EFFECT OF FIVE ALPHA DIHYDROTESTOSTERONE (5α-DHT) ON CYTOKINE PRODUCTION BY PERITONEAL MACROPHAGES OF NZB/BALBc MICE

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ABSTRACT

One of the mechanisms involved in the regulation of the immune system by steroid hormones could be the monocytic-macrophage system. In this study the effect of the male hormone 5α-DHT on cytokine release by peritoneal macrophages (mφ) of male and female NZB/BALBc mice was investigated. Macrophages from male mice activated with LPS produced a greater amount of IL-1β (21.8%) ($p<0.05$) and IL-6 (19.3%) ($p<0.01$) than age-matched females. However, the total amount of TNF-α released by LPS-activated macrophages was greater (24.8%) ($p<0.05$) in female than male mice. 5α-DHT enhanced IL-1β and reduced IL-6 release by activated macrophages in a dose-dependent fashion. The effect of 5α-DHT on TNF-α release was not exactly dose-dependent. Therefore, it is likely that the modulation of the immune system by steroid hormone may partly be due to the effect of 5α-DHT on the secretion of cytokines by the monocytic-macrophage system.

Keywords: Macrophage, Sex hormone, 5α-DHT, Cytokine.

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INTRODUCTION

Sex steroid hormones play a role in the complex network of immune responses. Generally it is believed that estrogens have immunostimulatory effects, at least on B cell activities, and elevate antibody production to T-dependent antigens. 17-beta estradiol has been shown to enhance immunoglobulin production in a T cell dependent way.[1] Clinical and experimental evidence demonstrated that androgens in general are immunosuppressive.[2,3] On the thymus, physiological and biochemical observations also suggested that androgenic hormones modulate the function of this organ.[4] For example, testosterone has been shown to cause atrophy of the thymus and some investigators believe that this effect may be mediated via the thymic epithelium since it has been shown to contain androgen receptors.[5-7] Androgens also alter the rate of production, migration and homing of lymphoid cells to the thymus.[7] Androgens cause lymphocyte depletion in cortical areas[8] and it is possible that androgens affect the induction of thymolysis. Androgens, as well as influencing stem cell differentiation in the thymus and bone marrow, have also been reported to elicit the production of soluble immunoregulatory factors by thymic epithelium.[9-10] The bursa and its presumed equivalent in the bone marrow cannot be excluded from androgen sensitivity. Treatment with testosterone during early embryonic life is known to produce bursal abnormalities in the adult chicken accompanied by absence

of normal B-lymphocyte function.[11] In adult male rabbits, it has been shown that androgen alters immunoglobulin synthesis.[12] Thus, recent investigations have demonstrated that dehydroepiandrosterone (DHEA) can also modulate the immune system via cytokine production and also nitric oxide release.[13] Dihydrotestosterone (DHT) has been shown to reduce the amount of IL-4, IL-5 and γ-IFN production by T cells after activation with anti-CD3 and leaving IL-2 production unaffected.[14] On the other hand exposure of isolated T cells to dihydroepiandrosterone (DHEA) resulted in an enhanced potential of activated T cells to produce IL-2 and γ-IFN after activation, with no effect on production of IL-4.[15] Testosterone has also been shown to inhibit IL-1 secretion by peripheral blood mononuclear cells (PBMCs) either in male normal or rheumatoid arthritis (RA) donors.[16] The aim of this investigation was to find out whether 5α-DHT affects cytokine release by peritoneal macrophages and in general to ascertain any sex and gender differences.

MATERIALS AND METHODS

Preparation of mouse resident peritoneal macrophages (Mφ)

Male and female NZB/BALBc mice (8w old) were killed by cervical dislocation and peritoneal cells were extracted immediately by washing with 5 mL ice-cold RPMI 1640 medium (Life Technology). Cells were kept at 2-8°C ice cold to avoid nonspecific attachment and were washed at 4°C for 2-3 times (1500 rpm for 5 minutes) with cold medium. Cells (1×10^6 cells/mL) were then resuspended in complete RPMI 1640 medium and allowed to adhere to the plastic tube (1mL/well) for 1-2 h at 37°C in 5% CO_2 in air followed by three more washings to remove non-adherent cells.

Trypan blue exclusion test

The sample of cell suspension was mixed with an equal volume of 0.4% (w/v) Trypan blue in PBS and incubated for 10 minutes. The cells failing to exclude the dye were counted and expressed as a percentage of the total cells present.

