

Short Analytical Review

## Estrogen as an immunomodulator

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### Abstract

Estrogen's role in the sex differences observed in autoimmune diseases such as systemic lupus, multiple sclerosis, and rheumatoid arthritis have remained unclear. Complicating the understanding of the immunomodulatory effects of estrogen are (1) the effects of estrogen on multiple components of the immune response; (2) its varied effects on different systems in which it appears pro-autoimmune, as in murine lupus, or anti-inflammatory, as in EAE; and (3) its effects on other hormones which are potentially immunomodulatory. Recent reports have shed light on the role of estrogen in the modulation of lymphocyte survival and expansion and in the elaboration of Th1 versus Th2 cytokines and on the mechanisms by which estrogen can activate via multiple signaling and genomic pathways in immune cells.

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### Introduction

Estrogens have attracted significant interest as potential modulators of autoimmune disease. This interest grew from the recognition that autoimmune disorders such as SLE, Sjogren's syndrome, and rheumatoid arthritis (RA) have a significant excess of affected females [1]. The potential role of estrogen as a contributor to this differential in disease incidence was also supported by anecdotal reports that SLE could frequently flare during pregnancy [2], a time when levels of estrogen are elevated, and remit with menopause [3]. Support from epidemiologic and prospective studies seeking to find links between exogenous estrogen use and the frequency of autoimmune diseases, such as SLE, has produced mixed results. A retrospective study of the Nurse's Health Study cohort [4] found a relationship between estrogen use and incidence SLE, while prospective studies have failed to find any correlation [5–7]. Nevertheless, the striking male–female differences in disease incidence still

make endogenous estrogen a potentially important risk factor in the increased female incidence of autoimmunity.

Studies of estrogen's effects on murine models of autoimmunity have supported an immunomodulatory role for estrogen. Early studies in the NZB/NZW F1 mouse, a SLE model that shows a female predilection to earlier and more severe disease, found that administration of estrogen to castrated males and females leads to increased mortality compared to sham-treated mice [8]. Oophorectomy alone did not reduce mortality in females. Castration of males increased mortality, with an additional increase occurring with administration of estradiol [8,9].

Estrogen enhancement of disease in the NZB/NZW F1 model is contrasted with its effects in models of multiple sclerosis (MS) and rheumatoid arthritis (RA). A different role for estrogen is suggested in humans. Though the majority of MS and RA patients are female, the effects of pregnancy on disease activity are quite different from that seen in SLE. Both diseases generally improve during pregnancy, suggesting, if estrogen has a role, that estrogen is an immunosuppressive factor [10,11]. While there has been concern that estrogen replacement and oral contraceptives could contribute to disease flares in SLE [5], estrogens have been investigated as a potential treatment in multiple sclerosis [12]. Admin-

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istration of estradiol in either EAE or collagen-induced arthritis resulted in disease amelioration [13,14]. An interpretation of these results and those in models of SLE is that estrogen drives a Th2 response, such as in NZB/NZW F1, and suppresses a Th1 response, such as EAE and CIA. This paradigm will be discussed later.

Understanding the mechanisms by which estrogen treatment modulates in such varied ways remains unclear. Studying the interactions between the hormonal environment and the immune system remains complicated by several considerations. First, estrogen can act on multiple immune processes, including lymphocyte and monocyte development, dendritic cell function, T cell responses, cytokine regulation, B cell expansion and survival, the development of the antibody response, and NK cell activity. Secondly, estrogen acts through multiple receptors, which may be differentially expressed in the immune system by cell type and state of cell differentiation, activate multiple signaling pathways, and induce transcription of multiple genes. To develop approaches to understanding the observed *in vivo* modulatory effects of estrogen, I will review the current understanding of its interactions with the various components of the immune response.

### Effects on lymphopoiesis and differentiation

It is well established that both thymus and bone marrow are affected by estrogen (Fig. 1). Pregnancy leads to a reduction in B lymphopoiesis and this reduction could be mimicked by a single injection of estrogen [15]. In contrast, B lymphopoiesis and splenic B cell numbers were enhanced in ovariectomized mice and rats [16,17]. Treatment with estrogen resulted in the rapid loss of IL-7-responsive B cells from murine bone marrow [18]. More recent data indicated a direct depletion by estrogen of a specific population of early B cell precursors, characterized as lineage marker  $\text{c-kit}^{\text{lo}}\text{IL-7R}\alpha^{\text{+}}\text{TdT}^{\text{+}}$  [19]. This effect was a direct action upon B cell progenitors because it occurred in the absence of bone marrow stromal cells.

