cells, macrophages, B cells (183, 186), hematopoietic cells, and non-hematopoietic tumor cells (183, 186). PD-L1 is expressed on freshly isolated and unstimulated antigen presenting cells (macrophages and dendritic cells), splenic T and B cells with the strongest expression in the marginal zone of the spleen. PD-L1 expression on antigen presenting cells is upregulated by IFN-γ and LPS upon stimulation (186, 192). PD-L1 mRNA expression is constitutively observed and upregulated in non-lymphoid tissues (heart, lung, placenta, kidney, liver) (182, 189, 193), spleen, thymus (189), endothelial cells (194), monocytes, and keratinocytes (182, 189). Freshly isolated T and B cells express negligible amounts of PD-L1 mRNA, but upon activation, the majority of the $CD3^+$ T cells and 5% of the $CD19^+$ B cells express PD-L1 (195).

PD-L2 (B7-DC) is another member of the B7 family, but its expression is strictly limited to dendritic cells and some macrophages after stimulation (183, 190, 191). PD-L2 expression is not detected within the spleens of naive BALB/c mice (192). While human mRNA for PD-L2 is expressed in nonlymphoid tissues, the expression of PD-L2 in murine tissues is weaker and more restricted than murine PD-L1 (190, 196). The mRNA expression of PD-L2 is seen in murine bone marrow DCs, splenic DCs, macrophages, and in macrophage lines (191).

Since PD-L1 and PD-L2 have distinct expression patterns in murine cells and tissues, it is likely that these ligands differentially regulate their target cells. Addition of IL-12 or IL-4 to dendritic cells upregulates the expression of PD-L1 and PD-L2, respectively (192). However, dendritic cells obtained from STAT-4 or STAT-6 knockout mice do not exhibit altered basal levels of PD-L1 or PD-L2 (192). Nevertheless, exposure of dendritic cells from STAT-6 knockout mice to exogenous IL-4 for 24 hrs results in lower PD-L2 expression compared to wild-type dendritic cells, but PD-L1 expression is not affected (192). This implies that PD-L2 upregulation in response to IL-4 in macrophages is dependent on STAT-6 (197).

The PD-1/PD-L Pathway and T Cell Function

The stage of immune response is important to the function of the PD-1/PD-L pathways. The constitutive expression of PD-L1 in peripheral tissues suggests that signaling between PD-L1 on antigen presenting cells and PD-1 on activated T cells happens at the effector phase of the immune response, which occurs after T cells migrate to the site of inflammation (193). It has been shown that effector T cells, which migrate into non-lymphoid tissues, are able to produce cytokines but cannot proliferate (198). The PD-1/PD-L1 pathway permits effector T cells to perform primary functions such as cytokine secretion but inhibits proliferation to prevent excessive immune reaction and autoreactivity in peripheral tissues (193). The whole mechanism of how the PD-1 /PD-L1 pathway regulates homeostasis of the immune system is not entirely apparent. The question of whether the PD-1/ PD-L pathway is costimulatory or inhibitory is currently a subject of debate. The mechanism of PD-L1 interaction in cell-mediated responses depends upon the phase of T cells. PD-L1 can stimulate naïve or resting T cells, whereas it can induce tolerance, anergy of T cells, or apoptosis of activated T cells in peripheral tissues (189, 195). By this means, PD-L1 can prevent inadvertent activation of self-reactive T cells and protect peripheral tissues from antigen specific destruction by either inducing anergy at their priming stage or delivering death signals to effector T cells through the PD-L1 receptor or PD-1 (195).

Studies on interactions of endothelial cells and T cells show that blockade of PD-L1 on endothelial cells using the PD-L1-Fc fusion protein allows PHA-stimulated naïve T cells to secrete high levels of IL-2 and IFN-γ (194, 199) and to increase the cytolytic activity of CD8$^+$ T cells in response to endothelial peptide antigen presentation (194). IFN-γ-induced upregulation of PD-L1 on macrophages and mouse endothelial cells depends on activation through TRL-4 and STAT-1 (194). Therefore, enhancement of PD-L1 expression on lymphocytes by increased levels of Th1 cytokines may be one of the responsible pathways in turning off Th1 type responses. On the other hand, purified T cells (CD4 or CD8) cultured in suboptimal doses of anti-CD3 antibody (TCR signaling) and a constant amount of PD-L2-Ig fusion protein have higher T cell proliferative responses compared to costimulation via B7-1 alone (191). PD-L2 costimulation in the presence of suboptimal TCR signaling also results in increased IFN-γ and repressed IL-4 and IL-10 protein levels. These results suggest that PD-L2 can drive cells into a Th1 response in the presence of low antigenic TCR stimulation (191). At higher optimal concentrations of anti-CD3 antibody increased PD-1 expression appears to inhibit T cell activation and induce apoptosis (191). The intensity of antigen stimulation through the TCR is important in the determination of the outcome of T cell responses. At low antigen concentrations, PD-L2/PD-1 costimulation inhibits strong costimulation through CD28/B7. As the concentration of the antigen increases, interaction of PD-L2 with PD-1 is no longer able to inhibit CD4$^+$ T cell proliferation, but partially suppresses cytokine production (190). In the presence of low antigenic stimulation, PD-1 engagement with PD-L2 inhibits IL-2, but does not drastically affect mRNA expression of IL-4 and IL-13, which implies that Th2 cytokines, unlike Th1 cytokines, may not be as sensitive to the PD-1 pathway (190).

PD-L2 knockout mice do not show signs of autoimmunity for up to one year of age. The phenotypes of the spleen, thymus, and lymph nodes, as well as cell numbers, are normal in the knockout mice. The bone marrow dendritic cells isolated from PD-L2 deficient mice cultured with unstimulated CD4$^+$ T cells with different concentrations of $OVA_{323\text{-}339}$ peptide show decreased proliferation and IFN-γ levels compared to wild-type BMDC

cultures (200). PD-L2 also potentiates dendritic cell function by increasing MHC complexes and IL-12p70 secretion, an important Th1 stimulating cytokine (201). Th2 type cytokines can increase PD-L2 expression on lymphocytes. The expression of PD-L2 on macrophages is enhanced by IL-4, which depends on activation via IL-4Rα and STAT-4 (197).

PD-L1 and PD-L2 bind to PD-1 and it was proposed that they fulfill their actions through PD-1 (182). The addition of PD-L1 results in inhibition of T cell proliferation and IFN-γ production in the presence of optimal and submitogenic stimulation with anti-CD3 antibodies (182). PD-1 knockout mice demonstrate splenomegaly and increased cellularity of both lymphoid and myeloid cells (202). Functions of T cells from PD-1 knockout mice are not blocked by PD-L1 demonstrating that PD-1 is important in delivering the inhibitory signals of PD-L1 to T cells (203).

The PD-1/ PD-L Pathway, B cells, and Autoimmunity

PD-1 is expressed on the surface of pro-B cells and may contribute to the differentiation of naïve B cells (204). Activated B cells also express PD-1 on their surface. The PD-1/PD-L pathway suppresses T cell function as well as B cell function. In the absence of PD-1, B cells produce increased levels of IgG2b, IgA, and IgG3 as detected in the sera of PD-1 knockout mice (202). IgG3 antibodies, also called nephritogenic antibodies, are deposited in kidneys and lead to autoimmune diseases such as lupus in MRL/lpr mice (205). CD5 expression, a negative regulator for B cell receptor (BCR)-mediated signaling in B-1 cells (206), is decreased on Mac-1^{+}B-1 peritoneal cells from PD-1 knockout mice suggesting a suppressive effect of PD-1 on the effector functions of B-1 cells (202). The suppressive effect of PD-1 on BCR-mediated signaling occurs as a result of coligation of receptors on B cells and by recruiting the scr homology 2-domain-containing tyrosine phosphatase-2 to phosphotyrosine (207). PD-1 knockout mice show a tendency to develop diverse symptoms of autoimmune diseases in different strains of mice. For example, PD-1 knockout mice with a C57BL/6 background develop lupus-like arthritis, glomerulonephritis, splenomegaly with increased numbers of B cells and myeloid cells,

increased serum IgG3, IgG2b, and IgA, as well as augmented B cell responses (203), while PD-1 deficient mice with a BALB/c background demonstrate auto-antibody-mediated cardiomyopathy with increased auto-antibody levels to cardiac proteins (207, 208).

In our previous studies, we have shown that blocking the interactions of CD28 on T cells with B7.1/B7.2 on APCs with a CTLA-4Ig fusion protein markedly downregulated the levels of IFN-γ and IFN-γ inducible iNOS/nitric oxide. This implies that the interactions of CD28 with B7.1/B7.2 molecules have a positive effect on IFN-γ and the IFN-γ-inducible iNOS gene. More recently, additional new costimulatory molecules have been identified that have been shown to influence IFN-γ and other aspects of the immune system. In an effort to understand more about the immunomodulatory effects of estrogen on the activation of splenic lymphocytes and the production of IFN-γ, we explored the PD-1/PD-L1-PD-L2 pathway. PD-1 and its ligands, PD-L1 and PD-L2, can determine the fate of cells of the immune system by affecting proliferation and differentiation, cell cycle arrest, cell death, or autoimmunity.

c.) MATERIALS AND METHODS

Mice: Three-to-four week old C57BL/6 wild-type male mice were obtained from Charles River Laboratories and maintained as described in first section of this chapter in accordance with the Virginia Polytechnic Institute and State University Institutional Animal Care guidelines.

Estrogen Treatment: Four-to-five week old male mice were orchiectomized and given placebo (control) or 17-β estradiol implants as explained previously in the first section of this chapter.

Isolation and Culture of Splenic Lymphocytes Splenic lymphocytes were collected and cultured with Con-A or left unstimulated in media as explained in the first section of this chapter.

Detection of PD-1, PD-L1, and PD-L2 Expression: For flow cytometric analysis, freshly isolated or cells that were cultured with or without Con-A (10 μg/ml) for 24 hrs were stained with appropriate monoclonal antibodies (mAbs) and analyzed by flow cytometry according to previously reported procedures (73, 75). The following mAbs were used in this study: fluorescein isothiocynate (FITC)- or phycoerythrin (PE)-conjugated anti-Thy1.2 (CD90.2, clone 53-21), anti-CD45RB (B220) (clone RA3-6B2) (BDPharmingen, San Diego, CA), anti-PD-1 (clone J43), anti-PD-L1 (B7-H1, clone MIH-5), and anti-PD-L2 (B7-H1, clone MIH-5) (eBioscience Inc. San Diego, CA). One-hundred microliters of 5 x10^6 cells/ml were plated in 96-well round bottom tissue culture plates (Corning, NY). For the detection of cell surface markers on fresh cells or lymphocytes, which were cultured with or without Con-A for 24 hrs, 100 μl of fluorochrome-conjugated monoclonal antibody in PBS were added to each well, followed by incubation at 4 C in the dark for 30 minutes. The samples were washed with PBS followed by analysis on an EPICS XL-MXL flow cytometer (Coulter, Hialeah, FL). Forward scatter (FS) and side scatter (SS) analysis was also included for both splenic lymphocytes in order to visualize various lymphocyte subsets. The data were represented as means of relative percent expression of the surface markers.

Statistics: Data were analyzed using SAS software (SAS Institute Inc., Cary, NC). The analysis method was described earlier in this chapter. P-values less than 0.05 were considered to be significant. Data are represented as means and standard error of means.

d.) RESULTS

PD-1 expression is decreased on B cells, increased on macrophages, but not altered on T cells from estrogen-treated mice:

The PD-1 expression showed a tendency to increase on whole unstimulated and Con-A-activated splenocytes from estrogen-treated mice when compared to placebo-treated mice (**Figure 1, Panel A**). Expression of PD-1 was significantly decreased on B cells (CD45

B220$^+$ cells) from unstimulated (p<0.001) and Con-A (p<0.01) stimulated whole splenic lymphocyte cultures from estrogen-treated mice (**Figure 1, Panel B**). Activation of splenic lymphocytes with Con-A led to an increase in the expression of PD-1 on B cells from placebo (p<0.001) or estrogen-treated mice (p<0.001) when compared to unstimulated cells. Although estrogen treatment did not alter PD-1 expression on T cells (Thy1.2$^+$ cells) from unstimulated or Con-A activated whole lymphocytes (**Figure 1, Panel C**), PD-1 expression was noticeably increased on unstimulated (p<0.001) and Con-A activated (p<0.01) predominantly macrophages (CD11b$^+$ cells) from estrogen-treated mice (**Figure 1, Panel D**) after 24 hrs of culture.

Estrogen treatment alters PD-L1 expression on B cells and macrophages but not on T cells:

We next examined the expression of PD-L1 (B7-H1) on macrophages, B, and T cells, an inhibitory molecule for T cell activation, which is able to downregulate T cell activation and functions though PD-1 (182) from whole splenic lymphocytes stimulated with or without Con-A for 24 hrs. Although there was no marked difference in PD-L1 expression on whole unstimulated and Con-A-activated splenocytes from estrogen-treated mice when compared to placebo-treated mice, the Con-A activation significantly increased the PD-L1 expression on splenocytes when compared to stimulated cells (media) (p<0.001; **Figure 2, Panel A**). As shown in **Figure 2, Panel B**, PD-L1 expression was decreased on unstimulated B cells (CD45 B220$^+$ cells, media, p<0.05) and has a tendency to decline on B cells after Con-A stimulation of splenocyte culture from estrogen-treated mice. Activation with Con-A elevated the expression of PD-L1 on B cells from placebo (p<0.01) and estrogen-treated mice (p<0.001) in comparison to the expression observed in unstimulated cells. T cells (Thy1.2$^+$ cells) from unstimulated or Con-A activated splenocytes from estrogen-treated mice did not exhibit a notable change in PD-L1 expression as compared to control mice (**Figure 2, Panel C**). Estrogen treatment clearly increased PD-L1 expression on predominantly macrophages (CD11b$^+$ cells) stimulated with (p<0.001) or without Con-A (p<0.01) (**Figure 2, Panel D**).

Expression of PD-L2 is upregulated on macrophages from estrogen-treated mice:

The expression of PD-L2 on macrophages from whole splenocytes stimulated with or without Con-A from placebo and estrogen-treated mice was next investigated. PD-L2 expression was markedly upregulated in Con-A activated splenocytes from estrogen-treated mice ($p<0.01$, **Figure 3, Panel A**). As shown in **Figure 3, Panel B** estrogen treatment upregulated PD-L2 expression on unstimulated predominantly macrophages ($p<0.05$) as well as Con-A-stimulated macrophages ($p<0.001$) detected as $CD11b^+$ cells from whole splenic lymphocyte cultures.

e.) DISCUSSION

Although existing data have shown that estrogen regulates the immune system influencing all the major cells such as T, B, macrophages, and dendritic cells (209-213), the specific immunological effects of estrogen treatment especially in reference to the expression of the costimulatory molecules are yet studied. The present study focused on whether estrogen regulates the expression of costimulatory molecules, PD-1, PD-L1, and PD-L2.

The complete activation of lymphocytes requires both antigen-specific signals, delivered via the T cell receptor (TCR), known as the “primary signal”, and classical costimulatiory molecules such as the well known CD-28/B7.1 (CD80), B7.2 (CD86) molecules, which deliver the “secondary signal” to both T and antigen presenting cells, (173). After activation of naïve T cells, activated and effector T cells can migrate to the sites of inflammation and activate major inflammatory cell macrophages resulting in innate immunity. The over activation of macrophages in the peripheral tissues by activated T cells can have serious consequences for the system. Therefore, costimulatory molecules such as the recently identified PD-1/PD-L1/L2 molecules are important in downregulation of immune responses to maintain immune homeostasis.

PD-1, which is expressed by activated B and T cells, myeloid cells, and low levels of NK cells, is classified as an inhibitory costimulatory molecule because its absence is associated with autoimmune diseases such as lupus like disease and arthritis in C57BL/6 mice (202, 203) or autoantibody mediated dilated cardiomyopathy in Balb/c mice (214). Recent investigations show that the PD-1/PD-L pathway has an important role in the development and/or regulation of autoimmune diseases. For example in NOD mice, a model for spontaneous autoimmune diabetes, blocking of PD-1 and PD-L1, but not PD-L2, suppresses symptoms in female prediabetic NOD mice. In the male NOD mice, inhibition of PD-1/PD-Ll signaling initiates early onset of insulitis with increased levels of IFN-γ secretion from splenic lymphocytes (215). The blockade of PD-L2 does not affect the progress of disease in male NOD mice. PD-1 blockade in mice with experimental autoimmune encephalomyelitis (EAE), which is largely a Th1-mediated disease in which IL-12 and IL-23 play crucial roles in development and maintenance of disease (216), resulted in accelerated and severe disease associated with increased lymphocyte infiltration into the central nervous system. C57BL/6 mice with MOG (myelin oligodendrocyte glycoprotein)-induced EAE develop severe EAE symptoms accompanied with increased PD-1 and PD-L1 expression but not PD-L2. The blocking of PD-1 and PD-L2, but not PD-L1, results in augmentation of EAE with increased IFN-γ producing T cells and anti-MOG antibody levels (217).

Unlike in mice, in humans, the expression of PD-1 on $CD4^{+}$T cells is also elevated in synovial fluid (218) and the PD-1 gene is significantly associated with the onset of disease in patients with Rheumatoid Arthritis (RA) (219). $CD4^{+}PD\text{-}1^{+}$ T cells from RA patients also produce higher levels of IL-10 compared to controls (218). In human SLE (systemic lupus erythematosus) patients, one single-nucleotide polymorphism (SNP) in PD-1 is associated with the progress of SLE in Europeans and Mexicans. In addition, the expression of PD-1 is increased on T cells in SLE (220, 221). In other autoimmune diseases, such as Sjögren's syndrome (222), inflammatory disease ulcerative colitis, and Crohn's disease (223), the level of PD-1 expression in patients is higher than in controls.

Overall, the obstruction or deficiency of PD-1 or PD-Ligands results in autoimmunity or worsening of the disease followed by increased B cell activity and Ig production. The previous studies in our laboratory demonstrated that estrogen treatment increased IgG secretion, anti-cardiolipin antibody levels, and plasma cell differentiation when compared to placebo-treated mice (224-226). Our present data clearly show that estrogen treatment diminishes the expression of PD-1 and PD-L1 on B lymphocytes, which can lead to augmentation in immunoglobulin secretion from splenic lymphocytes. This study is the first to demonstrate the immunomodulatory effect of estrogen on PD-1 and PD-Ligand molecules.

Loke and Allison found that increased PD-L1 expression on peritoneal macrophages correlated with increased levels of nitric oxide secretion upon stimulation with LPS and IFN-γ (197). These macrophages can be primed with low doses of IFN-γ (227) suggesting that PD-L1 is a marker for primed macrophages. In this study, we found that PD-L1 expression was significantly increased on $CD11b^{+}$ cells, which constitue a significant percentage of macrophages from estrogen-treated mice. Thus, estrogen treatment can "prime" splenic macrophages via increased IFN-γ production which is followed by increased nitric oxide levels in the supernatants of Con-A stimulated splenocytes from estrogen-treated mice (to be submitted). Estrogen treatment also increased PD-L2 expression on splenic macrophages. The expression of PD-L2 was lower than the expression of PD-L1 on macrophages from estrogen treated mice. Previous data showed that PD-L2 is mostly expressed on macrophages activated by IL-4 in the presence of Th2 cells suggesting an alternative method of activation (197). Blockade of increased PD-L2 expression on DCs from OVA-induced allergic asthmatic mice with anti-PD-L2 antibodies results in increased Th2 type cytokines, IL-5 and IL-13, and decreased IFN-γ (228), which suggests the importance of PD-L2 in IFN-γ production and Th1 cell differentiation. On the other hand, treatment with anti-PD-L1 or anti-PD-1 antibodies does not alter these particular cytokines (228). We find that the expression of PD-L2 is increased on $CD11b^{+}$ cells from estrogen-treated mice.

Furthermore, the expression of PD-1 on both unstimulated and Con-A-stimulated macrophages from estrogen-treated mice was significantly increased compared to controls. This elevation in PD-1 expression can lead to increased binding of PD-L1 or PD-L2 expression on neighboring macrophages or on self-macrophages. Overall, higher PD-L expression is paralleled with increased macrophage activity.

The expression of the proteins, PD-1 and PD-L1, on unstimulated or Con-A activated T cells was not altered due to estrogen treatment. Some studies suggest that PD-L1 can inhibit T cell mediated immune responses and T cell proliferation (182, 185), on the other hand in the presence of suboptimal doses of anti-CD3 stimulation of T cells and immobilized PD-L1-Ig fusion protein, supernatants from these cultures demonstrated an increase in IL-10 and IFN-γ secretion (189) and proliferation (229). Studies involving PD-L1 or PD-L2 and their fusion proteins demonstrated to regulate T cell activation; then again reports on whether they up or downregulate T cell cytokine synthesis are differing. This could be due to different stimulations of the cells, different cell types or stages of cells, or even culturing of cells in the presence of stable plate-coated or soluble fusion proteins. One other theory about the discrepancy between the data may be linked to PD-L stimulation of naïve T cells versus activated effector T cells. In our study, the expression of PD-1 and PD-L1 on unstimulated or Con-A activated T cells was not altered due to estrogen treatment. Wang et al have shown the presence of another receptor that binds PD-L1 and PD-L2 in the absence of PD-1, which maintains the costimulatory activity of T cells (230).

The studies performed using fusion proteins and conflicting data about the inhibitory or stimulatory role of PD-L1 and PD-L2 molecules suggest a possibility of another unknown and unnamed ligand. Since PD-L1 and PD-L2 bind to PD-1, the elevation of both PD-L1 and PD-L2 on macrophages from estrogen-treated mice may imply: **1**. PD-L1 predominantly binds to PD-1, bypasses the effect of PD-L2, and induces Th1 responses (197), **2**. IFN-γ-primed (due to estrogen treatment) or estrogen-primed macrophages from estrogen-treated mice can express PD-L1 earlier than PD-L2, **3**. Increased levels of IFN-γ can induce macrophages to express PD-L2 to promote the

differentiation into Th2 cells. **4**. The presence of this new unnamed receptor can be expressed on T cells from estrogen-treated mice and deliver the costimulatory signals from PD-Ligands on antigen presenting cells.

We have recently shown that splenic lymphocytes from estrogen-treated mice generate higher levels of IFN-γ, and especially macrophage derived IFN-γ inducible nitric oxide, iNOS, and Cox-2 when compared to that from placebo-treated control mice implying increased macrophage activity. The number of macrophages was increased in splenic lymphocyte cultures from estrogen-treated mice compared to controls.

We hypothesize that increased PD-1 expression helps to induce the secretion of Th1 cytokines such as IFN-γ, which in turn upregulate the expression of PD-L1. Increased PD-L1 bound to PD-1 results in diminished levels of IFN-γ and upregulation of Th2 cytokines, such as IL-4. IL-4 stimulates the expression of PD-L2 on antigen presenting cells, that upregulates PD-1. Further studies may be beneficial to investigate this complex loop of events. Therefore, it will be useful to explore whether: **1.** naïve T cells will behave differently compared to activated T cells due to PD-L1 expression after estrogen treatment, **2.** PD-L2 Ig fusion protein will behave like a stimulant in the presence of submitogenic TCR stimulation via anti-CD3 antibodies, which may result in increased IFN-γ and/or nitric oxide levels in cultures from estrogen-treated mice, **3.** Addition of PD-L1Ig fusion protein may alter the levels of IFN-γ secretion from estrogen-treated mice compared to cultures from control mice.

Overall, our results propose the presence of an alternate signaling pathway between PD-1, PD-L1, PD-L2, and raise the possibility of a new PD-Ligand binding costimulatory molecule for induction of Th1 responses and IFN-γ as a response to estrogen treatment. Estrogen has been related to many IFN-γ related autoimmune diseases and inflammatory disorders (231-235). Therefore, these new observations may have profound repercussions in the therapeutic manipulation of several diseases and transplantation due to the importance of costimulatory molecules.

