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Testosterone Acts Directly on CD4$^+$ T Lymphocytes to Increase IL-10 Production[1]

Stephanie M. Liva* and Rhonda R. Voskuhl[2†]

Males are less susceptible than females to experimental autoimmune encephalomyelitis and many other autoimmune diseases. Gender differences in cytokine production have been observed in splenocytes of experimental autoimmune encephalomyelitis mice stimulated with myelin proteins and may underlie gender differences in susceptibility. As these differences should not be limited to responses specific for myelin proteins, gender differences in cytokine production upon stimulation with Ab to CD3 were examined, and the mechanisms were delineated. Splenocytes from male mice stimulated with Ab to CD3 produced more IL-10 and IL-4 and less IL-12 than those from female mice. Furthermore, splenocytes from dihydrotestosterone (DHT)-treated female mice produced more IL-10 and less IL-12 than those from placebo-treated female mice, whereas there was no difference in IL-4. IL-12 knockout mice were then used to determine whether changes in IL-10 production were mediated directly by testosterone vs indirectly by changes in IL-12. The results of these experiments favored the first hypothesis, because DHT treatment of female IL-12 knockout mice increased IL-10 production. To begin to delineate the mechanism by which DHT may be acting, the cellular source of IL-10 was determined. At both the RNA and protein levels, IL-10 was produced primarily by CD4$^+$ T lymphocytes. CD4$^+$ T lymphocytes were then shown to express the androgen receptor, raising the possibility that testosterone acts directly on CD4$^+$ T lymphocytes to increase IL-10 production. In vitro experiments demonstrated increased IL-10 production following treatment of CD4$^+$ T lymphocytes with DHT. Thus, testosterone can act directly via androgen receptors on CD4$^+$ T lymphocytes to increase IL-10 gene expression. ***The Journal of Immunology,*** **2001, 167: 2060–2067.**

In general, females have a more responsive immune system than do males. Females have a greater humoral response, as evidenced by higher serum Ig concentrations than males (1) and a greater Ab response to various Ags after immunization (2–4). In addition, females reject skin allografts faster and have a reduced incidence of tumors, indicating that they also have a greater cellular immune response (5, 6). This difference in immune response is thought to be responsible for the greater susceptibility of females to autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus. This gender difference has also been observed in animal models of autoimmune disease such as experimental autoimmune encephalomyelitis (EAE)[3] in SJL mice, thyroiditis, adjuvant arthritis, Sjogren's syndrome in MRL/Mp-*lpr/lpr* mice, diabetes in nonobese diabetic mice, and spontaneous systemic lupus erythematosus in NZB×NZW mice (7–14).

A protective effect of testosterone is thought to underlie why males are less susceptible to autoimmune disease than females. This is based on studies that include removing testosterone from male mice via castration as well as by treatment of female mice with testosterone. For example, castration of male nonobese diabetic mice resulted in an increased prevalence of diabetes (15), and castration of male SJL mice increased EAE severity (16). Conversely, female nonobese diabetic or SJL mice implanted with testosterone pellets had a lower incidence of diabetes and less severe EAE, respectively, compared with those implanted with placebo pellets (17, 18). Also, castration of males increased the incidence and severity of thyroiditis and adjuvant arthritis, whereas testosterone treatment was protective (8, 9). Furthermore, testosterone treatment of female MRL/Mp-*lpr/lpr* mice with Sjogren's syndrome reduced lymphocyte infiltration into lacrimal tissue (10). Finally, in systemic lupus erythematosus in NZB/NZW mice, testosterone treatment improved survival (13).

Previous studies have indicated that gender differences in susceptibility may be due to gender differences in cytokine production upon autoantigen-specific stimulation. In males, compared with females, greater Th2 and less Th1 cytokine production has been observed (11, 18, 19). The balance between cytokines produced by Th1 and Th2 lymphocytes is considered central to the development of autoimmune disease. Th1 lymphocytes produce IFN-γ, IL-2, lymphotoxin-α, and TNF-α. Th2 lymphocytes secrete IL-4, IL-5, IL-6, IL-10, and IL-13. These two cell types are mutually inhibitory, and their development occurs under very specific conditions. If a naive T lymphocyte is initially stimulated with Ag in the presence of IL-12, the immune response is skewed toward Th1. However, if a naive T lymphocyte is initially stimulated with Ag in the presence of IL-4, the immune response is skewed toward Th2. In this manuscript we address the mechanisms responsible for gender differences in the Th1/Th2 balance.

Materials and Methods

Mice

Female and male SJL mice, aged 4 wk, were obtained from The Jackson Laboratory (Bar Harbor, ME). Female C57BL/6 and IL-10-, IFN-γ-, and

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DHT, 5α-dihydrotestosterone; AR, androgen receptor; MBP, myelin basic protein.

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IL-12b-deficient mice on the C57BL/6 background, aged 4 wk, were also obtained from The Jackson Laboratory.

Hormone pellets

Ninety-day release pellets of 5α-dihydrotestosterone (DHT) at doses of 5 and 15 mg, as well as placebo pellets that contain the carrier binder (cholesterol-methyl cellulose-α-lactulose) were purchased from Innovative Research of America (Sarasota, FL). Pellets were implanted s.c. in the scapular area of the neck of gonadally intact female mice at 4 wk of age using a trochar after methoxyflurane inhalation anesthesia. Mice were implanted with pellets for 2 wk before splenocyte isolation.

Serum testosterone levels

Two weeks after implantation, blood was obtained by intracardiac puncture from placebo- or DHT-treated mice. Serum testosterone levels of individual mice were determined by ELISA in duplicate according to the manufacturer's directions (Oxford Biomedical Research, Oxford, MI).

