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Testosterone Replacement Effectively Inhibits the Development of Experimental Autoimmune Orchitis in Rats: Evidence for a Direct Role of Testosterone on Regulatory T Cell Expansion

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Despite the immune-privileged status of the male genital tract, infection and inflammation of the male genital tract are important etiological factors in male infertility. A common observation in clinical and experimental orchitis as well as in systemic infection and inflammation are decreased levels of testosterone. Emerging data point to an immunosuppressive role of testosterone. In our study, we substituted testosterone levels in experimental autoimmune orchitis (EO) in rat by s.c. testosterone implants. EO development was reduced to 17% when animals were treated with low-dose testosterone implants (3 cm long, EO+T3) and to 33% when rats were supplied with high-dose testosterone implants (24 cm, EO+T24) compared with 80% of animals developing disease in the EO control group. In the testis, testosterone replacement in EO animals prevented the accumulation of macrophages and significantly reduced the number of CD4+ T cells with a strong concomitant increase in the number of regulatory T cells (CD4+ CD25+Foxp3+) compared with EO control. In vitro testosterone treatment of naive T cells led to an expansion of the regulatory T cell subset with suppressive activity and ameliorated MCP-1–stimulated chemotaxis of T lymphocytes in a Transwell assay. Moreover, expression of proinflammatory mediators such as MCP-1, TNF-α, and IL-6 in the testis and secretion of Th1 cytokines such as IFN-γ and IL-2 by mononuclear cells isolated from testicular draining lymph nodes were decreased in the EO+T3 and EO+T24 groups. Thus, our study shows an immunomodulatory and protective effect of testosterone substitution in the pathogenesis of EO and suggests androgens as a new factor in the differentiation of regulatory T cells. The Journal of Immunology, 2011, 186: 5162–5172.

Infertility and subfertility affect between 10 and 15% of couples with ∼50% of cases attributable to some form of male factor (1). Of the known causes and/or cofactors of male infertility, 13–15% of cases have been shown to result from infection or inflammation of the male reproductive tract (2, 3). This high prevalence marks infection and/or inflammation as key etiological factors of male reproductive dysfunction. Orchitis is characterized by inflammatory infiltrates in the testicular interstitium with associated damage to the seminiferous tubules, which can lead to focal and in its most severe form also to total impairment of spermatogenesis. In most cases, infection involves also the epididymis, resulting in a combined epididymo–orchitis (4). Whereas acute testicular inflammation is symptomatic and mainly related to infection, the subacute, or chronic, form of the disease is predominately asymptomatic (4–6). It should be noted that although the acute phase of the disease, which is the consequence of infection, can be successfully treated with antibiotics and anti-inflammatory drugs, the immunologic reaction is more insidious in that it may remain undetected in both testes slowly undermining testicular function (7–10).

Experimental autoimmune orchitis (EO) in the rodent is an established model of chronic testicular inflammation (11). Induced by immunization with testicular homogenate in adjuvant, EO is characterized by the production of auto-antibodies against testicular Ags, elevated levels of proinflammatory cytokines (e.g., TNF-α, IL-6) and chemokines (e.g., MCP-1), followed by the infiltration of leukocytes (mainly macrophages, dendritic cells, T lymphocytes) (12–14). An increase in the number of regulatory CD4+ CD25+Foxp3+ T cells is also observed (15). In the later stages of the disease, these infiltrates can form granulomas. The final phase of EO consists of the progressive apoptosis of germ cells, which leads to aspermatogenesis and thus sterility (16–20). Decreased levels of testosterone and increased FSH concentrations in serum indicate that the detrimental influence of EO is not limited to testicular germ cells alone but also extends to the dysfunction of the somatic, Sertoli, and Leydig cells (21). An increasing body of evidence suggests that sex hormones play an important role in the modulation of the onset and/or perpetuation of autoimmune disease. In particular, steroid hormones have been...
implicated as crucial contributors to the immune response either as enhancers, as is the situation with estrogens, or as endogenous inhibitors, as is the case with testosterone and glucocorticoids (22, 23). This is best exemplified by the much higher incidence of autoimmune diseases such as multiple sclerosis in women, where the female to male ratio is significantly higher (2:1 to 3:1) (24, 25). Beyond sex-linked genetic factors and gender differences in immune responsiveness, a major contributing factor to this sex bias is thought to be the lesser degree of protection afforded to females due to their lower testosterone levels and hence the diminution of its inhibitory/protective influence (26, 27). A corollary is the later onset of multiple sclerosis in males compared with females, which coincides with a decline in bioavailable testosterone in men (28) and which suggests that testosterone may provide a degree of protection to young men who are genetically susceptible to the disease. Further supporting evidence of testosterone’s influence is provided by studies of animal models such as the NOD mouse, where prepuberal castration results in an increased prevalence of immunologically mediated diabetes (29), and SJL mice, where castration increased the severity of experimental autoimmune encephalomyelitis (EAE) (30). Conversely, female NOD and SJL mice implanted with testosterone pellets reveal lower incidences of diabetes and lesser forms of EAE, respectively, than in those without hormonal intervention (31, 32). Similar findings have also been reported on the increased incidence and severity of thyroiditis and adjuvant arthritis after castration, whereas testosterone treatment was found to be protective in both cases (33, 34).

