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Review

Sex hormones as immunomodulators in health and disease

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Abstract

In addition to their effects on sexual differentiation and reproduction, sex hormones influence the immune system. This results in a gender dimorphism in the immune function with females having higher immunoglobulin levels and mounting stronger immune responses following immunization or infection than males. The greater immune responsiveness in females is also evident in their increased susceptibility to autoimmune diseases. However, a clear understanding of the myriad of effects that sex hormones have on the immune system is lacking. Studies in normal mice show that estrogen treatment induces polyclonal B cell activation with increased expression of autoantibodies characteristic of autoimmune diseases. Several mechanisms appear to contribute to the break in tolerance and the increase in plasma cell activity including a reduction of the mass of the bone marrow and the thymus, the emergence of sites of extrameduallary hematopoiesis and altered susceptibility of B cells to cell death. In addition, sex hormone levels in both humans and experimental models correlated with the activity of their cytokine-secreting cells indicating that sex hormones influence the cytokine milieu and suggesting that altered sex hormonal levels in autoimmune patients contribute to the skewed cytokine milieu characteristic of systemic lupus erythematosus (SLE).

While sex hormones alone do not cause autoimmune disease, abnormal hormone levels may provide the stage for other factors (genetic, infectious) to trigger disease. Understanding the physiology of the interaction between sex hormones and immune function and its potential pathological consequences may provide insight into the autoimmune diseases and new directions for their treatment. Published by Elsevier Science B.V.

Keywords: Estrogen; Dehydroepiandrosterone sulphate; Cytokines; Systemic lupus erythematosus

1. Introduction

The immune, endocrine and central nervous systems are integrated through a network of signal molecules—cytokines, hormones, and neurotransmitters—that act on a common set of receptors [1]. One of the consequences of this interaction is that, in addition to the profound effects on sexual differentiation and reproduction, sex steroids influence the development and function of the cells of the immune system [2,3]. This results in a dimorphic immune response, with females having higher immunoglobulin (Ig) levels and mounting stronger immune responses following immunization or infection than males. The greater immune responsiveness in females is also evident in their increased susceptibility to autoimmune diseases. Thus, gender emerges as

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one of the most important epidemiological risk factors for the development of autoimmunity, with females during their childbearing years having up to 9 times higher incidence than males of the same age.

These hormones, particularly estrogen and dehydroepiandrosterone (DHEA) affect the female predisposition to develop autoimmune disease. However, a clear understanding of the myriad of effects that sex hormones have on the immune system is lacking. Deciphering the mechanism(s) by which sex hormones regulate the immune system is quite important, given the increasing numbers of people worldwide who are using sex hormones as oral contraceptives, replacement therapy (HRT) or dietary supplements. An additional source of concern is the recent identification of hormone disrupting effects of a large number of pesticides and insecticides currently on the market [4–6].

2. Animal models

2.1. Female mice produce higher levels of antibodies than males

Studies of murine models provide considerable insight into the immunomodulatory effects of sex hormones (reviewed in Refs. [2,7–9]. Early studies showed that female mice had higher serum Ig levels and produced more antibodies to a variety of heteroantigens than males [9,10]. Further, female mice have reduced incidence of tumors and reject allografts more rapidly than males [9,11,12]. These findings are consistent with females mounting stronger humoral and cell-mediated immune responses than males.

2.2. The effect of sex hormones in murine models of systemic lupus erythematosus (SLE)

Early evidence for a contribution by sex hormones to autoimmune diseases was provided by studies of animal models of SLE. (NZB $\times$ NZW)F_1 mice (B/W mice) provide an excellent experimental model of SLE. Female B/W mice spontaneously developed autoantibodies, lymphadenopathy, arthritis, immune complex glomerulonephritis, and died earlier than their male counterparts. A "protective" effect of androgens was observed in studies showing that orchidectomy (with a corresponding drop in testosterone levels) of male B/W mice resulted in premature death. Further, administration of 5α-dehydrotestosterone (DHT), a male steroid that is not metabolized into estrogen, lowered anti-DNA autoantibody levels and prolonged the life span of female mice [13,14]. The beneficial effects of androgens were evident even in mice with established disease [15]. Other androgens such as dehydroisoandrosterone (DHA) and DHEA retarded the onset but not the progression of disease in B/W mice [16–18]. In contrast, in vivo administration of estrogen accelerated disease progression and death [14]. The accelerating effect of estrogen on disease progression was reported in other SLE models including MRL/lpr mice [19], and BALB/c mice injected with anti-DNA (16/6 id) antibodies [20]. A recent report suggests that the effects of estrogen on the development of disease in MRL/lpr mice could be mediated by altered number and affinity of estrogen receptors (ER) in lymphoid organs [21].