Prelabelled cells by methionine-[³⁵S]

Methionine-free RPMI 1640 (Life Technology) in a volume of 450 μL was added and incubated for 15 min at 37°C, 5% CO_2 in air, after which 50 μL of [³⁵S] methionine (5 μCi/well) diluted in methionine-free RPMI 1640 was added and the cells incubated for a further 6 h. Culture medium (methionine-free) was removed from all wells and replaced with fresh normal RPMI 1640 (maintained at 37°C) in 400 μL volume without any radiolabel.[17] The cells were then stimulated with 10 μg/mL LPS in 50 μL. Various concentrations of 5α-DHT (Sigma) ranging between

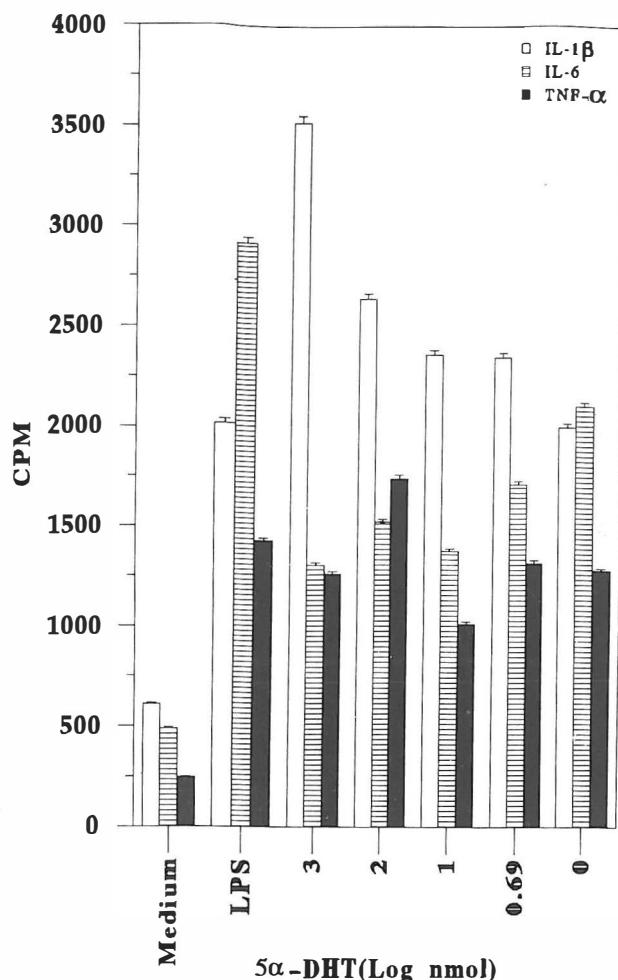**Fig. 1.** The effect of 5α-DHT on IL-Iβ, IL-6 and TNF-α production. Peritoneal macrophages from male NZB/BALBc mice were prelabelled with [³⁵S]-methionine (5μci/well) in 0.5 mL methionine-free medium and incubated for 6 h at 37°C at 5% CO_2 in air. The supernatant was discarded and replaced with normal medium containing different concentrations of 5α-DHT and incubated for 2 h. LPS (10 μg/mL) was added and cells were then incubated for a further 24 h as described in *Materials and Methods*. Supernatants were assayed for IL-Iβ, IL-6 and TNF-α. Values represent the mean±SD for n= 3.

1×10^{-6}M to 1×10^{-13}M was added 2h before LPS-stimulation and the final volume of each well adjusted to 0.5 mL and incubation continued for a further 24 h. The culture medium was removed from each well and stored.