Cytokine profiles

Splenocytes were cultured in 24-well plates (Costar 3524; Corning Glass, Corning, NY) at 5×10^6 cells/ml in 2 ml RPMI 1640, without phenol red (BioWhittaker, Walkersville, MD), supplemented with Nutridoma-NS (Roche, Indianapolis, IN), HEPES, L-glutamine, nonessential amino acids, sodium pyruvate, and antibiotics (all obtained from BioWhittaker). Cells were stimulated with Ab to CD3 (anti-CD3, 0.2 or 1 μg/ml) either alone or in conjunction with Ab to CD28 (anti-CD28) (2.5 μg/ml; anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51), both obtained from BD PharMingen, San Diego, CA) or with medium alone. Culture supernatants were harvested at 24 and 48 h, and ELISAs were performed for various cytokines. IFN-γ, IL-4, IL-10, and IL-12 p40 were quantified using a sandwich ELISA technique. The Ab pairs were DB-1 and a rabbit anti-mouse polyclonal (IFN-γ; BioSource International, Camarillo, CA), 11B11 and BVD6-24G2 (IL-4, BD PharMingen), JES5-2A5 and JES5-16E3 (IL-10; BD PharMingen), and C17.8 and C15.6 (IL-12 p40; BD PharMingen). The sensitivity ranges for the ELISA were 8–500 pg/ml for IFN-γ, 15–2000 pg/ml for IL-4, 15–2000 pg/ml for IL-10, and 30–4000 pg/ml for IL-12.

Enrichment for splenocyte cell subpopulations

Splenocytes were isolated from female SJL mice and stimulated with 1 μg/ml anti-CD3. After 24 h in culture, nonadherent cells were harvested to obtain T lymphocytes and B lymphocytes for further purification into subpopulations. Adherent cells were used as the macrophage-enriched subpopulation and were 70% MAC-1 positive cells as determined by flow cytometry. Nonadherent cells were purified further into $CD4^+$ T lymphocyte and $CD8^+$ T lymphocyte and B lymphocyte subpopulations by MACS as described previously (20, 21). Briefly, cells were resuspended in MACS buffer (PBS containing 2 mM EDTA, 0.5% BSA, and 0.01% sodium azide) and separated into four groups. The first group of cells was not manipulated further and constituted the unseparated cell population. It consisted of 31–47% B lymphocytes, 31–39% $CD4^+$ T lymphocytes, and 6–19% $CD8^+$ T lymphocytes. The second, third, and fourth groups of cells were incubated for 15 min at 4°C with magnetic bead-conjugated Abs specific for B220 to label B lymphocytes, CD4 to label $CD4^+$ T lymphocytes, or CD8 to label $CD8^+$ T lymphocytes (Miltenyi Biotec, Auburn, CA). After two washes in MACS buffer, cells were added to a positive selection column (VS+, Miltenyi Biotec). The column was washed four times with buffer. Cells retained in the column were then eluted, and flow cytometric analysis showed that cells incubated with B220 Ab were 94–96% B lymphocytes, cells incubated with CD4 Ab were 96–99% $CD4^+$ T lymphocytes, and cells incubated with CD8 Ab were 93–99% $CD8^+$ T lymphocytes.

Cell depletions

Spleens were isolated from female SJL mice that had been implanted with placebo or DHT pellets 2 wk before the experiment and dissociated into a single-cell suspension in MACS buffer. These cells were incubated for 15 min at 4°C with magnetic bead-conjugated Abs specific for CD4. After two washes in MACS buffer, cells were added to a depletion column (BS; Miltenyi Biotec). The cells that flowed through contained only 1–4% $CD4^+$ T lymphocytes as assessed by flow cytometry and were used as the $CD4^+$ T lymphocyte-depleted fraction. The cells that were retained in the columns were further purified on a positive selection column (RS+, Miltenyi Biotec) and were 90–94% $CD4^+$ T lymphocytes. These cells were used as the $CD4^+$ T lymphocyte fraction.

RT-PCR for IL-10 and androgen receptor (AR) within each enriched cell subpopulation

Total RNA from each subpopulation that had been enriched for a certain cell type or depleted of a certain cell type was prepared by guanidinium isothiocyanate extraction followed by phenol-chloroform extraction. One microgram of total RNA was reverse transcribed using the GeneAmp RNA PCR kit from PerkinElmer (Branchburg, NJ). cDNA was amplified for 30 cycles for IL-10 and actin or for 35 cycles for AR, using primers for murine IL-10 (CLONTECH, Palo Alto, CA), murine AR (sequence obtained from Ref. 19, primers synthesized by AnaGen Technologies, Palo Alto, CA), or for murine actin CLONTECH). Southern blots of the PCR products were hybridized using a ^{32}P-labeled murine IL-10 internal probe (CLONTECH) or a ^{32}P-labeled murine AR internal probe (5′-TAC CAG CTC ACC AAG CTC CT-3′, synthesized by AnaGen Technologies). The radiolabeled Southern blot was exposed to a phosphor storage screen (Molecular Dynamics, Sunnyvale, CA) and read using the PhosphorImager (Molecular Dynamics) as previously described (22).

In vitro treatments with DHT

In the primary stimulation, splenocytes from male SJL mice were placed in culture in the presence of 10^{-9} M DHT or the hormone diluent alone, and the cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (2.5 μg/ml) for 72 h. At that time, cells were harvested, pelleted, and replaced in culture in the presence of 25 U/ml IL-2. After 5 days cells were again harvested. A majority of these cells were pelleted and replaced in culture, either alone or in the presence of irradiated splenocytes. The remaining cells were analyzed by flow cytometry to determine the percentage of viable cells (10%), and the percentage of viable cells that were $CD4^+$ T lymphocytes (80%). During this secondary stimulation, the medium contained either 10^{-9} M DHT or hormone diluent alone as a control, and the cells were again stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (2.5 μg/ml). Culture supernatants were harvested at 24 h, and ELISAs were performed for IL-10 and IFN-γ.

Statistical analysis

Mean cytokine levels and serum testosterone levels were calculated from duplicate measurements and compared using paired *t* tests, one-way ANOVA, repeated measures ANOVA, and 2×2 factorial ANOVA.