An impact of sex hormones was reported in animal models of other autoimmune diseases. In NOD mice, which are prone to insulin-dependent diabetes mellitus [22], disease progression is accelerated in females. Similarly, sex hormones accelerate the development of polyarthritis in Lewis rats (LEW/N rats) and of autoimmune thyroiditis in PVG/c rats [9]. Sex hormones, however, do not play a role in every model for autoimmunity as shown for example by the BXSB mice, which develop an SLE-like disease predominantly in the males that has been associated to *yaa* gene.

2.3. Estrogen promotes B cell hyperactivity and the production of autoantibodies in non-autoimmune mice

The overproduction of pathogenic autoantibodies is a key feature of autoimmune diseases such as SLE, yet numerous studies indicate that the B cells in lupus are not abnormal [23,24]. Rather, they appear to be hyperstimulated by their environment [24].

A set of elegant studies where anti-DNA producing B cells from normal DBA/2 and autoimmune NZB mice were transferred into NZB.xid mice showed that autoreactive B cells expanded preferentially in female vs. male mice and in NZB vs. DBA/2 recipients [23,25]. This suggested that: (1) sex hormones play a role in regulating lymphocyte proliferation and/or activation, (2) a "female" environment is conducive to the expansion of autoantibody-secreting cells, and (3) B cell hyperactivity depends on the immunologic/endocrine environment [23,26].

The prevalence of autoimmune disease in females led us to examine the natural autoantibody levels in mice of each sex. Sera from male and female C57BL/6 mice were tested for the concentration of IgG autoantibodies against cardiolipin and dsDNA, which are characteristic of anti-phospholipid syndrome (APS) and SLE [27,28]. As shown in Table 1, only female mice had detectable levels of IgG autoantibodies. Unlike in autoimmune mice, the absence of autoantibodies from normal males was not due to the protective effect of male hormones, since pre-pubertal orchidectomy or administration of (DHT) did not induce autoantibody production (Table 2). In contrast, pre-pubertal chronic administration of estrogen markedly increased the incidence and concentration of autoantibodies in both female and male mice (Table 2). These antibodies remained elevated even after the implants were removed and estrogen levels returned to baseline [28,29]. These findings suggest that early rather than sustained exposure to estrogen is sufficient to induce autoantibody production in the absence of intentional autoantigen exposure.

Table 1
Normal female mice have higher incidence and levels of autoantibodies than normal males

	Anti-dsDNA antibodies		Anti-cardiolipin antibodies	
	Incidence (%)	Mean titer (S.E.)	Incidence (%)	Mean titer (S.E.)
Male (♂)	11	5 ± 2	22	3 ± 2
Female (♀)	62	32 ± 6	58	27 ± 2

Expression of IgG autoantibodies was determined by ELISA in serially diluted sera of untreated 6- to 11-month-old non-autoimmune female ($n = 18$) and male ($n = 18$) C57BL/6 mice [27,90]. Double stranded DNA used to coat the plates was digested with S1 nuclease to eliminate ssDNA as described [90].