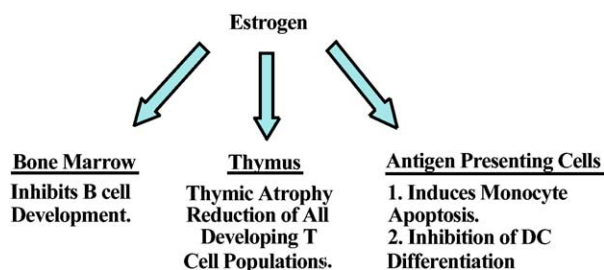


Fig. 1. Estrogen effects both B and T cell development as well as APC differentiation and homeostasis. Both B and T cell development are suppressed with estrogen administration. Likewise, *in vitro* dendritic cell differentiation is inhibited, though, the *in vivo* effects of estrogens on this population are unknown. Estrogen can also induce the apoptosis of monocytes, suggesting a role in myeloid homeostasis.

ER $\alpha$  and ER $\beta$  are both expressed in B cell progenitors in the mouse [20]. Estrogen receptor expression, though, is absent pre- and postnatally, explaining how B cell lymphopoiesis can occur in the fetus despite exposure to elevated levels of estrogen from the maternal environment. Both receptors are eventually expressed fully in the mouse between 3 and 8 weeks of age in both  $\text{c-kit}^{\text{hi}}$  and  $\text{c-kit}^{\text{lo}}$  populations. This represents an example of differential receptor expression over the life span of the organism leading to differential hormonal effects.

Mechanisms of estrogen-induced thymic atrophy are not as well characterized. Estrogen administration and pregnancy caused reversible thymic atrophy [21]. This atrophy is characterized by loss of all thymocyte subsets and disproportionate loss of the CD4 $^+$ CD8 $^+$  population [22]. Whether estrogen causes atrophy by effecting thymocytes directly or stromal elements has been addressed using mice chimeric for ER $\alpha^{-/-}$  thymic stromal cells and ER $\alpha^{+/+}$  thymocytes. These mice fail to undergo estrogen-induced thymic atrophy, while a mouse chimera of ER $\alpha^{-/-}$  thymocytes and ER $\alpha^{+/+}$  thymic stromal cells undergoes normal estrogen-dependent atrophy [23]. Thus the action of estrogen on thymic stromal elements appears of primary importance in estrogen-induced thymic atrophy.

### Estrogen effects on immune responses

Estrogen enhances humoral immune responses in several systems. One aspect of this enhancement is an increase in immunoglobulin production. Baseline sex differences in IgG and IgA levels have not been reproducibly found in humans [24]. IgM levels have been reported to be increased in adult females [25]. Estrogen enhanced *in vitro* IgG and IgM production by human PBMCs without effecting either viability or proliferation of PBMCs [26]. This enhancement was partially attributed to IL-10 because co-culture with neutralizing anti-IL-10 antibody partially inhibited the observed increase in immunoglobulin production.

Estrogen can also increase the serum titers of autoantibodies. An early observation of this effect *in vivo* was estrogen enhancement of anti-DNA IgG production in ovariectomized or castrated NZB/NZW F1 [8]. CD5 $^+$  B cells derived from a non-autoimmune mouse strain C57BL/6 and immunized with bromelain-treated mouse erythrocytes, as an auto-antigen, displayed augmented anti-erythrocyte antibody production after estrogen treatment [27]. This is not dependent on increased numbers of either total splenic B cells or peripheral CD5 $^+$  B cells, suggesting that estrogen has the capacity to either increase autoantibody production by individual cells or increase the number of autoantibody producing cells.

Studies in the R4A- $\gamma$ 2b Balb/c mouse, which is transgenic for an anti-DNA antibody heavy chain, revealed that estrogen treatment could increase the production of a high-affinity serum anti-dsDNA antibody that is normally deleted through

mechanisms of tolerance [28]. This was associated with increased expression of molecules involved in B cell activation and survival including bcl-2, shp-1, cd22, and VCAM-1. Interestingly, the prolactin secretion blocker bromocriptine could partially inhibit the estradiol-induced production of high-affinity anti-dsDNA antibodies [29]. Though estradiol still augmented the proliferation of transgene-expressing B cells, bromocriptine partially maintained tolerance, suggesting that the breaking is partially mediated by estradiol induction of prolactin activity or sensitivity. Estradiol also conferred resistance of splenic B cells to IgM-induced apoptosis [30]. This was associated with the accumulation of marginal zone splenic B cells, a population believed critical in the development of T cell dependent autoantibodies [31]. Substantiating the importance of the marginal zone B cell population, anti-DNA antibody secreting B cells were enriched within the marginal zone after estradiol treatment. These studies suggest estrogen may enhance autoantibody production by allowing high affinity auto-reactive B cell clones to escape normal mechanisms of tolerance.