Immunoprecipitation of [³⁵S]-labelled cytokines

Supernatants were subjected to immunoprecipitation for direct cytokine measurement. Aliquots (50 μL) of the supernatants were placed into Eppendorf tubes containing assay buffer (50μL of 25 mM phosphate buffer, 0.1 sodium azide, pH 7.4). Rat anti-mouse IL-6 monoclonal Ab (Genzyme Co.), hamster anti-mouse IL-1β monoclonal Ab (Genzyme Co.) or goat anti-mouse TNF-α monoclonal Ab
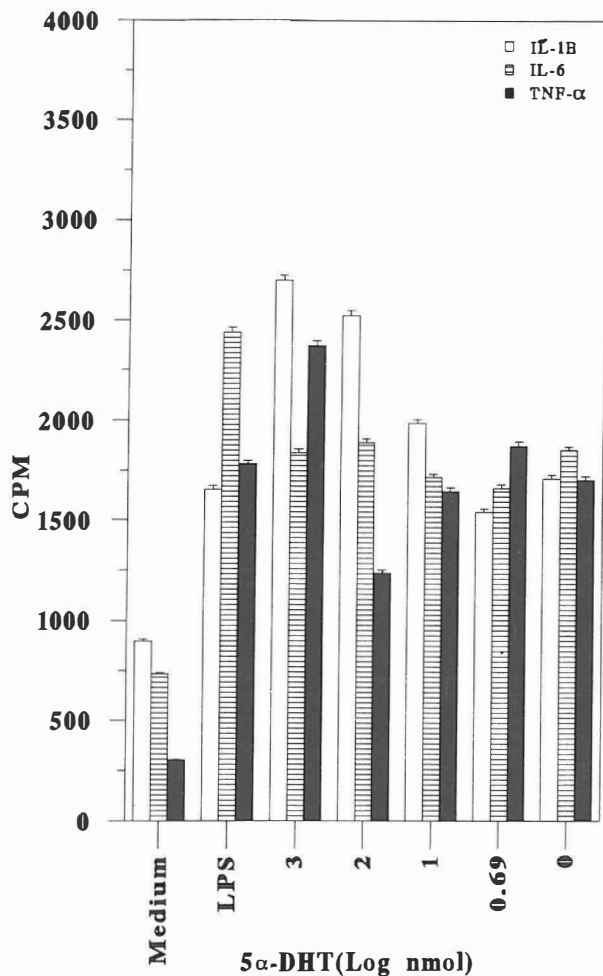
Fig. 2. The effect of 5α-DHT on IL-Iβ, IL-6 and TNF-α production. Peritoneal macrophages from female NZB/BALBc were prelabelled with [^{35}S]-methionine (5μci/well) in 0.5 mL methionine-free medium and incubated for 6 h at 37°C at 5% CO_2 in air. The supernatant was discarded and replaced with normal medium containing different concentrations of 5α-DHT and incubated for 2 h. LPS (10 μg/mL) was added and cells were then incubated for a further 24 h as described in *Materials and Methods*. Supernatants were assayed for IL-Iβ, IL-6 and TNF-α. Values represent the mean±SD for n= 3.

(R & D system) were added to respective tubes in 100 μL amounts (1 μg/mL). All tubes were vortexed, mixed and incubated at 4°C for 16 h after which 200 μL of protein A (Sigma) (10 μg/mL) was added followed by vortexing and incubation of tubes at room temperature for 2-3 h. Tubes were then centrifuged at 1500 rpm for 5 minutes and washed three times with PBS. The supernatant was discarded and radioactivity in the pellet was measured after solubilizing in solubilizing solution (0.1 M NaOH; 2% (w/v) sodium lauryl sulphate) followed by liquid scintillation counting.[17]

Measurement of [^{35}S]-labelled IL-6, IL-1β and TNF-α

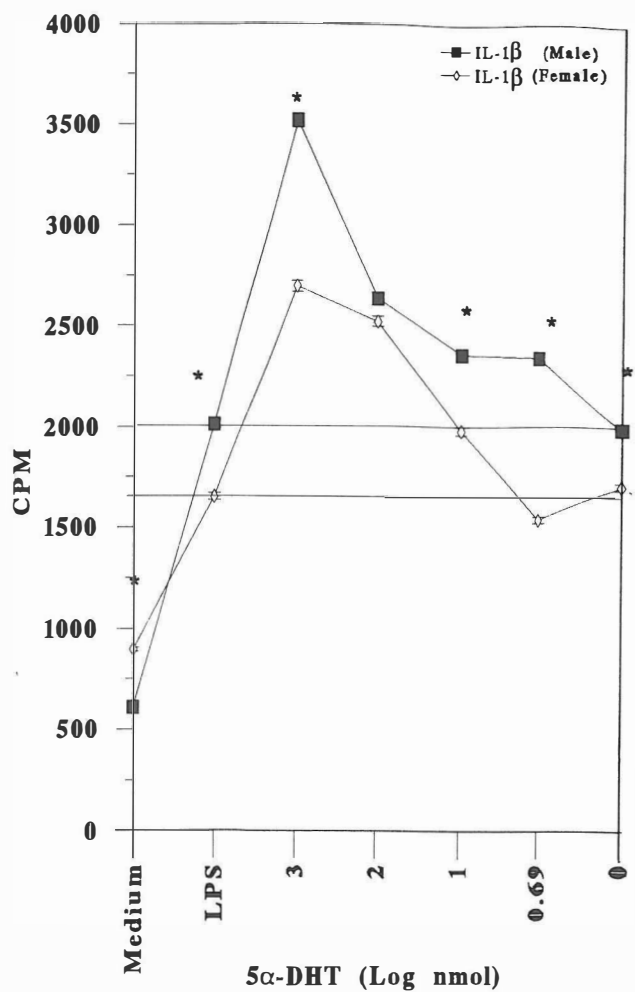Solubilizing solution (0.1 M NaOH; 2% (w/v) sodium