Table 2
Male hormones do not prevent the expression of autoantibodies in normal mice

	IgG Anti-cardiolipin antibodies	
	Incidence (%)	Mean titer
♂ P–S	30	5 + 2.3
♂ P–O	20	3 + 2.1
♂ DHT–O	33	6 + 2.2
♂ E2–O**	63	42 + 1.6
♂ E2–S**	60	36 + 2.7
♀ P	50	24 + 2.5
♀ E2***	83	298 + 2.6

Male (♂, $n = 18$) non-autoimmune C57Bl/6 mice were prepubertaly orchidectomized (O), and treated (90-day implants) with 5α-dehydrotestosterone (DHT), 17β-estradiol (E2) or placebo (P) as described [27]. Female mice (♀, $n = 19$) were treated with 17β-estradiol (E2) or placebo (P) 90 day implants. Mice that were sham orchiectomized and treated with placebo implants served as controls (P–S). $N = 9$–20 mice per group. Sera was collected and tested for antibodies to cardiolipin 6 months after treatment [29]. Similar results were obtained 2, 3, 4 and 8 months after treatment.

$p < 0.01$, $p < 0.001$, Non-parametric ANOVA and Mann–Whitney U-test.

The induction of antibodies in normal mice by in vivo treatment with estrogen was not restricted to cardiolipin and dsDNA. Estrogen increased the titers of IgG antibodies to anionic phospholipids, lysozyme, and ovalbumin as well as to complex microbial antigens indicating that estrogen acts as a polyclonal B cell activator [30]. This may partly explain the increased resistance to bacterial infection reported in women. Competition studies using DNA, cardiolipin and other negatively charged phospholipids showed very low cross-reactivity of estrogen-induced autoantibodies [30]. The isotype and sub-isotype of the antibodies induced was also examined. Animals treated with estrogen had autoantibodies predominantly of IgG (but not IgA or IgE) isotype, and the main sub-isotypes elicited were IgG2b and IgG1 consistent with T cell-dependent immune responses [29]. These results suggest that antibodies elicited by estrogen were not merely an expansion of natural antibodies, which are usually IgM, low affinity and broadly cross-reactive, but rather antigen-specific autoantibodies [31,32].

We reasoned that the estrogen-induced increase in antibody titers might result from: (a) an expansion of B cell numbers; (b) the accelerated maturation of B cells into plasma cells; or (c) increased amounts of antibody produced per cell. Flow cytometric analysis of spleen cells in estrogen-treated mice showed no increase in $B220^+$ $CD19^+$ cells, indicating that estrogen did not induce a general expansion of mature B cells. Histologic examination of the spleens of mice treated with estrogen showed a 10-fold increase in the number in plasma cells (which are $CD19^-$ and $CD220^-$). This increase in plasma cells produced a 6-fold increase in the total number of IgG-secreting cells in the spleen. Similar analysis performed on bone marrow revealed a 10-fold increase of the number of IgG-secreting cells [30]. Moreover, the antibody output per cell in spleen and bone marrow was elevated in mice treated with estrogen compared to placebo [30]. Therefore, the increase in antibody titer induced by estrogen treatment was the result of an increase in the number and activity of plasma cells in bone marrow and spleen.

Klinman and colleagues [33,34] showed that the B cell repertoire of young B/W and MRL/lpr mice was not biased towards autoantibody production, but that specific antigenic stimulation subsequently served to magnify and perpetuate the production of autoantibodies. Estrogen treatment increases cell death at the sites of lymphocyte maturation and these dying cells may expose anionic phospholipids on their membrane and release dsDNA. It is therefore possible that the expansion of autoreactive B cells is due to increased availability of self-antigens. However, the proportion of B cells secreting antibodies reactive with self- or hetero-antigens was similar in estrogen-treated and control mice. This suggests that the expansion of B cells was not due to increased exposure to antigen but rather the result of polyclonal B cell activation.

2.4. Sex hormones may act directly on immune cells, alter the sites of lymphocyte development and modify the immune milieu

Estrogen may override tolerance to allow the activation and expansion of autoreactive B cells during B cell development. Chronic administration of estrogen leads to reduced mass of bone marrow and thymus, organs where deletion of autoreactive cells normally occurs [5,30,35]. Kincade showed that estrogen induces the death of pre-B cells in the bone marrow at the IL-7 sensitive stage [36,37]. This effect was interpreted to be indirect, since no estrogen receptors had been identified on pre-B cells. However, since then another type of estrogen receptor, ERβ, has been identified, which is present primarily on CNS and immune cells [38]. In addition, recent studies using ERα knockout mice suggest that estrogen may have a direct effect on developing B cells [39]. Alternatively, estrogen could act on the stromal cells of bone marrow, which have ERα [36]. We found that estrogen-treated mice developed alternative sites of hematopoiesis in the spleen and liver [30]. It is possible that the autoreactive B cells that develop at these sites escape negative selection. Such a phenomenon was reported by Nakayama who showed that BALB/c mice treated with estrogen developed autoreactive ($V\beta3^+$ or $V\beta8^+$)$TcR^{intermediate}$ cells in the liver [40].