Although some progress has been made in deciphering the manner by which testosterone exerts its immunoprotective influence, the precise mechanism of its actions is still unknown. Some hints as to the nature of the anti-inflammatory process have been provided by studies showing that testosterone supplementation reduces the expression of IFN-γ and increases the expression of the anti-inflammatory cytokine IL-10 by autoantigen-specific T lymphocytes (32, 35). Complementary in vitro studies have found CD4+ T lymphocytes treated with 5α-dihydrotestosterone also respond with increased IL-10 production (36).

In the testis, high local testosterone concentrations (10-fold higher than in serum in rats) seem to play an important role in the maintenance of testicular immune privilege, an effect strikingly demonstrated by transplantation studies that showed that rats pretreated with estrogen to suppress Leydig cell testosterone production promptly rejected intratesticular allografts that were otherwise tolerated (37). As serum testosterone levels in EAO animals are significantly decreased (21) and androgens are important immunosuppressive factors, the rationale behind our study was to investigate the putative protective influence that testosterone may provide during the development and pathogenesis of EAO.

Materials and Methods

Animals

Adult male inbred Wistar Kyoto rats aged 50–70 d were purchased from Charles River Laboratories (Sulzfeld, Germany). Animals were kept at 22°C with a 14-h light, 10-h dark schedule and fed with standard food pellets and water ad libitum. All procedures conformed to National Institutes of Health guidelines for care and use of experimental animals and were approved by local animal ethics committees (Regierungspraesidium Giessen GI 20/23 - Nr. 33/2008) prior to their commencement.

Induction of EAO and testosterone replacement treatment (experimental design)

EAO was induced in 22 rats (the experimental group, E) by active immunization three times every 2 wk with syngeneic testicular homogenate as previously described (16, 19). Briefly, immunization on anesthetized rats was performed on day 0 by injecting 0.4 ml syngeneic testicular homogenate mixed with 0.4 ml CFA (Sigma-Aldrich) into the hind footpads (s.c.) and in different sites near the popliteal lymph nodes and the neck area (intradermally). These injections were repeated twice at 14-d intervals (day 14 and day 28, see Fig. 1). The first two immunizations (day 0, day 14) were followed by an i.v. injection of 10^5 inactivated Bordetella pertussis bacteria (strain DSM 4952; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) dispersed in 0.5 ml isotonic saline, and the third (day 28) was followed by an i.p. injection of B. pertussis at a concentration of 5 × 10^7 in 0.5 ml isotonic saline. Control animals received CFA only and B. pertussis following the same scheme.

Twenty days after the first immunization, testosterone capsules were implanted s.c. along the dorsal surface of the rats under anesthesia (day 20). The implants were prepared using medical-grade, polymethylsiloxane tubing (inner diameter 1.59 mm, outer diameter 3.18 mm; Dow Corning, Midland, MI) filled with testosterone powder (Sigma; T-1500) and sealed at each end with medical adhesive silicone type A as previously described (38, 38). The testosterone implants were either 3 cm (T3) or 24 cm (T24; 3 × 8 cm = 24 cm) in length and had a release rate of 30 µg/cm.d. A schematic demonstration of the immunization procedure including testosterone treatment is shown in Fig. 1.

In numerous studies, it has been established that low-dose T3 implants cause a reduction in serum luteinizing hormone (LH) levels, a subsequent decline in intratesticular testosterone, and loss of the developing spermatogenic cells in the testis. In contrast, high-dose T24 similarly causes a drop in LH, but supplies sufficient testosterone to maintain intratesticular T levels adequate to support qualitatively normal seminiferous tubule function (38, 39). The animals were divided into the following seven groups each comprising 6–10 rats: 1) E rats treated with T3 implant (EAO+T3); 2) E plus T24 implant (EAO+T24); 3) adjuvant controls treated with T3 implant (Adjuvant+T3); 4) adjuvant plus T24 implant (Adjuvant+T24); 5) EAO rats without hormone replacement (EAO control); 6) adjuvant control; 7) normal untreated rats (Normal). Fifty days after the first immunization, the animals were sacrificed by an overdose of isoflurane (Forene; Abbott, Wiesbaden, Germany), and the testes and seminal vesicles were removed under sterile conditions and weighed. One testis was snap frozen in liquid nitrogen or fixed in Bouin’s solution to evaluate the degree of germ cell loss as an indirect parameter of seminiferous tubule damage. EAO animals display characteristic pathological changes such as significantly smaller testis size and weight, inhomogeneous testicular parenchyma, and testis redness. Histopathologically, EAO testis reveal premature release of developing germ cells in the lumen resulting in impaired spermatogenesis and atrophy of germinal epithelium and leukocytic infiltration of the interstitium with occasional granuloma formation. The contralateral testis and the testicular draining lymph nodes (LNs) were processed to generate single-cell suspensions. To determine intratesticular testosterone levels, a piece of left or right testis (∼300 mg) was decapsulated and homogenized with 3 volumes of ice-cold PBS. The resultant protein extract was frozen at −20°C and subsequently used for hormone measurement. Testicular cys-teclets or paraffin-embedded sections were cut, processed, then stained with hematoxylin using standard procedures. Peripheral blood was collected by cardiac puncture and stored overnight at 4°C prior to the serum being collected by centrifugation (1000 × g for 15 min). Sera were then stored at −80°C for subsequent hormone assays.

