Effect of medical castration on CD4+CD25+ T cells, CD8+ T cell IFN-γ expression, and NK cells: a physiological role for testosterone and/or its metabolites

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antigens (protective immunity) (9, 34, 35). In humans, developmental defects in CD4⁺CD25⁺ regulatory T cells result in autoimmune disease in multiple endocrine organs, the immunodeficiency syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (20, 46). A recent study showed that estrogen can drive the expansion of suppressive CD4⁺CD25⁺FoxP3⁺ regulatory T cells (30); however, how androgens may affect regulatory T cells is not clear.

For protective host immunity, CD8⁺ T lymphocytes and NK cells are acknowledged as the major effectors (1, 13). CD8⁺ T cells constitute the major component of adaptive immunity and secrete proinflammatory cytokines such as IFN-γ when activated by foreign or tumor antigens (1), whereas NK cells play a key role in innate immunity against tumors and foreign antigens via homing to inflammatory sites and directly destroying tumor or infected cells through cell cytotoxicity or secretion of IFN-γ (13). Limited data in animal models suggest that sex steroids may contribute to the regulation of NK and T cell proliferation, including CD4⁺CD25⁺ regulatory T cells (26, 33); however, data in humans are lacking.

In this randomized, placebo-controlled study, we investigated the physiological role of T and its metabolites on immune function by assessing the effects of medical castration induced by a potent gonadotropin-releasing hormone (GnRH) antagonist, acyline (14, 15), with and without exogenous physiological T replacement, on various characteristics of circulating lymphocytes, including percentages of T cell subtypes, ex vivo cytotoxic T cell activation, and the population of NK cells and their functional receptors. Our findings may have implications for the understanding and treatment of autoimmune disease and the evaluation and modification of immune-based regimens for androgen-deprived men with prostate cancer.

MATERIALS AND METHODS

Acyline. Acyline, a 10-amin acid peptide that acts as a GnRH antagonist (14), was originally synthesized by Jean Rivier at the Sask Institute and is being distributed by the National Institute of Child Health and Human Development. Acyline lyophilized powder synthesized by NeoMDS (San Diego, CA) was suspended in bacteriostatic water to a final concentration of 2 mg/ml. In all cases, 300 µg/kg acyline was administered by subcutaneous injection in the abdomen. This dose has been previously demonstrated to render subjects medically castrate (T < 1.7 nmol/l) within 24 h, with maintenance of castrate T levels for 2 wk (15).

Subjects. All procedures involving human subjects were approved by the Institutional Review Board at the University of Washington and performed in accordance with the guidelines in The Declaration of Helsinki. Fourteen men (age 35–55 yr) were recruited by newspaper advertisement and posted flyers, and of these 13 met study criteria. He was not included in the analyses.

All subjects had normal baseline physical examinations, normal medical histories. All subjects were healthy men on no medications with normal medical chemistries, complete blood count and electrolytes and glucose (chemistry 7), calcium, and liver function tests were measured at the Department of Laboratory Medicine, University of Washington.

Phenotyping of PBLs. PBLs were thawed and stained with trypan blue to ensure that cell viability was >95% in all samples. To measure the subpopulation of T lymphocytes (CD3⁺) and characterize NK cells (CD3⁻CD56⁺), 0.5 × 10⁶ thawed PBLs from each subject at designated time points were incubated with a combination of fluorochrome-conjugated antibodies for 30 min on ice. After a washing, cells were analyzed using Becton-Dickinson FACScan cytometry. Data were analyzed using CellQuest software from BD Bioscience (San Jose, CA), because of the large variation in immune parameters among individual subjects, lymphocyte subpopulations during treatment were normalized to baseline (day 0) values that were set as “1” or “100%” for statistical analysis.

The following antibodies were used: anti-CD3-fluorescein isothiocyanate (FITC), anti-CD3-phycocerythrin (PE), anti-CD3-peridinin-chlorophyll-protein complex (PerCP), anti-CD4-FITC, anti-CD8-PerCP, anti-CD56-PerCP, anti-CD25-PerCP, anti-NK cell-activating receptor (NK2D)-PE, and anti-homing receptor (CXCR1)-PE. All antibodies specific to the CD antigens were from BD Biosciences. Anti-NKG2D-PE and anti-CXCR1-PE were from R&D systems (Minneapolis, MN).