An alternative mechanism by which sex hormones might induce B cell hyperactivity is by modifying the cytokine milieu. Since cytokines mediate lymphocyte growth and function, a defect in the cytokine network could disrupt the development of self-tolerance. In vitro stimulation of lymphocytes with sex hormones affects cytokine production by T cells and macrophages [41,42]. Indeed, hormone-responsive elements have been identified upstream of several cytokine encoding genes [43,44]. As shown in Fig. 1, estrogen treatment increased the frequency of IL-6 and IL-10-secreting cells in BALB/c mice. These findings complement those of Dayan et al. [45] who showed that in BALB/c mice immunized with 16/6 id, administration of Tamoxifen or anti-estrogen increased the type 1 cytokines IL-2 and IFNγ and reduced the levels of IL-10, IL-1 and TNFα. Interleukin-10 and -6 cytokines are the most potent activators of B lymphocytes in vitro, inducing both proliferation and strong Ig production by promoting the maturation of B cells into plasma cells [46,47]. Increased levels of IL-10 and IL-6 have been linked to disease severity in SLE models [47–50]. Administration of IL-10 or IL-6 accelerated, while anti-IL-10 or anti-IL-6 delayed the development of disease in B/W mice [51–53].

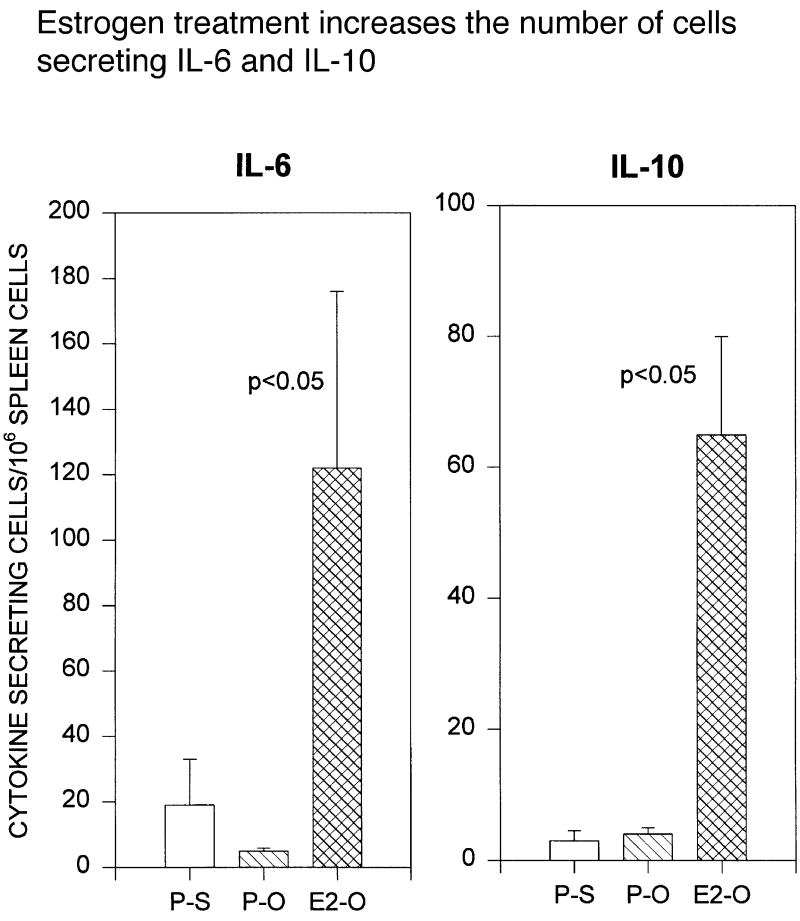

Fig. 1. Frequency of IL-6- and IL-10-secreting cells (as determined by ELIspot [88]) in the spleen of C57BL/6 female mice (3–6 per group) ovariectomized pre-pubertally (O) and treated with placebo (P) or estrogen (E2) [28]. Mice that were sham-ovariectomized (S) and received placebos served as controls. Results represent the mean ± S.E.M. All samples were tested blind and decoded retrospectively. Statistical significance tested with Mann–Whitney *U* non-parametric test.