Fig. 3. Comparison of IL-Iβ release by peritoneal macrophages from male (■) and female (◇) NZB/BALBc mice in response to different concentrations of 5α-DHT.

lauryl sulphate) in a volume of 0.5 mL was added to each tube of immunoprecipitation pellet as already described and left for 24 h at room temperature. Solubilized samples were transferred to 5 mL plastic scintillation vials and 3.33 mL (1mL/150 μL) of liquid scintillation fluid (Liquid Scintillation Ultima TMMW 6013151-Packard) was added to each vial. Radioactivity was then measured by a liquid scintillation counter (1217 rack BETA LKB WALLAC Pharmacia Diagnostic) and results expressed as mean counts per min (cpm).

RESULTS

The effect of 5α-DHT on cytokine secretion

Macrophages from male mice activated with LPS produced a greater amount of IL-1β (21.8%) ($p<0.05$) (Figure 3) and IL-6 (19.3%) ($p<0.01$) (Figure 4) than age matched females. However, the total amount of TNF-α released by LPS-activated macrophages was greater (24.8%)
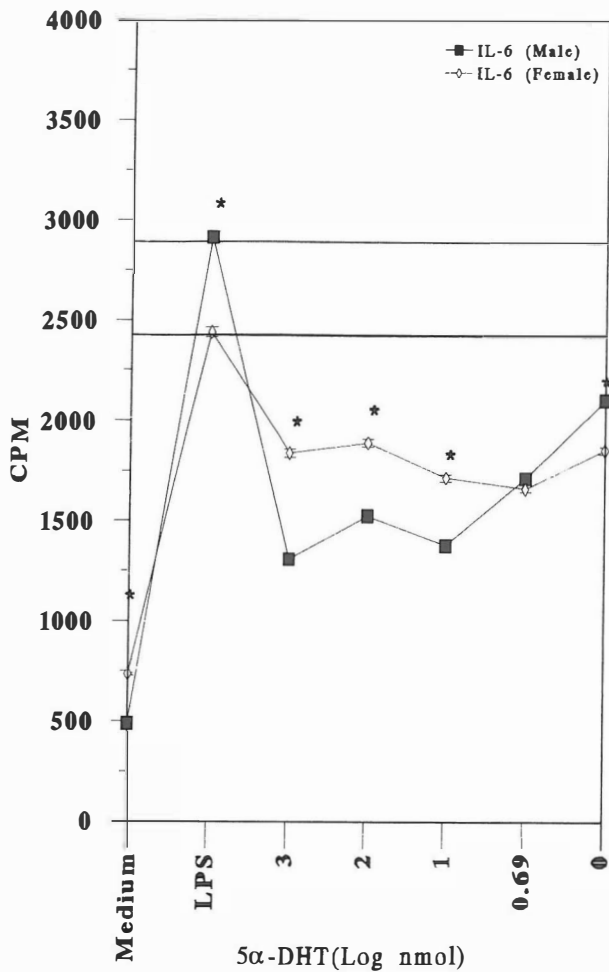
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Fig. 4. Comparison of IL-6 release by peritoneal macrophages from male (■) and female (◇) NZB/BALBc mice in response to different concentrations of 5α-DHT.
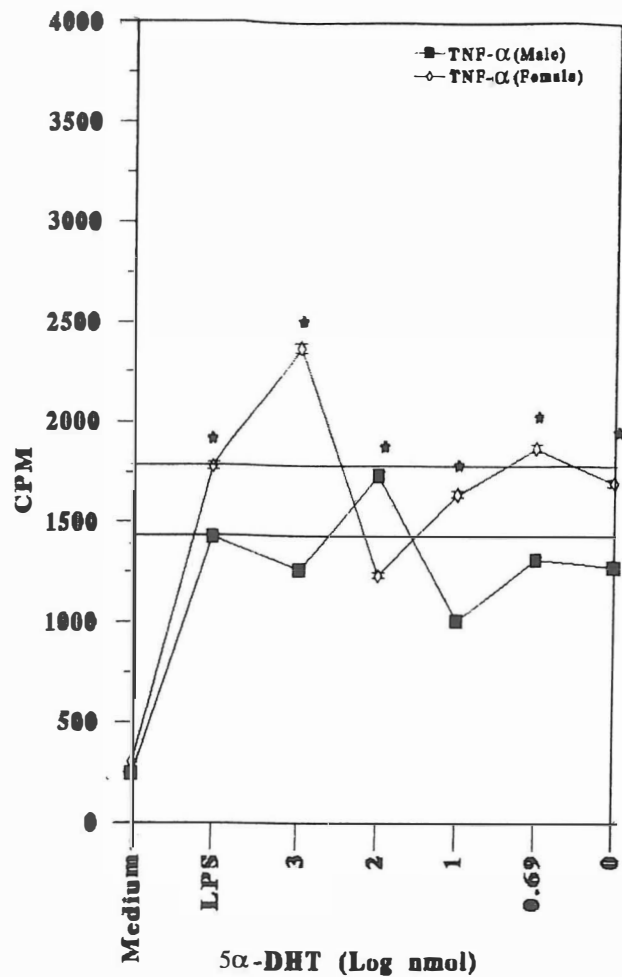