T cell activation and intracellular cytokine staining. For analysis of CD8⁺ T cell activation, 1 × 10⁶ thawed PBLs were incubated in RPMI supplemented with 10% FCS. Phorbol myristate acetate (PMA, 25 ng/ml) and ionomycin (1 µg/ml) were added to the media in the presence of 0.1 mM brefeldin A (a Golgi inhibitor). After stimulation at 37°C for 4 h, cells were collected and incubated with anti-CD3-
FITC and anti-CD8-PerCp antibodies. After several washes, cells were permeabilized with Cytofix/Cytoperm solution (BD Bioscience), and intracellular IFN-γ was measured by subsequent incubating with anti-IFN-γ-PE antibody (BD Biosciences). Cells were analyzed using Becton-Dickinson FACScan cytometry. Due to the large variation among individual subjects, populations of IFN-γ+CD8+ T cells at baseline (day 0) were normalized as "1" or "100%" for statistical analysis.

Statistical analysis. Hormone measurements were log transformed before analyses. For differences between groups, data were compared by ANOVA with a Scheffé correction for multiple comparisons. For within-group comparisons relative to baseline, paired t-tests with a Bonferroni correction for multiple comparisons (effective α = 0.01) were performed on the log-transformed values. For all comparisons, a P value of <0.05 was considered statistically significant. Statistical analyses were performed using STATA (College Park, TX).

RESULTS

Baseline hormones and clinical response to treatment. The baseline characteristics of the study subjects are summarized in Table 1. There were no significant differences in the groups at baseline. Subjects in the acyline-only group tended to have higher baseline androgen levels than the other groups (P = 0.07 vs. placebo and P = 0.11 vs. acyline + T), which did not reach significance. All baseline androgen values were within the normal physiological range (12). There were no significant differences between groups in measures of immune composition at baseline. No serious adverse events or changes in serum chemistries were seen during the study. The subjects receiving acyline had mild erythema and pruritis at the injection site, which resolved in 24 h as has been reported previously (15). Two men in the acyline group noted hot flashes over the preceding 2 wk on day 28, which resolved over the 1st wk of recovery. No men reported symptoms of infection (fever, cough, diarrhea) during the study, and there were no increases in white blood cell number above the normal range for any of the subjects at any time point.

Effect of medical castration and T replacement on serum hormone concentrations. Serum T and DHT declined significantly in subjects who received acyline only compared with those who received placebo or acyline + T (P < 0.05; Fig. 1, A and B) throughout the drug exposure period (days 7–28). Serum T and DHT in the acyline groups declined significantly compared with baseline at all time points during drug exposure (P < 0.05), with the exception of DHT on day 7 (P = 0.07 after correction for multiple comparisons). In the acyline-only group, T concentrations during treatment were near or below castrate levels (T < 1.7 nmol/l) throughout the 28 days of treatment. At recovery, there was a significant increase in serum T in the group receiving acyline only compared with the other groups, but not compared with baseline. There were no significant differences in serum T between the placebo and the acyline + T groups at any time point nor within these groups.

Table 1. Baseline characteristics of study subjects

<table>
<thead>
<tr>
<th>Sample size, n</th>
<th>Placebo</th>
<th>Acyline</th>
<th>Acyline + T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>46.5 (7.0)</td>
<td>39.8 (5.2)</td>
<td>41.8 (3.2)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.6 (5.8)</td>
<td>24.1 (1.0)</td>
<td>28.1 (3.0)</td>
</tr>
<tr>
<td>T, nmol/l</td>
<td>12.2 (7.8)</td>
<td>23.7 (4.8)</td>
<td>12.6 (4.4)</td>
</tr>
<tr>
<td>DHT, nmol/l</td>
<td>2.0 (1.3)</td>
<td>3.7 (1.0)</td>
<td>3.5 (3.8)</td>
</tr>
<tr>
<td>Estradiol, pmol/l</td>
<td>118 (26)</td>
<td>148 (26)</td>
<td>128 (22)</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>4.05 (1.58)</td>
<td>2.61 (1.17)</td>
<td>2.31 (0.92)</td>
</tr>
<tr>
<td>CD4+/CD25+ (%CD4+ T cells)</td>
<td>8.94 (1.39)</td>
<td>9.52 (1.09)</td>
<td>7.45 (3.12)</td>
</tr>
<tr>
<td>NK cells (%PBL)</td>
<td>12.27 (6.47)</td>
<td>10.31 (1.69)</td>
<td>15.95 (5.98)</td>
</tr>
</tbody>
</table>