Results

Splenocytes from males stimulated with anti-CD3 secrete more IL-4 and IL-10, and less IL-12

Our laboratory has previously demonstrated that male SJL mice develop less severe adoptive EAE (12), and that splenocytes from male mice in the effector phase of the disease secrete more IL-10 when stimulated with myelin basic protein (MBP) than splenocytes from female mice (18). Our initial experiments were designed to determine whether this difference in IL-10 secretion was specific to T lymphocytes stimulated with MBP or was a more generalized phenomenon. Naive splenocytes from healthy male and female mice were stimulated with anti-CD3, and cytokine secretion was measured by ELISA. Fig. 1 shows concentrations of IL-4, IL-10, IL-12, and IFN-γ in supernatants from such cultures of three male-female pairs. The Th2 cytokine IL-4 was secreted at close to 2-fold higher levels by splenocytes from male mice, which was a statistically significant difference ($p = 0.04$). The Th2-associated cytokine IL-10 was also secreted at close to 2-fold higher levels by splenocytes from male mice, also reaching statistical significance ($p = 0.02$). In contrast, the Th1-inducing cytokine IL-12 was secreted at significantly lower levels by anti-CD3-stimulated splenocytes isolated from males compared with females ($p = 0.04$). Secretion of the Th1 cytokine IFN-γ was not significantly different. Secretion of IL-5 and IL-6 was also not significantly different (data not shown).

Splenocytes from female mice treated with testosterone secrete more IL-10 and less IL-12.

The possibility that testosterone was the factor responsible for the difference in cytokine secretion between splenocytes from each

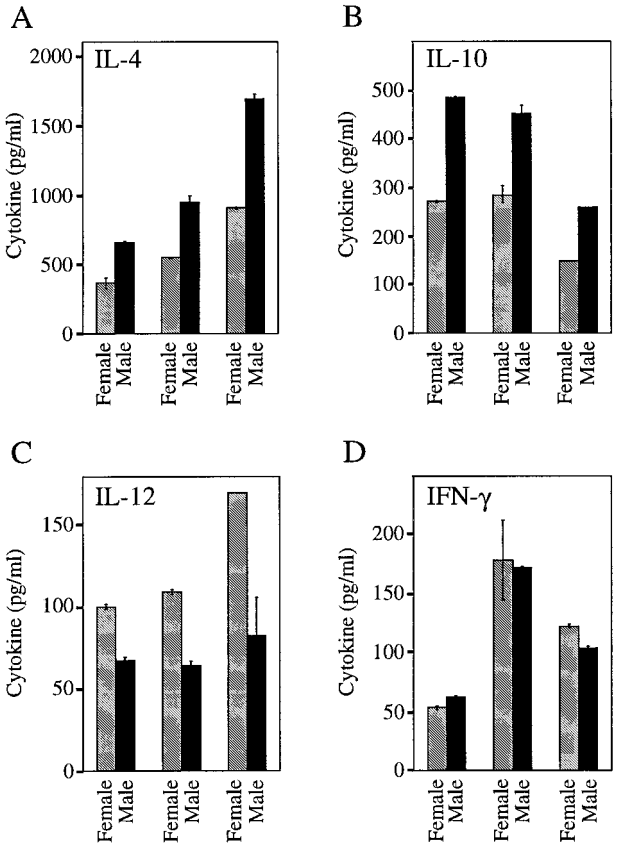

FIGURE 1. Splenocytes from male mice stimulated with anti-CD3 secrete more IL-4 and IL-10 and less IL-12 than splenocytes from female mice. Splenocytes from male and female SJL mice were placed in culture and stimulated with anti-CD3 (1 μg/ml). Culture supernatants were harvested at 24 and 48 h, and ELISAs were performed for IL-4 (*A*), IL-10 (*B*), IL-12 (*C*), and IFN-γ (*D*). Supernatants were tested for cytokine concentrations after 24 h of culture (IFN-γ) or 48 h of culture (IL-4, IL-10, IL-12). Cytokine values are presented as the mean concentration in picograms per milliliter from duplicate wells, with error bars indicating the SD. The differences in IL-4, IL-10, and IL-12 production were significantly different between three male mice and three female mice as assessed by paired *t* test. One-sided *p* values were $p = 0.04$, $p = 0.02$, and $p = 0.04$, respectively.

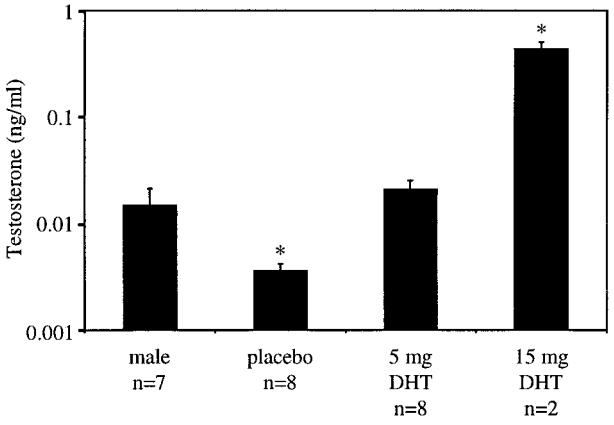

FIGURE 2. Treatment of female mice with 5 mg DHT pellets induces physiologic serum testosterone levels compared with those in males. Female SJL mice were implanted with either placebo or DHT pellets, and hormone levels were allowed to equilibrate for 2 wk before serum was collected. Serum was also collected from age-matched male SJL mice. Testosterone levels were measured by ELISA. Data are presented as the mean serum testosterone concentration ± SD for the number of mice indicated in each group. The data were statistically analyzed using one-way ANOVA. Mean serum testosterone concentrations were significantly different between male mice and placebo-treated female mice ($p = 0.0003$) and between male mice and female mice treated with 15 mg DHT ($p < 0.0001$), whereas there was no difference between levels in female mice treated with 5 mg DHT and male mice.

gender was tested by implanting normal female mice with 90-day release DHT pellets 2 wk before an experiment. DHT was chosen instead of testosterone because testosterone can be converted to estrogen, whereas DHT cannot. Therefore, the effect of DHT is purely androgenic. In initial experiments mice were treated with either of two doses of DHT (5- or 15-mg pellets) to determine which would increase the serum testosterone level in female mice to that in male mice. As Fig. 2 shows, implanting female mice with either the 5- or the 15-mg pellet caused an increase in the serum testosterone level compared with that in female mice implanted with a placebo pellet. Most importantly, there was no significant difference between the serum testosterone level in female mice treated with 5 mg DHT and that in untreated male mice, whereas the serum testosterone level in the female mice treated with 15 mg DHT was supraphysiologic. Because of these findings, the physiologic DHT dose of 5 mg was used in subsequent experiments.