In summary, PD/PD-L costimulation of T, antigen presenting cells and whole splenocytes due to estrogen exposure may be important not only for homeostasis of the immune system but for autoimmune and inflammatory diseases as well.

g.) FIGURES

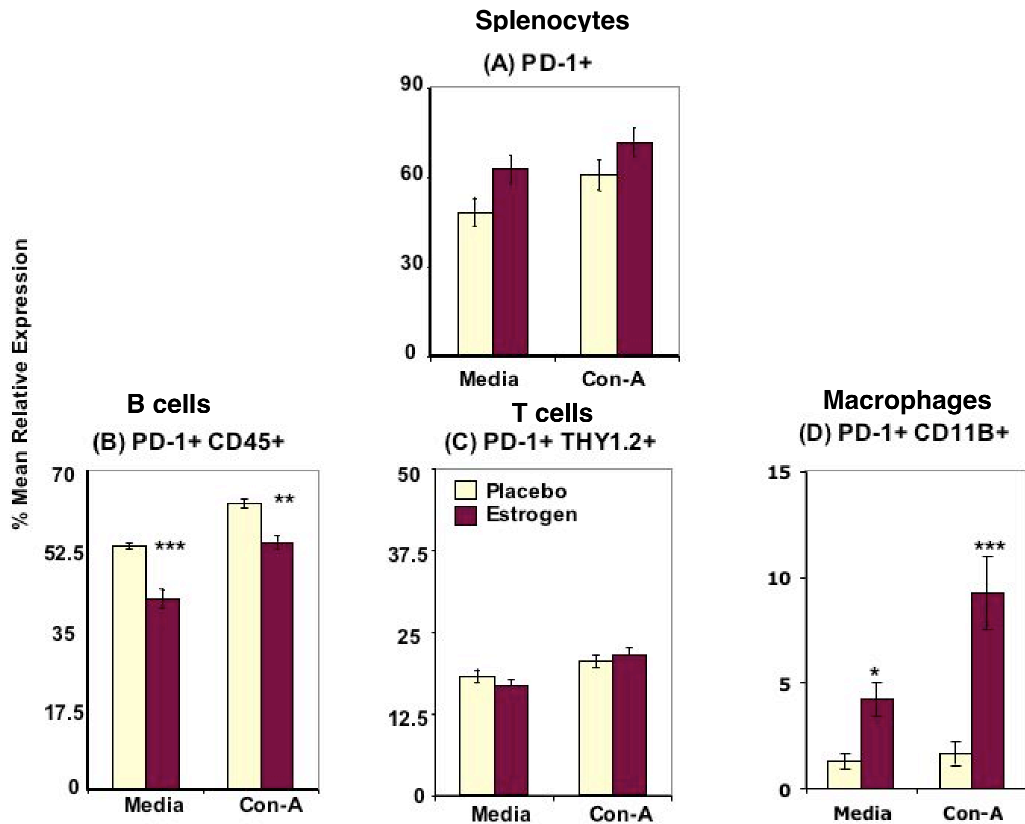


Figure 1. Estrogen alters expression of PD-1 on B cells and macrophages, but not on T cells. Splenic lymphocytes from placebo and estrogen-treated mice were cultured with or without Con-A (10 μg/ml) for 24hrs and subjected to FACS analysis with PD-1 mAb and mAb to CD45 B220 (B cells), Thy1.2 (T cells) and CD11b (predominantly macrophages). Panel A shows the whole expression of PD-1 on splenocytes (n=11 mice per placebo or estrogen treatment). The numbers indicate the percentage of PD-1 and CD45B220 (**Panel B**), Thy1.2 (**Panel C**), or CD11b (**Panel D**) double positive cells. Panel B shows that PD-1 expression on B cells was decreased on unstimulated and Con-A activated splenocytes from estrogen treated mice compared to controls (Media***$p<0.001$; Con-A **$p<0.01$; n=12 mice per placebo or estrogen treatment). Panel C shows PD-1 expression on T cells in unstimulated and Con-A activated splenocytes from placebo and estrogen treated mice (n=11 mice per placebo or estrogen treatment). Panel D depicts increased PD-1 expression on macrophages from unstimulated and Con-A activated splenocytes from estrogen treated mice compared to controls (media *$p<0.05$, Con-A ***$p<0.001$**;** n=4 mice per placebo or estrogen treatment). Data are presented as means with standard error bars.

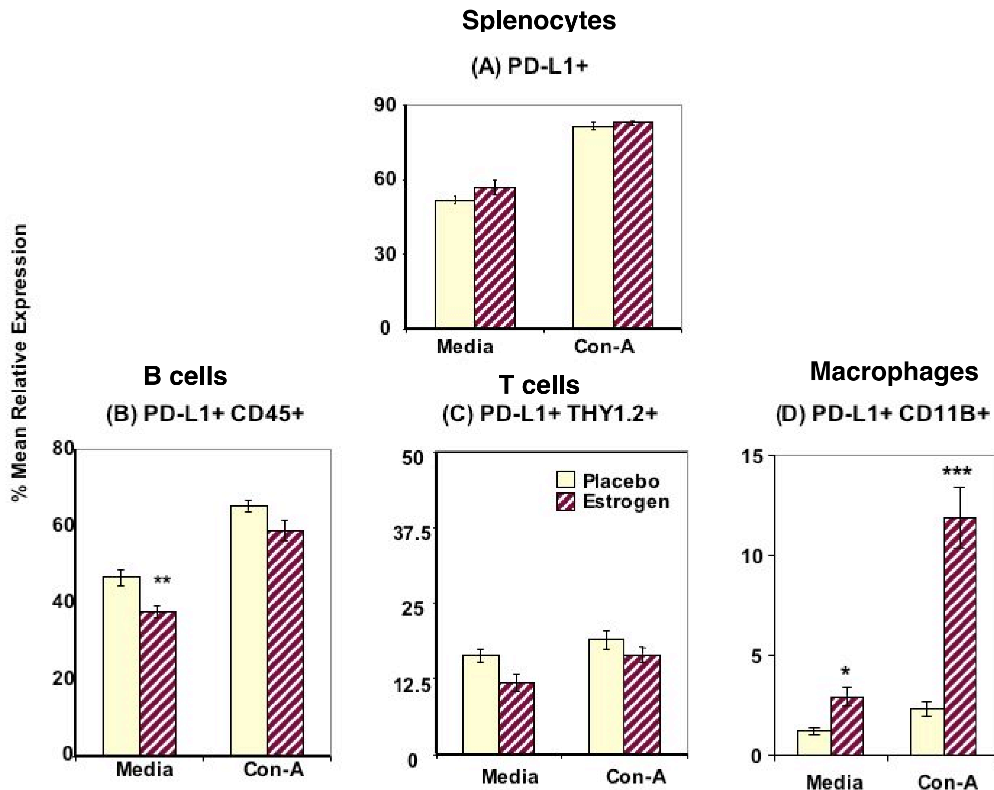


Figure 2. Estrogen alters expression of PD-L1 on B cells and macrophages but not on T cells. Splenic lymphocytes from placebo and estrogen-treated mice were cultured with or without Con-A (10 µg/ml) for 24hrs and subjected to FACS analysis with PD-L1 mAb and mAb to CD45B220 (B cells), Thy1.2 (T cells) and CD11b (predominantly macrophages). Panel A shows the whole expression of PD-L1 on splenocytes (n=11 mice per placebo or estrogen treatment). The numbers indicate the percentage of PD-L1 and CD45B220 (**Panel B**), Thy1.2 (**Panel C**), or CD11b (**Panel D**) double positive cells. Panel B that shows PD-1 expression on B cells was decreased from unstimulated but not from Con-A activated splenocytes from estrogen treated mice compared to controls (Media**p<0.01; n=12 mice per placebo or estrogen treatment). Panel C shows that PD-L1 expression on T cells in unstimulated and Con-A activated splenocytes from placebo and estrogen treated mice (n=11 mice per placebo or estrogen treatment). Panel D depicts increased PD-L1 expression on predominantly macrophages from Con-A-activated splenocytes from estrogen-treated mice compared to controls (media not significant, Con-A ***p<0.001; n=4 mice per placebo or estrogen treatment). Data are presented as means with standard error bars.

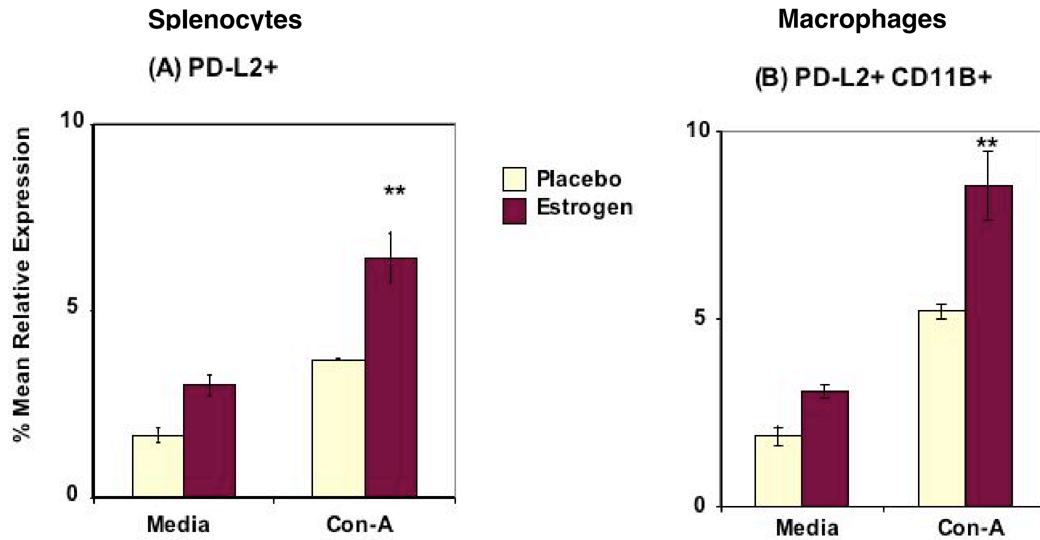


Figure 3. Estrogen upregulates PD-L2 expression on macrophages. Splenic lymphocytes from placebo and estrogen-treated mice were cultured with or without Con-A (10 μg/ml) for 24 hrs and subjected to FACS analysis with PD-1 mAb and CD11b (predominantly macrophages) mAb. Panel A shows the whole expression of PD-L1 on splenocytes (n=4 mice per placebo or estrogen treatment). As demonstrated in Panel B, estrogen treatment noticeably increased PD-L2 expression on macrophages from Con-A activated splenocytes when compared to controls (Con-A ***p*<0.01; n=4 mice per placebo or estrogen treatment). Data are presented as means with standard error bars.

g.) REFERENCES

1. Dinarello, C. A., and G. Fantuzzi. 2003. Interleukin-18 and host defense against infection. *J Infect Dis 187 Suppl 2:S370*.
2. Pflanz, S., J. C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W. M. Blumenschein, J. D. Mattson, J. L. Wagner, W. To, S. Zurawski, T. K. McClanahan, D. M. Gorman, J. F. Bazan, R. de Waal Malefyt, D. Rennick, and R. A. Kastelein. 2002. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity 16:779*.
3. Chen, Q., N. Ghilardi, H. Wang, T. Baker, M. H. Xie, A. Gurney, I. S. Grewal, and F. J. de Sauvage. 2000. Development of Th1-type immune responses requires the type I cytokine receptor TCCR. *Nature 407:916*.
4. Yoshida, H., S. Hamano, G. Senaldi, T. Covey, R. Faggioni, S. Mu, M. Xia, A. C. Wakeham, H. Nishina, J. Potter, C. J. Saris, and T. W. Mak. 2001. WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection. *Immunity 15:569*.
5. Smits, H. H., A. J. van Beelen, C. Hessle, R. Westland, E. de Jong, E. Soeteman, A. Wold, E. A. Wierenga, and M. L. Kapsenberg. 2004. Commensal Gram-negative bacteria prime human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development. *Eur J Immunol 34:1371*.
6. Pflanz, S., L. Hibbert, J. Mattson, R. Rosales, E. Vaisberg, J. F. Bazan, J. H. Phillips, T. K. McClanahan, R. de Waal Malefyt, and R. A. Kastelein. 2004. WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27. *J Immunol 172:2225*.
7. Lucas, S., N. Ghilardi, J. Li, and F. J. de Sauvage. 2003. IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. *Proc Natl Acad Sci U S A 100:15047*.
8. Hibbert, L., S. Pflanz, R. De Waal Malefyt, and R. A. Kastelein. 2003. IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells. *J Interferon Cytokine Res 23:513*.

9. Takeda, A., S. Hamano, A. Yamanaka, T. Hanada, T. Ishibashi, T. W. Mak, A. Yoshimura, and H. Yoshida. 2003. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. *J Immunol 170:4886*.
10. Yoshimoto, T., K. Okada, N. Morishima, S. Kamiya, T. Owaki, M. Asakawa, Y. Iwakura, F. Fukai, and J. Mizuguchi. 2004. Induction of IgG2a class switching in B cells by IL-27. *J Immunol 173:2479*.
11. Trinchieri, G., and P. Scott. 1999. Interleukin-12: basic principles and clinical applications. *Curr Top Microbiol Immunol 238:57*.
12. Schulz, O., A. D. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher, and C. Reis e Sousa. 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity 13:453*.
13. Reis e Sousa, C., A. D. Edwards, S. P. Manickasingham, and O. Schulz. 2001. Conditioning of dendritic cells by pathogen-derived stimuli. *Immunobiology 204:595*.
14. D'Andrea, A., M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young, and E. Nickbarg. 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J Exp Med 176:1387*.
15. Robinson, D., K. Shibuya, A. Mui, F. Zonin, E. Murphy, T. Sana, S. B. Hartley, S. Menon, R. Kastelein, F. Bazan, and A. O'Garra. 1997. IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity 7:571*.
16. Micallef, M. J., T. Ohtsuki, K. Kohno, F. Tanabe, S. Ushio, M. Namba, T. Tanimoto, K. Torigoe, M. Fujii, M. Ikeda, S. Fukuda, and M. Kurimoto. 1996. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur J Immunol 26:1647*.

17. Dickensheets, H. L., S. L. Freeman, and R. P. Donnelly. 2000. Interleukin-12 differentially regulates expression of IFN-gamma and interleukin-2 in human T lymphoblasts. *J Interferon Cytokine Res 20:897*.

18. Lawless, V. A., S. Zhang, O. N. Ozes, H. A. Bruns, I. Oldham, T. Hoey, M. J. Grusby, and M. H. Kaplan. 2000. Stat4 regulates multiple components of IFN-gamma-inducing signaling pathways. *J Immunol 165:6803*.

19. Yoshimoto, T., H. Okamura, Y. I. Tagawa, Y. Iwakura, and K. Nakanishi. 1997. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells. *Proc Natl Acad Sci U S A 94:3948*.

20. Yoshida, A., Y. Koide, M. Uchijima, and T. O. Yoshida. 1994. IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. *Biochem Biophys Res Commun 198:857*.

21. Frucht, D. M., T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea, and S. Koyasu. 2001. IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends Immunol 22:556*.

22. Fukao, T., D. M. Frucht, G. Yap, M. Gadina, J. J. O'Shea, and S. Koyasu. 2001. Inducible expression of Stat4 in dendritic cells and macrophages and its critical role in innate and adaptive immune responses. *J Immunol 166:4446*.

23. Sinigaglia, F., D. D'Ambrosio, P. Panina-Bordignon, and L. Rogge. 1999. Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function. *Immunol Rev 170:65*.

24. Wu, C., X. Wang, M. Gadina, J. J. O'Shea, D. H. Presky, and J. Magram. 2000. IL-12 receptor beta 2 (IL-12R beta 2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J Immunol 165:6221*.

25. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol 3:133*.

26. Thibodeaux, D. K., S. E. Hunter, K. E. Waldburger, J. L. Bliss, W. L. Trepicchio, J. P. Sypek, K. Dunussi-Joannopoulos, S. J. Goldman, and J. P. Leonard. 1999.

Autocrine regulation of IL-12 receptor expression is independent of secondary IFN-gamma secretion and not restricted to T and NK cells. *J Immunol 163:5257*.

27. Szabo, S. J., A. S. Dighe, U. Gubler, and K. M. Murphy. 1997. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med 185:817*.
28. Nguyen, K. B., W. T. Watford, R. Salomon, S. R. Hofmann, G. C. Pien, A. Morinobu, M. Gadina, J. J. O'Shea, and C. A. Biron. 2002. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science 297:2063*.
29. Park, W. R., M. Nakahira, N. Sugimoto, Y. Bian, Y. Yashiro-Ohtani, X. Y. Zhou, Y. F. Yang, T. Hamaoka, and H. Fujiwara. 2004. A mechanism underlying STAT4-mediated up-regulation of IFN-gamma induction inTCR-triggered T cells. *Int Immunol 16:295*.
30. Zhang, S., and M. H. Kaplan. 2000. The p38 mitogen-activated protein kinase is required for IL-12-induced IFN-gamma expression. *J Immunol 165:1374*.
31. Lund, R. J., Z. Chen, J. Scheinin, and R. Lahesmaa. 2004. Early target genes of IL-12 and STAT4 signaling in th cells. *J Immunol 172:6775*.
32. Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld, and J. N. Ihle. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature 382:171*.
33. Kaplan, M. H., Y. L. Sun, T. Hoey, and M. J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature 382:174*.
34. Puren, A. J., G. Fantuzzi, Y. Gu, M. S. Su, and C. A. Dinarello. 1998. Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14+ human blood mononuclear cells. *J Clin Invest 101:711*.
35. Puren, A. J., G. Fantuzzi, and C. A. Dinarello. 1999. Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in

human blood mononuclear cells and mouse spleen cells. *Proc Natl Acad Sci U S A 96:2256*.

36. Stoll, S., G. Muller, M. Kurimoto, J. Saloga, T. Tanimoto, H. Yamauchi, H. Okamura, J. Knop, and A. H. Enk. 1997. Production of IL-18 (IFN-gamma-inducing factor) messenger RNA and functional protein by murine keratinocytes. *J Immunol 159:298*.
37. Hyodo, Y., K. Matsui, N. Hayashi, H. Tsutsui, S. Kashiwamura, H. Yamauchi, K. Hiroishi, K. Takeda, Y. Tagawa, Y. Iwakura, N. Kayagaki, M. Kurimoto, H. Okamura, T. Hada, H. Yagita, S. Akira, K. Nakanishi, and K. Higashino. 1999. IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor. *J Immunol 162:1662*.
38. Tsutsui, H., K. Matsui, N. Kawada, Y. Hyodo, N. Hayashi, H. Okamura, K. Higashino, and K. Nakanishi. 1997. IL-18 accounts for both TNF-alpha- and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice. *J Immunol 159:3961*.
39. Okamoto, I., K. Kohno, T. Tanimoto, H. Ikegami, and M. Kurimoto. 1999. Development of CD8+ effector T cells is differentially regulated by IL-18 and IL-12. *J Immunol 162:3202*.
40. Dao, T., K. Ohashi, T. Kayano, M. Kurimoto, and H. Okamura. 1996. Interferon-gamma-inducing factor, a novel cytokine, enhances Fas ligand-mediated cytotoxicity of murine T helper 1 cells. *Cell Immunol 173:230*.
41. Nakanishi, K., T. Yoshimoto, H. Tsutsui, and H. Okamura. 2001. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev 12:53*.
42. Nakanishi, K., T. Yoshimoto, H. Tsutsui, and H. Okamura. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol 19:423*.
43. Chang, J. T., B. M. Segal, K. Nakanishi, H. Okamura, and E. M. Shevach. 2000. The costimulatory effect of IL-18 on the induction of antigen-specific IFN-gamma production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor beta2 subunit. *Eur J Immunol 30:1113*.

44. Son, Y. I., R. M. Dallal, R. B. Mailliard, S. Egawa, Z. L. Jonak, and M. T. Lotze. 2001. Interleukin-18 (IL-18) synergizes with IL-2 to enhance cytotoxicity, interferon-gamma production, and expansion of natural killer cells. *Cancer Res 61:884*.
45. Fantuzzi, L., P. Puddu, B. Varano, M. Del Corno, F. Belardelli, and S. Gessani. 2000. IFN-alpha and IL-18 exert opposite regulatory effects on the IL-12 receptor expression and IL-12-induced IFN-gamma production in mouse macrophages: novel pathways in the regulation of the inflammatory response of macrophages. *J Leukoc Biol 68:707*.
46. Yoshimoto, T., K. Takeda, T. Tanaka, K. Ohkusu, S. Kashiwamura, H. Okamura, S. Akira, and K. Nakanishi. 1998. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J Immunol 161:3400*.
47. Sareneva, T., I. Julkunen, and S. Matikainen. 2000. IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J Immunol 165:1933*.
48. Neumann, D., and M. U. Martin. 2001. Interleukin-12 upregulates the IL-18Rbeta chain in BALB/c thymocytes. *J Interferon Cytokine Res 21:635*.
49. Smeltz, R. B., J. Chen, J. Hu-Li, and E. M. Shevach. 2001. Regulation of interleukin (IL)-18 receptor alpha chain expression on CD4(+) T cells during T helper (Th)1/Th2 differentiation. Critical downregulatory role of IL-4. *J Exp Med 194:143*.
50. Xu, D., W. L. Chan, B. P. Leung, D. Hunter, K. Schulz, R. W. Carter, I. B. McInnes, J. H. Robinson, and F. Y. Liew. 1998. Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *J Exp Med 188:1485*.
51. Smeltz, R. B., J. Chen, R. Ehrhardt, and E. M. Shevach. 2002. Role of IFN-gamma in Th1 differentiation: IFN-gamma regulates IL-18R alpha expression by preventing the negative effects of IL-4 and by inducing/maintaining IL-12 receptor beta 2 expression. *J Immunol 168:6165*.

52. Kim, S. H., L. L. Reznikov, R. J. Stuyt, C. H. Selzman, G. Fantuzzi, T. Hoshino, H. A. Young, and C. A. Dinarello. 2001. Functional reconstitution and regulation of IL-18 activity by the IL-18R beta chain. *J Immunol 166:148*.
53. Thomas, J. A., J. L. Allen, M. Tsen, T. Dubnicoff, J. Danao, X. C. Liao, Z. Cao, and S. A. Wasserman. 1999. Impaired cytokine signaling in mice lacking the IL-1 receptor-associated kinase. *J Immunol 163:978*.
54. Hoshino, K., H. Tsutsui, T. Kawai, K. Takeda, K. Nakanishi, Y. Takeda, and S. Akira. 1999. Cutting edge: generation of IL-18 receptor-deficient mice: evidence for IL-1 receptor-related protein as an essential IL-18 binding receptor. *J Immunol 162:5041*.
55. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity 9:143*.
56. Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol 12:141*.
57. Schindler, H., M. B. Lutz, M. Rollinghoff, and C. Bogdan. 2001. The production of IFN-gamma by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *J Immunol 166:3075*.
58. Munder, M., M. Mallo, K. Eichmann, and M. Modolell. 2001. Direct stimulation of macrophages by IL-12 and IL-18 - a bridge built on solid ground. *Immunol Lett 75:159*.
59. Munder, M., M. Mallo, K. Eichmann, and M. Modolell. 1998. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *J Exp Med 187:2103*.
60. Ohteki, T. 2002. Critical role for IL-15 in innate immunity. *Curr Mol Med 2:371*.
61. Waldmann, T. A., and Y. Tagaya. 1999. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol 17:19*.
62. Weng, N. P., K. Liu, M. Catalfamo, Y. Li, and P. A. Henkart. 2002. IL-15 is a growth factor and an activator of CD8 memory T cells. *Ann N Y Acad Sci 975:46*.