Fig. 5. Comparison of TNF-α release by peritoneal macrophages from male (■) and female (◇) NZB/BALBc mice in response to different concentrations of 5α-DHT.

(p<0.05) in female than male mice (Figures 1-5).

Interleukin-1 (IL-1)

5α-DHT enhanced IL-1β production by activated macrophages in a dose and sex dependent fashion (Figure 3). In male mice, 5α-DHT at concentrations of less than 5×10^{-9}M did not have a significant effect on IL-1β release by LPS-activated macrophages. A significant enhancement in IL-1β was induced (16.3%) (p<0.01) when cells were treated with at least 5×10^{-9}M. Moreover, maximum enhancement (74%) (p<0.01) in radioactivity precipitation was seen in the presence of 1×10^{-6}M 5α-DHT compared with LPS-stimulated cells receiving no hormone (Figures 1, 3). In contrast, in female mice, androgen hormone 5α-DHT had no effect on IL-1β release at concentrations below 1×10^{-8}M. But at 1×10^{-8}M, IL-1β was increased by 20% (p<0.05) and maximum increase occurred when LPS stimulated cells were treated with 1×10^{-6}M of 5α-DHT (Figures 2, 4).

Interleukin-6 (IL-6)

IL-6 production by LPS-stimulated macrophages in both sexes was significantly inhibited by 5α-DHT (Figure 4). As illustrated in Figures 1, 2 and 4, nonactivated peritoneal macrophages from female mice produced a greater quantity of IL-6 (33%) than male macrophages (p<0.05). In contrast, in LPS-stimulated macrophages the amount of IL-6 was greater in male macrophages than female (19.3%) (p<0.05) (Figure 4). In macrophages from male mice the level of IL-6 was reduced by 55.1%, 47.7%, 52.7%, 41.3% and 27.93%, in response to 5α-DHT at concentrations of 10^{-6}, 10^{-7}, 10^{-8}, 5×10^{-9} and 10^{-9}M, respectively (Figure 1).

The inhibitory effect of androgen on IL-6 production was found to be greater in males than females. The reduction over female mice was as follows; 24.8%, 22.7%, 29.83%, 32% and 24.1% respectively at the same concentrations (Figure 2).

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Tumor necrosis factor-α (TNF-α)

As shown in Figures 1, 2 and 5, when cells of male and female mice were stimulated with 10 μg/mL LPS, the amount of TNF-α secretion was significantly greater in females than males (19.8%) ($p<0.02$) (Figure 5).

In female macrophages, the variability of the effect of 5α-DHT on TNF-α production was greater than in males, so that at 1×10^{-6}M it enhanced by 33%, and at a concentration of 10^{-7}M, reduced by 30.4% (Figure 5). 5α-DHT at less than 10^{-7}M appeared to have no significant effect on TNF-α production (Figure 2, 5). These results demonstrated that 5α-DHT can modulate cytokine production (IL-1β, IL-6 and TNF-α) in response to LPS in both sexes. In each experiment, values represent the mean±S.D. for n=4.