To determine whether treatment of female mice with physiologic doses of DHT had an effect on cytokine secretion, cytokine concentrations were measured on supernatants from cultures of splenocytes isolated from female mice treated with 5-mg DHT pellets or placebo DHT pellets and stimulated with anti-CD3. As Fig. 3*B* indicates, splenocytes from female mice treated with DHT secreted significantly more IL-10 than splenocytes from placebo-treated female mice ($p < 0.0001$). Splenocytes from female mice treated with DHT also secreted significantly less IL-12 ($p = 0.03$; Fig. 3*C*). Therefore, treatment of female mice with DHT recapitulated the increase in IL-10 and the decrease in IL-12 secretion seen in Fig. 1 in male mice compared with female mice. These data indicate that differences in the levels of testosterone in male mice vs female mice are probably responsible for the difference in IL-10 and IL-12 secretion between genders. In contrast, treatment of female mice with DHT did not recapitulate the difference in IL-4 secretion seen between genders, indicating that increased testosterone levels in males were not responsible for the increased IL-4 secretion (Fig. 3*A*). As in Fig. 1*D*, treatment of female mice with DHT did not alter IFN-γ production (Fig. 3*D*).

Testosterone increases IL-10 secretion independently of changes in IL-12 secretion

To address the mechanism by which testosterone altered cytokine production, the first step was to determine how DHT caused both an increase in IL-10 and a decrease in IL-12 secretion. It was possible that DHT was acting independently to increase IL-10 secretion and decrease IL-12 secretion. However, because IL-10 and IL-12 can regulate each other (23, 24), it was also possible that DHT was acting to increase IL-10 secretion, with increased IL-10 levels causing a decrease in IL-12 secretion. Conversely, DHT may have initially decreased IL-12 secretion, with decreased IL-12 levels then causing an increase in IL-10 secretion.

To investigate whether DHT was altering IL-10 secretion independently of changes in IL-12 secretion, female IL-12 knockout mice (IL-12$^{-/-}$) and female C57BL/6 wild-type mice were implanted with DHT or placebo pellets. After 2 wk, their splenocytes were isolated and stimulated in culture with anti-CD3. Splenocytes from IL-12$^{-/-}$ female mice treated with DHT secreted significantly more IL-10 than splenocytes from placebo-treated IL-12$^{-/-}$ female mice ($p = 0.03$; Fig. 4*A*). This indicated that DHT increased IL-10

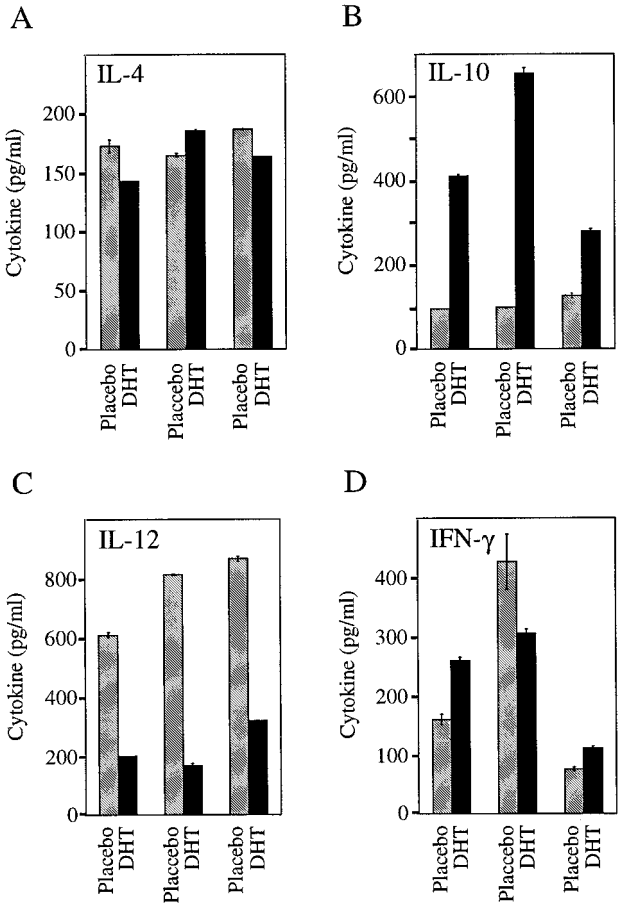

FIGURE 3. Splenocytes from female mice treated with physiologic levels of testosterone secrete more IL-10 and less IL-12 than splenocytes from placebo-treated female mice. Splenocytes from DHT- or placebo-treated female SJL mice were placed in culture and stimulated with anti-CD3 (1 μg/ml). Cytokine levels were determined by ELISA of supernatants after 24 h of culture (IFN-γ) or 48 h of culture (IL-4, IL-10, IL-12). Cytokine values are presented as the mean concentration in picograms per milliliter from duplicate wells, with error bars indicating the SD. The cytokine values shown for three placebo-treated and three DHT-treated female mice are representative of eight placebo-treated mice and eight DHT-treated female mice from two experiments. The differences in IL-10 and IL-12 production between placebo- and DHT-treated female mice were significantly different as assessed by a 2 $\times$ 2 factorial ANOVA ($p < 0.0001$, $n = 8$ and $p = 0.03$, $n = 8$, respectively).