Data are expressed as means (SD). T, testosterone; DHT, dihydrotestosterone; PBL, peripheral blood lymphocytes; NK, natural killer.
compared with their baseline values. T treatment maintained physiological serum T levels throughout the treatment period in acyline + T-treated men, whereas their serum DHT was increased during treatment compared with baseline and with subjects receiving placebo at days 7 and 21.

Serum E2 declined significantly in the subjects receiving acyline only compared with baseline at all time points and compared with the acyline + T group throughout the drug exposure period (P < 0.05; Fig. 1C). Although E2 levels tended to be lower in subjects receiving acyline than in the placebo group, this difference reached statistical significance only on day 7. There were no significant differences in E2 between the placebo and acyline + T groups throughout the study period nor within these groups compared with baseline. Results for E2 are similar to results for T, which is to be expected because T is the hormone precursor (via aromatization in tissues) for E2.

**Decrease in CD4^+CD25^+ T cells with medical castration and prevention by T replacement.** We measured the percentage of circulating CD8^+ and CD4^+ T lymphocyte subpopulations during and after treatment and compared them with pretreatment (baseline) levels or subjects in any other treatment groups. No significant changes occurred in the total number of lymphocytes, the number of CD4^+ or CD8^+ T cell subpopulations (data not shown), and the CD4^+/CD8^+ ratio (Fig. 2A) in subjects in any treatment group. However, there was a significant decrease (~30%) in the percentage of CD4^+CD25^+ T cells in subjects receiving acyline treatment compared with baseline (P < 0.05; Fig. 2B) and subjects in the placebo or acyline + T groups. This decline normalized during recovery with the return of normal hormone levels. Similar changes were seen when gating specifically on the CD4^+CD25^bright cells (data not shown). No significant change in the percentage of CD4^+CD25^+ T cells occurred in subjects treated with placebo or T replacement, acyline + T (Fig. 2B).

**Impairment of CD8^+ T cell activation by medical castration and prevention by T replacement.** In response to antigen stimulation, CD8^+ T cells secrete IFN-γ and become cytotoxic. To evaluate whether medical castration affects the function of CD8^+ T cells, we activated PBLs from the study subjects ex vivo with mitogenic stimulus PMA and ionomycin and assessed CD8^+ T cell activation by measuring intracellular IFN-γ expression. In subjects who received acyline-only treatment, although no significant change was observed in the level of IFN-γ production per cell among IFN-γ-producing CD8^+ T cells (IFNγ^+CD8^+; Fig. 3A), the percentage of IFNγ^+CD8^+ T cells was decreased by ~75% in these subjects (P < 0.01; Fig. 3B) compared with baseline and the control groups. Four weeks after treatment was completed, the percentage of IFNγ^+CD8^+ T cells returned to pretreatment levels. In individuals who received placebo or T replacement (acycline + T), no significant change in the percentage of IFNγ^+CD8^+ T cells was detected during treatment or recovery.