Estrogen's effects on T cell responses have not been as extensively studied as those upon B cells. The behaviors of antigen-specific T cells are particularly difficult to study in most models of autoimmunity such as NZB/NZW F1 because the auto-reactive T cells that are driving the disease are difficult to identify. There is evidence, though, implicating estrogen modulation of T cell function. As noted earlier the pregnant state is associated with a Th2 bias. This Th2 bias has been illustrated by the inability of pregnant C57BL/6 mice, a strain relatively resistant to leishmania infection due to a strong Th1 response, to clear cutaneous infection by leishmania [32]. Spleen cells from leishmania-infected pregnant C57BL/6 mice produced less IFN- $\gamma$  and more IL-4, IL-5, and IL-10 when stimulated by leishmania Ag *in vitro* than infected non-pregnant C57BL/6.

These data suggest that female hormones may act to bias T cells toward a Th2 phenotype. Support for estrogen enhancement of Th2 responses, specifically, is provided by studies in both EAE and CIA models. Treatment of EAE mice with the estrogen estradiol not only reduced clinical activity of EAE, but also led to reduction of IFN- $\gamma$ -dependent anti-myelin basic protein IgG2a and increased production of IL-10 [13]. The amelioration of EAE by estradiol was strongly correlated with enhanced *in vitro* IL-10 production by MBP-specific T cells [13]. T cell clones specific for the myelin antigen PLP isolated from multiple sclerosis patients, stimulated *in vitro* with antigen and in the presence of varying concentrations of estradiol, also displayed enhanced production of IL-10 but not IL-4 [33]. A recent study in collagen-induced arthritis found that estradiol shifted the anti-collagen antibody isotype profile from one dominated by IgG2a to one dominated by IgG1 [34]. This was correlated with a reduction in IFN- $\gamma$  expression, though, Th2 cytokine expression was not increased. IL-10 production was actually decreased, while IL-4 and IL-5 remained the same. In this case, disease

amelioration was more associated with suppression of a Th1 response.

In contrast to the paradigm that estrogen leads to a Th2 bias is evidence that estrogen can enhance Th1 cytokine production. In a study previously cited, antigen-specific T cell clones isolated from multiple sclerosis patients and treated with estradiol *in vitro* not only increased IL-10 but also IFN- $\gamma$  expression [33]. Earlier studies have reported the capacity of estradiol to enhance IFN- $\gamma$  transcription by direct estrogen receptor interaction with an estrogen response element in the 5'-prime flanking region of the IFN- $\gamma$  gene [35]. A recent report indicated that estradiol can enhance IFN- $\gamma$  production as well as antigen-specific T cell expansion [36]. C57BL/6 and Balb/c mice were ovariectomized and then treated with either estradiol or placebo, followed by immunization with ova peptide. Spleen and lymph nodes were then restimulated in culture with ova peptide. T cell expansion and IFN- $\gamma$  production were then assessed. Estradiol enhanced *in vitro* antigen-stimulated T cell proliferation and IFN- $\gamma$  production. Thus, estradiol not only appeared to influence T cell differentiation, but also to enhance the accumulation of Th1 CD4+ T cells in response to antigen.

Supporting a female hormonal influence on T cell expansion, studies in the parent-into-F1 model of chronic GVHD, a model in which allo-reactive donor CD4+ T cells can be analyzed during an *in vivo* immune response, revealed that allogeneic donor CD4+ T cells expand to a greater degree when injected into female hosts as opposed to male hosts [37]. This difference is primarily dependent on enhanced proliferation of CD4+ T cells in female hosts, as well as on the sex of the host and not the donor. Supporting the role of estrogen as an enhancer of CD4+ T cell expansion, administration of estradiol to male hosts increased the expansion of donor CD4+ T cells (unpublished data). Whether the observed effects are by direct interaction of estrogen with T cells or by indirect effects on APCs is not yet clear.

The role of estrogen in enhancing T cell responsive and T-B cell interaction has also been suggested by studies in patients with SLE. Estrogen has been reported to cause ER-dependent enhancement of calcineurin expression in T cells cultured *in vitro* from SLE patients but not from normals [38]. Culture of unstimulated or anti-CD3-stimulated SLE T cells in the presence of 2-fluoroestradiol increased the surface expression of CD40 ligand [39]. A similar increase was not reported in normal T cells. These reports suggest an important role for estrogen as stimulatory to T cell responses, T-B cell interactions, B cell activation, and antibody production.