Hormone assays

Intratesticular and serum testosterone levels were measured by RIA after extraction of the samples with tolune as previously described (41). For testosterone, the intra-assay and interassay coefficients of variation were between 7.8 and 9% with the lower limit of detection being 0.1 ng/ml. Serum LH and FSH were determined by RIAs using reagents provided by DRG Instruments (Marburg, Germany) according to the manufacturer’s protocols. Serum samples were assayed in duplicate across a single assay. The sensitivity of the assay was 0.18 ng/ml for LH and 2 ng/ml for FSH.

Preparation of testicular interstitial cell suspensions

Testicular interstitial cells were obtained by enzymatic digestion as described previously (42). Briefly, decapsulated testes were incubated with 1 mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany) plus 0.1% BSA (fraction V; Sigma Chemical) in PBS in a shaking water bath at 34°C for 15 min. The enzymes was inactivated by adding ice-cold PBS, and the tissue fragments were washed with PBS, then the supernatant containing the pellet, which contained interstitial cells, was washed with PBS and erythrocytes depleted by osmotic lysis using RBC lysis buffer (Genta Systems, Minneapolis, MN) for 5 min.
at room temperature. The final cell suspension was washed with PBS at 300 × g for 10 min at 4 °C and processed directly for flow cytometric analysis.

**Flow cytometric analysis**

The following mAbs were used: mouse anti-rat CD4-PH (clone OX-35), anti-rat CD25-FITC (clone OX-39), PE-conjugated mouse IgG1k, mouse IgG1k-FITC. Background staining was evaluated using appropriate isotype controls. All Abs were purchased from BD Biosciences (Heidelberg, Germany), except for anti mouse/rat Foxp3-Alexa Fluor 647 (clone FJK-16s) and mouse IgG2a-Alexa Fluor 647 obtained from eBioscience (San Diego, CA) and AbD Serotech (Oxford, U.K.), respectively. All incubation steps were conducted at 4 °C for 30 min. Briefly, a maximum of 10^6 intestinal cells were incubated in PBS with 5% normal FCS, 1% BSA, and 0.1% sodium azide for 10 min at 4 °C. After blocking with anti-CD16/32(Fc block; BD Biosciences), CD25 and CD4 Abs were added. Cells were washed with PBS/BSA buffer. For intracellular staining of Foxp3, cells were fixed and permeabilized for 30 min using Foxp3 staining buffer set (Becton Dickinson Immunocytometry Systems, San Jose, CA).

**Preparation of monoclonal cell suspensions from testicular draining LNs**

Suspensions of monoclonal cells (MNCs) from renal LNs were prepared by grinding the organs through a 70-μm nylon mesh. After a subsequent washing step, the cells were suspended at a concentration of 2 × 10^7/mL in complete RPMI 1640 medium (PAA, Colbe, Germany) supplemented with 10% FCS (PAA), 1% MEM (Sigma), 1 mM sodium pyruvate (Life Technologies), 10 mM HEPES (Life Technologies), 100 U penicillin, 100 μg/mL streptomycin (PAA), and 50 μg/mL mercaptoethanol (Life Technologies).

**Rat IL-2, IFN-γ, and IL-10 ELISA**

For cytokine assays, 2 × 10^6 MNCs from renal LNs were cultured in 1 ml complete RPMI 1640 medium at 37 °C, 5% CO_2_. After 96 h, the supernatant was collected, and cell monolayers were treated with 10-μl portions of 15 s at 94 °C, annealing 30 s at 55 °C and 30 s at 72 °C. The primers for β-actin message.

**RNA isolation and real-time RT-PCR**

Total RNA was obtained using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. To remove genomic DNA contamination, isolated RNA samples were treated with RNase-free DNase I (Qiagen) for 25 min at room temperature while on the column. The total testis mRNA (2.5 μg) and 2 μl oligo(dT)15 primer were preincubated at 70°C for 10 min. The RT-PCR reaction mixture (buffer, 10 mM of each deoxyribonucleotide triphosphate, 40U recombinant RNasin RNase inhibitor) was preheated at 42°C for 2 min before mRNA, oligo(dT)15 primer, and 200 U Moloney murine leukemia virus reverse transcriptase were added and incubation continued for 75 min at 42°C. The reaction was inactivated by incubation at 70°C for 15 min. All RT-PCR reagents were provided by Promega (Mannheim, Germany). The cDNA quality was checked by amplifying the β-actin message.