**Increase in circulating NK cells with medical castration and prevention by T replacement.** To evaluate whether physiological T levels might impact NK cell immune surveillance, we assessed the number of circulating NK cells, and expression levels of the major NK cell homing receptor CXCR1 and the activating receptor NK2D, both of which are expressed by all human NK cells (13, 32). In subjects who received acyline-only treatment, the number of CD3^-CD56^- NK cells among total PBLs was significantly elevated, approximately twofold, during treatment compared with baseline (P < 0.05; Fig. 4A) and the control groups. After 4 wk of recovery, the number of circulating NK cells was restored to pretreatment levels. There were no significant changes in the number of CD3^-CD56^- NK cells in subjects treated with placebo or T replacement (acyline + T). There were no significant changes in the expression levels of CXCR1 or NK2D in subjects in any treatment group during the study period (Fig. 4, B and C).
DISCUSSION

Although the influence of hormones on cell-mediated immunity has been studied in various mouse models and human autoimmune diseases, this is the first placebo-controlled longitudinal study to investigate how sex steroids, specifically T and/or its metabolites E2 and DHT, may modulate cell-mediated immunity in healthy men. Our data demonstrate three significant modifications in the cellular immune composition in medically castrated men (compared both with their baseline and with the two control groups). First, the population of CD4<sup>+</sup>/CD8<sup>+</sup> T cells was significantly reduced, whereas the percentages of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells remained unchanged. Second, when PBLs were stimulated ex vivo with mitogenic stimulus, the ability of CD8<sup>+</sup> T cells to be activated, as measured by the expression of the proinflammatory cytokine IFN-γ, significantly declined. Finally, NK cell numbers significantly increased in medically castrated subjects. In addition, we demonstrate that these changes were prevented by T replacement. Together, our data suggest that T and/or its metabolites play an important physiological role in regulating immunity at the cellular level.

Consistent with the recent findings that E₂ increases the regulatory CD4<sup>+</sup>/CD25<sup>+</sup> compartment in mice (30), we show that the combination of T and E₂ deprivation results in a decrease in the population of CD4<sup>+</sup>/CD25<sup>+</sup> T cells, which contains the CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cell compartment, and that T (and E₂) replacement prevented this reduction in medically castrated men. Deficiency in either the number or function of CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells has been implicated in a number of human autoimmune diseases. Data suggest that deficiency in the number of CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells correlates with disease activity in human inflammatory bowel disease and systemic lupus erythematosus (SLE) (7, 27). Studies also suggest that changes in the function of CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells may impact autoimmune diseases such as multiple sclerosis, myasthenia gravis, and type 1 diabetes (4, 22, 45). Androgen administration has recently been demonstrated to improve disease activity in women with SLE (29) and is associated with decreased levels of proinflammatory cytokines in hypogonadal men when given to the elderly (17, 23). In the current study, our data suggest that both androgens and E₂ may be important in maintaining the number of potential regulatory

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**Fig. 3. Changes in CD8<sup>+</sup> T cell activation as measured by intracellular IFN-γ staining.** PBLs were stimulated with phorbol myristate acetate (PMA) and ionomycin in the presence of Golgi inhibitor for 4 h. Cells were stained with anti-CD3-FITC, anti-CD8-PerCP, and anti-IFN-γ-PE antibody after permeabilization. A: representative dot plot showing the IFN-γ-producing CD8<sup>+</sup> population among CD3<sup>+</sup>/CD8<sup>+</sup> subsets and levels of IFN-γ expression. Numbers represent percentage (%) of CD8<sup>+</sup>/IFN-γ<sup>+</sup> population. B: relative percentage of CD8<sup>+</sup>/IFN-γ<sup>+</sup> population among CD8<sup>+</sup> T cells. Data are presented as means (SD). Baseline (day 0) population was defined as 1 = 100% in all groups. *P < 0.01 vs. baseline (day 0).
T cells, a mechanism through which T and/or its active metabolites might preserve immune tolerance and protect men from autoimmune diseases.

Other studies have suggested that men with androgen deficiency due to KS have increased CD4+/CD8+ T cell ratios compared with normal controls, which are normalized with androgen replacement (5, 19). We did not observe an increase in CD4+/CD8+ ratio with acyline-mediated hypogonadism. This discrepancy may be due to the increased LH and FSH levels in KS patients that do not occur with acyline treatment (14, 15). Furthermore, subjects receiving acyline + T, who had changes in gonadotropins comparable to those receiving acyline alone (Page ST, Matsumoto AM, and Bremner WJ, in preparation), had no changes in immune composition. Therefore, it is unlikely that changes in GnRH, which has been implicated in immune regulation (42), or gonadotropins, account for the changes in immune composition that we observed.