### Effects on monocytic and dendritic cells

Estrogen produces a variety of effects on both macrophage and dendritic cells. Estrogens can effect inflamma-

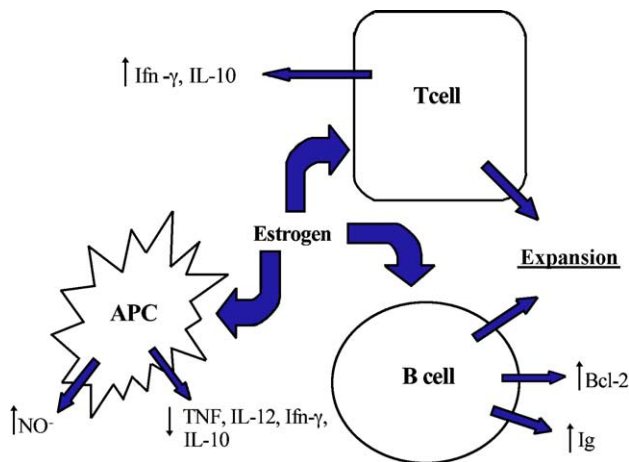


Fig. 2. Estrogen's effects on major components of the immune system are varied and inconsistent between experimental systems. It appears to enhance T cell and B cell expansion, attributable, at least in part to increased bcl-2 expression in the B cell. Estrogen has varied effects on cytokines both increasing and decreasing their expression depending on cell type.

tory mediator production by macrophage and macrophage-like cells both positively and negatively. For instance, estrogen increases TNF- $\alpha$  synthesis and decreases IL-10 synthesis from PMA-differentiated U937 cells [40]. Estrogen also increases expression of iNOS and production of nitric oxide in rat peritoneal macrophages [41]. In contrast, estrogen treatment of murine monocytes reduces expression of the chemokine receptors CCR2 and CXCR3, as well as reducing migratory activity in response to MIP-1 $\alpha$  and MCP-1/JE, suggesting an anti-inflammatory effect [42].

Estrogen also appears to play a role in monocyte differentiation. Using the monocytic cell line U937, estrogen induced apoptosis in vitro. If the cells are first differentiated with phorbol ester to a macrophage phenotype, estradiol no longer causes apoptosis [43]. This was correlated with differential expression of estrogen receptors. Undifferentiated U937 cells only express ER $\beta$ , while differentiated U937 expresses both ER $\alpha$  and ER $\beta$ .

Resistance to apoptosis in differentiated U937 was correlated with estrogen-induced downregulation of FasL [43]. ER $\alpha$  interacts with an estrogen response element contained in the FasL promoter [43]. Murine monocytes have been reported to undergo apoptosis when treated with estrogen [42]. In addition to highlighting a role for estrogen in myeloid homeostasis, these examples emphasize the importance of the state of cell differentiation and ER expression in understanding responses to estrogen.

Estrogen's effects on dendritic cell differentiation and cytokine production have also been reported. Studies of mature dendritic cells from animals with EAE and treated in vitro with estrogen lead to reduced capacity of dendritic cells to present antigen to myelin basic protein-specific T cells [44]. There was an associated reduction in production of TNF- $\alpha$ , IFN- $\gamma$ , and IL-12 by mature DCs as well as a

shift of co-cultured MBP-specific T cells to a Th2 phenotype. This observation suggests that the Th2 bias imparted by estrogens may at least partially be related to estrogen's effects on dendritic cells.

Estrogen also effects the differentiation of dendritic cells. Human synovial or peripheral blood monocytes treated in vitro with GM-CSF and IL-4 in the presence of the anti-estrogens tamoxifen and toremifene fail to normally differentiate into dendritic cells in vitro [45,46].

### Molecular mechanisms of estrogen action

An understanding of the mechanisms of sex hormone action requires, as demonstrated previously, an understanding of which estrogen receptors are expressed in which cell types and at which stages of differentiation. The more general expression of estrogen receptors in various components of the immune system has become clearer over the past decade (Fig. 2). Estrogen receptors can be categorized into the nuclear receptors ER $\alpha$  and ER $\beta$ , which interact directly with estrogen-regulated genes, and a membrane-associated estrogen receptor, which appears to mediate non-genomic effects such as increased Ca<sup>2+</sup> fluxes. This membrane receptor is likely to be either identical to ER $\alpha$  or closely related due to the ability of several ER $\alpha$ -specific