**Real-time quantitative PCR**

Real-time quantitative PCR was performed using an I-cycler IQ detection system (Bio-Rad, Munich, Germany) in combination with QuantiTect SYBR Green PCR Master Mix (Qiagen). The thermal cycling program consisted of an initial denaturation at 15 min at 95 °C, followed by 45 cycles of 15 s at 94°C, annealing 30 s at 55°C and 30 s at 72°C. The primers for rat MCP-1, TGF-β1, and IL-10 were purchased as QuantimTect Primer Assays from Qiagen. Primer sequences and amplicon sizes are shown in Table I. All analyses were conducted in duplicate and the mean of the results used for comparisons. The mRNA expression of all investigated genes was normalized based on the expression of the two housekeeping genes (HKGs) β-actin and hypoxanthine phosphoribosyltransferase 1 (HPRT-1). The relative expression levels were calculated by the equation 2^(-ΔΔCt) where ΔCt is the difference in the Ct (threshold cycle) value between the gene of interest (GOI) and the HKG as calculated by (GOI Ct) – (HKG Ct). ΔΔCt = ΔCt (SACCt - CAt) and the control ΔCt (CAt - uncontrolled treatment). Comparison of both HKGs showed no significant differences between experimental and control groups were expressed in relation to the expression found in the corresponding untreated (N) controls. All data are presented as the mean ± SEM of six to seven different testicular samples for each treatment group.

**Immunofluorescence microscopy**

Cryosections (12 μm) were cut, air dried, and fixed in isopropanol for 15 min at −20°C. To prevent nonspecific binding, the sections were incubated in blocking solution (5% BSA plus 5% normal horse serum in PBS) at room temperature for 2 h. After blocking, the sections were washed three times with PBS for 10 min and incubated with the primary Ab (mouse anti-rat CD68 (ED1) and CD163 (ED2) 1:50; Serotec, Oxford, U.K.) at 4°C overnight. After rinsing in PBS/0.05% Tween 20, the slides were incubated with the corresponding donkey anti-mouse Cy3-conjugated secondary Ab (Dianova, Hamburg, Germany) diluted in PBS for 1 h at room temperature. In controls, the primary Abs were omitted. Finally, washed sections were mounted with Vectashield Mounting Medium containing DAPI (Vector, Burlingame, CA).

**Isolation of T cells and migration (chemotaxis) assay**

To test the influence of testosterone on the chemotactic activity of lymphocytes, T cells from healthy Wistar rats were isolated after centrifugation of a spleocyte suspension on a Ficoll–Paque PLUS gradient (GE Healthcare, Uppsala, Sweden). Spleocytes were prepared by injecting the spleen with RPMI 1640 medium and grinding through a 100-μm nylon mesh. All erythrocytes present were osmotically lysed. The leukocyte cell suspension was washed and further purified using magnetic beads coated with monoclonal mouse anti-rat pan T cell Ab and a MACS column (Miltenyi Biotech). The positively selected cells were collected as T cells. Cells were washed and suspended in complete-serum-free RPMI 1640 medium. Purity (>95%) was examined by flow cytometry using FITC-conjugated anti-CD3 and PE-conjugated anti-CD4 Abs (BD Biosciences Pharmingen, Erembodegem, Belgium). For the chemotaxis assay, testis cell culture incubated for 3 days with purified CD4+CD25+ cells from healthy Wistar rats (Sigma) were placed in serum-free RPMI 1640 medium containing 25 ng/ml rat recombinant MCP-1 (Biosource, Camarillo, CA) as chemoattractant in the lower compartment. For each experiment, 3 × 10^5 cells were seeded on the inserts and allowed to migrate overnight while being incubated with increasing doses of testosterone (10–1000 nM) at 37 °C. The cells that migrated into the lower well were counted using EasyCounter TT (Schaere Systems, Reutlingen, Germany).

**Influence of testosterone on T cells**

To determine the direct influence of testosterone on the differentiation of regulatory T cells (CD4+CD25+Foxp3+), T cells from healthy Wistar rat spleens were purified using the magnetic bead selection described earlier. Isolated T cells were stimulated every 24 h with different doses of testosterone (10–100 nM) or with 1 μM of the androgen antagonist flutamide (Sigma) in complete RPMI 1640 medium supplemented with 10% heat-inactivated serum (PAA) for 92 h at 37 °C. Cells treated with vehicle (0.1% DMSO) were used as control. The counting of regulatory T cells (Tregs) was performed by flow cytometry as described earlier. A total of eight independent experiments was assessed.

**Isolation of CD4+CD25− (Tresp) cells and CD4+CD25+ (Tregs)**

LN cells from renal and iliacal LNs draining the testis were isolated from EAO+T24, Adjuvant+T24, and from EAO and adjuvant control groups. Moreover, splenic T cells from healthy Wistar rats cultivated in vitro in the presence of 100 nM testosterone or vehicle for 92 h at 37 °C as described earlier were also sorted. Cell preparations were stained with anti-CD4–PE, anti-CD25–FITC (BD Biosciences), and subsequently sorted by flow cytometry (FACS aria II Cell Sorter; BD Biosciences). The cell purity was routinely greater than 98%.