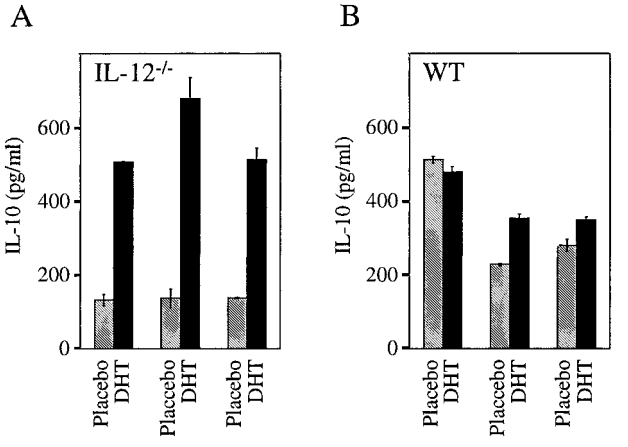

FIGURE 4. Testosterone increases IL-10 secretion independently of changes in IL-12 secretion. IL-10 production is increased in splenocytes isolated from DHT-treated IL-12$^{-/-}$ female mice compared with placebo-treated IL-12$^{-/-}$ female mice (*A*), but is not different in DHT-treated C57BL/6 wild-type female mice and placebo-treated C57BL/6 wild-type female mice (*B*). Splenocytes from DHT- or placebo-treated mice were placed in culture and stimulated with anti-CD3 (1 μg/ml) for 48 h. Levels of IL-10 were determined by ELISA and are presented as the mean concentration in picograms per milliliter from duplicate wells, with error bars indicating the SD. The experiments shown are representative of experiments repeated twice. The differences in IL-10 production between placebo- and DHT-treated IL-12$^{-/-}$ mice were significantly different as assessed by a 2 $\times$ 2 factorial ANOVA ($p = 0.03$, $n = 5$).

secretion in a manner that was not dependent upon differences in IL-12.

Interestingly, DHT treatment did not cause an increase in IL-10 production in splenocytes derived from wild-type C57BL/6 mice compared with placebo treatment (Fig. 4*B*). In addition, there was no gender difference in IL-10 and IL-12 production upon stimulation with anti-CD3 of C57BL/6 female mice vs male mice (data not shown). There was also no difference in IL-12 production upon stimulation of splenocytes from DHT vs placebo-treated IL-10$^{-/-}$ mice and no difference in IL-10 and IL-12 production upon stimulation of splenocytes from DHT vs placebo-treated IFN-$\gamma^{-/-}$ C57BL/6 mice (data not shown). However, conclusions concerning the role of IL-10 and IFN-γ in DHT-induced differences in IL-12 production cannot be drawn from these data because the absence of a difference in cytokine production between DHT and placebo treatment in the IL-10$^{-/-}$ and IFN-$\gamma^{-/-}$ mice may merely reflect the absence of a difference in cytokine production inherent to the C57BL/6 background.

In contrast, although the absence of differences in cytokine production upon DHT treatment of the IL-10$^{-/-}$ and IFN-$\gamma^{-/-}$ mice is not informative, the presence of cytokine differences upon DHT treatment of IL-12$^{-/-}$ mice is informative. Indeed, the observation that IL-10 production is increased upon DHT treatment of IL-12$^{-/-}$ mice confirms that the increase in IL-10 during DHT treatment is not dependent upon DHT-induced differences in IL-12 production. Furthermore, these data provide new insights. Removing IL-12, as occurs in the IL-12 knockout, unmasked the ability of DHT to increase IL-10 in the C57BL/6 strain. In contrast, removing IFN-γ, as occurs in the IFN-γ knockout, did not unmask this ability of DHT to increase IL-10 in this strain. This suggests that IL-12 regulation of IL-10 may override the ability of testosterone to increase IL-10 in some strains (C57BL/6), but not in others (SJL). Further studies in our laboratory will pursue the role of IL-12 in suppressing the effect of testosterone in some strains but not others.

IL-10 is expressed by CD4$^+$ T lymphocytes

Our data indicated that DHT was affecting IL-10 secretion by splenocytes, independently of changes in IL-12 secretion. T lymphocytes, B lymphocytes, and macrophages all have the potential to produce IL-10. Thus, in further delineating the mechanism of action of testosterone, it became important to determine which cell type within splenocyte cultures was expressing IL-10. Following 24 h of stimulation with anti-CD3, splenocytes from female placebo- and DHT-treated mice were enriched, using MACS, into populations of CD4$^+$ T lymphocytes, CD8$^+$ T lymphocytes, and B lymphocytes. Adherent cells were used as a macrophage-enriched fraction. Each enriched cell subpopulation was then assessed for IL-10 production by RT-PCR. As demonstrated in Fig. 5, RT-PCR analysis for IL-10 within each enriched cell population demonstrated the greatest IL-10 signal in the CD4$^+$ T lymphocyte population. Furthermore, when the splenocyte population was depleted of CD4$^+$ T lymphocytes, the majority of the IL-10 signal

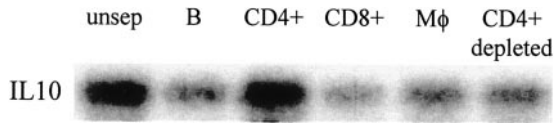

FIGURE 5. IL-10 is expressed by $CD4^+$ T lymphocytes. Splenocytes from gonadally intact female mice were stimulated in culture with anti-CD3 for 24 h, then harvested and used unseparated (unsep) or enriched for B lymphocytes (B), $CD4^+$ T lymphocytes ($CD4^+$), $CD8^+$ T lymphocytes ($CD8^+$), or depleted of $CD4^+$ T lymphocytes ($CD4^+$ depleted). Adherent cells were used as a macrophage-enriched population (Mϕ). RNA was isolated from each cell subpopulation, and 1 μg RNA was reverse transcribed with primer sets specific for IL-10. Data shown are from a Southern blot of the RT-PCR products hybridized with an IL-10 internal probe and are representative of experiments repeated twice.

was eliminated. The low level signal in the $CD8^+$ T lymphocyte, B lymphocyte, and macrophage fractions could be due to either low level expression of IL-10 by these cell populations or low level contamination of these fractions with $CD4^+$ T cells. Identical results were obtained from DHT-treated mice (data not shown). Together these data strongly suggest that IL-10 is predominantly expressed by $CD4^+$ T cells.