A third mechanism by which estrogen may induce polyclonal B cell activation in normal mice is by making activated B cells resistant to cell death. Splenic B cells from normal mice undergo apoptosis unless rescued by stimulation [54]. Recent results from our lab show that B cells from estrogen-treated C57BL/6 mice are resistant to apoptosis in vitro (Fig. 2) [5]. The possibility that estrogen interferes with apoptosis of autoreactive B cells is supported by a recent study showing that estrogen upregulates Bcl-2 expression in B cell of mice treated with estrogen [55].

Several other mechanisms have been postulated for the effects of sex hormones on immune function including the down-regulation of NK [56,57] or suppressor T cell functions [9,57], or indirect effects via the hypothalamic–pituitary–adrenal axis [7,58]. Regardless of the mechanism involved, exposure to exogenous estrogen overcame tolerance and induced B cell hyperactivity in normal mice, with increased number and activity of plasma cells (including autoreactive cells). This supports the concept that estrogen facilitates the development/activation of B cells regardless of their specificity and thus could

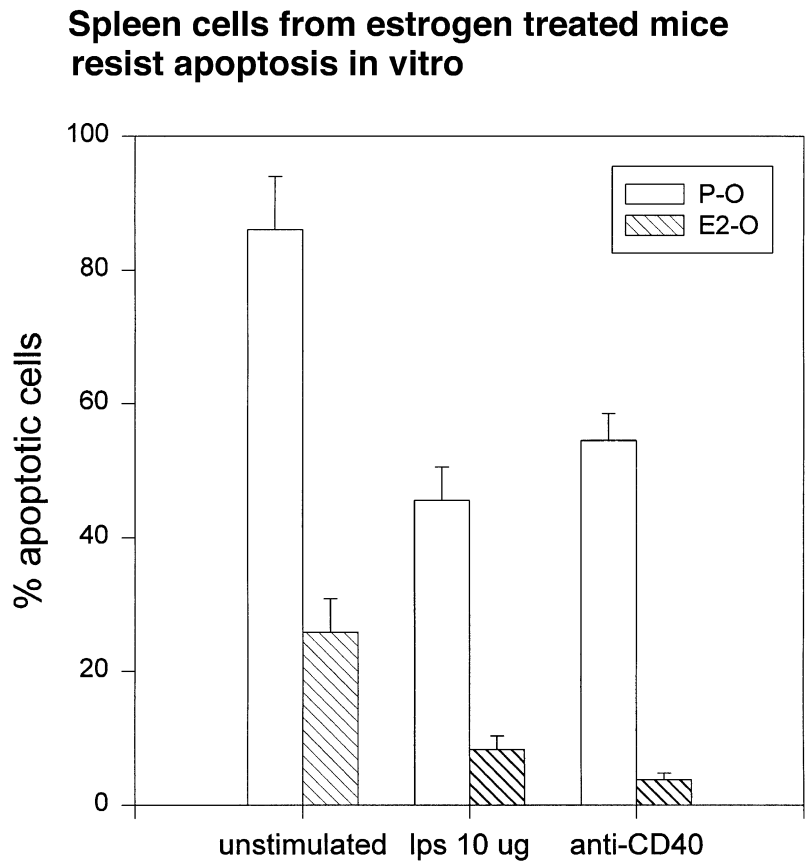

Fig. 2. Purified B cells from mice treated with estrogen or placebo were cultured for 48 h in complete RPMI media with 5% FCS alone or stimulated with LPS or anti-CD40 (D. Verthelyi and S. Ansar Ahmed, manuscript in preparation) [30]. Percent of hypodiploid cells was assessed by propidium iodide staining [89]. Similar results were obtained using acridine-orange and ethidium bromide. Results are representative of five individual experiments.

foster/accelerate the development of autoimmunity and autoimmune disease.