63. Doherty, T. M., R. A. Seder, and A. Sher. 1996. Induction and regulation of IL-15 expression in murine macrophages. *J Immunol 156:735*.

64. Vankayalapati, R., P. Klucar, B. Wizel, S. E. Weis, B. Samten, H. Safi, H. Shams, and P. F. Barnes. 2004. NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen. *J Immunol 172:130*.

65. Tagaya, Y., J. D. Burton, Y. Miyamoto, and T. A. Waldmann. 1996. Identification of a novel receptor/signal transduction pathway for IL-15/T in mast cells. *Embo J 15:4928*.

66. Seder, R. A. 1996. High-dose IL-2 and IL-15 enhance the in vitro priming of naive CD4+ T cells for IFN-gamma but have differential effects on priming for IL-4. *J Immunol 156:2413*.

67. Avice, M. N., C. E. Demeure, G. Delespesse, M. Rubio, M. Armant, and M. Sarfati. 1998. IL-15 promotes IL-12 production by human monocytes via T cell-dependent contact and may contribute to IL-12-mediated IFN-gamma secretion by CD4+ T cells in the absence of TCR ligation. *J Immunol 161:3408*.

68. Matthys, P., H. Dooms, P. Rottiers, T. Mitera, L. Overgergh, G. Leclercq, A. Billiau, and J. Grooten. 2002. Induction of IL-15 by TCR/CD3 aggregation depends on IFN-gamma and protects against apoptosis of immature thymocytes in vivo. *Clin Exp Immunol 130:379*.

69. Borger, P., H. F. Kauffman, D. S. Postma, M. T. Esselink, and E. Vellenga. 1999. Interleukin-15 differentially enhances the expression of interferon-gamma and interleukin-4 in activated human (CD4+) T lymphocytes. *Immunology 96:207*.

70. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol 162:4511*.

71. Strengell, M., T. Sareneva, D. Foster, I. Julkunen, and S. Matikainen. 2002. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. *J Immunol 169:3600*.

72. Strengell, M., S. Matikainen, J. Siren, A. Lehtonen, D. Foster, I. Julkunen, and T. Sareneva. 2003. IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. *J Immunol 170:5464*.

73. Ansar Ahmed, S., M. J. Dauphinee, A. I. Montoya, and N. Talal. 1989. Estrogen induces normal murine CD5+ B cells to produce autoantibodies. *J Immunol 142:2647*.

74. Ansar Ahmed, S., and D. Verthelyi. 1993. Antibodies to cardiolipin in normal C57BL/6J mice: induction by estrogen but not dihydrotestosterone. *J Autoimmun 6:265*.

75. Donner, K. J., K. M. Becker, B. D. Hissong, and S. A. Ahmed. 1999. Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. *Cytometry 35:80*.

76. Larousserie, F., S. Pflanz, A. Coulomb-L'Hermine, N. Brousse, R. Kastelein, and O. Devergne. 2004. Expression of IL-27 in human Th1-associated granulomatous diseases. *J Pathol 202:164*.

77. Carson, W. E., M. E. Ross, R. A. Baiocchi, M. J. Marien, N. Boiani, K. Grabstein, and M. A. Caligiuri. 1995. Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. *J Clin Invest 96:2578*.

78. Ye, W., J. D. Young, and C. C. Liu. 1996. Interleukin-15 induces the expression of mRNAs of cytolytic mediators and augments cytotoxic activities in primary murine lymphocytes. *Cell Immunol 174:54*.

79. Karpuzoglu-Sahin, E., B. D. Hissong, and S. Ansar Ahmed. 2001. Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. *J Reprod Immunol 52:113*.

80. Karpuzoglu-Sahin, E., Y. Zhi-Jun, A. Lengi, N. Sriranganathan, and S. Ansar Ahmed. 2001. Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. *Cytokine 14:208*.

81. Kamiya, S., T. Owaki, N. Morishima, F. Fukai, J. Mizuguchi, and T. Yoshimoto. 2004. An indispensable role for STAT1 in IL-27-induced T-bet expression but not proliferation of naive CD4+ T cells. *J Immunol 173:3871*.

82. Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C. Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty, and M. K. Gately. 1996. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity 4:471*.

83. Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler, and D. H. Presky. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol 16:495*.

84. Nakahira, M., H. J. Ahn, W. R. Park, P. Gao, M. Tomura, C. S. Park, T. Hamaoka, T. Ohta, M. Kurimoto, and H. Fujiwara. 2002. Synergy of IL-12 and IL-18 for IFN-gamma gene expression: IL-12-induced STAT4 contributes to IFN-gamma promoter activation by up-regulating the binding activity of IL-18-induced activator protein 1. *J Immunol 168:1146*.

85. Barbulescu, K., C. Becker, J. F. Schlaak, E. Schmitt, K. H. Meyer zum Buschenfelde, and M. F. Neurath. 1998. IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-gamma promoter in primary CD4+ T lymphocytes. *J Immunol 160:3642*.

86. Struhl, K. 1998. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev 12:599*.

87. Morinobu, A., Y. Kanno, and J. J. O'Shea. 2004. Discrete roles for histone acetylation in human T helper 1 cell-specific gene expression. *J Biol Chem 279:40640*.

88. Zhou, W., S. Chang, and T. M. Aune. 2004. Long-range histone acetylation of the Ifng gene is an essential feature of T cell differentiation. *Proc Natl Acad Sci U S A 101:2440*.

89. Brown, C. E., T. Lechner, L. Howe, and J. L. Workman. 2000. The many HATs of transcription coactivators. *Trends Biochem Sci 25:15*.

90. Sterner, D. E., and S. L. Berger. 2000. Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev 64:435*.

91. Matejuk, A., J. Dwyer, A. Zamora, A. A. Vandenbark, and H. Offner. 2002. Evaluation of the effects of 17beta-estradiol (17beta-e2) on gene expression in experimental autoimmune encephalomyelitis using DNA microarray. *Endocrinology 143:313*.

92. Torigoe, K., S. Ushio, T. Okura, S. Kobayashi, M. Taniai, T. Kunikata, T. Murakami, O. Sanou, H. Kojima, M. Fujii, T. Ohta, M. Ikeda, H. Ikegami, and M. Kurimoto. 1997. Purification and characterization of the human interleukin-18 receptor. *J Biol Chem 272:25737*.

93. Born, T. L., E. Thomassen, T. A. Bird, and J. E. Sims. 1998. Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. *J Biol Chem 273:29445*.

94. Neumann, D., E. Del Giudice, A. Ciaramella, D. Boraschi, and P. Bossu. 2001. Lymphocytes from autoimmune MRL lpr/lpr mice are hyperresponsive to IL-18 and overexpress the IL-18 receptor accessory chain. *J Immunol 166:3757*.

95. Yang, J., H. Zhu, T. L. Murphy, W. Ouyang, and K. M. Murphy. 2001. IL-18-stimulated GADD45 beta required in cytokine-induced, but not TCR-induced, IFN-gamma production. *Nat Immunol 2:157*.

96. Chi, H., B. Lu, M. Takekawa, R. J. Davis, and R. A. Flavell. 2004. GADD45beta/GADD45gamma and MEKK4 comprise a genetic pathway mediating STAT4-independent IFNgamma production in T cells. *Embo J 23:1576*.

97. Lu, B., H. Yu, C. Chow, B. Li, W. Zheng, R. J. Davis, and R. A. Flavell. 2001. GADD45gamma mediates the activation of the p38 and JNK MAP kinase pathways and cytokine production in effector TH1 cells. *Immunity 14:583*.

98. Suzuki, H., G. S. Duncan, H. Takimoto, and T. W. Mak. 1997. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. *J Exp Med 185:499*.

99. Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *J Exp Med 180:1395*.

100. Armitage, R. J., B. M. Macduff, J. Eisenman, R. Paxton, and K. H. Grabstein. 1995. IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *J Immunol 154:483*.

101. Okada, H., T. Nakajima, M. Sanezumi, A. Ikuta, K. Yasuda, and H. Kanzaki. 2000. Progesterone enhances interleukin-15 production in human endometrial stromal cells in vitro. *J Clin Endocrinol Metab 85:4765*.

102. Meazza, R., S. Verdiani, R. Biassoni, M. Coppolecchia, A. Gaggero, A. M. Orengo, M. P. Colombo, B. Azzarone, and S. Ferrini. 1996. Identification of a novel interleukin-15 (IL-15) transcript isoform generated by alternative splicing in human small cell lung cancer cell lines. *Oncogene 12:2187*.

103. Yamada, Y., K. Sugawara, T. Hata, K. Tsuruta, R. Moriuchi, T. Maeda, S. Atogami, K. Murata, K. Fujimoto, T. Kohno, K. Tsukasaki, M. Tomonaga, Y. Hirakata, and S. Kamihira. 1998. Interleukin-15 (IL-15) can replace the IL-2 signal in IL-2-dependent adult T-cell leukemia (ATL) cell lines: expression of IL-15 receptor alpha on ATL cells. *Blood 91:4265*.

104. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell 100:655*.

105. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science 295:338*.

106. Lametschwandtner, G., T. Biedermann, C. Schwarzler, C. Gunther, J. Kund, S. Fassl, S. Hinteregger, N. Carballido-Perrig, S. J. Szabo, L. H. Glimcher, and J. M. Carballido. 2004. Sustained T-bet expression confers polarized human TH2 cells with TH1-like cytokine production and migratory capacities. *J Allergy Clin Immunol 113:987*.

107. Finotto, S., M. F. Neurath, J. N. Glickman, S. Qin, H. A. Lehr, F. H. Green, K. Ackerman, K. Haley, P. R. Galle, S. J. Szabo, J. M. Drazen, G. T. De Sanctis, and L. H. Glimcher. 2002. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science 295:336*.

108. Snapper, C. M., and W. E. Paul. 1987. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science 236:944*.

109. Hasbold, J., J. S. Hong, M. R. Kehry, and P. D. Hodgkin. 1999. Integrating signals from IFN-gamma and IL-4 by B cells: positive and negative effects on CD40 ligand-induced proliferation, survival, and division-linked isotype switching to IgG1, IgE, and IgG2a. *J Immunol 163:4175*.

110. Peng, S. L., S. J. Szabo, and L. H. Glimcher. 2002. T-bet regulates IgG class switching and pathogenic autoantibody production. *Proc Natl Acad Sci U S A 99:5545*.

111. Gerth, A. J., L. Lin, and S. L. Peng. 2003. T-bet regulates T-independent IgG2a class switching. *Int Immunol 15:937*.

112. Mellman, I., and R. M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell 106:255*.

113. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol 18:767*.

114. Ohteki, T., T. Fukao, K. Suzue, C. Maki, M. Ito, M. Nakamura, and S. Koyasu. 1999. Interleukin 12-dependent interferon gamma production by CD8alpha+ lymphoid dendritic cells. *J Exp Med 189:1981*.

115. Fukao, T., S. Matsuda, and S. Koyasu. 2000. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN-gamma production by dendritic cells. *J Immunol 164:64*.

116. Lugo-Villarino, G., R. Maldonado-Lopez, R. Possemato, C. Penaranda, and L. H. Glimcher. 2003. T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. *Proc Natl Acad Sci U S A 100:7749*.

117. Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul, and J. J. O'Shea. 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A 98:15137*.

118. Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, and S. L. Reiner. 2001.

Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science 292:1907*.

119. Matsuoka, K., N. Inoue, T. Sato, S. Okamoto, T. Hisamatsu, Y. Kishi, A. Sakuraba, O. Hitotsumatsu, H. Ogata, K. Koganei, T. Fukushima, T. Kanai, M. Watanabe, H. Ishii, and T. Hibi. 2004. T-bet upregulation and subsequent interleukin 12 stimulation are essential for induction of Th1 mediated immunopathology in Crohn's disease. *Gut 53:1303*.

120. Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy, and K. M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol 3:549*.

121. Avni, O., D. Lee, F. Macian, S. J. Szabo, L. H. Glimcher, and A. Rao. 2002. T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat Immunol 3:643*.

122. Zheng, W. P., Q. Zhao, X. Zhao, B. Li, M. Hubank, D. G. Schatz, and R. A. Flavell. 2004. Up-regulation of Hlx in immature Th cells induces IFN-gamma expression. *J Immunol 172:114*.

123. Mullen, A. C., A. S. Hutchins, F. A. High, H. W. Lee, K. J. Sykes, L. A. Chodosh, and S. L. Reiner. 2002. Hlx is induced by and genetically interacts with T-bet to promote heritable T(H)1 gene induction. *Nat Immunol 3:652*.

124. Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, H. Shen, N. Cereb, S. Y. Yang, T. Lindsten, J. Rossant, C. A. Hunter, and S. L. Reiner. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science 302:1041*.

125. Leonard, W. J., and J. J. O'Shea. 1998. Jaks and STATs: biological implications. *Annu Rev Immunol 16:293*.

126. Ortmann, R. A., T. Cheng, R. Visconti, D. M. Frucht, and J. J. O'Shea. 2000. Janus kinases and signal transducers and activators of transcription: their roles in cytokine signaling, development and immunoregulation. *Arthritis Res 2:16*.

127. Ramana, C. V., M. Chatterjee-Kishore, H. Nguyen, and G. R. Stark. 2000. Complex roles of Stat1 in regulating gene expression. *Oncogene 19:2619*.

128. Darnell, J. E., Jr. 1997. STATs and gene regulation. *Science 277:1630*.

129. Ramana, C. V., N. Grammatikakis, M. Chernov, H. Nguyen, K. C. Goh, B. R. Williams, and G. R. Stark. 2000. Regulation of c-myc expression by IFN-gamma through Stat1-dependent and -independent pathways. *Embo J 19:263*.

130. Frucht, D. M., M. Aringer, J. Galon, C. Danning, M. Brown, S. Fan, M. Centola, C. Y. Wu, N. Yamada, H. El Gabalawy, and J. J. O'Shea. 2000. Stat4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. *J Immunol 164:4659*.

131. Murphy, K. M., W. Ouyang, J. D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T. L. Murphy. 2000. Signaling and transcription in T helper development. *Annu Rev Immunol 18:451*.

132. Bacon, C. M., E. F. Petricoin, 3rd, J. R. Ortaldo, R. C. Rees, A. C. Larner, J. A. Johnston, and J. J. O'Shea. 1995. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. *Proc Natl Acad Sci U S A 92:7307*.

133. Jacobson, N. G., S. J. Szabo, R. M. Weber-Nordt, Z. Zhong, R. D. Schreiber, J. E. Darnell, Jr., and K. M. Murphy. 1995. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J Exp Med 181:1755*.

134. Cho, S. S., C. M. Bacon, C. Sudarshan, R. C. Rees, D. Finbloom, R. Pine, and J. J. O'Shea. 1996. Activation of STAT4 by IL-12 and IFN-alpha: evidence for the involvement of ligand-induced tyrosine and serine phosphorylation. *J Immunol 157:4781*.

135. Xu, X., Y. L. Sun, and T. Hoey. 1996. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. *Science 273:794*.

136. Galon, J., C. Sudarshan, S. Ito, D. Finbloom, and J. J. O'Shea. 1999. IL-12 induces IFN regulating factor-1 (IRF-1) gene expression in human NK and T cells. *J Immunol 162:7256*.

137. Visconti, R., M. Gadina, M. Chiariello, E. H. Chen, L. F. Stancato, J. S. Gutkind, and J. J. O'Shea. 2000. Importance of the MKK6/p38 pathway for interleukin-12-

induced STAT4 serine phosphorylation and transcriptional activity. *Blood 96:1844*.

138. Morinobu, A., M. Gadina, W. Strober, R. Visconti, A. Fornace, C. Montagna, G. M. Feldman, R. Nishikomori, and J. J. O'Shea. 2002. STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. *Proc Natl Acad Sci U S A 99:12281*.
139. Bao, M., Y. Yang, H. S. Jun, and J. W. Yoon. 2002. Molecular mechanisms for gender differences in susceptibility to T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Immunol 168:5369*.
140. Nguyen, H., J. Hiscott, and P. M. Pitha. 1997. The growing family of interferon regulatory factors. *Cytokine Growth Factor Rev 8:293*.
141. Harada, H., T. Fujita, M. Miyamoto, Y. Kimura, M. Maruyama, A. Furia, T. Miyata, and T. Taniguchi. 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell 58:729*.
142. Fujita, T., Y. Kimura, M. Miyamoto, E. L. Barsoumian, and T. Taniguchi. 1989. Induction of endogenous IFN-alpha and IFN-beta genes by a regulatory transcription factor, IRF-1. *Nature 337:270*.
143. Fujita, T., J. Sakakibara, Y. Sudo, M. Miyamoto, Y. Kimura, and T. Taniguchi. 1988. Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-beta gene regulatory elements. *Embo J 7:3397*.
144. Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi. 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell 54:903*.
145. Maruyama, M., T. Fujita, and T. Taniguchi. 1989. Sequence of a cDNA coding for human IRF-1. *Nucleic Acids Res 17:3292*.
146. Harada, H., T. Taniguchi, and N. Tanaka. 1998. The role of interferon regulatory factors in the interferon system and cell growth control. *Biochimie 80:641*.
147. Au, W. C., P. A. Moore, W. Lowther, Y. T. Juang, and P. M. Pitha. 1995. Identification of a member of the interferon regulatory factor family that binds to

the interferon-stimulated response element and activates expression of interferon-induced genes. *Proc Natl Acad Sci U S A 92:11657*.

148. Juang, Y. T., W. Lowther, M. Kellum, W. C. Au, R. Lin, J. Hiscott, and P. M. Pitha. 1998. Primary activation of interferon A and interferon B gene transcription by interferon regulatory factor 3. *Proc Natl Acad Sci U S A 95:9837*.

149. Yamagata, T., J. Nishida, S. Tanaka, R. Sakai, K. Mitani, M. Yoshida, T. Taniguchi, Y. Yazaki, and H. Hirai. 1996. A novel interferon regulatory factor family transcription factor, ICSAT/Pip/LSIRF, that negatively regulates the activity of interferon-regulated genes. *Mol Cell Biol 16:1283*.

150. Barnes, B. J., P. A. Moore, and P. M. Pitha. 2001. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes. *J Biol Chem 276:23382*.

151. Zucchero, T. M., M. E. Cooper, B. S. Maher, S. Daack-Hirsch, B. Nepomuceno, L. Ribeiro, D. Caprau, K. Christensen, Y. Suzuki, J. Machida, N. Natsume, K. Yoshiura, A. R. Vieira, I. M. Orioli, E. E. Castilla, L. Moreno, M. Arcos-Burgos, A. C. Lidral, L. L. Field, Y. E. Liu, A. Ray, T. H. Goldstein, R. E. Schultz, M. Shi, M. K. Johnson, S. Kondo, B. C. Schutte, M. L. Marazita, and J. C. Murray. 2004. Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med 351:769*.

152. Au, W. C., P. A. Moore, D. W. LaFleur, B. Tombal, and P. M. Pitha. 1998. Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. *J Biol Chem 273:29210*.

153. Weisz, A., P. Marx, R. Sharf, E. Appella, P. H. Driggers, K. Ozato, and B. Z. Levi. 1992. Human interferon consensus sequence binding protein is a negative regulator of enhancer elements common to interferon-inducible genes. *J Biol Chem 267:25589*.

154. Kanno, Y., C. A. Kozak, C. Schindler, P. H. Driggers, D. L. Ennist, S. L. Gleason, J. E. Darnell, Jr., and K. Ozato. 1993. The genomic structure of the murine ICSBP gene reveals the presence of the gamma interferon-responsive element, to which an ISGF3 alpha subunit (or similar) molecule binds. *Mol Cell Biol 13:3951*.

155. Bluyssen, A. R., J. E. Durbin, and D. E. Levy. 1996. ISGF3 gamma p48, a specificity switch for interferon activated transcription factors. *Cytokine Growth Factor Rev 7:11*.

156. Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol 19:623*.

157. Taniguchi, T., N. Tanaka, K. Ogasawara, S. Taki, M. Sato, and A. Takaoka. 1999. Transcription factor IRF-1 and its family members in the regulation of host defense. *Cold Spring Harb Symp Quant Biol 64:465*.

158. Fujita, T., L. F. Reis, N. Watanabe, Y. Kimura, T. Taniguchi, and J. Vilcek. 1989. Induction of the transcription factor IRF-1 and interferon-beta mRNAs by cytokines and activators of second-messenger pathways. *Proc Natl Acad Sci U S A 86:9936*.

159. Flodstrom, M., and D. L. Eizirik. 1997. Interferon-gamma-induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. *Endocrinology 138:2747*.

160. Kimura, T., K. Nakayama, J. Penninger, M. Kitagawa, H. Harada, T. Matsuyama, N. Tanaka, R. Kamijo, J. Vilcek, T. W. Mak, and et al. 1994. Involvement of the IRF-1 transcription factor in antiviral responses to interferons. *Science 264:1921*.

161. Coccia, E. M., E. Stellacci, G. Marziali, G. Weiss, and A. Battistini. 2000. IFN-gamma and IL-4 differently regulate inducible NO synthase gene expression through IRF-1 modulation. *Int Immunol 12:977*.

162. Harada, H., E. Takahashi, S. Itoh, K. Harada, T. A. Hori, and T. Taniguchi. 1994. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. *Mol Cell Biol 14:1500*.

163. Mittrucker, H. W., T. Matsuyama, A. Grossman, T. M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P. S. Ohashi, and T. W. Mak. 1997. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science 275:540*.

164. Matsuyama, T., A. Grossman, H. W. Mittrucker, D. P. Siderovski, F. Kiefer, T. Kawakami, C. D. Richardson, T. Taniguchi, S. K. Yoshinaga, and T. W. Mak.

1995. Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). *Nucleic Acids Res 23:2127*.

165. Marecki, S., M. L. Atchison, and M. J. Fenton. 1999. Differential expression and distinct functions of IFN regulatory factor 4 and IFN consensus sequence binding protein in macrophages. *J Immunol 163:2713*.

166. Gupta, S., M. Jiang, A. Anthony, and A. B. Pernis. 1999. Lineage-specific modulation of interleukin 4 signaling by interferon regulatory factor 4. *J Exp Med 190:1837*.

167. Mamane, Y., C. Heylbroeck, P. Genin, M. Algarte, M. J. Servant, C. LePage, C. DeLuca, H. Kwon, R. Lin, and J. Hiscott. 1999. Interferon regulatory factors: the next generation. *Gene 237:1*.

168. Lehtonen, A., R. Lund, R. Lahesmaa, I. Julkunen, T. Sareneva, and S. Matikainen. 2003. IFN-alpha and IL-12 activate IFN regulatory factor 1 (IRF-1), IRF-4, and IRF-8 gene expression in human NK and T cells. *Cytokine 24:81*.

169. Lu, R., K. L. Medina, D. W. Lancki, and H. Singh. 2003. IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development. *Genes Dev 17:1703*.

170. Lohoff, M., H. W. Mittrucker, S. Prechtl, S. Bischof, F. Sommer, S. Kock, D. A. Ferrick, G. S. Duncan, A. Gessner, and T. W. Mak. 2002. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A 99:11808*.

171. Tominaga, N., K. Ohkusu-Tsukada, H. Udono, R. Abe, T. Matsuyama, and K. Yui. 2003. Development of Th1 and not Th2 immune responses in mice lacking IFN-regulatory factor-4. *Int Immunol 15:1*.

172. Rengarajan, J., K. A. Mowen, K. D. McBride, E. D. Smith, H. Singh, and L. H. Glimcher. 2002. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J Exp Med 195:1003*.

173. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu Rev Immunol 14:233*.

174. Frauwirth, K. A., and C. B. Thompson. 2002. Activation and inhibition of lymphocytes by costimulation. *J Clin Invest 109:295*.