Splenocytes, harvested from syngeneic rats, depleted of T cells by magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany), were irradiated (20 Gy) and used as costimulatory cells at 10^5 cells/well.

**Suppression assay**

CD4+CD25+ Tregs were analyzed for their suppressive capacity by using a coculture assay in 96-well round-bottom plates (Nunc, Roskilde, Denmark). CD4+CD25+ T cells (Tresp) were cultured at constant number (10^5 cells/well) with 10^5 irradiated T cell-depleted splenocytes in the presence of varying amounts of CD4+CD25+ T cells (Tregs) in 200 μl complete RPMI 1640 medium. Cell cultures were stimulated with 2 μg/ml anti-CD3 Ab (clone G4.18; BD Biosciences) for 5 d. At the end of day 4, cells were pulsed with 18–20 h with 1 μCi/ml of [3H]thymidine (GE Healthcare, England). Subsequently, the medium was removed; cells were washed twice with cold PBS and then solubilized in 0.5 M NaOH. The amount of radioactivity incorporated into DNA was determined by liquid scintillation spectrometry.
(Tri-Carb 1500; Packard) and expressed as counts per minute. The suppressive capacity of CD4+CD25+ Tregs toward responder cells in coculture was calculated relative to the maximum proliferation of the responder cells alone (100 × 1 – mean cpm coculture/mean cpm responder cells).

Statistical analysis
Results are expressed as mean ± SEM. Comparisons between groups were assessed using the non-parametric Kruskal–Wallis one-way ANOVA or the one-way ANOVA accompanied by the Bonferroni test when applicable. A p value <0.05 was considered statistically significant.

Results
Testosterone supplementation inhibits development of EAO and the inflammatory response in the testis
The histological architecture of rat testis was analyzed in animals supplemented 20 d after the first immunization with different doses (T3 and T24) of testosterone versus EAO animals having no testosterone treatment (Fig. 1). The time point of intervention was chosen to coincide with the first signs of disease (i.e., autoantibody formation becomes evident, data not shown). The histopathological appearance of the testes (Fig. 2) showed 80% of animals developed EAO in the control group (Fig. 2F) compared with only 17% in the EAO+T3 group and 33% in the EAO+T24 group (Fig. 2G). The histopathological changes seen were characteristic of orchitis and included reduced height of the seminiferous epithelium due to germ cell loss (Fig. 2) and lymphocytic infiltration and/or granuloma formation in the interstitium. It is noteworthy that of the 17% of EAO+T3 animals that showed signs of orchitis, the histopathological alterations were milder (e.g., the seminiferous epithelium remained largely intact and no granuloma formation was observed). Testosterone substitution caused a clear-cut reduction of the incidence of EAO with 83% of EAO animals supplemented with T3 (Fig. 2D) or 67% of EAO rats substituted with T24 (Fig. 2E) protected from the disease. Control adjuvant and testosterone-supplemented adjuvant animals showed no pathological changes in the testis (Fig. 2A, 2B, 2C, respectively). Thus, interventive testosterone treatment either completely inhibited the development of orchitis (most cases) or strongly reduced the severity of disease (Table I).

Effect of exogenous testosterone administration on weight of testis and seminal vesicles
As a measure of the androgenic effect of the testosterone implants, the weight of the testes and seminal vesicles was measured. Compared with normal and control adjuvant groups, EAO and adjuvant rats with the T3 implants had significantly decreased testicular weights commensurate with the observed loss of spermatogenic activity (Fig. 3A, Fig. 2D). In contrast, adjuvant and EAO rats implanted with the T24 capsule showed no difference in testicular weight (Fig. 3A) nor was spermatogenesis seemingly affected (Fig. 2R, 2E). The weight of seminal vesicles was significantly increased after supplementation with either T3 or T24 in all treated groups compared with that in nontreated rats. No significant changes were seen between the groups of untreated animals (Fig. 3B).

Hormone levels
The elevation of testicular testosterone levels seen in EAO and adjuvant groups was significantly suppressed after 30 d of treatment with exogenous testosterone (Fig. 4D).

EAO+T3 (7.78 ± 2.41 ng/g testis), EAO+T24 (10.69 ± 0.79 ng/g testis), as well as adjuvant+T3 (3.28 ± 0.16 ng/g testis) and adjuvant+T24 (8.15 ± 0.2 ng/g testis) groups all showed significantly lower testosterone levels compared with those in normal (38.04 ± 8.83 ng/g testis), adjuvant (62.48 ± 16.87 ng/g testis), and EAO (109.61 ± 25.99 ng/g testis) control groups (Fig. 4D). As a consequence of the exogenous application of testosterone, LH concentrations in all groups treated with implants were also significantly decreased (0.3 ± 0.02, 0.28 ± 0.03, 0.35 ± 0.02, 0.28 ± 0.02 ng/ml in EAO+T3, EAO+T24, adjuvant+T3, and adjuvant+T24, respectively) compared with those in EAO (0.77 ± 0.07 ng/ml), adjuvant (0.61 ± 0.03 ng/ml), and untreated controls (0.52 ± 0.03 ng/ml). Conversely, LH levels in the EAO group were significantly increased compared with those in all other groups (Fig. 4B). Notably, in contrast to testicular homogenates,

FIGURE 1. Schematic diagram illustrating the time course of treatments for the in vivo experiments.