DISCUSSION

As shown in Figures 1-5, 5α-DHT enhanced the production of IL-1β. Our observation is in agreement with a study by Li et al.[16] and also a very recent investigation by McLachlan[13] which reported that androgens can modulate the functions of a variety of macrophage cytokine products. IL-1 activity has been shown to be higher in the plasma of women and correlates with increased estrogen and progesterone levels in the luteal phase of their menstrual cycle.[18] Moreover, many investigations indicate increased IL-1 synthesis/secretion by rat peritoneal macrophages and blood mononuclear cells when incubated with estradiol.[16,19,20] However, Magnusson[21] reported that estradiol at high concentrations inhibits secretion of IL-1 by endotoxin-stimulated porcine mononuclear cells. In vitro experiments demonstrated that 5α-DHT significantly enhanced the release of IL-1β by activated murine peritoneal macrophages of both sexes, indicating that the effect of 5α-DHT on IL-1β release is not sex-linked. These results are also comparable to those of Yoshida,[22] who demonstrated that the number of macrophages of golden hamsters and their response to LH to produce IL-1 was increased by estradiol treatment but not by progesterone or testosterone treatment.

5α-DHT reduced IL-6 production in both sexes. In contrast, Gulshan et al.[23] demonstrated that 5α-DHT enhanced LPS-induced IL-6 in rat macrophages and the J111 cell line in biological assays using IL-6-sensitive G8 cells. It is well known that even small changes in the levels of physiologically active compounds can have enormous effects on the overall system and therefore changes under the influence of the sex steroids could be highly significant. This is not altogether surprising as different cell types may react differently to a given stimulus and the pleiotropic nature of IL-6 could mean that its regulation varies in different tissues. The work of Gulshan et al.[23] and Pesseri et al.[24] provide support for the belief that the sex steroids regulate the secretion of IL-6 from cells producing it and in this respect is supportive of the finding reported here. IL-6 is a cytokine involved in the regulation of inflammatory and immunologic responses. Some investigators have shown that estradiol and testosterone have no direct effect on IL-6 production. However, other researchers have shown that these steroid hormones may modulate the IL-6 release induced by IL-1.[25] Studies of hormonal influence on chondrocyte IL-6 production have shown that testosterone and estradiol synergized with IL-1 in the induction of IL-6.[25] In our study, we found that the male hormone 5α-DHT inhibited LPS-induced IL-6 release by peritoneal macrophages. Regarding the inhibitory effect of 5α-DHT on LPS-induced IL-6, more work is required to understand whether male hormone has the ability to inhibit IL-1β or TNF-α induced IL-6 production by peritoneal macrophages. In support of this study, Girasole et al.[26] reported that testosterone inhibited IL-1 and/or TNF-induced production of IL-6 from osteoclasts. Further evidence for a role played by androgen in cytokine release by osteoclasts has been provided by Mizuno et al.[27] who have identified an androgen receptor in mouse osteoclast-like multinucleated cells. Establishing the presence of androgen receptors in osteoclasts indicates that 5α-DHT might exert its effect directly through this receptor. It is also supported by the finding of DHEA receptor in monocytes.[13] Recently,[28] Revel reported a higher peritoneal fluid (PF) and serum IL-6 in patients with ovarian hyperstimulation syndrome. There is also evidence indicating that the level of IL-6 production by bone marrow macrophages has not been changed by treatment with beta estradiol or medroxyprogesterone acetate.[29]

The effect of 5α-DHT on TNF-α release was inconclusive. Supporting our data, Ralston et al.[30] found no changes in TNF release with testosterone at concentrations up to 10^{-6}M. Moreover, this study used murine resident peritoneal macrophages, whereas Ralston and coworkers used LPS-stimulated PBMCs from patients with postmenopausal osteoporosis. This also highlights the difficulty in interpreting the levels of cytokines released from cultured murine cells in vitro as an index of what may occur in vitro or in vivo with human PBMCs. At present, the reasons for increased TNF levels in patients with post-menopausal osteoporosis are unclear; however, it might be related to the lack of estrogen or change in the ratio of estrogen/testosterone. Another possibility is the variable 5α-DHT effect on cytokine release in different individuals and the variations in the amounts of LPS used to stimulate cytokine production. Further studies on the effect of the male hormone 5α-DHT on TNF-α release in normal subjects and patients with inflammatory disorders such as RA would be of interest since the effect of 5α-DHT on IL-1 and IL-6 release in normal males and RA patients has already been studied.[16] It can be concluded that the immunological

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mechanisms underlying differences in disease progression between sexes and also determination of how sex hormones influence these pathways and finally how sex hormones modulate immune function may partly be due to the modulation of cytokine production by these hormones.

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