175. Abbas, A. K., and A. H. Sharpe. 1999. T-cell stimulation: an abundance of B7s. *Nat Med 5:1345*.

176. Lenschow, D. J., Y. Zeng, J. R. Thistlethwaite, A. Montag, W. Brady, M. G. Gibson, P. S. Linsley, and J. A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science 257:789*.

177. Finck, B. K., P. S. Linsley, and D. Wofsy. 1994. Treatment of murine lupus with CTLA4Ig. *Science 265:1225*.

178. Najafian, N., and M. H. Sayegh. 2000. CTLA4-Ig: a novel immunosuppressive agent. *Expert Opin Investig Drugs 9:2147*.

179. Egen, J. G., M. S. Kuhns, and J. P. Allison. 2002. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol 3:611*.

180. Rothstein, D. M., and M. H. Sayegh. 2003. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol Rev 196:85*.

181. Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *Embo J 11:3887*.

182. Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser, L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med 192:1027*.

183. Ishida, M., Y. Iwai, Y. Tanaka, T. Okazaki, G. J. Freeman, N. Minato, and T. Honjo. 2002. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunol Lett 84:57*.

184. Agata, Y., A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita, and T. Honjo. 1996. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol 8:765*.

185. Carter, L., L. A. Fouser, J. Jussif, L. Fitz, B. Deng, C. R. Wood, M. Collins, T. Honjo, G. J. Freeman, and B. M. Carreno. 2002. PD-1:PD-L inhibitory pathway

affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. *Eur J Immunol 32:634*.

186. Yamazaki, T., H. Akiba, H. Iwai, H. Matsuda, M. Aoki, Y. Tanno, T. Shin, H. Tsuchiya, D. M. Pardoll, K. Okumura, M. Azuma, and H. Yagita. 2002. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol 169:5538*.

187. Nishimura, H., Y. Agata, A. Kawasaki, M. Sato, S. Imamura, N. Minato, H. Yagita, T. Nakano, and T. Honjo. 1996. Developmentally regulated expression of the PD-1 protein on the surface of double-negative (CD4-CD8-) thymocytes. *Int Immunol 8:773*.

188. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol 167:1245*.

189. Dong, H., G. Zhu, K. Tamada, and L. Chen. 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med 5:1365*.

190. Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, E. A. Greenfield, K. Bourque, V. A. Boussiotis, L. L. Carter, B. M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A. H. Sharpe, and G. J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol 2:261*.

191. Tseng, S. Y., M. Otsuji, K. Gorski, X. Huang, J. E. Slansky, S. I. Pai, A. Shalabi, T. Shin, D. M. Pardoll, and H. Tsuchiya. 2001. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med 193:839*.

192. Liang, S. C., Y. E. Latchman, J. E. Buhlmann, M. F. Tomczak, B. H. Horwitz, G. J. Freeman, and A. H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol 33:2706*.

193. Iwai, Y., S. Terawaki, M. Ikegawa, T. Okazaki, and T. Honjo. 2003. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J Exp Med 198:39*.

194. Rodig, N., T. Ryan, J. A. Allen, H. Pang, N. Grabie, T. Chernova, E. A. Greenfield, S. C. Liang, A. H. Sharpe, A. H. Lichtman, and G. J. Freeman. 2003.

Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. *Eur J Immunol 33:3117*.

195. Dong, H., and L. Chen. 2003. B7-H1 pathway and its role in the evasion of tumor immunity. *J Mol Med 81:281*.

196. Ozkaynak, E., L. Wang, A. Goodearl, K. McDonald, S. Qin, T. O'Keefe, T. Duong, T. Smith, J. C. Gutierrez-Ramos, J. B. Rottman, A. J. Coyle, and W. W. Hancock. 2002. Programmed death-1 targeting can promote allograft survival. *J Immunol 169:6546*.

197. Loke, P., and J. P. Allison. 2003. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. *Proc Natl Acad Sci U S A 100:5336*.

198. Harris, N. L., V. Watt, F. Ronchese, and G. Le Gros. 2002. Differential T cell function and fate in lymph node and nonlymphoid tissues. *J Exp Med 195:317*.

199. Mazanet, M. M., and C. C. Hughes. 2002. B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. *J Immunol 169:3581*.

200. Shin, T., G. Kennedy, K. Gorski, H. Tsuchiya, H. Koseki, M. Azuma, H. Yagita, L. Chen, J. Powell, D. Pardoll, and F. Housseau. 2003. Cooperative B7-1/2 (CD80/CD86) and B7-DC costimulation of CD4+ T cells independent of the PD-1 receptor. *J Exp Med 198:31*.

201. Nguyen, L. T., S. Radhakrishnan, B. Ciric, K. Tamada, T. Shin, D. M. Pardoll, L. Chen, M. Rodriguez, and L. R. Pease. 2002. Cross-linking the B7 family molecule B7-DC directly activates immune functions of dendritic cells. *J Exp Med 196:1393*.

202. Nishimura, H., N. Minato, T. Nakano, and T. Honjo. 1998. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int Immunol 10:1563*.

203. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity 11:141*.

204. Finger, L. R., J. Pu, R. Wasserman, R. Vibhakar, E. Louie, R. R. Hardy, P. D. Burrows, and L. G. Billips. 1997. The human PD-1 gene: complete cDNA,

genomic organization, and developmentally regulated expression in B cell progenitors. *Gene 197:177*.

205. Takahashi, S., M. Nose, J. Sasaki, T. Yamamoto, and M. Kyogoku. 1991. IgG3 production in MRL/lpr mice is responsible for development of lupus nephritis. *J Immunol 147:515*.

206. Bikah, G., J. Carey, J. R. Ciallella, A. Tarakhovsky, and S. Bondada. 1996. CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells. *Science 274:1906*.

207. Okazaki, T., A. Maeda, H. Nishimura, T. Kurosaki, and T. Honjo. 2001. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci U S A 98:13866*.

208. Nishimura, H., and T. Honjo. 2001. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol 22:265*.

209. Ansar Ahmed, S., B. D. Hissong, D. Verthelyi, K. Donner, K. Becker, and E. Karpuzoglu-Sahin. 1999. Gender and risk of autoimmune diseases: possible role of estrogenic compounds. *Environ Health Perspect 107 Suppl 5:681*.

210. Ansar Ahmed, S., W. J. Penhale, and N. Talal. 1985. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol 121:531*.

211. Neurath, M. F., B. Weigmann, S. Finotto, J. Glickman, E. Nieuwenhuis, H. Iijima, A. Mizoguchi, E. Mizoguchi, J. Mudter, P. R. Galle, A. Bhan, F. Autschbach, B. M. Sullivan, S. J. Szabo, L. H. Glimcher, and R. S. Blumberg. 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. *J Exp Med 195:1129*.

212. Ansar Ahmed, S., and N. Talal. 1989. Sex hormones and autoimmune rheumatic disorders. *Scand J Rheumatol 18:69*.

213. Verthelyi, D., M. Petri, M. Ylamus, and D. M. Klinman. 2001. Disassociation of sex hormone levels and cytokine production in SLE patients. *Lupus 10:352*.

214. Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science 291:319*.

215. Ansari, M. J., A. D. Salama, T. Chitnis, R. N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S. J. Khoury, H. Auchincloss, Jr., and M. H. Sayegh. 2003. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med 198:63*.

216. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S. A. Lira, D. Gorman, R. A. Kastelein, and J. D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature 421:744*.

217. Salama, A. D., T. Chitnis, J. Imitola, M. J. Ansari, H. Akiba, F. Tushima, M. Azuma, H. Yagita, M. H. Sayegh, and S. J. Khoury. 2003. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med 198:71*.

218. Hatachi, S., Y. Iwai, S. Kawano, S. Morinobu, M. Kobayashi, M. Koshiba, R. Saura, M. Kurosaka, T. Honjo, and S. Kumagai. 2003. CD4+ PD-1+ T cells accumulate as unique anergic cells in rheumatoid arthritis synovial fluid. *J Rheumatol 30:1410*.

219. Lin, S. C., J. H. Yen, J. J. Tsai, W. C. Tsai, T. T. Ou, H. W. Liu, and C. J. Chen. 2004. Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis, but not systemic lupus erythematosus. *Arthritis Rheum 50:770*.

220. Prokunina, L., and M. Alarcon-Riquelme. 2004. The genetic basis of systemic lupus erythematosus--knowledge of today and thoughts for tomorrow. *Hum Mol Genet 13 Spec No 1:R143*.

221. Prokunina, L., C. Castillejo-Lopez, F. Oberg, I. Gunnarsson, L. Berg, V. Magnusson, A. J. Brookes, D. Tentler, H. Kristjansdottir, G. Grondal, A. I. Bolstad, E. Svenungsson, I. Lundberg, G. Sturfelt, A. Jonssen, L. Truedsson, G. Lima, J. Alcocer-Varela, R. Jonsson, U. B. Gyllensten, J. B. Harley, D. Alarcon-

Segovia, K. Steinsson, and M. E. Alarcon-Riquelme. 2002. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nat Genet 32:666*.

222. Bolstad, A. I., H. G. Eiken, B. Rosenlund, M. E. Alarcon-Riquelme, and R. Jonsson. 2003. Increased salivary gland tissue expression of Fas, Fas ligand, cytotoxic T lymphocyte-associated antigen 4, and programmed cell death 1 in primary Sjogren's syndrome. *Arthritis Rheum 48:174*.
223. Kanai, T., T. Totsuka, K. Uraushihara, S. Makita, T. Nakamura, K. Koganei, T. Fukushima, H. Akiba, H. Yagita, K. Okumura, U. Machida, H. Iwai, M. Azuma, L. Chen, and M. Watanabe. 2003. Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. *J Immunol 171:4156*.
224. Verthelyi, D., and S. Ansar Ahmed. 1994. 17 beta-estradiol, but not 5 alpha-dihydrotestosterone, augments antibodies to double-stranded deoxyribonucleic acid in nonautoimmune C57BL/6J mice. *Endocrinology 135:2615*.
225. Verthelyi, D., and S. Ansar Ahmed. 1997. Characterization of estrogen-induced autoantibodies to cardiolipin in non-autoimmune mice. *J Autoimmun 10:115*.
226. Verthelyi, D. I., and S. Ansar Ahmed. 1998. Estrogen increases the number of plasma cells and enhances their autoantibody production in nonautoimmune C57BL/6 mice. *Cell Immunol 189:125*.
227. Hu, X., C. Herrero, W. P. Li, T. T. Antoniv, E. Falck-Pedersen, A. E. Koch, J. M. Woods, G. K. Haines, and L. B. Ivashkiv. 2002. Sensitization of IFN-gamma Jak-STAT signaling during macrophage activation. *Nat Immunol 3:859*.
228. Matsumoto, K., H. Inoue, T. Nakano, M. Tsuda, Y. Yoshiura, S. Fukuyama, F. Tsushima, T. Hoshino, H. Aizawa, H. Akiba, D. Pardoll, N. Hara, H. Yagita, M. Azuma, and Y. Nakanishi. 2004. B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. *J Immunol 172:2530*.
229. Tamura, H., H. Dong, G. Zhu, G. L. Sica, D. B. Flies, K. Tamada, and L. Chen. 2001. B7-H1 costimulation preferentially enhances CD28-independent T-helper cell function. *Blood 97:1809*.

230. Wang, S., J. Bajorath, D. B. Flies, H. Dong, T. Honjo, and L. Chen. 2003. Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. *J Exp Med 197:1083*.

231. Balomenos, D., R. Rumold, and A. N. Theofilopoulos. 1998. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. *J Clin Invest 101:364*.

232. Carvalho-Pinto, C. E., M. I. Garcia, M. Mellado, J. M. Rodriguez-Frade, J. Martin-Caballero, J. Flores, A. C. Martinez, and D. Balomenos. 2002. Autocrine production of IFN-gamma by macrophages controls their recruitment to kidney and the development of glomerulonephritis in MRL/lpr mice. *J Immunol 169:1058*.

233. Haas, C., B. Ryffel, and M. Le Hir. 1998. IFN-gamma receptor deletion prevents autoantibody production and glomerulonephritis in lupus-prone (NZB x NZW)F1 mice. *J Immunol 160:3713*.

234. Peng, S. L., J. Moslehi, and J. Craft. 1997. Roles of interferon-gamma and interleukin-4 in murine lupus. *J Clin Invest 99:1936*.

235. Pollard, K. M., P. Hultman, and D. H. Kono. 2003. Using single-gene deletions to identify checkpoints in the progression of systemic autoimmunity. *Ann N Y Acad Sci 987:236*.

CHAPTER 5. GENERAL CONCLUSIONS and FUTURE WORK

Intense research in several disciplines, coupled with the availability of molecular techniques including gene knockout mice, revealed broad effects of estrogen on diverse tissues (including the immune tissues) in both sexes. It is clear that estrogen can no longer be "pigeon-holed" as merely a reproductive hormone and there is an accumulating data on the effects of estrogen on tissues such as the immune system of normal and autoimmune individuals (1-3). Today, due to increased exposure to estrogen via oral contraceptives in pre-menopausal women and estrogen replacement therapy in menopausal women, as well as the increased risk of exposure to environmental and/or phyto-estrogens in the general population have raised health and concerns including its effects on the immune system. It is acknowledged that estrogen has complex effects on the immune system (1, 2, 4, 5). One mechanism by which estrogen affects the immune system is through alteration of levels or response to cytokines such as IFN-γ. This Ph.D. dissertation focused on effects of estrogen on IFN-γ and IFN-γ-mediated downstream events. Alteration of IFN-γ by estrogen is a significant finding, since IFN-γ is a master cytokine that affects all key cells of the immune system. It is critical for intracellular immunity and is incriminated in several autoimmune and inflammatory disorders. Therefore, it is critical to decipher the effects of and the molecular mechanisms by which estrogen affects IFN-γ. For the most part, neither the molecular means by which estrogen modulates IFN-γ nor the cellular and molecular immune outcomes of estrogen-induced IFN-γ are known. Furthermore, upregulation of IFN-γ-inducing cytokines and transcription factors as a result of estrogen treatment, which results in estrogen-induced IFN-γ and/or nitric oxide, may provide an explanation for the increased incidence of autoimmune diseases in females.

We found that estrogen treatment tended to increase the secretion of IFN-γ protein and mRNA expression from splenic lymphocytes when activated by the pan T cell stimulant, Con A (**Chapter 2**). This upregulation of IFN-γ by estrogen is in agreement with other studies, which employed either an *in vitro* system (6) or *in vivo* (7). However, no studies thus far have looked at the molecular effects of estrogen on IFN-γ-inducing cytokines,

transcription factors, or downstream IFN-γ-mediated events. IFN-γ can induce the expression of various proteins such as iNOS, iNOS-inducible nitric oxide, and COX-2. It is currently well established that IFN-γ-inducible iNOS-derived nitric oxide and Cycloxygenase-2 (COX-2), produced primarily by macrophages, play important roles in the physiological functions of various tissues and are involved in pathological situations. In spite of this, there is little information on the physiological or pathological role of iNOS, nitric oxide, or COX-2 in cells of the immune system relative to gender or sex hormones such as estrogen. Since IFN-γ is known to induce iNOS and iNOS-derived nitric oxide, it became important to investigate the effects of natural estrogen, 17-β estradiol, on the functions of macrophages, particularly on the release of nitric oxide and the expression of iNOS and COX-2. We showed that Con-A activated splenic lymphocytes from estrogen-treated mice had higher levels of nitric oxide and increased expression of iNOS protein and mRNA, as well as COX-2 protein compared to placebo-treated mice. We further demonstrated the importance of the CD-28/B7 costimulatory pathway by using a CTLA-4Ig fusion protein. Blocking with the CTLA-4Ig fusion protein diminished IFN-γ, nitric oxide, and the expression of iNOS mRNA and COX-2 protein in Con-A-stimulated splenocytes from both placebo and estrogen-treated mice (**Chapter 3**).

Since we have shown that estrogen treatment promotes IFN-γ, the role of IFN-γ was further examined using IFN-γ knockout mice. Preliminary results showed that these mice, did not release nitric oxide nor did they express iNOS suggesting that estrogen is not directly acting on macrophages to cause release of nitric oxide. The deliberate addition of rIFN-γ to splenic lymphocytes stimulated with Con-A for a brief period of time from estrogen-treated wild type resulted in elevated expression of iNOS protein, which implies the importance of IFN-γ to nitric oxide induction. IFN-γ, iNOS, and COX-2 are known to be regulated and induced by Interferon Regulatory Factor-1 (IRF-1) (8). Estrogen treatment of IRF-1 knockout mice marginally increased IFN-γ protein but does not induce iNOS protein or nitric oxide in Con-A-stimulated splenocytes (**Chapter 3**).

Analysis of IFN-γ inducing cytokines showed that *in vivo* estrogen treatment results in increased mRNA and/or protein expression of IFN-γ inducing cytokines and their receptors: IL-18, IL-15, IL-27, IL-12Rβ2, and IL-18Rβ. The decreased IL-12p70 protein levels observed in the supernatants from Con-A-activated splenocytes from estrogen-treated mice may be due to increased binding or turnover of the protein (**Chapter 4, Part 1**). We also found that T-bet, a critical Th1 transcription factor, and STAT-4 phosphorylation, a key in IL-12 signaling, are both increased. Not suprisingly, IRF-4, an important player in Th2 differentiation, is diminished in Con-A activated splenocytes from mice treated with estrogen (**Chapter 4, Part 2**).

Taken all together, these studies report that estrogen regulates IFN-γ and therefore the immune system via altering IFN-γ inducible genes, IFN-γ inducing cytokines, and transcription factors in normal C57BL/6 mice. Importantly, these are the *first studies* to demonstrate estrogen upregulation of iNOS, nitric oxide, and IFN-γ inducing cytokines: IL-27, IL-15, and IL-18, as well as the receptors of IFN-γ inducing cytokines: IL-12Rβ2 and IL-18β, transcription factors: T-bet, phosphorylated STAT-4, and downregulation of IRF-4 in splenic lymphocytes.

The modulation of the immune system by estrogen could induce susceptibility or resistance to autoimmune diseases, infections, allergies, and cancer, and as a consequence has extensive repercussions. Since estrogen-induced IFN-γ and/or nitric oxide, and IFN-γ inducing cytokines function as molecular messengers between lymphoid and non-lymphoid cells of the immune system to perform various functions such as cell differentiation and regulation, we hope this study will bring to light and explore one aspect of the “Pandora’s Box”: The immunomodulatory effects of estrogen on IFN-γ, which may influence the state of cancer, inflammatory, and autoimmune diseases.

These studies, thus, add new and valuable knowledge regarding the effects of estrogen on the normal immune system, a concept that is not yet well understood. The information provided in this dissertation is especially important since IFN-γ plays a critical role in

normal functions of the immune system as well as in many autoimmune, inflammatory, and infectious conditions.

Future studies

Future investigations of the immunomodulatory effects of estrogen can focus on following areas:

1. Determination of IFN-γ secreting cells: We have recently shown that Con-A or anti-CD3-activated splenic lymphocytes from estrogen-treated mice secrete higher levels of IFN-γ compared to similar cultures from placebo-treated mice. The precise mechanisms underlying this estrogen-induced increase in IFN-γ secretion and the secretors of IFN-γ are not known. It is known that the major IFN-γ secreting cells are $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, dendritic cells, and macrophages (9-11). It was recently found that B cells ("effector" Bel cells) can also produce IFN-γ (12). Therefore, it is critical to decipher whether estrogen-treated mice have increased numbers of IFN-γ secreting cells and/or individual T cells or even B cells that are secreting more IFN-γ by using intracellular staining and flow cytometric analysis.

2. Expression of IFN-γ receptors: IFN-γ binds to IFN-γ receptors that exist as heterodimers of IFN-γRα and IFN-γRβ chains (13-15) resulting in activation of members of the Janus Kinase family, Jak-1 by IFN-γRα and Jak-2 by IFN-γRβ in turn activating STAT-1 via phosphorylation. As a result, phosphorylated STAT-1 dimerizes and binds to specific DNA response elements of target genes to regulate STAT-1-regulated genes. We have shown that the expression of iNOS and nitric oxide, which are inducible by IFN-γ, were increased from Con-A or anti-CD3-activated splenocytes from estrogen-treated mice. For that reason, it is important to determine whether splenic lymphocytes (macrophages, $CD4^+$ and $CD8^+$ T cells, and B cells) from estrogen-treated mice have increased expression of IFN-γRα and/or IFN-γRβ receptors and/or enhanced signaling.

3. iNOS and COX-2 activity: Complete activation of lymphocytes requires both antigen-specific signals delivered via the T cell receptor (TCR) and costimulatory signals both on T and antigen presenting cells, while TCR stimulation alone leads to an unresponsive state, called anergy (16, 17). One of the well known costimulatory pathways is between CD28 and B7.1 (CD80)/B7.2 (CD86) molecules (18), which can be blocked by a fusion protein called CTLA-4Ig. Our study demonstrates that obstruction of the CD28-B7.1/B7.2 pathway by CTLA-4Ig inhibits the production of IFN-γ and IFN-γ inducible nitric oxide, relative iNOS mRNA expression, and COX-2 protein from lymphocytes stimulated with Con-A from both estrogen and placebo treated mice (**Chapter 3**). Protein expression of iNOS was not affected by CTLA-4Ig treatment (data not shown). Due to the fact that iNOS can be transcriptionally regulated (19, 20), the decrease in mRNA, but not in the protein of iNOS, could be due to decreased levels of the iNOS-inducer, IFN-γ. Other possibilities include diminution in arginine supply by arginase (21-23), decreased arginine uptake by cells (24, 25), or altered levels of iNOS cofactor tetrahydrobioptherin (BH4), which post-transcriptionally stabilizes iNOS mRNA (26, 27). Therefore, the activity of iNOS enyzme should be investigated in Con-A and Con-A with CTLA-4Ig stimulated splenic lymphocytes from placebo and estrogen-treated mice to explore these possibilities. The activity of COX-2 and the production of PGE_2 can also be investigated in Con-A and CTLA-4Ig stimulated splenic lymphocytes from estrogen-treated mice to emphasize the effect of costimulation on various functions of the cells.

4. Absolute quantification with Real Time PCR: We showed, using RT-PCR, that IL-27 and IL-18Rβ are increased in Con-A activated splenocytes from estrogen-treated mice when compared to placebo controls. The expression of other Th1 inducing transcription factors such as Eomes and HLX were also determined with RT-PCR. Since RT-PCR provides only a relative estimate of gene expression, Real Time PCR assays should be done to achieve absolute quantification of these genes.

5. Detection of expression of IL-12p70 and IL-18-mediated signaling: The decrease in IL-12p70 protein in supernatants from Con-A stimulated splenocytes from estrogen treated mice was suprising and could be due to: late time point (24 hours of incubation)

and/or internalization of IL-12p70. Therefore, a kinetic incubation of cells with time points such as 3, 6, 12, and 24 hours (as control) should be performed to determine whether the increase in the amount of free IL-12p70 in the supernatants takes place at an earlier time point. The detection of intracellular IL-12p70 can also be performed by intracellular cytokine staining and flow cytometric analysis at these time points. Cells exposed to estrogen may have enhanced signaling in response to IL-18. The signaling through IL-18 and IL-18 Receptors could be deciphered via analyzing intracellular signaling components including MyD88 and Interleukin-1 receptor-associated kinase (IRAK).

6. Determination of Th1 defining markers: Since IFN-γ organizes a vast array of cellular responses through transcriptional regulation of immunologically relevant genes, it is important to explore transcription factors other than T-bet or transcription factors that work in combination with T-bet such as E26 transformation-specific-1 (Ets-1) which helps T-bet to induce interferon-gamma production. This could be accomplished by using the Mouse Th1-Th2 Gene Array, which includes various Th1 markers and cytokines such as Igsf6, IL-12Rβ2, IL-18rα, Il2, Il2rα (CD25), IRF-8, T-bet, TCCR (IL-27 receptor), Timd3, TNF, and Tim-3.