FIGURE 2. Representative testicular histology of animals substituted with testosterone during the course of EAO. Adjuvant control animals were supplemented with T3 (A) and T24 (B) testosterone implants 20 d after the first immunization or no testosterone treatment was used (C). EAO group was treated either with T3 (D) or T24 (E) testosterone implants or left untreated (F). The table (G) shows the prevalence of animals developing EAO in total numbers and percentages. Importantly, 83% of EAO animals supplemented with T3 (D) or 67% of EAO rats substituted with T24 (E) compared with 20% of EAO control rats (F) showed no pathological changes characteristic for EAO. Adjuvant+T3 (A), adjuvant+T24 (B), as well as adjuvant control (C) groups showed no pathological alterations. Scale bars, 100 μm.
the levels of testosterone measured directly in the interstitial fluid surrounding the tubules and interstitial cells did not differ significantly in the EAO group compared with those in adjuvant and normal control animals (Supplemental Fig. 1).

Serum testosterone was found to be significantly elevated to supraphysiological levels in animals supplemented with T24 (16.2 ± 1.24 ng/ml and 15.9 ± 1.38 ng/ml in EO and adjuvant groups, respectively). Whereas the animals treated with T3 (3.4 ± 0.27 and 2.74 ± 0.32 ng/ml in EAO+T3 and adjuvant+T3 groups, respectively) did not show any significant changes compared with EAO control animals (2.04 ± 0.19 ng/ml), the levels were significantly lower than those of the normal (5.3 ± 1.12 ng/ml) and adjuvant (4.35 ± 0.89 ng/ml) control groups (Fig. 4C).

Serum FSH was significantly suppressed after treatment with T3 (3.40 ng/ml) compared with that in normal (4.35 ± 0.54 ng/ml) control animals. In EAO animals with chronic inflammatory conditions, serum FSH level was ∼2-fold higher (22.63 ± 1.78 ng/ml) compared with that in normal untreated rats (Fig. 4A).

**Testicular macrophages, CD4⁺, and CD4⁺CD25⁺Foxp3 T cells**

The infiltration of the testicular interstitial space by macrophages and T cells represents a characteristic hallmark of EAO histopathology. In the large cohort of EAO animals, where disease development was prevented by supplementation with T3 or T24, the accumulation of macrophages (ED1/ED2) was unchanged in comparison with that of adjuvant or untreated control groups. As a consequence, substantially less infiltration of testicular macrophages was seen compared with that in the orchitis animals (EO controls, Fig. 5). The histological assessment was corroborated by the flow cytometric measurement of the number of CD4⁺ T cells, which showed significantly fewer cells in EAO rats supplemented with T24. A moderate decrease was also found in the EAO+T3 group compared with that in the EAO-only animals (Fig. 6A).

Notably, the number of immunosuppressive Tregs (CD4⁺CD25⁺Foxp3) in the testis of EAO+T24 animals was significantly increased compared with the numbers seen in the EO controls (Fig. 6B).

**Quantification of testicular mRNA expression for MCP-1, IL-6, IL-10, TNF-α, and TGF-β1**

The expression of the chemokine MCP-1 mRNA was highly up-regulated (80-fold) in the EO group compared with the levels in adjuvant and normal controls. After testosterone supplementation, MCP-1 was significantly reduced in both the EO+T3 and EO+T24 groups compared with that in the EO control group (Fig. 7A). In EAO rats, expression levels of the proinflammatory cytokines TNF-α (Fig. 7B) and IL-6 (Fig. 7C) were elevated ∼20-fold in comparison with untreated and adjuvant controls. Supplementation with T3 and T24 (EO+T3 and EO+T24 groups) resulted in substantial but statistically non-significant reduction (∼50%) of TNF-α and IL-6 expression compared with that in EAO rats without hormone intervention (Fig. 7B, 7C). The mRNA expression of the regulatory cytokine IL-10 was significantly suppressed in the EO+T3 group and decreased substantially in the EO+T24 group compared with that in EAO controls (Fig. 7D). The expression of constitutively expressed TGF-β1 in the testis remained unaffected in all investigated groups (Fig. 7E).

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FIGURE 3. Weight (g) of testosterone-dependent organs: (A) testis, (B) seminal vesicles in EAO and adjuvant animals supplemented with testosterone (EO+T3, EO+T24, adjuvant+T3, adjuvant+T24, respectively), as well as in EAO, adjuvant, and normal control rats without testosterone substitution. Data are expressed as mean ± SEM (n = 5 to 7 animals/group). Values with different superscript letters differ significantly compared with EAO control group: *p > 0.05 (NS), †p < 0.01, ‡p < 0.001.
Secretion of Th1/Th2 cytokines (IL-2, IFN-γ, and IL-10) by MNCs from testicular draining LNs

In EAO+T3 animals, the level of the proinflammatory Th1 cytokines IL-2 and IFN-γ was significantly downregulated as was the expression of IL-2 in EAO+T24 rats (Fig. 8A, 8B). EAO rats showed a strong upregulation of IL-10 compared with that in normal or adjuvant controls (Fig. 8C), which was reduced (not to the level of statistical significance) in EAO rats treated with testosterone (EAO+T3, EAO+T24) (Fig. 8C). LN cells from adjuvant and normal control groups showed no significant differences in the secretion level of investigated cytokines (Fig. 8).