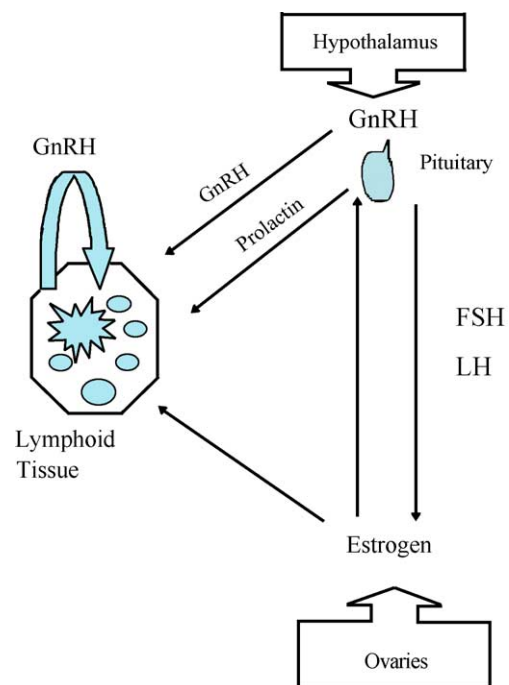


Fig. 3. Estrogen may play a role in modulating the expression or responsiveness of other potential immunomodulatory hormones. Estrogen can enhance sensitivity to GnRH, which is made by lymphoid cells, and can bind to GnRH receptors on T cells. Estrogens can also enhance prolactin expression that in turn has been implicated as an immunomodulator. The interactions between these components have not been extensively studied and their ramifications are unclear.

antibodies to recognize it. This receptor is present in caveolar rafts [47] and associates with various signally proteins including G proteins [48], Src, Ras [49], and B-Raf [50].

ER $\alpha$  is expressed in CD4+ and CD8+ T cells, B cells, NK cells, and macrophages [30,43,51–53]. ER $\beta$  has been reported in DN T cells in the *lpr/lpr* mouse, B cells, monocytes, and macrophages [30,43,51]. Using estrogen conjugated to BSA, calcium influx has been reported in T cells and macrophages, suggesting that these cells possess the membrane-associated form of the ER [54,55]. By the same methodology, B cells did not manifest calcium fluxes and, therefore, are not believed to have the membrane-associated receptor [56]. Given that these receptors are present in immune cells, it is possible that estrogen can induce intracellular signals similar to those described above. Additionally, because many of the proteins implicated in estrogen signal transduction are associated with activities of cell survival and growth, it is possible that these pathways may be critical in estrogen's role as a pro-autoimmune factor. This must be considered with the caveat that estrogen can clearly act as a downmodulator of the immune response. Reconciling these apparent contradictions is a critical aspect of understanding the immunobiology of estrogen.

### Estrogen–endocrine–immune interactions

Another aspect of studying estrogen's immunomodulatory effects is how it interacts with other hormones, which have also been implicated as immunomodulators including androgens, progesterone, prolactin, and gonadotropin-releasing hormone (GnRH) (Fig. 3). This interaction can occur at several different levels. (1) Estradiol enhances the production of some hormones including prolactin. (2) Estradiol can regulate the responsiveness of cells to hormones including GnRH. Because T cells have been reported to express the GnRH receptor, some effects of estrogen could be modulated through GnRH [57]. (3) Hormones may act together through a common signaling pathway. For instance, both progesterone and androgen have been reported to act synergistically with estrogen to activate the Erk pathway and cause proliferation in prostate and breast tumor cells [58,59]. (4) Multiple hormones can act on the same target cell population such as the prior case of estradiol and prolactin having roles in the breaking of B cell tolerance [31,60]. Each of these interactions should be considered in experimental design and interpretation.

### Summary

In summary, estrogen has been shown to effect multiple cellular components of the immune system in ways that are not at first glance consistent, as in the case of Th1 and Th2 phenotype. Explanations for these differences may reside

in variations in the immune mechanisms involved in these models and differences in responses to varied doses of estrogen. Several instances have been reported where estrogens display nonlinear dose responses. Additionally, understanding how estrogen can produce varied results in different systems, one should consider the general biology of estrogens and how it would be expected to interact with the immune system. A critical aspect of estrogen's biology is its capacity to support cell proliferation and survival. In keeping with this biology, estrogens appear to augment B cell survival and T cell proliferation, though not in every system studied. The impact upon the development of autoimmunity in females could reside in estrogen permitting auto-reactive clones to escape normal mechanisms of regulation and thereby accumulate in sufficient numbers to produce disease. Whether this could provide a mechanism for explaining sex differences in disease frequency will require a better understanding of estrogen's effects on the normal cellular responses of lymphocyte and APCs in various experimental systems.

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