7. T-bet silencing: The importance of T-bet in the induction of IFN-γ in splenocytes from estrogen-treated mice can be explored by suppressing T-bet expression using siRNA assays. Small interfering RNA (siRNA) is a short RNA duplex between 15 and 21 nucleotides in length, which once transfected into cells, targets messenger RNA molecules containing an identical sequence for degradation in a catalytic manner. The degradation of mRNA of the target gene leads to loss of protein therefore, silencing the corresponding gene. The effects of silencing can be assayed by a number of different molecular and cellular assays to understand the role of the target gene. To determine if suppression of T-bet may downregulate IFN-γ production in splenocytes from estrogen-treated mice, small interfering RNAs (siRNA) specific for T-bet can be designed, which will silence genes utilizing an evolutionarily conserved mechanism of degrading mRNA complementary to any double-stranded RNA in the cell. The presence of T-bet mRNA

expression can then be evaluated with Real-Time PCR assays. We expect to see decreased IFN-γ and IFN-γ induced nitric oxide levels in siRNA T-bet-suppressed splenocytes from estrogen-treated mice compared to untreated cells from estrogen-treated mice. It is also possible that estrogen treatment may induce an unknown transcription factor or directly control gene transcription by binding to ERE elements and in return upregulate IFN-γ production. If the latter is the case, the effect of estrogen receptors, ER-α and/or ER-β, can be explored using placebo or estrogen-treated ER-α and/or ER-β knockout mice.

8. Switching from Th1 to Th2 type response: Although the cellular effects of IFN-γ (including increased antigen processing and presentation, induction of the antiviral state, activation of microbiocidal effector functions inhibition of cellular proliferation and effects on apoptosis, immunomodulation, delayed-type hypersensitivity, and leukocyte trafficking) the prolonged secretion of IFN-γ, IFN-γ induced nitric oxide, and other Th1 cytokines can likely result in severe dysregulation of the normal immune system. For an effective and stable Th1 response, inflammatory cytokines are produced simultaneously by Th1 type cells to suppress the expression of anti-inflammatory cytokines. In return, increased levels of nitric oxide are able to suppress IFN-γ and IL-2, and induce costimulatory molecules, cytokines such as IL-4, to induce a Th2 response (28). In addition, recent studies have shown that prolonged nitric oxide release by nitric oxide releasing-nonsteroidal anti-inflammatory drugs (NO-NSAIDs), widely prescribed drugs with anti-inflammatory, antipyretic, and analgesic properties, suppresses production of IFN-γ and IL-18 as well as nitric oxide inducible COX-2 (29, 30). Therefore, we hypothesize that increased levels of IFN-γ and IFN-γ-inducible nitric oxide can "switch" Con-A-stimulated splenic lymphocytes from estrogen-treated mice from Th1 type cells into Th2 type cells after long periods of incubation. Splenocytes from placebo or estrogen-treated mice should be cultured for 48, 72, and 96 hours in the presence or absence of Con-A. Cells can be washed at 72 hours and new media or Con-A can be added to supplement these cell cultures. At the end of the incubation, key cytokines for Th1, IFN-γ, other Th1 cytokines, and markers as stated above can be examined. Th2 markers and cytokines such as IL-4 can be explored, as well as CIITA, RANTES, MCP-

3, MCP-1, GATA-3, IL-10, IL-13, IL-13Rα1, IL-13Rα2, IL-5, IL-9, c-Maf, and STAT-6 both at protein and gene transcription levels in splenocytes from both placebo and estrogen-treated mice.

These studies may provide valuable information about how estrogen induces progression of several autoimmune and inflammatory diseases and how the immune system regulates itself in the presence of estrogen. Furthermore, these will lay the ground work for understanding and assisting in the design of NO-NSAIDs for women, which have anti-inflammatory, analgesic and anti-thrombotic properties with protective effects in terms of cardiovascular and renal tissues and a number of inflammatory disorders.

References

1. Ansar Ahmed, S., W. J. Penhale, and N. Talal. 1985. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol 121:531*.
2. Ansar Ahmed, S., B. D. Hissong, D. Verthelyi, K. Donner, K. Becker, and E. Karpuzoglu-Sahin. 1999. Gender and risk of autoimmune diseases: possible role of estrogenic compounds. *Environ Health Perspect 107 Suppl 5:681*.
3. Lahita, R. G. 1996. The connective tissue diseases and the overall influence of gender. *Int J Fertil Menopausal Stud 41:156*.
4. Beeson, P. B. 1994. Age and sex associations of 40 autoimmune diseases. *Am J Med 96:457*.
5. Roubinian, J. R., N. Talal, J. S. Greenspan, J. R. Goodman, and P. K. Siiteri. 1978. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice. *J Exp Med 147:1568*.
6. Fox, H. S., B. L. Bond, and T. G. Parslow. 1991. Estrogen regulates the IFN-gamma promoter. *J Immunol 146:4362*.
7. Maret, A., J. D. Coudert, L. Garidou, G. Foucras, P. Gourdy, A. Krust, S. Dupont, P. Chambon, P. Druet, F. Bayard, and J. C. Guery. 2003. Estradiol enhances primary antigen-specific CD4 T cell responses and Th1 development in vivo. Essential role of estrogen receptor alpha expression in hematopoietic cells. *Eur J Immunol 33:512*.
8. Kroger, A., M. Koster, K. Schroeder, H. Hauser, and P. P. Mueller. 2002. Activities of IRF-1. *J Interferon Cytokine Res 22:5*.
9. Frucht, D. M., T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea, and S. Koyasu. 2001. IFN-gamma production by antigen-presenting cells: mechanisms emerge. *Trends Immunol 22:556*.
10. Billiau, A., H. Heremans, K. Vermeire, and P. Matthys. 1998. Immunomodulatory properties of interferon-gamma. An update. *Ann N Y Acad Sci 856:22*.

11. O'Garra, A. 1998. Checkpoints for regulation of development and interferon gamma production by Th1 cells in TCR-transgenic models. *Eur Cytokine Netw 9:41*.
12. Lund, F. E., B. A. Garvy, T. D. Randall, and D. P. Harris. 2005. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. *Curr Dir Autoimmun 8:25*.
13. Bach, E. A., S. J. Szabo, A. S. Dighe, A. Ashkenazi, M. Aguet, K. M. Murphy, and R. D. Schreiber. 1995. Ligand-induced autoregulation of IFN-gamma receptor beta chain expression in T helper cell subsets. *Science 270:1215*.
14. Rigamonti, L., S. Ariotti, G. Losana, R. Gradini, M. A. Russo, E. Jouanguy, J. L. Casanova, G. Forni, and F. Novelli. 2000. Surface expression of the IFN-gamma R2 chain is regulated by intracellular trafficking in human T lymphocytes. *J Immunol 164:201*.
15. Larkin, J., 3rd, H. M. Johnson, and P. S. Subramaniam. 2000. Differential nuclear localization of the IFNGR-1 and IFNGR-2 subunits of the IFN-gamma receptor complex following activation by IFN-gamma. *J Interferon Cytokine Res 20:565*.
16. Bretscher, P. 1992. The two-signal model of lymphocyte activation twenty-one years later. *Immunol Today 13:74*.
17. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu Rev Immunol 14:233*.
18. June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol Today 15:321*.
19. Forstermann, U., E. I. Closs, J. S. Pollock, M. Nakane, P. Schwarz, I. Gath, and H. Kleinert. 1994. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension 23:1121*.
20. Kleinert, H., P. M. Schwarz, and U. Forstermann. 2003. Regulation of the expression of inducible nitric oxide synthase. *Biol Chem 384:1343*.
21. Bruch-Gerharz, D., O. Schnorr, C. Suschek, K. F. Beck, J. Pfeilschifter, T. Ruzicka, and V. Kolb-Bachofen. 2003. Arginase 1 overexpression in psoriasis: limitation of inducible nitric oxide synthase activity as a molecular mechanism for keratinocyte hyperproliferation. *Am J Pathol 162:203*.

22. Mori, M., and T. Gotoh. 2004. Arginine metabolic enzymes, nitric oxide and infection. *J Nutr 134:2820S*.
23. Morris, S. M., Jr. 2004. Enzymes of arginine metabolism. *J Nutr 134:2743S*.
24. Closs, E. I., J. S. Scheld, M. Sharafi, and U. Forstermann. 2000. Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol 57:68*.
25. Hardy, T. A., and J. M. May. 2002. Coordinate regulation of L-arginine uptake and nitric oxide synthase activity in cultured endothelial cells. *Free Radic Biol Med 32:122*.
26. Kasai, K., Y. Hattori, N. Banba, S. Hattori, S. Motohashi, S. Shimoda, N. Nakanishi, and S. S. Gross. 1997. Induction of tetrahydrobiopterin synthesis in rat cardiac myocytes: impact on cytokine-induced NO generation. *Am J Physiol 273:H665*.
27. Linscheid, P., A. Schaffner, and G. Schoedon. 1998. Modulation of inducible nitric oxide synthase mRNA stability by tetrahydrobiopterin in vascular smooth muscle cells. *Biochem Biophys Res Commun 243:137*.
28. Singh, V. K., S. Mehrotra, P. Narayan, C. M. Pandey, and S. S. Agarwal. 2000. Modulation of autoimmune diseases by nitric oxide. *Immunol Res 22:1*.
29. Fiorucci, S., E. Antonelli, J. L. Burgaud, and A. Morelli. 2001. Nitric oxide-releasing NSAIDs: a review of their current status. *Drug Saf 24:801*.
30. Wallace, J. L., N. Vergnolle, M. N. Muscara, S. Asfaha, K. Chapman, W. McKnight, P. Del Soldato, A. Morelli, and S. Fiorucci. 1999. Enhanced anti-inflammatory effects of a nitric oxide-releasing derivative of mesalamine in rats. *Gastroenterology 117:557*.

APPENDIX RELATED TO DISSERTATION

"Short-Term Administration of 17-β Estradiol to Outbred Male CD-1 Mice induces changes in the Immune System, but not in Reproductive Organs. E. Karpuzoglu-Sahin, R. M. Gogal Jr, C. Hardy , P. Sponenberg., S. Ansar Ahmed. *Immunological Investigations*, 34 (1): 1-26 2005 " In this paper, we demonstrate that short-term administration of relatively low doses of estrogen to a less estrogen-responsive outbred mouse strain, CD-1, also upregulated IFN-γ. This increase in IFN-γ secretion was accompanied by increase in nitric oxide and was not related to alterations in cell numbers. Copyright (2005) From (Short-Term Administration of 17-β Estradiol to Outbred Male CD-1 Mice Induces Changes in the Immune System, but not In Reproductive Organs) by (E. Karpuzoglu-Sahin et al)Reproduced by permission of Taylor & Francis Group LLC., http://www.taylorandfrancis.com

SHORT-TERM ADMINISTRATION OF 17-β ESTRADIOL TO OUTBRED MALE CD-1 MICE INDUCES CHANGES IN THE IMMUNE SYSTEM, BUT NOT IN REPRODUCTIVE ORGANS

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Key words: 17-β estradiol (estrogen, E_2), immunity, short-term, CD-1 mice, IFN-γ, nitric oxide

a.) ABSTRACT

The magnitude of an immune response to many foreign and/or self-antigens is known to be gender-dependent and influenced by sex hormones. While the immune consequences of long-term exposure (3 to 5 months) to natural 17-β estradiol in an in-bred mouse model (e.g. C57BL/6, Balb/c) are relatively well-documented, the immunological effects of shorter-term 17 β-estradiol exposure in an out-bred mouse model (CD-1) have not been thoroughly evaluated. The male out-bred-CD-1 mouse was chosen as a relevant human model for endocrine disruption by 17-β estradiol, because it is considered to be less 17-β estradiol-responsive (in terms of reproductive changes) compared to the in-bred mouse. In the present study, CD-1 male mice were dosed with vehicle, or 17-β estradiol at 2 or 4 μg / 100g body weight on alternate days over a seven day period. The immune changes in the developmental organ (thymus) and mature lymphoid organ (spleen) were determined. Thymic organ weight / body weight ratio and thymocyte cellularity decreased with increasing 17-β estradiol, reaching significance at the 4μg dose. Although 17-β estradiol decreased thymocyte numbers, no differences were measured in the relative percentages of major thymocyte subsets ($CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, $CD4^-CD8^-$) and no evidence of enhanced apoptosis was found. In contrast to the diminished thymocyte numbers, 17-β estradiol increased splenic lymphocyte cellularity, especially in mice given the at 4 μg 17-β estradiol dose. The functionality of splenocytes from mice exposed to 17-β estradiol was also altered. Supernatants from Con-A activated splenocytes from 17-β estradiol-treated mice had increased IFN-γ and decreased IL-4 levels (*$P<0.05$* at the 4 μg dose). This increase in IFN-γ in 17-β estradiol treated mice was not due to an increase in the relative percentages of T cells, since they were comparable to relative percentages of T cells from oil-treated control mice. In addition, supernatants from cultured splenocytes (both Con A-activated and unstimulated) also had significantly higher levels of nitric oxide activity, especially at the 4 μg 17-β estradiol dose. These results indicate that short-term 17-β estradiol treatment in out-bred mice, at relatively modest doses (2-4 μg / 100 g body weight), altered both thymocytes and splenocytes. These immune changes are compelling, since in these mice, post-17-β estradiol

exposure did not demonstrate robust changes in the male reproductive system (testicular and seminal vesical weights to body weight ratios).

b.) INTRODUCTION

It is now well accepted that there are marked sex differences in the level of immune responsiveness including susceptibility to autoimmune diseases. For example, in many autoimmune diseases involving both human and animal models, females have a higher predisposition to develop these disorders compared to males (Ansar Ahmed et al., 1989; Ansar Ahmed et al., 1985; Ansar Ahmed et al., 1999; Ansar Ahmed et al., 1985). These sex differences in immune response to a variety of antigens are thought to be largely due to the effects of sex hormones.

Many studies have shown that sex hormones such as 17-β estradiol are profoundly immunomodulatory (Ansar Ahmed et al., 1999; Cutolo et al., 1995). The 17-β estradiol has been shown to affect all major cells of the immune system. Thymic atrophy is a common consequence of long-term 17-β estradiol exposure in wild type C57BL/6 mice (Ansar Ahmed, 2000; Mor et al., 2001; Screpanti et al., 1991; Screpanti et al., 1989; Staples et al., 1999; Yao and Hou, 2004). The 17-β estradiol promotes B cell hyperactivity in wild type mice as demonstrated by the increased secretion of immunoglobulins and autoantibodies to mouse erythrocytes (Ansar Ahmed et al., 1989), dsDNA (Verthelyi and Ahmed, 1994), and anionic phospholipids (Ansar Ahmed and Verthelyi, 1993; Verthelyi and Ansar Ahmed, 1997). This implies that 17-β estradiol can break B cell tolerance, even in normal mice, an aspect also shown in Balb/c mice transgenic for the heavy chain of pathogenic anti-double stranded DNA antibodies (Bynoe et al., 2000). Further, 17-β estradiol appears to enhance B cell differentiation, as evidenced by the increased number of plasma cells in the spleen (Verthelyi, 2001; Verthelyi and Ahmed, 1998). In mice or rodents, the administration of 17-β estradiol to mice decreases natural killer cell (NK) activity (Ansar Ahmed et al., 1989; Seaman et al., 1978; Seaman and Gindhart, 1979), and in female mice a reduction in NK cell numbers and activity correlates with increased levels of 17-β estradiol (Furukawa et al., 1984; Hrushesky et

al., 1988). Furthermore, 17-β estradiol has been shown to affect macrophages by altering proliferation, phagocytosis (Schreiber et al., 1988; Vernon-Roberts, 1969; Zuckerman and Bryan, 1996), and lysosomal activities (Yu et al., 1996), and enhancing the levels of IL-1 (Flynn, 1984; Flynn, 1986; Hu et al., 1988).

The mechanisms by which 17-β estradiol modulates the immune system are complex. One important mechanism is through the regulation of the secretion and/or response to cytokines. Studies from our laboratory, as well as others, have shown that 17-β estradiol markedly augment the secretion of IFN-γ (Fox et al., 1991; Karpuzoglu-Sahin et al., 2001; Sarvetnick and Fox, 1990). These observations are of biological and clinical importance, since IFN-γ (primarily secreted by T-helper 1 (Th1), $CD8^+$ cells, and NK cells), plays a central role in the host defense against intracellular infection (Gallin et al., 1995; Trinchieri, 1997) and many proinflammatory conditions including autoimmune diseases (Sarvetnick and Fox, 1990; Theofilopoulos et al., 2001). Other investigators have shown that 17-β estradiol also suppresses the production of T helper 2 type cytokines, such as IL-6 (Koka et al., 1998; Messingham et al., 2001), and TNF-α (Ito et al., 2001).

Taken together, the above findings demonstrate that studies addressing the immunomodulatory effect of 17-β estradiol on the immune system are of high health significance, since this hormone affects all of the major players of the immune system, and regulates normal and autoimmune responses. Of clinical relevance, humans are exposed to estrogenic compounds through diverse sources. Women are exposed, not only to endogenous 17-β estradiol, but also exogenous estrogens in the form of oral contraceptives or estrogen replacement therapy. Moreover, both men and women, and animals are inadvertently exposed to estrogenic compounds that are universally present in the environment, both naturally and as contaminants. Humans are likely exposed to estrogenic compounds, either acutely or chronically, thus exhibiting different sensitivities. Immunomodulation by an estrogenic compound is dependent upon both the dose and duration of exposure (Ansar Ahmed, 2000). In this study, we investigated the immunological consequences of short-term exposure to relatively modest doses of 17-β estradiol in out-bred CD-1 mice which are considered to be

less sensitive to even high doses of 17-β estradiol compared to in-bred mice (Spearow et al., 1999; Spearow et al., 2001).

c.) MATERIALS AND METHODS

Mice: Nine-month-old male CD-1 out-bred mice (Charles River, Wilmington, MA) were housed in standard cages (3-4 animals per cage) at the Center for Molecular Medicine and Infectious Diseases (CMMID) Laboratory Animal Facility. All mice were fed on a commercial pellet diet devoid of synthetic estrogens (Special diet # 7013 Harlan Teklad, Madison, WI) with water *ad libitum*. The mice were kept on a 14 h light, 10 h dark cycle. All procedures and treatments were performed in accordance with the guidelines of the Animal Care Committee of the Virginia Polytechnic Institute and State University.

The 17-β Estradiol Treatment: Commercial estrogen, 17-β estradiol (Sigma, St. Louis, MO) was dissolved in autoclaved, tocopherol-stripped corn oil (ICN, Auro, Ohio). Mice were given three subcutaneous injections of 17-β estradiol at 2 or 4 μg / 100g body weight (b.w.) in 50 μl on alternate days. The mice that received oil vehicle only served as controls. Mice were euthanized by cervical dislocation 7 days post-treatment and bled before termination.

Analysis of Reproductive Organ Changes by 17-β Estradiol: Reproductive organs (seminal vesicles and testes) were removed, trimmed of excess body fat, and pre-weighed. The net wet weight and the ratio of reproductive organ to body weight were analyzed. Seminal vesicles and testes were placed in 10 % buffered formalin (Sigma, St. Louis, MO) and processed in a coded fashion for histopathologic analysis by staining paraffin-embedded tissue sections with hematoxylin and eosin. The height of the epithelial cells was measured by micrometer in a coded fashion. The data were then decoded and statistical analyzed.

Isolation of Lymphocytes: Thymus and spleen were collected under aseptic conditions and weighed. The lymphoid organ weights of each individual mouse were normalized to their respective body weight. The cells from thymus, and spleen were isolated by gently dissociating on a 60-mesh steel screen (Sigma, St. Louis, MO) in sterile phenol-red free

incomplete RPMI-1640 media (CellGro, Mediatech, Herndon, VA), as described in our previous studies (Donner et al., 1999). Splenic lymphocytes were enriched by removing erythrocytes using lysis in ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) (Ansar Ahmed et al., 1994). Thymocytes and enriched-splenocytes were resuspended in complete RPMI-1640 free of estrogenic phenol red supplemented with 10% charcoal-stripped fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (ICN, Costa Mesa, CA), 50 IU/ml penicilin (Mediatech, Herndon, VA), 50 μg/ml streptomycin (Mediatech, Herndon, VA), and non-essential amino acids (Fisher, Pittsburg, PA). The enriched lymphocytes were then enumerated with a CASY-1 Cell Counter and Analyzer System (Scharfe System GmbH, Reutingen, Germany). In our experience, cells ranging from 5 to 10 microns constitute the major lymphocyte populations (Calemine et al., 2002; Donner et al., 1999). The cells were then adjusted to a concentration of $5x10^6$ cells/ml in complete phenol-red free complete RPMI as described in our earlier studies (Donner et al., 1999).

Determination of Cell Surface Marker Expression and Apoptosis via Triple-Color 7-AAD Method: Staining of thymocytes with 7-amino-actinomycin (7-AAD, Molecular Probes, Eugene, OR) is a reliable and sensitive method for identifying apoptotic thymocytes, and in particular thymocytes in early stages of apoptosis (Donner et al., 1999; Schmid et al., 1994). Cells that do not take up 7-AAD dye are identified as "live cells" or 7-AAD^{dull}, the cells that are in early stages of apoptosis are identified as 7-$AAD^{intermediate}$, while the cells in the late stage of apoptotic or necrotic phase are identified as 7-AAD^{bright} (Schmid et al., 1994). To identify whether 17-β estradiol selectively affects apoptosis of thymocyte subsets, thymocytes were triple stained with FITC-anti-CD8a, PE-anti-CD4 antibodies (BD Pharmingen, San Diego, CA) and 7-AAD (Molecular Probes, Eugene, OR). Briefly, 7-AAD at 10 μg/ml in PBS supplemented with 0.1% sodium azide, 0.1% BSA, and 1% FBS was added to 100 μl of thymocytes ($5x10^6$ cells/ml) from control, and. 17-β estradiol-treated (2 and 4 μg / 100g body weight) mice and incubated 20 min at 4 C in the dark. These stained cells were subjected to triple-color on an EPICS XL flow cytometer using Immuno 4 program. The gates were set on each thymocyte subset ($CD4^-CD8^+$, $CD4^+CD8^-$, $CD4^+CD8^+$,

$CD4^{-}CD8^{-}$) and the percentage of live, early apoptotic, and late apoptotic/necrotic cells were determined according to 7-AAD uptake, which is directly proportional to the extent of apoptosis (Donner et al., 1999). The percentages of live, early and late apoptotic cells were also determined in the total population of thymocytes. The effects of 17-β estradiol treatment on apoptosis of mature B cells ($CD45RB^{+}$ cells) and T cells ($CD90^{+}$ cells) in the spleen were also determined. The absolute recoveries of the cells were obtained via multiplying mean percent relative expression with cell recoveries. Briefly, freshly-isolated and 48 hours Con-A (10 μg/ml) activated splenic lymphocytes were analyzed by triple staining with FITC-anti-Thy1.2, PE-anti-CD45RB (B220) antibodies (BD Pharmingen, San Diego, CA) and 7-AAD method as described above.