Influence of testosterone on migration of T cells

In the presence of increasing concentrations of testosterone, a dose-dependent migration of T cells toward the MCP-1 gradient was observed. Incubation of cells with 100 nM testosterone significantly increased the MCP-1–stimulated chemotactic activity of T cells, whereas 1000 nM testosterone had no influence (Fig. 9). Testosterone alone showed no effect on the chemotaxis of T cells (data not shown). Thus, testosterone required the presence of MCP-1 to show its additive effect on chemotaxis.

Testosterone stimulates in vitro generation of Tregs

As the in vivo supplementation of EAO animals with testosterone inhibited the development of testicular inflammation and induced an increase in the number of CD4+CD25+Foxp3+ Tregs in the testes, we further investigated whether testosterone has a direct effect on the generation of Tregs from naive T cells. In vitro treatment of purified splenic T cells from healthy animals given different doses of testosterone showed a prominent expansion of the CD4+CD25+Foxp3 population within CD4+ T cells. Doses of 10 and 100 nM testosterone induced a 3- and 2.7-fold increase in the percentage of Tregs in the total population of T cells, respectively. This effect was abolished by the addition of the androgen antagonist flutamide (Fig. 10).

Suppressive capacity of Tregs expanded by testosterone in vitro

To determine the suppressive capacity of in vitro-cultivated Tregs on the proliferative activity of T effector cells, highly purified CD4+ CD25− and CD4+CD25+ T cells were cocultivated and polyclonal
stimulated in the presence of irradiated splenocytes and anti-CD3 Ab. In our setup, the CD4+CD25+ cells sorted from vehicle as well as testosterone-treated T cell in vitro culture showed typical Treg characteristics (i.e., hyporesponsiveness toward TCR stimulation and the ability to suppress the proliferation of Tresp cells) (Fig. 11). Coculture of Tregs with Tresp cells from vehicle- as well as testosterone-treated cells resulted in reduced proliferation of Tresp cells with the strongest suppression of proliferation (~90%) at the cell ratio 1:1. Tregs sorted from in vitro testosterone-treated T cells showed a similar level of suppression; however, suppressive activity was stronger in view of the 3-fold higher proliferative capacity of CD4+CD25+ T cells from testosterone-treated culture (Fig. 11C) compared with vehicle-treated cells (Fig. 11A).

Moreover, CD4+CD25+ T cells purified from testicular draining LNs from EAO and adjuvant animals supplemented with T24 as well as CD4+CD25+ T cells purified from EAO and adjuvant control animals demonstrated inhibitory potential on the proliferation of CD4+CD25- T cells from testosteronetreated culture (Fig. 11C) compared with vehicle-treated cells (Fig. 11A).

Discussion
In the past few years, various experimental and clinical studies have provided evidence for a possible immunosuppressive role for testosterone. These recent studies have built upon the findings of previous animal investigations that showed that castration and the associated deprivation of androgens aided in the development and exacerbated the consequences of experimental autoimmune diseases such as EAE, diabetes, thyroiditis, and adjuvant arthritis (29–31, 34, 43). In contrast, the provision of testosterone to females,
generally more prone to autoimmunity than males, was shown to ameliorate symptoms in a variety of autoimmune disease models (31, 32, 44). As the reduction of serum testosterone levels is a common feature in systemic and acute testicular inflammation in humans and experimental animal models alike, we investigated whether androgen supplementation at the onset of EAO could influence the course and severity of the disease.

Testosterone secreted by Leydig cells under the influence of pituitary LH acts on the seminiferous epithelium to promote spermatogenesis. In our study, the administration of T3 implant as expected resulted in the reduction of serum gonadotropin levels, but the testicular levels) to support qualitatively normal seminiferous tubule function (40, 45, 46). The histological assessment of the treated animals clearly showed that testosterone replacement provided a significant degree of protection against the development of orchitis with only 17% of EAO+T3 and 33% of EAO+T24 animals developing the disease, compared with 80% when no hormone intervention was performed. Furthermore, in the treated animals that did develop EAO, testosterone was found to reduce strongly the severity of disease, a finding that mirrors that seen in EAE where oral testosterone administration in castrated animals reversed the clinical symptoms of the disease (47).