3. The human data

3.1. Sex hormones and sexual dimorphism in immune function

Analogous to the murine models, sex hormones play a central role in the gender dimorphism among humans. Women tend to have higher levels of Ig, and mount stronger humoral immune responses to a variety of antigens than men (reviewed in Refs. [2,7,8,59]. This allows them to respond better to most microbial (e.g., *Escherichia coli*, *Brucella*, measles, rubella and hepatitis B) and non-microbial antigens than their male counterparts [7,8,57]. These differences become evident after menarche and diminish after menopause [57,60,61].

3.2. The effects of sex hormones on SLE

The phenomenon of gender bias in the susceptibility to autoimmune diseases has been recognized for many years. Women are 3- to 9-fold more susceptible to lupus, Sjogren's syndrome (SS), and rheumatoid arthritis (RA) than men [8,57]. Abundant clinical

evidence connects estrogen to the pathogenesis of SLE: SLE patients, in general, have increased levels of estrogen and active estrogen metabolites in serum [62,63]. The majority (80% according to Steinberg and Steinberg) of the symptomatic episodes in women with regular menstrual cycles occurred during the luteal phase of the menstrual cycle [64]. Flares commonly occur during or immediately after pregnancy [65,66]. In addition, two large studies show that HRT in post-menopausal women doubles the risk of developing SLE [67,68]. Additional evidence for the pathogenic role of steroid hormones on SLE include a report by Ben-Chetrit and Ben-Chetrit [69] describing three cases of previously healthy women who developed SLE after repeated cycles of ovulation induction. Repeated cycles of ovulation induction therapy in SLE patients resulted in severe (even fatal) flares in SLE patients with anti-cardiolipin antibodies [70,71]. It should be noted, however, that most post-menopausal patients with SLE do not have increased number of flares [65,72] or thromboembolic events while on oral contraceptives or HRT [73], and that hundreds of women undergo repeated cycles of ovulation induction every year without developing autoimmune diseases. This underscores that fact that increased estrogen levels are just one factor in the complex pathogenesis of SLE.

Several lines of investigation suggest a pathogenic role for the reduced levels of DHEAS in SLE female patients [74,75] including an association with the reduced levels of IL-2 and IFNγ which are characteristic of SLE [76,77]. Recent clinical trials showed that supplementation with exogenous DHEAS resulted in modest clinical improvement in patients with moderate disease [78]. The proposed mechanistic basis for their therapeutic success is modulation of T cell activity and cytokine secretion in vivo.

3.3. Estrogen induces polyclonal B cell activation in humans

Similar to what had been described in mice, estrogen increased levels of IgM and IgG secretion by human PBMC in vitro [79,80]. Estrogen also magnified B cell response to Pokeweed mitogen, while testosterone reduced it [61,81]. These effects were independent of the hormonal status of the lymphocyte donor (male, female, luteal or follicular phase of menstrual cycle, or post-menopausal) and were partially dependent on IL-10 secretion by monocytes [80,81]. This suggested that the effect of estrogen on human B cells is mediated in part by modulating cytokine production.

3.4. Sex hormone levels are associated with cytokine secretion by PBMC from normal subjects but not with the activity of PBMC from SLE patients

The association of sex hormone levels with the activity of cytokine-secreting cells in vivo was explored in a study that correlated estrogen, progesterone and DHEAS levels in the sera of 88 subjects with the activity of their cytokine-secreting cells (Table 3). This was done using an ELISpot assay, which allowed us to estimate the number of cells that were activated to secrete cytokines in vivo. It showed that serum DHEAS levels closely correlated with the

Table 3
Association of sex hormone levels with number of cytokine-secreting cells in peripheral blood

			IFNγ and DHEAS		IL-4* and Estrogen	
			r	*p* Value	*r*	*p* Value
Control	$n = 38$	♀	0.4	0.001	0.6	0.001
SLE inactive	$n = 25$	♀	< 0.01	ns	0.3	0.01
SLE active	$n = 27$	♀	< 0.01	ns	0.06	ns
Control	$n = 28$	♂	0.5	0.001	0.1	ns
SLE	$n = 8$	♂	0.02	ns	0.1	ns

Correlation between hormone levels (as determined by RIA) and frequency of cytokine-secreting cells (as determined by ELIspot) in pre-menopausal controls and SLE patients with inactive or active disease and male control and SLE patients [82,86]. The number of cytokine-secreting cells was correlated with serum hormone levels in each individual. Correlations determined using Pearson rank order analysis.