IL-10 is secreted by $CD4^+$ T lymphocytes

To determine whether IL-10 was also predominantly expressed by $CD4^+$ T lymphocytes at the protein level, cell populations obtained from either placebo-treated female mice or DHT-treated female mice were enriched for $CD4^+$ T lymphocytes or were depleted of $CD4^+$ T lymphocytes using MACS. The four populations of cells were placed in culture and stimulated for 48 h with anti-CD3 and anti-CD28. ELISAs were then performed to determine the IL-10 levels in the culture supernatants. Significantly more IL-10 was secreted from cultures of cells enriched for $CD4^+$ T lymphocytes than from cultures of cells depleted of $CD4^+$ T lymphocytes (Fig. 6). This was true for both cells obtained from mice treated with placebo pellets as well as cells obtained from mice treated with DHT pellets. Consistent with previous results, cultures of cells enriched for $CD4^+$ T lymphocytes produced significantly more IL-10 if they were isolated from mice treated with DHT pellets as opposed to mice treated with placebo pellets. Conversely, the lack of a difference in IL-10 secretion between the $CD4^+$ T lymphocyte-depleted population treated with DHT vs placebo indicated a lack of a DHT treatment effect on other cell types within the spleen. Together these results further support the conclusion that it is $CD4^+$ T lymphocytes that are producing IL-10, and that it is the $CD4^+$ T lymphocytes that are reacting either directly or indirectly to a change in testosterone levels in the mice.

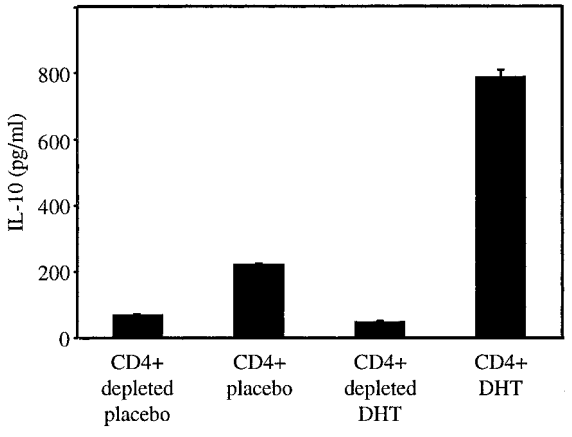

FIGURE 6. IL-10 is secreted by $CD4^+$ T lymphocytes. Splenocytes from female mice implanted with either placebo or DHT pellets were harvested and enriched for or depleted of $CD4^+$ T lymphocytes. Four cell fractions were obtained: $CD4^+$ T lymphocyte-depleted fractions from mice treated with placebo pellets ($CD4^+$ depleted placebo), $CD4^+$ T lymphocyte-enriched fractions from mice treated with placebo pellets ($CD4^+$ placebo), $CD4^+$ T lymphocyte-depleted fractions from mice treated with DHT pellets ($CD4^+$ depleted DHT), and $CD4^+$ T lymphocyte-enriched fractions from mice treated with DHT pellets ($CD4^+$ DHT). These four fractions were individually placed in culture and stimulated with anti-CD3 (0.2 or 1 μg/ml) and anti-CD28 (2.5 μg/ml). Levels of IL-10 were determined by ELISA and are presented as the mean concentration in picograms per milliliter from duplicate wells, with error bars indicating the SD. Data are from one representative experiment of four that showed similar results. Statistical significance was assessed by comparing mean IL-10 values in $CD4^+$ depleted vs $CD4^+$ and placebo vs DHT using repeated measures ANOVA where the repeated factor was the $CD4^+$ depleted vs $CD4^+$. The differences between $CD4^+$ depleted placebo and $CD4^+$ placebo were significant ($p = 0.0001$, $n = 4$). The differences between $CD4^+$ depleted DHT and $CD4^+$ DHT were significant ($p < 0.0001$, $n = 4$), as were the differences between $CD4^+$ placebo and $CD4^+$ DHT ($p = 0.04$, $n = 4$).

AR is expressed on $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and macrophages

For testosterone to have a direct effect on $CD4^+$ T lymphocytes, these cells would need to express the receptor for testosterone, the AR. Therefore, AR expression was analyzed by RT-PCR on RNA obtained from enriched splenocyte populations. The AR was expressed by enriched populations of $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and macrophages (Fig. 7), consistent with previous reports (19, 25). Enriched populations of B lymphocytes expressed only low levels of AR. Because it is likely that AR expression at the mRNA level indicates AR expression at the protein level, these data give further support to the hypothesis that testosterone could be acting directly on $CD4^+$ T lymphocytes via the AR to increase IL-10 expression.