Detection of Cytokines: The levels of IFN-γ, and IL-4 protein were determined by a sandwich, enzyme linked immunosorbent assay (ELISA) as per our previous publications. (Karpuzoglu-Sahin et al., 2001; Karpuzoglu-Sahin et al., 2001). Briefly, 500 μl ($2.5x10^{6}$ cells/well) of splenic lymphocytes from 17- β estradiol and control treated mice were stimulated for 24 or 48 hrs with an equivalent volume of Con-A (10 μg/ml) or left unstimulated in complete phenol-red free RPMI-1640 media. Following this incubation, the supernatants were collected and stored at -20^{0}C until assayed. Ninety-six well Maxisorp high binding immunoassay plates were coated overnight with anti-cytokine antibodies (anti-IFN-γ clone R4-6A2, ATCC, HB-170; or anti-IL-4 antibody, clone 11B11, ATCC, HB-188, BD Pharmingen, San Diego, CA) in PBS and then blocked with 2% BSA for 1 hr. Plates were incubated for 2 hrs with supernatants or recombinant cytokines with serial dilutions. Biotin conjugated anti-cytokine antibodies (anti-IL-4 or anti-IFN-γ; BD Pharmingen, San Diego, CA) were aliquoted into wells and incubated for 1 hr. Avidin-horseradish peroxidase solution (Vector Labs, Burlingame, CA) was added and incubated for 30 mins. The development of color was initiated with substrate TMB (3,3',5,5'-tetra-methylbenzidine), (KPL, Gaithersburg, MD) followed with 0.18 M sulfuric acid to stop the reaction. Plates were washed between each step with wash buffer (50mM Tris, 0.2% Tween 20). Plates were read at 450 nm by microplate ELISA reader (Molecular Devices Sunnyvale, CA). The IL-4 and IFN-γ protein

levels were calculated by the formula obtained from standards using SoftMax Pro software from Molecular Devices Inc.

Detection of Nitric Oxide: The levels of nitric oxide in the supernatants derived from the splenic lymphocyte cultured for 24 or 48 hr were determined by the Greiss Assay, which measures nitrite, an end product of oxidation of nitric oxide. Briefly, 50 μl of nitric oxide standards with serial dilutions beginning at 1000 μM to 0.5 μM or supernatants were added to a 96-well plate. Equal volumes of Griess assay reagents, 50μl 1%(w/v) sulfanilamide (Sigma St. Louis, MO) and 50 μl 0.1% (w/v) naphthylenediamine dihydrochloride (Sigma, St. Louis, MO) were then added to an optical density plate. The color change in the plates was immediately measured at 550 nm by ELISA reader (Molecular Devices Sunnyvale, CA). The nitric oxide levels in the supernatants were calculated using SoftMax Pro software from Molecular Devices Inc.

Detection of Immunoglobulins: A sandwich enzyme linked immunosorbent assay was employed to measure IgG, IgM, and IgG2a levels in the blood (Verthelyi and Ansar Ahmed, 1997). Briefly, peripheral blood obtained from each CD-1 mice retro-orbitally, was allowed to clot, then centrifuged at 7000 rpm for 7 min. Sera were collected from each sample then frozen at –70°C until analyzed. Medium binding plates (Costar, Cambrigde, MA) were coated with 50 μl of IgG, IgG2a, or IgM (0.25 μg/ml, Southern Biotech, Birmingham, AL) in bicarbonate buffer and incubated overnight at 4°C. The plates were rinsed with PBS with Tween 20 between each step. The plates were blocked with 1% BSA in PBS and incubated at room temperature for 1 hour in a humidified chamber. After blocking, the sera were diluted in 1% BSA in PBS and the standards (Southern Biotech, Birmingham, AL) were prepared. A 100 μl aliquot of sera or standard were added to each well incubated for 3 hrs at room temperature in a humidified chamber. One hundred microliters of AP-conjugated goat anti-mouse IgG, IgG2a, or IgM (Southern Biotech, Birmingham, AL) in 1% BSA PBS was added per well and incubated at 37°C for 1 hr in a humidified chamber. Plates were washed with PBS with 5% Tween between each step. The color was determined using pNPP (Sigma, St.Louis, MO) after incubating for 45 min and plates were read at 405 nm by ELISA reader

(Molecular Devices Sunnyvale, CA). The IgG, IgG2a, or IgM antibody levels were calculated by the formula obtained from standards using SoftMax Pro software from Molecular Devices Inc.

Statistics: Data were analyzed by using INSTAT software (GraphPad Software Inc., San Diego, CA). The analysis of variance (ANOVA) was used to assess main effects of doses and their interaction. The treatments were compared to each other using Bonferroni multiple comparisions test. Data are represented as mean ± standard error of mean (S.E.M.).

d.) RESULTS

Short-Term 17-β Estradiol Treatment does not affect Body Weights, Reproductive Organ Weight or Organ/Body Weight Ratio: Low-dose, short-term 17-β estradiol treatment of intact male CD-1 mice did not affect body weight (Vehicle control-0 μg /100g body weight (b.w.) 43.93 g ± 1.95; 17-β estradiol - 2 μg / 100g body weight 45.28 g ± 1.41; 17-β estradiol - 4 μg / 100 g body weight 45.77 g ± 3.04). The 17-β estradiol treatment of male CD-1 mice did not alter the seminal vesicle net weights (Vehicle control-0 μg / 100g body weight 0.45 g ± 0.03; 17-β estradiol - 2 μg / 100g body weight 0.45 g ± 0.03; 17-β estradiol - 4 μg / 100 g body weight 0.49 g ± 0.05). The ratios of seminal vesicle weights to body weights were also not changed (Vehicle control - 0 μg / 100g body weight 0.01 g ± 0.01; 17-β estradiol - 2 μg / 100g body weight 0.01 g ± 0.01; 17-β estradiol - 4 μg / 100 g body weight 0.011 g ± 0.01). The testicular net weights (Vehicle control - 0 μg / 100g body weight 0.32 g ± 0.02; 17-β estradiol - 2 μg / 100g body weight 0.3 g ± 0.02; 17-β estradiol - 4 μg / 100 g body weight 0.34 g ± 0.02) or their ratio to the body weight were not changed after administration of 17-β estradiol for 7 days (Vehicle control-0 μg / 100g body weight 0.01 g ± 0.01; 17-β estradiol -2 μg / 100g body weight 0.01 g ± 0.01; 17-β estradiol - 4 μg / 100 g body weight 0.01 g ± 0.01). The histologic changes in the seminal vesicles were unremarkable and not significant. There was an increasing, but not significant, trend in epithelial cell height with increasing dose of 17-β estradiol (Vehicle control - 0 μg / 100g body weight 6.33 μm ±

0.48; 17-β estradiol -2 μg / 100g body weight 7.15 μm ± 0.56; 17-β estradiol -4 μg / 100 g body weight 7.32 μm ± 0.78, statistically not significant).

Short-Term 17-β Estradiol Treatment decreases Thymocyte Cellularity: The total thymic cellularity was significantly decreased in CD1 mice given 4 μg of 17-β estradiol / 100g body weight. Thymic-body weight ratio was decreased in mice given 4 μg / 100g body weight of 17-β estradiol when compared to controls (**Figure 1**).

The Effect of Short-Term 17-β Estradiol Treatment on the Subset Composition and Apoptosis of Thymocytes: Since short-term 17-β estradiol treatment in out-bred CD-1 mouse decreased the thymocyte cellularity, we next explored whether there was an alteration in thymocyte subsets or increased apoptosis in these subsets. Short-term 17-β estradiol treatment did not alter the relative mean percentages of thymic subsets of $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{+}$, $CD4^{+}CD8^{+}$, $CD4^{-}CD8^{-}$ T cells (**Table 1**). Although the relative percentages of these subsets were unchanged by 17-β estradiol, since this hormone decreased the thymocyte cellularity, the absolute numbers of thymocytes subsets per thymus were decreased (**Table 1**). The 7-AAD triple color analysis of these subsets revealed no significant change in the relative percentages of live or apoptotic cells (**Table 2**).

The Cellularity, Cellular Composition, and Apoptosis of Fresh Splenic Lymphocytes: The 17-β estradiol treatment at 4 μg / 100g body weight showed a significant increase in the cellularity of splenic lymphocytes gated between 5.0 and 10 mm of size. A trend toward increased splenic cellularity was also observed in mice treated with 2 μg of 17-β estradiol (Figure 2). The cell numbers between 5.0 and 7.5 mm were also significantly increased with increasing doses of 17-β estradiol. Lymphocytes at 7.5 to 10 microns, representing increased size, were significantly increased after treatment with 4 μg of 17-β estradiol dose for 7 days. The number of cells in the quadrant between 2.5 and 5.0 mm, which may reflect increase in number of erythrocytes, platelets, debris or apoptosis cells, was also increased by 17-β estradiol -treatment (**Figure 2**).

The Effect of Short-Term 17-β Estradiol Treatment on the Subset Composition and Apoptosis of Fresh Splenic Cells: In the spleen, the percentages of lymphocytes expressing T cell marker, Thy1.2^{+} (CD90^{+}), or B cell marker CD45RB^{+} (B220^{+}) were not altered after short-term of 17-β estradiol treatment. Even though 17-β estradiol treatment of out-bred CD-1 mice had increased numbers of splenocytes, there were no relative differences in the percentages of Thy1.2^{+} (pan T cells) or CD45R (B220^{+}, pan B cells). The absolute numbers of subpopulations per spleen were increased in the numbers of non-T and non-B cells in both doses, 2 and 4 μg of 17-β estradiol / 100g body weight, but not in T or B cells (**Table 3**). The short-term 17-β estradiol treatment of freshly-isolated splenic lymphocytes did not alter viability of total splenocytes or T or B splenic subsets when compared to controls (**Table 4**).

Short-Term 17-β Estradiol Treatment alters IFN-γ and IFN-γ: IL-4 Ratios: Even though the relative percentages of splenic T cells were not altered by 17-β estradiol, it is likely that these cells may be functionally altered. We, therefore, measured the levels of IFN-γ in Con-A activated splenocytes of mice given short-term 17-β estradiol treatment. Interferon-γ was chosen, because in our previous long-term 17-β estradiol studies (3-5 months), we reported that this cytokine is upregulated by this hormone (Karpuzoglu-Sahin et al., 2001; Karpuzoglu-Sahin et al., 2001). In the present study, IFN-γ protein levels were significantly increased in the supernatants of Con-A-stimulated splenic lymphocytes from mice given 4 μg 17-β estradiol dose ($P<0.05$, **Figure 3A**). There was a significant decrease in IL-4 protein levels in these 24 hour cultures of Con-A activated splenocytes from mice exposed to increasing doses of 17-β estradiol (**Figure 3B**). The ratio of IFN-γ to IL-4 protein was increased significantly in 17-β estradiol-treated mice, after 4 μg of 17-β estradiol treatment (**Figure 3C**).

Short-Term 17-β Estradiol Treatment induced IFN-γ-Dependent Nitric Oxide, a Product of Inducible NOS: Given that 17-β estradiol increased IFN-γ levels, we next determined whether nitric oxide levels were also increased following short-term 17-β estradiol treatment, since macrophages are known to release nitric oxide in response to IFN-γ. Low levels of nitric oxide were detected even in the supernatants of unstimulated cultures from 17-β estradiol -

treated mice. The levels of nitric oxide were significantly increased in the supernatants of Con-A-stimulated splenocytes from 4 μg of 17-β estradiol-treated mice (**Figure 4**).

Cellular Composition and Apoptosis of Splenic Lymphocytes from 17-β Estradiol Treated Mice were not altered after 48 Hr of Culture: The17-β estradiol treatment of mice did not alter the mean relative expression of splenic T or B cell subsets that were cultured for 48 hr (**Table 5**). Apoptosis seemed to be variably influenced by culturing conditions and treatment. Cells cultured in medium showed a trend toward increased apoptosis. However, when the same cells when stimulated with Con-A, they were rescued from apoptosis , but on the other hand the controls had increased apoptosis (**Table 6**).

Effect of Short-Term 17-β Estradiol Treatment on the Serum Immunoglobulins: As shown in **Figure 5**, the levels of IgM, IgG and IgG2a were decreased in the sera from mice treated with 2 μg 17-β estradiol dose.

e.) DISCUSSION

This study was performed to explore whether short-term, low-dose 17-β estradiol treatment (4 μg 17-β estradiol / 100g body weight over 7 days) would alter the immune system of "estrogen-resistant" out-bred CD-1 male mice (Spearow et al., 1999; Spearow et al., 2001). Our findings demonstrate that even a short period of treatment with 17-β estradiol produced significant qualitative and quantitative effects on primary (thymus) and secondary (spleen) lymphoid organs in these "estrogen-resistant" mice. Spearow et al, 1999 (Spearow et al., 1999) demonstrated the differential effects of 17-β estradiol on the disruption of reproductive development in different strains of mice, including the CD-1 strain, as an out-bred mouse model, and the C57BL/6J strain, as an in-bred mouse model.The magnitude of reproductive organ changes varied between C57BL/6 and CD-1 mice, when exposed to same dose and duration of 17-β estradiol. Although C57BL/6 mice had susceptibility to 17-β estradiol at doses as low as 0.25 μg / gram body weight, CD-1 mice were resistant to 17-β estradiol levels as high as 40 μg / gram body weight. CD-1 mice were shown to be 16X more resistant to the endocrine disruption of male reproductive development in relation to the effect of estrogen on

the inhibition of testes and vesicular gland weight, and on spermatogenesis (Spearow et al., 1999; Spearow et al., 2001). In agreement with these studies, our studies with low-doses of 17-β estradiol given for a short-term show that, CD-1 mice are resistant to the effect of 17-β estradiol on gonadal functions; 17-β estradiol did not cause any change in the body weights or net weights of the seminal vesicles or testes, or in the reproductive organ to body weight ratios. In contrast, our studies show that CD-1 mice are sensitive to immunomodulatory effects of even these short- term low-doses of 17-β estradiol. Interestingly, while this dose of 17-β estradiol had no obvious effects on the reproductive system, the immune changes were clearly evident.

One important mechanism by which 17-β estradiol (estrogen) could modulate the immune system is via its effect on thymic and splenic cellularity and on the rate of apoptosis of thymic and splenic lymphocytes. Long-term estrogen treatment (3–5 months) has been shown to diminish thymic cellularity, decrease thymic weight, induce thymic atrophy, and alter the subsets of thymocytes, such $CD4^+CD8^+$ T cells, which were decreased (Forsberg, 1996; Okasha et al., 2001; Seiki and Sakabe, 1997; Silverstone et al., 1994; Staples et al., 1999). In our study, we observed that short-term treatment of out-bred CD-1 mice with 4 μg 17-β estradiol / 100g body weight resulted in a significant decrease in thymic cellularity and thymic to body weight ratio. Possible mechanisms by which estrogen could induce thymic atrophy are by altering the thymocyte subsets, accelerating thymocyte emigration, decreasing thymocyte proliferation, or increasing the apoptosis of thymocytes. Therefore, we evaluated the effect of short-term low-dose 17-β estradiol on the T cell subsets and apoptosis of freshly-isolated thymocytes from 17-β estradiol and corn oil (control)-treated CD-1 male mice. Previous studies (Rijhsinghani et al., 1996; Screpanti et al., 1989; Staples et al., 1999), showed that long-term estrogen treatment depletes subsets of $CD4^+CD8^+$ double-positive (DP) thymocytes and alters the relative proportion of $CD4^+CD8^-$ and $CD4^-CD8^+$ single-positive (SP) thymocytes. In our study, short-term 17-β estradiol treatment did not alter the relative percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, and $CD4^-CD8^-$ thymocyte subsets. However, the absolute numbers of fresh-thymocyte subsets per thymus showed a significant decrease in all subsets after treatment with 4 μg 17-β estradiol / 100g body weight.

The differences in the effect of 17-β estradiol on the percentage of thymic subsets between this study and the previous studies could be due to differences in the strains of mice (for example: the use of out-bred CD-1 strain in this study compared to in-bred strains, C57BL/6 Balb/c, employed in other studies), in the dose and duration of estrogen administration. A possible reason for the decreased thymic cellularity in our study and the previous studies (Mor et al., 2001; Okasha et al., 2001) could be due to spontaneous or steroid-induced apoptosis. Although our data demonstrate decreased thymic cellularity after 17-β estradiol treatment, no significant change was measured in the apoptosis of whole fresh thymocytes by, the 7-AAD analysis. The possibility that 17-β estradiol -induced apoptotic cells were engulfed prior to analysis still needs to be considered. Silverstone et al. 1994 (Silverstone et al., 1994) demonstrated that a single 17-β estradiol dose (7.5 mg / 100g body weight) resulted in thymic atrophy and a persistent decrease in the thymic $Rag1^{+}$ TdT^{+} $CD4^{+}8^{+}3^{+}$ population. This 17-β estradiol dose also caused a slow reduction in the bone marrow stem cell markers, Rag-1 and TdT. This effect of estrogen on thymic atrophy was found to be driven by an alteration in the bone marrow thymocyte precursors, rather than by apoptosis (Silverstone et al., 1994). Furthermore, estrogen treatment also decreased bone marrow RAG-1 mRNA, which is important in thymus development. Thus, a decrease in thymic cellularity following short-term 17-β estradiol exposure could be due to alterations in bone marrow thymocyte precursors.

In contrast to the effect of short-term, low-dose 17-β estradiol treatment (4μg /100 g body weight over 7 days) on thymic cellularity, our studies showed that this treatment produced a significant increase in splenic cellularity in out-bred male CD-1 mice. Mice treated with 17-β estradiol at 2 μg / 100 g body weight also showed a similar non-significant trend. Interestingly, this increase in the splenic cellularity was not attributed to an increase in $Thy1.2^{+}$ or $CD45R^{+}$ cells based on the relative percentages of these cells in from fresh lymphocyte preparations. The absolute numbers of splenic lymphocyte subsets that were calculated, using the cell numbers from each spleen, demonstrated, instead, that the double negative cells (non-B and non-T cells/$CD45RB^{-}$ $Thy1.2^{-}$) increased with 17-β estradiol treatment. These double negative cells could be macrophages or plasma cells, or B and/or T cells that shed their markers after treatment. Studies in our laboratory demonstrated that long-

term 17-β estradiol treatment [3-5 months using silastic implants (7 mm long, containing 4-6 mg of 17-β estradiol)] of gonadectomized mice decreased the number of $CD45RB^{+}$ ($B220^{+}$) cells and small lymphocytes, while the number of plasma cells increased (Verthelyi and Ahmed, 1998). Medina et al (1993, 1994) showed that high-dose of estrogen (2.5 mg of 17-β estradiol pellets given for 21 days) or pregnancy suppress B lymphopoesis and B lineage precursors in bone marrow (Medina and Kincade, 1994; Medina et al., 1993). They also showed that mature splenic B cell subsets are not changed during pregnancy (Medina and Kincade, 1994; Medina et al., 1993). On the other hand, mice that were deficient in sex steroids had increased B cell numbers (Masuzawa et al., 1994).

The levels of sex hormones, such as 17-β estradiol, have been shown to influence the outcome of apoptosis in a number of cell types (Ansar Ahmed, 2000; Ansar Ahmed et al., 1999). Previous *in-vitro* estrogen treatment studies showed that estrogen modulates apoptosis, increasing in T cell death and promoting the survival of B cells (Jenkins et al., 2001; McMurray et al., 2001), and breast cancer cells (LTED cell line) (Song and Santen, 2003). Zajchowski and Hoffman-Goetz (Zajchowski and Hoffman-Goetz, 2000) showed that supra-physiological levels of 17-β estradiol (pellets releasing 71.4 μg per day for 14 days), given *in vivo* to gonadectomized female C57BL/6 mice, induce the apoptosis of lymphocytes. At physiological levels, 17-β estradiol plays a critical role in the protection from apoptosis of several cell types and tissues, such as brain, endothelium, testes, uterus, aortic endothelial cells (Liu et al., 2002; Sudoh et al., 2001), bone marrow mesenchymal stem cells (Zhou et al., 2001), neuronal cells (PC12 cell line) (Hosoda et al., 2001), cortical neuronal cells (Honda et al., 2001), osteocytes (Tomkinson et al., 1998), B lymphocytes (Grimaldi et al., 2002; Verthelyi, 2001) In contrast to these studies, the current study did not show any significant effect of short-term, low-dose 17-β estradiol (2-4 μg 17-β estradiol / 100g body weight over 7 days) on the apoptosis of the splenic lymphocyte subsets. Further studies should be performed using multiple assays for apoptosis to definitely ascertain the effect of short-term, low-dose 17-β estradiol treatment on the apoptosis of splenic lymphocytes. In the present study, 7-AAD was employed, which cannot distinguish between late apoptosis and necrosis. Further, this assay does not provide precise information on DNA fragmentation.

Extensive studies from our laboratory (Ansar Ahmed, 2000; Ansar Ahmed et al., 1999) and others (Bilbo and Nelson, 2001; Cutolo et al., 1995; Olsen and Kovacs, 1996) have recognized the sensitivity of the immune system to the effects of estrogen. One important mechanism by which estrogen modulates the immune system is by altering the secretion and/or response to cytokines. Studies in our laboratory have demonstrated that chronic exposure to 17-β estradiol results in increased IFN-γ mRNA expression and protein secretion (Karpuzoglu-Sahin et al., 2001; Karpuzoglu-Sahin et al., 2001). We showed that female C57BL7/6 mice had more IFN-γ protein levels than males (Karpuzoglu-Sahin et al., 2001). Fox et al (1991) demonstrated that *in vitro* 17-β estradiol treatment (10^{-7} M of 17-β estradiol for 10 hrs) increased IFN-γ mRNA expression in Con-A-treated spleen cells from CD-1 mice through a direct effect on the IFN-γ promoter (Fox et al., 1991). The current study demonstrated that short-term, low-dose 17-β estradiol treatment (2-4 μg 17-β estradiol / 100g body weight over 7 days) increased the level of IFN-γ protein, which is a T helper 1 cytokine, and decreased the level of IL-4, which is a T helper 2 cytokine. This study shows that short-term, low-dose 17-β estradiol treatment leads to functional alterations in cytokine release by splenic lymphocytes.

In the present study, we also found that the supernatants of Con-A activated splenic lymphocytes treated with 4 μg 17-β estradiol / 100 g body weight had increased nitric oxide levels after 48 hours of incubation compared to supernatants from cells treated with 2 μg 17-β estradiol / 100g body weight or corn oil (vehicle control). The level of nitric oxide in unstimulated cultures (media instead of Con-A) also increased in splenic lymphocytes treated with the 4 μg dose. Nitric oxide (NO), an end product of the IFN-γ-inducible iNOS (inducible nitric oxide synthase), plays an important role in immune regulation and cell differentiation. For example, it provides both, anti-microbial and cytotoxic effects for the immune system (MacMicking et al., 1997). The activity of iNOS is controlled at the transcription level and regulated by steroid hormones, such as glucocorticoids and progestrone (Kunz et al., 1996; Miller et al., 1996; Radomski et al., 1990). Dixit and Parvizi (Dixit and Parvizi, 2001) showed that nitric oxide secretion from peripheral blood lymphocytes was significantly increased in

all stages of pregnancy compared to that in cyclic and cystic cows. These investigators also showed that physiological concentrations of estrogen caused the release of NO by iNOS in macrophages, but that iNOS protein was decreased in IFN-γ and LPS stimulated J774 cells that were co-incubated with 17-β estradiol. The 17-β estradiol has been shown to increase iNOS expression in cardiac myocytes (Nuedling et al., 1999), ovine coronary artery (Mershon et al., 2002), and uterine leukocytes (Hunt et al., 1997).

Many previous studies have demonstrated that estrogen alters B cell differentiation and/or function. We previously showed that chronic 17-β estradiol treatment [3-5 months using silastic implants (7 mm long, containing 4-6 mg of 17-β estradiol)] induces hyperactivity in B cells, resulting in increased immunoglobulins, autoantibodies, and plasma cells (Verthelyi and Ahmed, 1998). Although the long-term 17-β estradiol treatment of C57BL/6 mice caused a decrease in numbers of $CD45^+$ ($B220^+$) cells, it increased the serum titers of IgG by 6-fold and IgM by 3-fold (Verthelyi and Ahmed, 1998). This may be due to a direct effect of estrogen on mature B cells or the regulation of the synthesis of T cell-derived cytokines that in turn regulate B cell functions. In the present study, the levels of IgG, IgG2a and IgM were not significantly increased by treatment with 4μg 17-β estradiol / 100g body weight. Possible explanations for the differences between our study and the previous study (Verthelyi and Ahmed, 1998) may involve the shorter duration of exposure, the lower 17-β estradiol dose, and the different strain of mice used in out current study.