Our data suggest that the presence of heightened testosterone levels exerts a multifactorial influence resulting in a number of significant modifications, all of which collectively constitute the observed immunoprotective response. First, we found that testosterone effectively inhibited the accumulation of macrophages and CD4+ lymphocytes while simultaneously selectively increasing the number of immunosuppressive Tregs (CD4+CD25+Foxp3+) in the testicular interstitial space. Second, testosterone supplementation inhibited mRNA expression and as a corollary the presence of proinflammatory cytokines (TNF-α, IL-6) and chemokines (MCP-1) in the testis. Finally, we found that its influence goes beyond the local (testicular) level, as systemically testosterone decreased the secretion of the Th1 cytokines IFN-γ and IL-2 seen in MNCs from testicular draining LN. In shifting the balance of Th1/Th2 cytokines, a T cell-driven autoimmune response is inhibited thus preventing an excessive proinflammatory response and the ensuing tissue damage.

The subtly of testosterone actions is illustrated by the finding that although it prevented accumulation, the number of macrophages present in the testis was unaffected, yet the composition of immune cells present was altered; namely, heightened numbers of Tregs. The ability of testosterone to induce this transformation was clearly demonstrated by our novel finding that in vitro after testosterone treatment, CD4+CD25+Foxp3+ Tregs were derived from splenic CD4+ T lymphocytes. Furthermore, we showed that testosterone-derived Tregs were very effective in the inhibition of proliferative responses of effector T cells showing a higher capacity than Tregs derived from vehicle control. This is of particular importance, considering Tregs are known both for their potent regulatory capacity in controlling autoimmunity and their protective role against immunity-induced pathology (48). In conjunction with our functional studies, our finding that the CD4+CD25+ cells from the treated animals express Foxp3, the master regulator for the Treg lineage, indicates that these cells fulfill their role in the maintenance of self-tolerance and tissue homeostasis in vivo. Conversely, others have shown that although increased numbers of CD4+ and CD8+ effector Tregs are found in EAO (49) and various autoimmune diseases (50–52), their presence alone was insufficient to inhibit disease development. As in our study, Jacobo et al. (49) reported increased CD4+ T cells with a concomitant proportional increase of CD4+CD25+Foxp3+ Tregs in EAO testes. Thus, the relative proportion of two subsets of cells remained unchanged. This could explain why in EAO, the recorded absolute increase in Treg...
numbers was ineffective in preventing disease outbreak. The addition of testosterone, regardless of quantity, not only resulted in the increased absolute number of Tregs, but also, compared with that of the EAO-only animals, the Tregs constituted a higher proportion of the CD4+ lymphocyte population. Considering their purported inhibitory abilities, this increase could provide an explanation as to how testosterone prevented the onset of EAO in the majority of treated rats. A further insight into the importance of the relative proportions of the differing types of T lymphocyte is shown by the finding that the CD8+ effector population increased more than CD4+Foxp3+ Tregs during EAO. This imbalance could be sufficient to overwhelm the immunosuppressive actions of CD4+Foxp3+ Treg function and thus lead to chronic testicular inflammation (49).

Further evidence of testosterone’s involvement in a multifaceted immunoprotective mechanism during EAO is the dual nature of its subversion of the role of the chemokine MCP-1 during inflammation. We found that the presence of testosterone, on the
mRNA level, significantly decreased the level of expression and on the functional level ameliorated MCP-1–induced chemotaxis in vitro. In orchitis, high MCP-1 levels ameliorated by testosterone, which is found strongly elevated in testicular homogenates and close to normal in testicular interstitial fluid, may concert the migration of leukocytes. In contrast, in testosterone-supplemented macrophages, we found that mRNA expression levels for these genes were significantly inhibited by 1000 nM testosterone in vitro (data not shown). Systemically, a T cell autoimmune response is initiated in the draining LNs where dendritic cells present captured autoantigens to naive T lymphocytes in the presence of Th1 cytokines such as IL-2 and IFN-γ. We were able to demonstrate clearly that the strongly elevated secretion of IL-2 and IFN-γ, which is characteristic of orchitis, was suppressed almost to adventage levels in testosterone-treated EAO animals with the exception of IFN-γ in EAO+T24. Thus, an essential stimulus to trigger a T cell-based cellular autoimmune response is lacking and may add on the systemic level to the local immunosuppressive mechanism elicited by testosterone treatment. In support, the increased incidence of EAO in animals treated with T24 (33% responders) compared with T3 (17% responders) may be attributed to the larger implant’s inability to suppress the expression of IFN-γ with a concomitant inhibited IL-2 synthesis seen for both implants.

In summary, our results demonstrate that testosterone supplementation exerts a multifaceted protective effect during EAO development. Mechanistically, this is mediated systemically by the inhibition of Th1-specific cytokine production in testicular draining LNs. In the tests itself, testosterone appears to induce an expansion of suppressive Tregs from naive T cells leading to an overproportional representation of Tregs within the CD4+ T cell population while simultaneously inhibiting the synthesis of proinflammatory mediators MCP-1, TNF-α, and IL-10. Clearly, the exact proportion of systemic and local actions of testosterone requires further elucidation. Taken together, these findings suggest that testosterone plays an important role in the maintenance of immunological balance in the tests and point to a previously unrecognized role of testosterone in the differentiation of Tregs.

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Disclosures
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