IL-10 secretion by $CD4^+$ T lymphocytes is increased by in vitro treatment with testosterone

To fully test the hypothesis that testosterone is acting directly on $CD4^+$ T lymphocytes, we treated naive $CD4^+$ T lymphocytes in vitro with DHT. Splenocytes were stimulated in the presence of a physiologic dose of DHT in both a primary and a secondary stimulation. Flow cytometry verified that cells used in the secondary

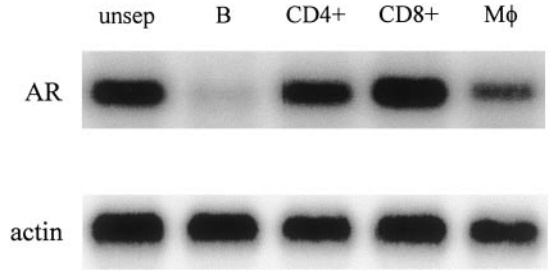

FIGURE 7. The AR is expressed on $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and macrophages. Splenocytes were used unseparated (unsep) or enriched for B lymphocytes (B), $CD4^+$ T lymphocytes ($CD4^+$), or $CD8^+$ T lymphocytes ($CD8^+$). Adherent cells were used as a macrophage-enriched population (Mϕ). RNA was isolated from each cell subpopulation, and 1 μg RNA was reverse transcribed with primer sets specific for the AR or a housekeeping gene (actin). Data shown are Southern blots of the RT-PCR products hybridized with AR or actin internal probes. The relative ratios of AR to actin in each cell population were: unsep, 1.87; B, 0.35, $CD4^+$, 2.00; $CD8^+$, 5.16; Mϕ, 0.99. Data are representative of experiments repeated twice.

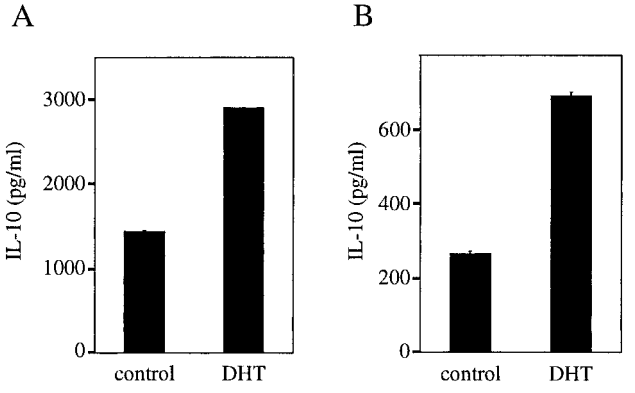

FIGURE 8. IL-10 secretion by $CD4^+$ T lymphocytes is increased by in vitro treatment with testosterone. Splenocytes were stimulated for 72 h with anti-CD3 (1 μg/ml) and anti-CD28 (2.5 μg/ml) in the presence of 10^{-9} M DHT or hormone diluent alone. Cells from male mice were then replated and cultured with IL-2 for 5 days. Cells were again replated either in the presence of irradiated splenocytes (*A*) or alone (*B*) and restimulated for 24 h with anti-CD3 (1 μg/ml) and anti-CD28 (2.5 μg/ml) in the presence of 10^{-9} M DHT or hormone diluent alone (Con). Levels of IL-10 were determined by ELISA and are presented as the mean concentration in picograms per milliliter from duplicate wells, with error bars indicating the SD. The differences in IL-10 production were significantly different between control and DHT-treated cells as assessed by paired *t* test ($p < 0.05$). Results are from one of two independent experiments that gave similar results.

stimulation were comprised predominantly of $CD4^+$ T lymphocytes. As shown in Fig. 8*A*, addition of DHT to these cultures of $CD4^+$ T lymphocytes caused a significant increase in IL-10 secretion. IFN-γ secretion was not significantly changed (data not shown). This first set of data was obtained from cells cultured on irradiated splenocytes, raising the question as to which cells were responding to DHT with an increase in IL-10: the $CD4^+$ T lymphocytes or the irradiated splenocytes. Therefore, these experiments were repeated in the absence of irradiated splenocytes (Fig. 8*B*). Again, cultured $CD4^+$ T lymphocytes, in the absence of irradiated splenocytes, produced significantly more IL-10 when DHT was added in vitro. Therefore, it is clear that DHT can act directly on $CD4^+$ T lymphocytes to cause an increase in IL-10 secretion.

Discussion

Gender differences have been observed in a number of models of autoimmune disease. In trying to determine the cause of gender differences in autoimmune disease, studies have demonstrated gender differences in cytokine expression. For example, in EAE, studies have collectively shown that cells from males produce more IL-4 and IL-10 and less IFN-γ and IL-12, supporting the conclusion that the male immune system is shifted toward Th2 immunity (7, 18, 19, 26). Mechanisms underlying why there is a gender difference in cytokine production remain unknown. Many possibilities exist. They include differences in levels of male sex hormones such as testosterone, differences in female sex hormones such as estrogen and progesterone, and differences in genes located on sex chromosomes.

To delineate such mechanisms, we believed it was necessary to use a simple system. Indeed, although cytokine studies of gender differences in EAE collectively showed increased Th2 cytokines and decreased Th1 cytokines in male cells compared with female cells, studies differed slightly with regard to which cytokines were altered. This was most likely due to differences in experimental methods: the use of peptide vs whole molecule as stimulatory autoantigens, the use of proteolipid protein vs MBP, active vs adoptive EAE, and the presence or absence of adjuvant, which induces artificial Th1 conditions. Comparing cytokine production between splenocytes derived from normal male and female mice after stimulation with anti-CD3 eliminated all these complicating factors. Thus, we first wanted to determine whether there were gender differences in cytokine production in this simpler system. Similar to studies in EAE, we found that levels of the Th2 cytokines IL-4 and IL-10 were higher and the IL-12 level was lower in splenocytes from males compared with females. However, in this study there was no difference in IFN-γ production.

Having established that there were gender differences in our system, we next wanted to determine which cytokine differences were due to testosterone. Splenocytes from female mice implanted with testosterone pellets, like splenocytes from male mice, secreted more IL-10 and less IL-12. However, treatment with testosterone did not cause increased IL-4 production. This clearly indicates that testosterone does not recapitulate all the cytokine differences seen in male vs female mice, and that the increase in IL-4 must be due to gender differences in other sex hormones and/or genes found on sex chromosomes.