Taken together, this study demonstrates that the immune system is a sensitive target for 17-β estradiol even in CD-1 mice whose reproductive tissues are less responsive to this hormone. Short-term, low-dose 17-β estradiol treatment induced both qualitative (decreased thymocyte cellularity and increased splenic cellularity) and quantitative (enhanced IFN-γ and nitric oxide levels) changes to the immune system.

f.) REFERENCES

Ansar Ahmed, S. (2000). The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. *Toxicology* 150: 191-206.

Ansar Ahmed, S., Dauphinee, M. J., Montoya, A. I., and Talal, N. (1989). Estrogen induces normal murine CD5+ B cells to produce autoantibodies. *J Immunol* 142: 2647-2653.

Ansar Ahmed, S., Dauphinee, M. J., and Talal, N. (1985). Effects of short-term administration of sex hormones on normal and autoimmune mice. *J Immunol* 134: 204-210.

Ansar Ahmed, S., Gogal, R. M., Jr., and Walsh, J. E. (1994). A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *J Immunol Methods* 170: 211-224.

Ansar Ahmed, S., Hissong, B. D., Verthelyi, D., Donner, K., Becker, K., and Karpuzoglu-Sahin, E. (1999). Gender and risk of autoimmune diseases: possible role of estrogenic compounds. *Environ Health Perspect* 107 Suppl 5: 681-686.

Ansar Ahmed, S., Penhale, W. J., and Talal, N. (1985). Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol* 121: 531-551.

Ansar Ahmed, S., and Verthelyi, D. (1993). Antibodies to cardiolipin in normal C57BL/6J mice: induction by estrogen but not dihydrotestosterone. *J Autoimmun* 6: 265-279.

Bilbo, S. D., and Nelson, R. J. (2001). Sex steroid hormones enhance immune function in male and female Siberian hamsters. *Am J Physiol Regul Integr Comp Physiol* 280: R207-213.

Bynoe, M. S., Grimaldi, C. M., and Diamond, B. (2000). Estrogen up-regulates Bcl-2 and blocks tolerance induction of naive B cells. *Proc Natl Acad Sci U S A* 97: 2703-2708.

Calemine, J. B., Gogal, R. M., Jr., Lengi, A., Sponenberg, P., and Ahmed, S. A. (2002). Immunomodulation by diethylstilbestrol is dose and gender related: effects on thymocyte apoptosis and mitogen-induced proliferation. *Toxicology* 178: 101-118.

Cutolo, M., Sulli, A., Seriolo, B., Accardo, S., and Masi, A. T. (1995). Estrogens, the immune response and autoimmunity. *Clin Exp Rheumatol* 13: 217-226.

Dixit, V. D., and Parvizi, N. (2001). Pregnancy stimulates secretion of adrenocorticotropin and nitric oxide from peripheral bovine lymphocytes. *Biol Reprod* 64: 242-248.

Donner, K. J., Becker, K. M., Hissong, B. D., and Ahmed, S. A. (1999). Comparison of multiple assays for kinetic detection of apoptosis in thymocytes exposed to dexamethasone or diethylstilbesterol. *Cytometry* 35: 80-90.

Flynn, A. (1984). Stimulation of interleukin-1 production from placental monocytes. *Lymphokine Res* 3: 1-5.

Flynn, A. (1986). Expression of Ia and the production of interleukin 1 by peritoneal exudate macrophages activated in vivo by steroids. *Life Sci* 38: 2455-2460.

Forsberg, J. G. (1996). The different responses of the female mouse thymus to estrogen after treatment of neonatal, prepubertal, and adult animals. *Acta Anat (Basel)* 157: 275-290.

Fox, H. S., Bond, B. L., and Parslow, T. G. (1991). Estrogen regulates the IFN-gamma promoter. *J Immunol* 146: 4362-4367.

Furukawa, K., Itoh, K., Okamura, K., Kumagai, K., and Suzuki, M. (1984). Changes in NK cell activity during the estrous cycle and pregnancy in mice. *J Reprod Immunol* 6: 353-363.

Gallin, J. I., Farber, J. M., Holland, S. M., and Nutman, T. B. (1995). Interferon-gamma in the management of infectious diseases. *Ann Intern Med* 123: 216-224.

Grimaldi, C. M., Cleary, J., Dagtas, A. S., Moussai, D., and Diamond, B. (2002). Estrogen alters thresholds for B cell apoptosis and activation. *J Clin Invest* 109: 1625-1633.

Honda, K., Shimohama, S., Sawada, H., Kihara, T., Nakamizo, T., Shibasaki, H., and Akaike, A. (2001). Nongenomic antiapoptotic signal transduction by estrogen in cultured cortical neurons. *J Neurosci Res* 64: 466-475.

Hosoda, T., Nakajima, H., and Honjo, H. (2001). Estrogen protects neuronal cells from amyloid beta-induced apoptotic cell death. *Neuroreport* 12: 1965-1970.

Hrushesky, W. J., Gruber, S. A., Sothern, R. B., Hoffman, R. A., Lakatua, D., Carlson, A., Cerra, F., and Simmons, R. L. (1988). Natural killer cell activity: age, estrous- and circadian-stage dependence and inverse correlation with metastatic potential. *J Natl Cancer Inst* 80: 1232-1237.

Hu, S. K., Mitcho, Y. L., and Rath, N. C. (1988). Effect of estradiol on interleukin 1 synthesis by macrophages. *Int J Immunopharmacol* 10: 247-252.

Hunt, J. S., Miller, L., Roby, K. F., Huang, J., Platt, J. S., and DeBrot, B. L. (1997). Female steroid hormones regulate production of pro-inflammatory molecules in uterine leukocytes. *J Reprod Immunol* 35: 87-99.

Ito, A., Bebo, B. F., Jr., Matejuk, A., Zamora, A., Silverman, M., Fyfe-Johnson, A., and Offner, H. (2001). Estrogen treatment down-regulates TNF-alpha production and reduces the severity of experimental autoimmune encephalomyelitis in cytokine knockout mice. *J Immunol* 167: 542-552.

Jenkins, J. K., Suwannaroj, S., Elbourne, K. B., Ndebele, K., and McMurray, R. W. (2001). 17-beta-estradiol alters Jurkat lymphocyte cell cycling and induces apoptosis through suppression of Bcl-2 and cyclin A. *Int Immunopharmacol* 1: 1897-1911.

Karpuzoglu-Sahin, E., Hissong, B. D., and Ansar Ahmed, S. (2001). Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. *J Reprod Immunol* 52: 113-127.

Karpuzoglu-Sahin, E., Zhi-Jun, Y., Lengi, A., Sriranganathan, N., and Ansar Ahmed, S. (2001). Effects of long-term estrogen treatment on IFN-gamma, IL-2 and IL-4 gene expression and protein synthesis in spleen and thymus of normal C57BL/6 mice. *Cytokine* 14: 208-217.

Koka, S., Petro, T. M., and Reinhardt, R. A. (1998). Estrogen inhibits interleukin-1beta-induced interleukin-6 production by human osteoblast-like cells. *J Interferon Cytokine Res* 18: 479-483.

Kunz, D., Walker, G., Eberhardt, W., and Pfeilschifter, J. (1996). Molecular mechanisms of dexamethasone inhibition of nitric oxide synthase expression in interleukin 1 beta-stimulated mesangial cells: evidence for the involvement of transcriptional and posttranscriptional regulation. *Proc Natl Acad Sci U S A* 93: 255-259.

Liu, C. Q., Wang, Z. X., and Yuan, Y. (2002). Effect of mifepristone on uterine receptivity in guinea pigs. *Acta Pharmacol Sin* 23: 177-182.

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu Rev Immunol* 15: 323-350.

Masuzawa, T., Miyaura, C., Onoe, Y., Kusano, K., Ohta, H., Nozawa, S., and Suda, T. (1994). Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. *J Clin Invest* 94: 1090-1097.

McMurray, R. W., Suwannaroj, S., Ndebele, K., and Jenkins, J. K. (2001). Differential effects of sex steroids on T and B cells: modulation of cell cycle phase distribution, apoptosis and bcl-2 protein levels. *Pathobiology* 69: 44-58.

Medina, K. L., and Kincade, P. W. (1994). Pregnancy-related steroids are potential negative regulators of B lymphopoiesis. *Proc Natl Acad Sci U S A* 91: 5382-5386.

Medina, K. L., Smithson, G., and Kincade, P. W. (1993). Suppression of B lymphopoiesis during normal pregnancy. *J Exp Med* 178: 1507-1515.

Mershon, J. L., Baker, R. S., and Clark, K. E. (2002). Estrogen increases iNOS expression in the ovine coronary artery. *Am J Physiol Heart Circ Physiol* 283: H1169-1180.

Messingham, K. A., Heinrich, S. A., and Kovacs, E. J. (2001). Estrogen restores cellular immunity in injured male mice via suppression of interleukin-6 production. *J Leukoc Biol* 70: 887-895.

Miller, L., Alley, E. W., Murphy, W. J., Russell, S. W., and Hunt, J. S. (1996). Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. *J Leukoc Biol* 59: 442-450.

Mor, G., Munoz, A., Redlinger, R., Jr., Silva, I., Song, J., Lim, C., and Kohen, F. (2001). The role of the Fas/Fas ligand system in estrogen-induced thymic alteration. *Am J Reprod Immunol* 46: 298-307.

Nuedling, S., Kahlert, S., Loebbert, K., Doevendans, P. A., Meyer, R., Vetter, H., and Grohe, C. (1999). 17 Beta-estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo. *Cardiovasc Res* 43: 666-674.

Okasha, S. A., Ryu, S., Do, Y., McKallip, R. J., Nagarkatti, M., and Nagarkatti, P. S. (2001). Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus. *Toxicology* 163: 49-62.

Olsen, N. J., and Kovacs, W. J. (1996). Gonadal steroids and immunity. *Endocr Rev* 17: 369-384.

Radomski, M. W., Palmer, R. M., and Moncada, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci U S A* 87: 10043-10047.

Rijhsinghani, A. G., Thompson, K., Bhatia, S. K., and Waldschmidt, T. J. (1996). Estrogen blocks early T cell development in the thymus. *Am J Reprod Immunol* 36: 269-277.

Sarvetnick, N., and Fox, H. S. (1990). Interferon-gamma and the sexual dimorphism of autoimmunity. *Mol Biol Med* 7: 323-331.

Schmid, I., Uittenbogaart, C. H., Keld, B., and Giorgi, J. V. (1994). A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods* 170: 145-157.

Schreiber, A. D., Nettl, F. M., Sanders, M. C., King, M., Szabolcs, P., Friedman, D., and Gomez, F. (1988). Effect of endogenous and synthetic sex steroids on the clearance of antibody-coated cells. *J Immunol* 141: 2959-2966.

Screpanti, I., Meco, D., Morrone, S., Gulino, A., Mathieson, B. J., and Frati, L. (1991). In vivo modulation of the distribution of thymocyte subsets: effects of estrogen on the expression of different T cell receptor V beta gene families in CD4-, CD8- thymocytes. *Cell Immunol* 134: 414-426.

Screpanti, I., Morrone, S., Meco, D., Santoni, A., Gulino, A., Paolini, R., Crisanti, A., Mathieson, B. J., and Frati, L. (1989). Steroid sensitivity of thymocyte subpopulations during intrathymic differentiation. Effects of 17 beta-estradiol and dexamethasone on subsets expressing T cell antigen receptor or IL-2 receptor. *J Immunol* 142: 3378-3383.

Seaman, W. E., Blackman, M. A., Gindhart, T. D., Roubinian, J. R., Loeb, J. M., and Talal, N. (1978). beta-Estradiol reduces natural killer cells in mice. *J Immunol* 121: 2193-2198.

Seaman, W. E., and Gindhart, T. D. (1979). Effect of estrogen on natural killer cells. *Arthritis Rheum* 22: 1234-1240.

Seiki, K., and Sakabe, K. (1997). Sex hormones and the thymus in relation to thymocyte proliferation and maturation. *Arch Histol Cytol* 60: 29-38.

Silverstone, A. E., Frazier, D. E., Jr., Fiore, N. C., Soults, J. A., and Gasiewicz, T. A. (1994). Dexamethasone, beta-estradiol, and 2,3,7,8-tetrachlorodibenzo-p-dioxin elicit thymic atrophy through different cellular targets. *Toxicol Appl Pharmacol* 126: 248-259.

Song, R. X., and Santen, R. J. (2003). Apoptotic action of estrogen. *Apoptosis* 8: 55-60.

Spearow, J. L., Doemeny, P., Sera, R., Leffler, R., and Barkley, M. (1999). Genetic variation in susceptibility to endocrine disruption by estrogen in mice. *Science* 285: 1259-1261.

Spearow, J. L., O'Henley, P., Doemeny, P., Sera, R., Leffler, R., Sofos, T., and Barkley, M. (2001). Genetic variation in physiological sensitivity to estrogen in mice. *Apmis* 109: 356-364.

Staples, J. E., Gasiewicz, T. A., Fiore, N. C., Lubahn, D. B., Korach, K. S., and Silverstone, A. E. (1999). Estrogen receptor alpha is necessary in thymic development and estradiol-induced thymic alterations. *J Immunol* 163: 4168-4174.

Sudoh, N., Toba, K., Akishita, M., Ako, J., Hashimoto, M., Iijima, K., Kim, S., Liang, Y. Q., Ohike, Y., Watanabe, T., *et al.* (2001). Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. *Circulation* 103: 724-729.

Theofilopoulos, A. N., Koundouris, S., Kono, D. H., and Lawson, B. R. (2001). The role of IFN-gamma in systemic lupus erythematosus: a challenge to the Th1/Th2 paradigm in autoimmunity. *Arthritis Res* 3: 136-141.

Tomkinson, A., Gevers, E. F., Wit, J. M., Reeve, J., and Noble, B. S. (1998). The role of estrogen in the control of rat osteocyte apoptosis. *J Bone Miner Res* 13: 1243-1250.

Trinchieri, G. (1997). Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). *Curr Opin Immunol* 9: 17-23.

Vernon-Roberts, B. (1969). The effects of steroid hormones on macrophage activity. *Int Rev Cytol* 25: 131-159.

Verthelyi, D. (2001). Sex hormones as immunomodulators in health and disease. *Int Immunopharmacol* 1: 983-993.

Verthelyi, D., and Ahmed, S. A. (1994). 17 beta-estradiol, but not 5 alpha-dihydrotestosterone, augments antibodies to double-stranded deoxyribonucleic acid in nonautoimmune C57BL/6J mice. *Endocrinology* 135: 2615-2622.

Verthelyi, D., and Ansar Ahmed, S. (1997). Characterization of estrogen-induced autoantibodies to cardiolipin in non-autoimmune mice. *J Autoimmun* 10: 115-125.

Verthelyi, D. I., and Ahmed, S. A. (1998). Estrogen increases the number of plasma cells and enhances their autoantibody production in nonautoimmune C57BL/6 mice. *Cell Immunol* 189: 125-134.

Yao, G., and Hou, Y. (2004). Thymic atrophy via estrogen-induced apoptosis is related to Fas/FasL pathway. *Int Immunopharmacol* 4: 213-221.

Yu, L., Ma, R., and Yu, T. (1996). Antitumor and antitumor-promoting actions of macrophages and their relationship with estrogen. *Oncology* 53: 322-326.

Zajchowski, S., and Hoffman-Goetz, L. (2000). Supraphysiological level of estrogen exposure in vivo increases lymphoid cell death in mice. *Life Sci* 66: 1451-1459.

Zhou, S., Zilberman, Y., Wassermann, K., Bain, S. D., Sadovsky, Y., and Gazit, D. (2001). Estrogen modulates estrogen receptor alpha and beta expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. *J Cell Biochem* 81: 144-155.

Zuckerman, S. H., and Bryan, N. (1996). Inhibition of LDL oxidation and myeloperoxidase dependent tyrosyl radical formation by the selective estrogen receptor modulator raloxifene (LY139481 HCL). *Atherosclerosis* 126: 65-75.

g.) FIGURES

A. Mean relative expression				B. Mean absolute thymocyte numbers (10^6 cells)			
	17-β-estradiol (μg/100 g b.w.)				17-β-estradiol (μg/100g b.w.)		
	0	**2**	**4**		**0**	**2**	**4**
$CD4^+CD8^-$	18.5 ± 1.13^{a}	14.04 ± 1.15^{b}	15.32 ± 1.23^{b}	**$CD4^+CD8^-$**	2.12 ± 0.45^{c}	1.32 ± 0.38^{cd}	0.18 ± 0.06^{d}
$CD4^-CD8^+$	4.6±0.58	3.03±0.39	3.71±0.82	**$CD4^-CD8^+$**	0.56 ± 0.14^{a}	0.27 ± 0.08^{ab}	0.03 ± 0.01^{b}
$CD4^+CD8^+$	69.38±3.09	78.06±2.74	75.85±2.48	**$CD4^+CD8^+$**	8.46 ± 2.26^{a}	8.29 ± 2.67^{ab}	0.51 ± 0.08^{b}
$CD4^-CD8^-$	7.50±2.06	4.87±1.41	5.08±0.54	**$CD4^-CD8^-$**	0.79±0.26	0.39±0.12	0.05±0.01

Table 1. Mean relative expression (Panel A) and mean absolute cell numbers (Panel B) of fresh thymocyte subsets after short-term estrogen treatment. Freshly isolated thymocytes were stained with anti-CD4, T helper cell marker and anti-CD8, marker for cytotoxic T cells and analyzed by flow cytometry. Panel A demonstrates the mean relative expression of fresh thymocyte subsets ($CD4^+CD8^-$). Panel B shows the absolute recoveries of thymic subsets from male CD1 mice (mean of % expression multiplied with cell recovery). The data are presented as means ± S.E.M. Means with different superscript per row differed at *P<0.05* (a, b) or *P<0.01* (c, d).

	17-β-estradiol (μg/100 g b.w.)		
!	**0**	**2**	**4**
Total thymocytes			
Live cells	89.75 ± 0.49	89.80 ± 1.9	91.97 ± 0.87
Early apoptotic cells	6.42 ± 1.09	4.62 ± 0.40	3.12 ± 0.29
Late apoptotic cells	3.12 ± 0.29	3.77 ± 0.84	3.40 ± 0.48
Thymocyte subsets			
$CD4^-CD8^-$			
Live cells	85.20 ± 0.94	87.36 ± 2.3	87.82 ± 1.83
Early apoptotic cells	8.07 ± 0.87	5.81 ± 1.18	5.39 ± 0.42
Late apoptotic cells	6.73 ± 0.71	6.81 ± 1.54	6.80 ± 1.44
$CD4^+CD8^-$			
Live cells	90.72 ± 0.23	88.16 ± 1.79	89.55 ± 1.17
Early apoptotic cells	5.54 ± 0.19	7.10 ± 1.0	4.94 ± 0.63
Late apoptotic cells	3.75 ± 0.22	4.75 ± 1.06	5.51 ± 0.60
$CD4^-CD8^+$			
Live cells	93.72 ± 0.38 ^{a}	90.88 ± 0.96 ^{b}	92.12 ± 0.98 ^{ab}
Early apoptotic cells	3.83 ± 0.40 ^{a}	5.86 ± 0.58 ^{b}	4.44 ± 0.46 ^{ab}
Late apoptotic cells	2.44 ± 0.27	3.26 ± 0.49	3.43 ± 0.58
$CD4^+CD8^+$			
Live cells	90.33 ± 0.67	90.52 ± 2.01	93.20 ± 0.81
Early apoptotic cells	6.50 ± 0.55	5.43 ± 0.75	4.54 ± 0.75
Late apoptotic cells	3.17 ± 0.42	4.03 ± 1.14	3.43 ± 0.58

Table 2. Effects of short-term estrogen treatment on apoptosis of total thymocyte and thymocyte subsets: Isolated thymocytes from 6 CD1 mice per treatment group of short-term 17-β-estradiol with 0, 2, 4 μg/100 g body weight were stained with anti-CD4 and anti-CD8-FITC antibodies and 7-AAD. The whole thymocyte population was analyzed for apoptosis via gating on 7-AAD staining. The 7-AAD defines programmed cell death via binding DNA and identifies as 7-AAD^{dull}, live, 7-AAD^{intermediate}, early apoptotic, or 7-AAD^{bright}, late apoptotic/ necrotic cells. The thymocyte populations were analyzed by flow cytometer. The data are presented as means ± S.E.M. Means with different superscripts per row differed at *P<0.05* (a, b).

A. Mean relative expression

	17-β-estradiol (μg/100 g b.w.)		
	0	**2**	**4**
CD45R$^+$ Thy1.2$^-$	63.45 ± 1.77	62.70 ± 3.43	61.13 ± 4.26
CD45R$^-$ Thy1.2$^+$	22.67 ± 1.03	13.39 ± 4.82	21.40 ± 4.69
CD45R$^-$ Thy1.2$^-$	10.89 ± 0.73	20.12 ± 3.86	14.71 ± 4.75

B. Mean absolute splenocyte numbers (10^6 cells)

	17-β-estradiol (μg/100g b.w.)		
	0	**2**	**4**
CD45R$^+$ Thy1.2$^-$	13.42±2.09	24.40±5.33	24.73±4.58
CD45R$^-$ Thy1.2$^+$	4.95±1.16	4.85±1.62	8.40±1.95
CD45R$^-$ Thy1.2$^-$	2.40±0.55	7.70±1.76	6.90±3.30

Table 3. Effect of short-term estrogen treatment on th e subsets of fresh splenic lymphocytes-based on live cells. Freshly isolated splenic lymphocytes from 6 mice per treatment group were stained with anti-CD45R (B220) and anti-Thy1.2 antibodies and analyzed by flow cytometry. Panel A shows the mean relative expression of fresh lymphocyte subsets. Panel B shows the absolute recoveries of splenic lymphocyte subsets from male CD1 mice (% relative expression multiplied with total recovery of splenic lymphocytes). The data are presented as means ± S.E.M.

!	17-β-estradiol (μg/100 g b.w.)		
	0	**2**	**4**
Total splenocytes			
Live cells	77.72 ± 2.7	75.90 ± 5.22	74.88 ± 5.04
Early apoptotic cells	16.38 ± 1.95	13.17 ± 1.71	20.44 ± 2.48
Late apoptotic cells	5.90 ± 0.76	10.94 ± 4.17	8.70 ± 1.87
$CD45R^{+}$ $Thy1.2^{-}$			
Live cells	73.60 ± 4.85	73.05 ± 5.82	70.60 ± 6.49
Early apoptotic cells	19.52 ± 3.64	15.01 ± 2.35	19.20 ± 4.08
Late apoptotic cells	6.90 ± 1.2	11.94 ± 4.44	10.20 ± 2.46
$CD45R^{-}$ $Thy1.2^{+}$			
Live cells	90.40 ± 2.93	82.43 ± 5.40	85.05 ± 2.50
Early apoptotic cells	7.06 ± 1.80	8.28 ± 1.95	7.13 ± 2.55
Late apoptotic cells	2.54 ± 1.14	9.30 ± 3.54	7.06 ± 1.10
$CD45R^{-}$ $Thy1.2^{-}$			
Live cells	85.80 ± 0.91	84.75 ± 2.44	84.75 ± 2.19
Early apoptotic cells	9.93 ± 1.17	8.82 ± 0.78	10.04 ± 1.81
Late apoptotic cells	4.28 ± 0.60	6.42 ± 1.81	5.21 ± 0.65

Table 4. Effect of short-term estrogen treatment on apoptosis of fresh splenic lymphocytes: Freshly isolated splenic lymphocytes from six CD1 mice per treatment group of 17-β estradiol with 0, 2, 4 μg/100 g body weight were stained with anti-CD45R (B220), and anti-Thy1.2 antibodies, and 7-AAD and analyzed by flow cytometry. The data are presented as means ± S.E.M.