* Cells stimulated with PHA.

activity of IFNγ-secreting cells in men and premenopausal women [82]. Post-menopausal women, had reduced levels of DHEAS and fewer IFNγ-secreting cells but no direct correlation between the two was observed [82]. Post-menopausal women treated with hormone replacement therapy, however, had higher DHEAS levels and higher frequency of IFNγ-secreting cells. This indicated that sex hormones rather than increased age were responsible for the reduced activity of IFNγ-secreting cells. The finding that DHEAS levels are associated with the activity of IFNγ-secreting cells is consistent with reports that DHEAS promotes the production of IL-2, another type 1 cytokine [76], and with reports that DHEAS stimulates IFNγ production by murine lymphocytes [83].

The same study showed that the number of PBMC capable of secreting IL-4 (as determined by PHA stimulation) was closely associated with the estrogen levels and oscillated with the menstrual cycle [82]. In addition, the number of cells-secreting IL-6 was higher during the follicular phase than the luteal phase, confirming a previous report by Angstrum et al. [84]. Since IL-4 and IL-6 are co-inducers of B cell activation, these results may explain the stronger humoral immune response of women [2,8,59], and suggest that the type and magnitude a woman's immune response to immune challenge (such as infection or vaccination) may vary with the menstrual cycle.

The activity of cytokine-secreting cells in SLE patients is altered, with reduced number of IFNγ-secreting cells and normal or increased number of IL-10-secreting cells [85,86]. As a result, their IL-10/IFNγ-secreting cell ratio is increased, with higher ratios being present in patients with the most severe disease [85]. Since sex hormones modulate cytokine secretion and SLE patients have increased estrogen and reduced DHEAS levels, this raised the possibility that a cause–effect relationship may exist between the hormonal imbalance, the skewed cytokine milieu and the development of SLE. To explore this possibility we studied serum sex hormone levels and the number of cytokine-secreting cells in the peripheral blood of 128 SLE patients. Although in these patients, both DHEAS levels and IFNγ production were significantly reduced, no direct correlation was evident between serum DHEAS levels and the number of cells secreting IFNγ in peripheral blood, regardless of disease activity (Table 3). In the same study, the correlation between estrogen levels and IL-4-secreting cells that was previously found in healthy individuals was lost as patients developed active SLE. While it is possible that the failure of lymphocytes to be modulated by changes in estrogen level is due to abnormalities in the expression estrogen receptors in SLE [87], these results are more consistent a progressive failure of lymphocytes to respond to sex hormones in patients with increasingly severe disease.

4. Conclusion

Sex steroids are pleiotropic hormones that act on multiple targets including the central nervous system, bone, reproductive organs, and the immune system among others. Sex hormones influence the development, maturation, activation and death of immune cells. Studies show that DHEAS promotes the production of type I cytokines (IL-2 and IFNγ) and the development of cell-mediated immunity, while estrogen fosters B cell activation, in part by stimulating type 2 cytokines (IL-4, IL-6, IL-10).

While sex hormones alone are not responsible for the gender dimorphism of the immune response or the development of autoimmune disease, abnormal hormone levels may provide fertile ground for other factors (genetic, infectious) to trigger disease and may modulate their course. Understanding the physiology of the interaction between sex hormones and immune function and its potential pathological consequences may provide insight into autoimmune diseases and new directions for their treatment. However, this may require us to change our focus/vision from assessing the effects of sex hormones on immune cells, to understanding the mechanics of a system in which sex hormones and immune cells are just two parts of an integrated whole. Complex diseases such as SLE, which have genetic, infectious, hormonal, and psychological factors, make this need apparent.

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