Regarding the relevance of these cytokine changes to EAE pathogenesis, IL-12 can mediate EAE through an IFN-γ-independent mechanism (24, 27, 28), whereas the role of IFN-γ in EAE is controversial (29–34). Thus, a decrease in IL-12 production associated with testosterone treatment may indeed be important in the decreased susceptibility of male mice to EAE. The finding of increased IL-10 production is equally as important as the finding of decreased IL-12 production upon testosterone treatment. Numerous studies have shown that IL-10 is essential in the regulation of EAE. Specifically, treatment of EAE with IL-10 has been shown to ameliorate disease (35–37), whereas administration of Abs to IL-10 has exacerbated disease (35, 38). Although treatment of EAE with IL-4 also ameliorated disease (39, 40), studies of IL-4- and IL-10-deficient mice and IL-4 and IL-10 transgenic mice have shown that IL-10 may play a more critical role in protection from EAE. Indeed, IL-$10^{-/-}$ mice developed more severe EAE compared with wild-type mice, and overexpression of IL-10 rendered mice resistant to EAE (41). In contrast, although some studies have shown increased susceptibility of IL-$4^{-/-}$ mice to EAE, others have not (41–43), and transgenic mice overexpressing IL-4 had disease similar to that in wild-type mice (41). Because IL-10 has been shown to play a protective role and IL-12 a disease-promoting role in EAE, and because testosterone increases IL-10 and decreases IL-12, testosterone would appear to play an important role in gender differences in susceptibility to this disease.

Having shown that testosterone increases IL-10 and decreases IL-12 production, it is important to determine the mechanism through which this occurs. There are three central questions that need to be answered: 1) is the decrease in IL-12 secretion upon treatment with testosterone responsible for the increase in IL-10 secretion? 2) what cell type within spleen has altered cytokine production? and 3) are the effects of testosterone on this cell direct or indirect? In answer to the first question, our data from IL-$12^{-/-}$ mice showed that the increase in IL-10 secretion is independent of changes in IL-12 secretion. Furthermore, it is possible that in the SJL strain, the decrease in IL-12 production was secondary to the increase in IL-10 production. This hypothesis is supported by a recent report in which treatment of male SJL mice with Abs to IL-10 resulted in increased IL-12 production (44). Taken together, these studies indicate that testosterone initially increases IL-10 production, which may then lead to a decrease in IL-12 production.

To address the second question of what cell type within spleen has altered cytokine production, we have presented evidence in

this manuscript at both the RNA and protein levels indicating that $CD4^+$ T lymphocytes are the predominant cell type within spleen that produce and secrete IL-10. Furthermore, it is the IL-10 produced by the $CD4^+$ T lymphocytes that is responsive to alterations in testosterone levels both in vivo and in vitro.

Finally, the third question, is the effect of testosterone direct or indirect? To this end, it is essential to determine which cells within the splenocyte population express the AR. RT-PCR analysis showed that $CD4^+$ T lymphocytes express the AR, supporting the possibility of direct action of testosterone on these cells. However, the AR is also expressed by $CD8^+$ T lymphocytes and macrophages. Thus, an indirect action of testosterone mediated through these cells was also possible. To definitely demonstrate that testosterone can act directly on $CD4^+$ T lymphocytes, we showed that in vitro stimulation of $CD4^+$ T lymphocytes in the presence of testosterone and in the absence of other cells resulted in increased IL-10 production. This is consistent with previous studies that have shown that treatment of naive T lymphocytes from Vβ8.2 TCR transgenic mice with testosterone in vitro makes more IL-10 upon stimulation with MBP Ac1–11 peptide (19). Taken together, these studies show that testosterone can act directly on $CD4^+$ T lymphocytes to increase IL-10 production.

In summary, although many cells within spleen express the AR, we have shown that testosterone can act directly upon $CD4^+$ T lymphocytes to increase IL-10 expression during stimulation with anti-CD3. The increase in IL-10 from $CD4^+$ T lymphocytes may then lead to reduced IL-12 production by macrophages. We have no evidence to suggest that testosterone can act directly on macrophages to cause a decrease in IL-12. However, the mechanism presented here does not rule out the possibility that testosterone may also affect $CD8^+$ T lymphocytes or macrophages. Indeed, a recent report using in vitro cultures of T lymphocytes and peritoneal exudate cells at a ratio of 9:1 has suggested that this can occur following stimulation with 10- to 50-fold higher concentrations of anti-CD3 (10 μg/ml) (44). Notably, however, we found that testosterone treatment had no effect on IL-10 production by $CD8^+$ T lymphocytes and macrophages cultured ex vivo (Fig. 6). Furthermore, the increase in IL-10 during in vitro treatment of $CD4^+$ T lymphocytes with testosterone occurred in the absence of these other cells and thus was not dependent upon interactions with them.

The differences in cytokine production between males and females (increased IL-4 and IL-10, decreased IL-12) could be hypothesized to be due to a testosterone-induced shift in the immune system toward Th2 immunity. Furthermore, it could be hypothesized that to cause a general shift, testosterone may be affecting one or more of the key molecules involved in establishing cell lineage, such as GATA-3 or STAT6. However, this is not the case. Although the expression of many cytokines is different between genders, testosterone initially affects only IL-10. Thus, testosterone is not acting on molecules involved in establishing cell lineage; rather, it is acting by altering regulation of the IL-10 gene. In support of this idea, a search for an androgen response element in the promoter for the IL-10 gene revealed a putative response element in the human IL-10 gene promoter, ~300 bp upstream of the TATA box. However, this putative response element in not well conserved in the mouse IL-10 gene promoter, with only 8 of the 12 nucleotides being identical with the consensus sequence. Although other nonconserved sequences in various genes have been shown to be androgen responsive (45–47), it is difficult to speculate about whether the putative androgen response element in the IL-10 promoter is indeed androgen responsive until promoter analysis experiments are conducted. Nonetheless, the observations presented in this manuscript regarding the effect of testosterone on up-regulation of IL-10 in $CD4^+$ T lymphocytes are informative in and of themselves and are likely to be relevant to a variety of autoimmune disease models characterized by gender differences.

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