!	17-β-estradiol (μg/100 g b.w.)		
	0	**2**	**4**
Media			
CD45R$^+$ Thy1.2$^-$	19.62 ± 0.39	20.90 ± 1.7	20.88 ± 2.03
CD45R$^-$ Thy1.2$^+$	5.23 ± 0.37	3.89 ± 0.67	4.48 ± 0.63
CD45R$^-$ Thy1.2$^-$	72.88 ± 105	72.44 ± 2.15	72.08 ± 2.17
Concanavalin-A (Con-A)			
CD45R$^+$ Thy1.2$^-$	17.23 ± 1.29	19.60 ± 1.7	20.90 ± 1.01
CD45R$^-$ Thy1.2$^+$	4.16 ± 0.35	3.68 ± 0.76	5.01 ± 0.90
CD45R$^-$ Thy1.2$^-$	72.84 ± 1.43	70.84 ± 2.3	70.03 ± 0.21

Table 5 Mean relative expression of splenocyte subsets after 48 hours of culture. Freshly isolated splenocytes from six CD-1 mice per treatment group were cultured with or w ithout Con-A (10 μg/ml) for 48 hours. The cultured cells were stained with anti-CD45R (B220) and anti-Thy1.2 antibodies and evaluated by flow cytometer. The relative expression of media-alone and Con-A-stimulated lymphocyte subsets are presented as means ± S.E.M.

A. Media

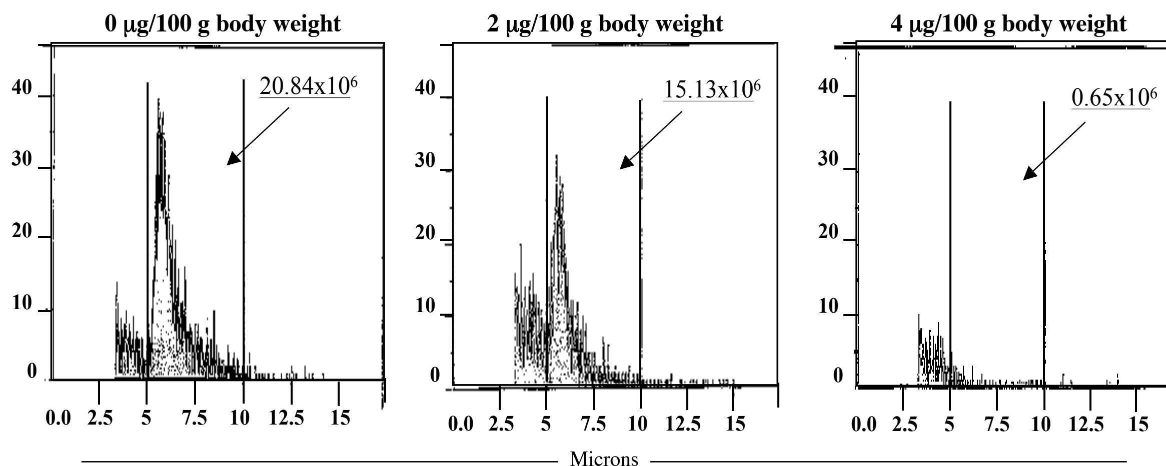
	17-β-estradiol	(μg/100g bw)	
	0	2	4
Total splenocytes			
Live cells	37.5±3.04	24.88±4.11	25.83±5.16
Early apoptotic cells	63.92±2.25	70.83±3.46	70.50±5.18
Late apoptotic cells	2.3±1.03	4.31±0.67	3.69±0.61
Total apoptotic cells	66.23±3.04	75.14±4.11	74.18±5.17
Splenic subsets			
CD45R (B220) $^{+}$***Thy1.2*** $^{-}$			
Live cells	54.77±4.89	34.60±7.67	39.65±5.18
Early apoptotic cells	33.88±2.07	43.50±7.97	41.08±3.12
Late apoptotic cells	11.36±3.97	21.88±4.0	19.26±4.06
Total apoptotic cells	45.24±4.88	65.38±7.67	60.34±5.19
CD45R (B220) $^{-}$***Thy1.2*** $^{+}$			
Live cells	73.0±6.52	45.78±10.95	55.15±13.43
Early apoptotic cells	19.77±3.12	37.03±9.52	31.58±9.2
Late apoptotic cells	7.22±3.63	17.17±5.0	13.3±5.45
Total apoptotic cells	26.99±6.53	54.19±10.95	47.38±15.93
CD45R (B220) $^{-}$***Thy1.2*** $^{-}$			
Live cells	29.12±2.06	24.22±3.64	22.73±4.73
Early apoptotic cells	67.98±0.85	70.93±3.01	72.38±5.07
Late apoptotic cells	2.91±1.23	4.84±0.67	4.93±0.8
Total apoptotic cells	70.89±2.05	75.77±3.62	77.30±4.71

B. Con-A

	17-β-estradiol	(μg/100g bw)	
	0	2	4
Total splenocytes			
Live cells	27.85±1.01	28.50±3.24	27.20±5.15
Early apoptotic cells	70.50±0.52	68.70±2.8	70.0±4.11
Late apoptotic cells	1.66±0.53	2.82±0.67	2.82±1.08
Total apoptotic cells	72.16±0.99	71.52±3.23	72.82±5.16
Splenic subsets			
CD45R (B220) $^{+}$***Thy1.2*** $^{-}$			
Live cells	45.90±2.71	38.30±6.7	43.75±7.56
Early apoptotic cells	35.77±1.08	39.55±5.74	33.42±2.14
Late apoptotic cells	18.30±1.81	22.05±1.24	22.80±5.57
Total apoptotic cells	54.08±2.69	61.60±6.75	56.23±7.57
CD45R (B220) $^{-}$***Thy1.2*** $^{+}$			
Live cells	66.50±4.22	50.70±8.98	58.33±14.39
Early apoptotic cells	24.95±2.72	31.35±6.7	25.03±5.81
Late apoptotic cells	8.55±2.11	17.95±2.53	16.69±8.82
Total apoptotic cells	33.49±4.22	49.30±8.98	41.72±14.38
CD45R (B220) $^{-}$***Thy1.2*** $^{-}$			
Live cells	27.88±0.51	31.33±3.01	24.92±4.37
Early apoptotic cells	70.28±0.63	66.22±3.14	71.98±3.11
Late apoptotic cells	1.81±0.67	2.43±0.19	3.10±1.3
Total apoptotic cells	72.08±0.51	68.65±3.0	75.07±4.37

Table 6 Effect of short-term estrogen treatment on splenocytes cultured for 48 hours: Splenic lymphocytes that were isolated from nine month old out-bred CD1 mice treated for short-term with 17-β-estradiol 0, 2, 4 μg/100 g body weight were cultured in phenol red free complete RPMI for 48 hrs and stained with anti-CD45R (B220) and anti-Thy1.2 antibodies and 7-AAD. The total splenic population, the B and T cell subsets were analyzed for apoptosis via gating on 7-AAD staining. The data are reprinted as means ± S.E.M.

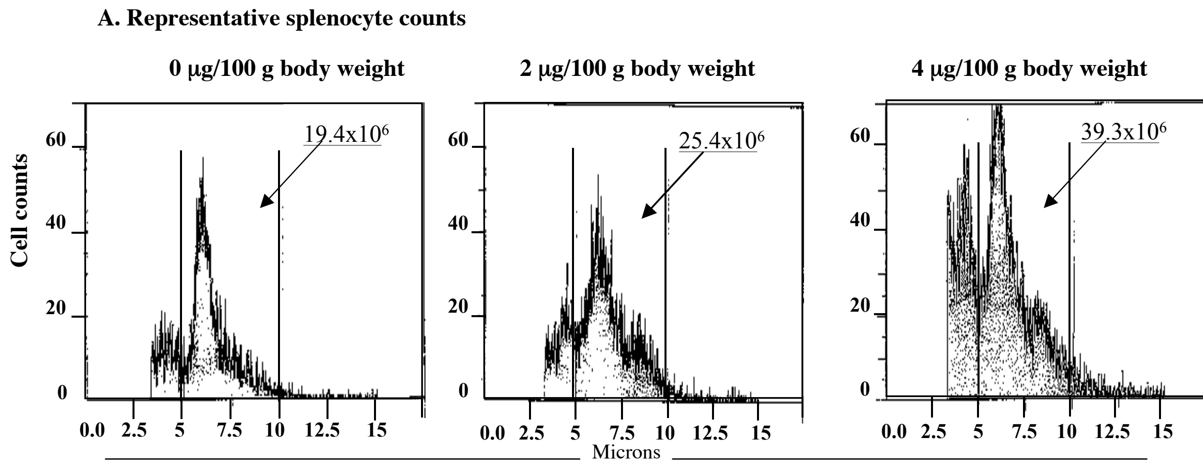
A. Representative thymocyte cell counts



B. Thymic weight per body weight and thymocyte cell counts

	17-β estradiol (μg/100 g body weight)		
	0	2	4
Thymic weight/body weight	0.0009±0.0001 [a]	0.0010±0.0001 [b]	0.0007±0.0001 [c]
Thymic cellularity ($x10^6$)	13.79±3.46 [a]	12.18±2.77 [b]	0.93±0.14 [c]

Figure 1: Immunomodulatory effects of short-term 17-β estradiol treatment on the thymus of CD1 mice. Three subcutaneous doses of 17-β estradiol at 2 μg/100g body weight or 4 μg/100g body weight were given to 6 mice per treatment group on alternate days. Control mice received an equivalent volume of oil only (0 μg/100g body weight; n=5 mice). Thymocytes were isolated and cells were analyzed using a CASY cell counter. The cells gated between 5.0 and 10 μM were quantitated. Panel A demonstrates representative thymocyte cell counts and profiles from vehicle-control 0, 2 and 4 μg/100g body weight 17-β estradiol given male CD-1 mice. Panel B shows the thymic weights per body weight and thymocyte cell counts. Data are represented as mean ± standard error of mean (S.E.M.). Means with superscripts differ at *P<0.05*.



B. Splenocyte counts

	Splenic cellularity($x10^6$)		
	17- β -estradiol (µg/100 g body weight)		
Gate settings (microns)	0	2	4
2.5-5.0	4.52 ± 0.69 ^{a}	6.76 ± 1.44 ^{b}	11.4 ± 2.1 ^{c}
5.0-7.5	10.72 ± 2.02 ^{a}	19.85 ± 5.16 ^{b}	27.06 ± 4.99 ^{c}
7.5-10.0	3.93 ± 0.34 ^{a}	6.79 ± 1.6 ^{b}	9.24 ± 1.75 ^{c}
5.0-10.0	14.32 ± 2.44 ^{a}	26.63 ± 6.71 ^{b}	36.30 ± 6.71 ^{c}

Figure 2: Immunomodulatory effects of short-term 17-β estradiol treatment on the spleen of CD1 mice. Isolated splenocytes were analyzed with a CASY cell counter from 6 or 7 mice per treatment group. Panel A demonstrates representative profiles of splenocyte counts from vehicle-control (0), 2 and 4 µg/100g body weight 17-β estradiol given to mice. Panel B shows the splenic cell counts by gate. Data are represented as mean ± standard error of mean (S.E.M.). Means with different superscript per row differ at $P<0.05$.

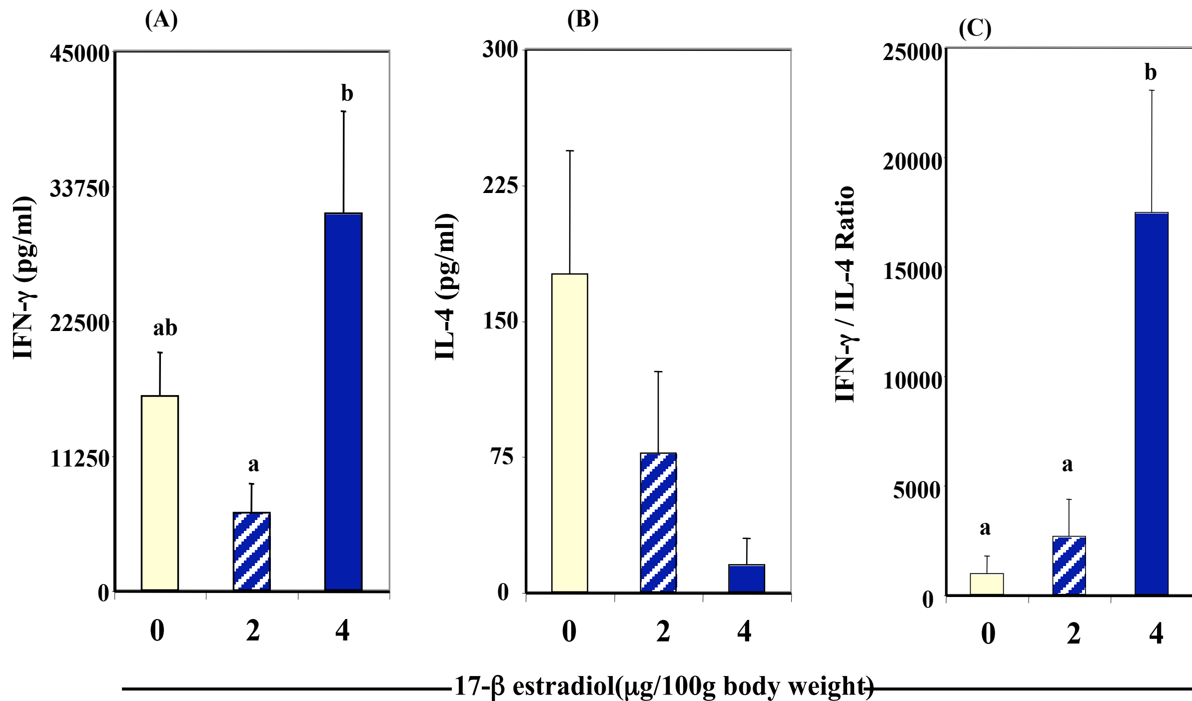


Figure 3. Short-term 17-β estradiol treatment affects levels of IFN-γ, IL-4 and IFN-γ/IL-4 ratio. Splenic lymphocytes isolated from 6 male CD-1 mice per treatment group of 17-β-estradiol (2 and 4 μg/100g body weight) or oil were stimulated with Con-A (10 μg/ml) for 24 hrs. The supernatants were collected and analyzed for concentrations of IFN-γ (pg/ml) and IL-4 (pg/ml). The 17-β-estradiol treatment affected IFN-γ (*P=0.03*; Panel A), IL-4 (*P=0.09*; Panel B), and the ratio of IFN-γ/IL-4 (*P=0.009*; Panel C). Bars represent means ± S.E.M. Bars marked with different letters differ at *P<0.05*.

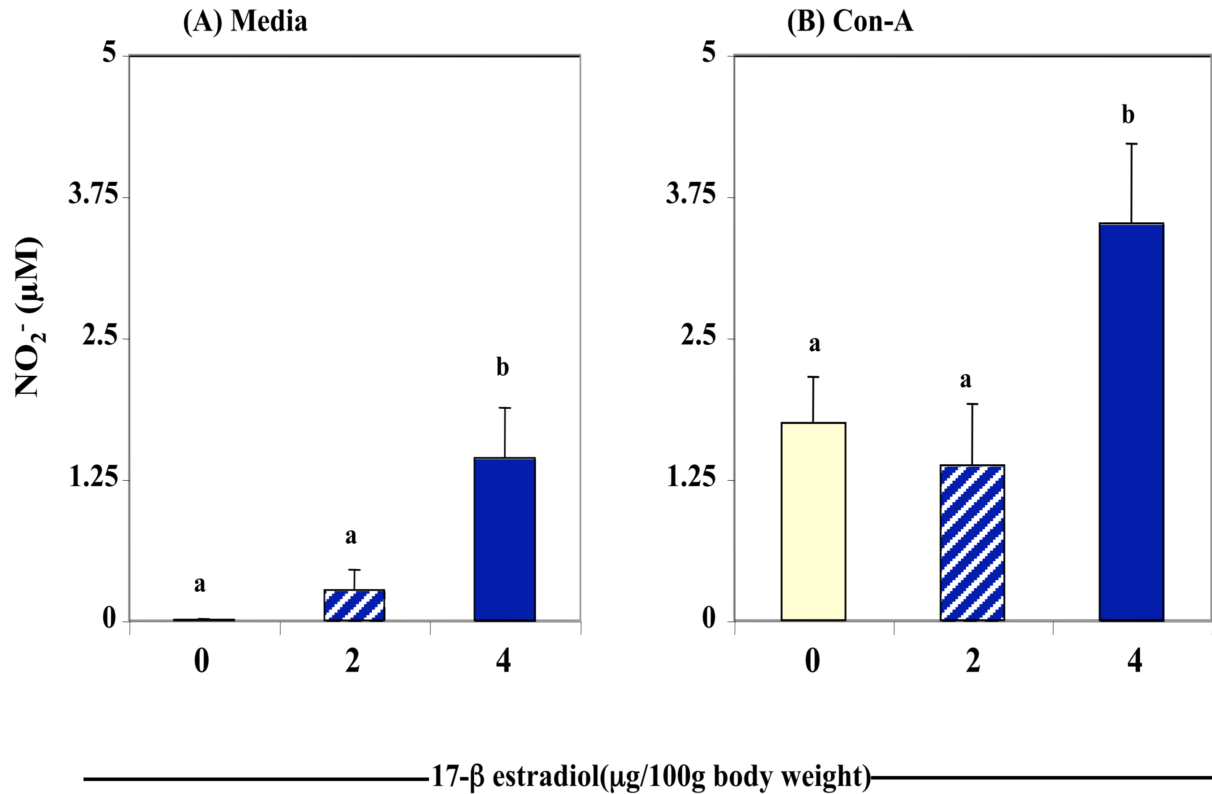


Figure 4. Short-term 17-β estradiol treatment increases nitric oxide levels. Splenic lymphocytes isolated from 6 male CD-1 mice per treatment group of 17-β estradiol (2 and 4 μg/100g body weight) or oil as control were cultured in media alone Panel A or with Con-A (10 μg/ml) Panel B for 48 hrs. The 17-β estradiol treatment affected the level of nitric oxide in the supernatants which were measured with the Griess Assay after incubation with media (*P=0.005*, Panel A) and Con-A (*P=0.04*, Panel B). Bars represent means ± S.E.M. Bars marked with different letters differ at *P<0.05*.

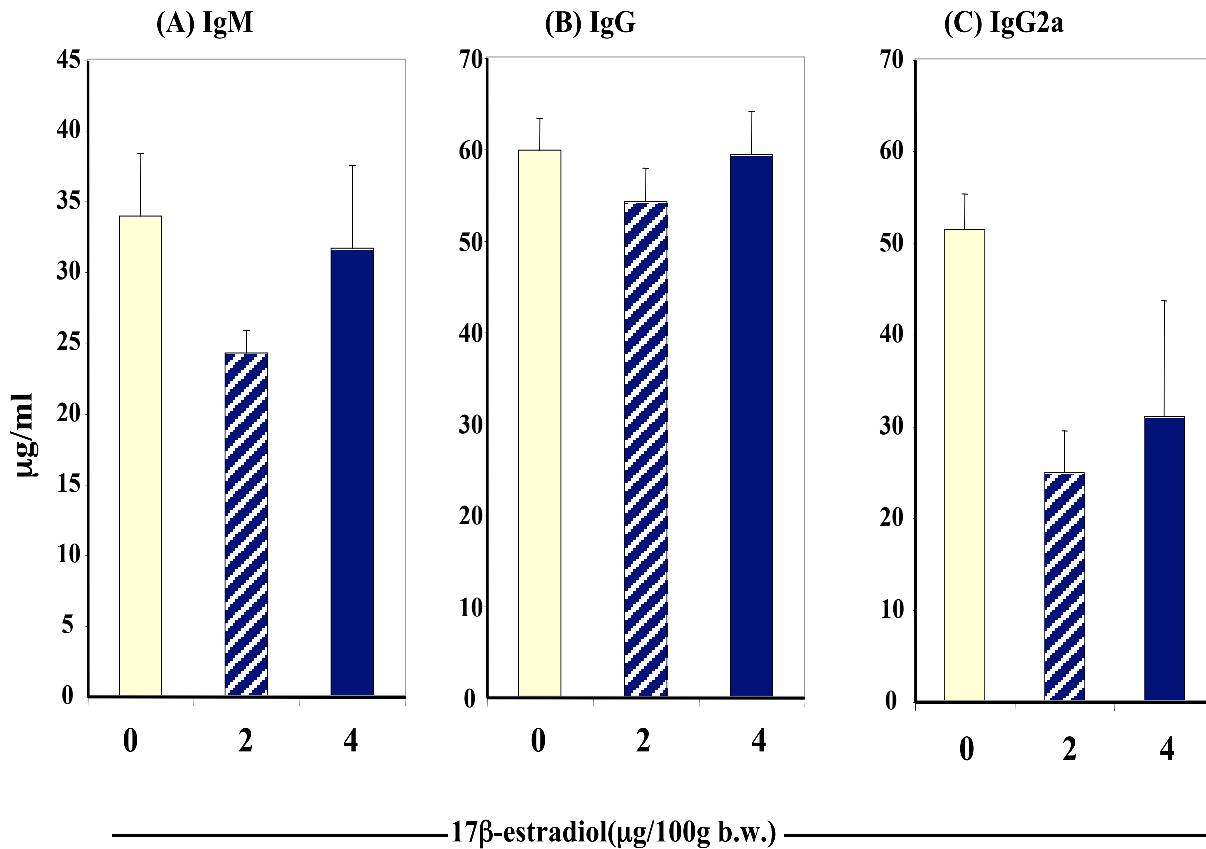


Figure 5. Short-term 17-β estradiol treatment and levels of IgM, IgG and IgG2a. Three subcutaneous doses of 17-β estradiol at 2 µg/100g body weight or 4 µg/100g body weight were given to 6 mice per treatment group on alternate days. Control mice received an equivalent volume of oil only (0 µg/100g body weight; n=5 mice). Sera from 6 male CD-1 mice per treatment group were analyzed for levels of Panel A IgM; Panel B IgG; and Panel C IgG2a. Data are represented as mean ± standard error of mean (S.E.M.).

VITA

Ebru Karpuzoglu-Sahin was born on March 6, 1971, in New York, USA. She received her high school diploma from Antalya Anatolian High School in Antalya, Turkey, in 1989. She graduated with her Bachelors of Science degree in Biology with Honors from Middle East Technical University Ankara, Turkey, 1994. She enrolled in a Masters of Science program in Biotechnology in Akdeniz University, Antalya, Turkey. She completed her M.Sc. Thesis "Early identification of CMV infections due to immunosuppressive drug therapy in kidney transplant patients by PCR" in 1996 with Honors. During her masters, she received a fellowship funded by UNIDO for scientific research and training program at Institute G. Gaslini Genoa, Italy to work on RET gene of Hirschsprung disease. Subsequently, she enrolled in a Doctor of Philosophy program at the VA-MD Regional College of Veterinary Medicine, Department of Biomedical Sciences and Pathobiology. She continued her academic pursuit under Dr. S. Ansar Ahmed's supervision in immunology. She is the daughter of Tuncer Karpuzoglu, Professor of General Surgery, and Gulten Karpuzoglu, Professor of Pathology in Akdeniz University, Antalya, Turkey.