Studies have suggested that E2 can directly upregulate IFN-γ gene expression in activated lymphocytes (10). Consistent with this, we demonstrate a marked decrease in activated CD8+ T cell IFN-γ expression in medically castrated subjects who have reduced serum T and E2 levels. IFN-γ is a major inflammatory cytokine functioning in host defense against viral infections and tumor development (36, 43). Hence, our observations of impaired activation of CD8+ T cells of castrated men suggests a possible mechanism by which T and/or its metabolites impact adaptive immunity. Because CD8+ T cell-mediated immunity constitutes one of the main components of the immune response to tumor antigens (1), the reduced ability of CD8+ T cells to respond to a mitogenic stimulus with sex steroid deprivation may impair the effectiveness of anticancer immune therapy in patients undergoing concomitant androgen ablation. Immune-therapy trials for hormone-sensitive cancers such as prostate cancer should examine the effects of hormone ablation on these emerging treatment modalities.

The current literature suggests that immunomodulation of NK cells by sex steroids is complex (21, 37, 39, 41). E2 has been reported to suppress NK proliferation in mice and transsexual men (37, 41) and increase NK cell proliferation in vitro (36), whereas T had no effect on NK cell proliferation in mice (41). Here, we demonstrate an increase in peripheral NK cells in the setting of both reduced T and E2 levels associated with medical castration, the former levels being much more greatly suppressed. Furthermore, we found that NK cells were unaffected when the T and E2 levels were maintained in the physiological range with T replacement. These data indicate that T and/or its metabolites may suppress NK cell proliferation in healthy men.

NK cells execute their protective function against tumors by homing to tumor sites, through their major homing receptor CXCR1 and directly destroying tumors via two known mechanisms. One mechanism is via the frequent downmodulation of tumor cell surface major histocompatibility complex (MHC) class I molecule, the ligand for NK cell inhibitory receptors (13). The other mechanism is via tumor-specific ligand-induced activation of the stimulatory NK cell receptor NKG2D (13). The expression of NKG2D is of particular interest to us, as we have previously shown that NK cell antitumor activity in men with prostate cancer is mediated predominantly via the latter mechanism (47). It is possible that hormone ablation...
therapy may impact NKG2D expression or the homing ability of NK cells. Our data in this study demonstrated that medical castration had no significant effect on surface NKG2D expression or the homing receptor CXCR1 in normal men.

The mechanism by which sex steroids influence immune composition or cellular immune function is not well understood. Whereas immature thymocytes are androgen receptor positive and clearly androgen sensitive (28, 42), it is generally accepted that human peripheral lymphocytes express estrogen receptors but not the androgen receptor (6, 28, 40). Studies in mice have suggested that androgens may act on mature lymphocytes via non-genomic pathways that initiate transcription-independent signaling (26, 48).

This study has some limitations. In particular, small sample size might have resulted in skewed results if one subject experienced a transient viral infection during treatment; however, there were no clinical indications that this occurred, nor were any significant outliers in the immune parameters measured. Despite randomization, subjects who received acyline alone tended to have higher levels of T at baseline (p = 0.07). Given the degree of change we observed and the fact that there were no measured differences in baseline immune composition, it is unlikely that any baseline differences in T levels influenced our results, although we cannot exclude this possibility. Finally, although subjects treated with acyline alone experienced a >90% reduction in circulating T and an 80% reduction in DHT, they also experienced a 55% reduction in serum E2 concentrations. Therefore, although the changes in lymphocyte subsets and function that we observed in this study are clearly related to alterations in sex steroids, whether they resulted from androgen deprivation, estrogen deprivation, or a combination of these is not clear from our data. Further studies combining GnRH antagonist treatment with non-aromatizable androgens or androgen blockade, or T with or without an aromatase inhibitor, will be required to further delineate the effects of a specific sex steroid on these cellular immune components.

In summary, data from this prospective, randomized, placebo-controlled study support a physiological role for T and/or its active metabolites in cellular immune function in healthy normal men. Future studies designed to characterize the role of sex hormones on antigen-specific effector functions and their impact on tumor surveillance and autoimmune processes are warranted.

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