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# Role of testosterone in regulating induction of TNF-α in rat spleen *via* ERK signaling pathway

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ABSTRACT

Spleen is a pivotal organ for regulating immune homeostasis. It has been shown that testosterone diminishes secretion of various inflammatory molecules under multiple conditions. However, the mechanisms of action of endogenous testosterone affecting immune responses in the spleen remain unknown. The aim of the present study was to evaluate the immune functions of the spleen in response to testosterone withdrawal after orchidectomy, and the impact of splenocytes on the bacterial endotoxin lipopolysaccharide (LPS)-induced secretion of inflammatory molecules. Male rats were divided into 3 groups, i.e. intact, orchidectomized (Orch) and orchidectomized plus replacement of testosterone propionate (TP) (Orch + TP). The Orch and Orch + TP rats underwent bilateral orchidectomy one week before TP replacement (2 mg/kg body weight) or sesame oil in intact rats as controls for seven days. Orch resulted in a significant increase of spleen weight and basal secretion of nitric oxide (NO) from splenocytes. Additionally, LPS up-regulated cell proliferation and the secretion of tumor necrosis factor-alpha (TNF-α) in splenocytes of Orch rats. Orch further up-regulated phosphorylation of extracellular signal-regulated kinases. Interestingly, the plasma corticosterone concentration in the Orch group was higher than that in the intact and Orch + TP groups. Deficiency of testosterone-elevated TNF-α and NO secretion in response to LPS were confirmed in the rat splenocytes. Testosterone also significantly attenuated LPS-elicited release of TNF-α and NO in a dose-dependent manner. However, testosterone did not suppress splenic blastogenesis at doses in the $10^{-10}$–$10^{-7}$ M concentration range. In this context, testosterone might have a protective role against inflammatory responses in the spleen. The present study provides evidence to indicate that testosterone might modulate the immune system.



## 1. Introduction

Testosterone, synthesized and secreted mainly by the Leydig cells of the testes [1], is responsible for maintaining spermatogenesis and secondary sexual characteristics [2,3] such as increased bone and muscle mass [4], and sex drive in the male. Recently, there is an increasing interest in the immuno-endocrine system, including the relationship between testosterone and chronic inflammatory diseases. Low levels of testosterone have been linked to the increase of cardiovascular risk factor [5], mortality [6], and diabetes mellitus [7,8]. These observations raised the possibility that testosterone exerts immunomodulatory effects.

On stimulation by the bacterial endotoxin lipopolysaccharide (LPS) [9], immune cells secrete pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 6 [10,11] and/or other inflammatory molecules. In turn, these cytokines induce other host immune responses associated with inflammatory

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diseases, including sepsis [12], asthma [13], rheumatoid arthritis and inflammatory bowel disease [14]. TNF-α is a key mediator of inflammation. Recently TNF-α has been attributed a major role in the development of pathological processes in the promotion of expression of genes associated with inflammation [15] and in leukocyte extravasation [16]. Intriguingly, regardless of whether it is a physiological consequence of aging or a surgical intervention, low levels of testosterone have been associated with increased levels of TNF-α [11] or other inflammatory mediators [17,18]. These findings, taken together with the observation of cytokine attenuations by testosterone, suggest that testosterone may exert potent anti-inflammatory effects.

Toll-like receptor 4 (TLR-4) recognizes specific patterns of microbial conserved components such as LPS, and activates mitogen-activated protein kinases, including extracellular signal regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and the p38 subfamilies consequently leading to the release of pro-inflammatory cytokines, such as TNF-α and IL-1β [19,20]. Several experimental studies have documented that testosterone not only decreases the synthesis of pro-inflammatory cytokines [11,21,22], but also promotes the secretion of anti-inflammatory mediators such as IL-10 [23]. On the other hand, it has been shown that testosterone could abrogate LPS-induced cell proliferation [21] and down-regulate TLR-4 expression in macrophages [11] and non-immune cell types such as prostate smooth muscle cells [21]. Nevertheless, few works have studied the mechanism of the effects of testosterone on the signaling downstream of TLR-4. Moreover, little is known about the impact of immune responses in the spleen caused by testosterone withdrawal. The mechanisms underlying alterations of TLR-4 downstream proteins by testosterone deficiency are also unclear. Accordingly, the present study was carried out to determine the effects of testosterone depletion via orchidectomy on the secretion of TNF-α and TLR-4 in the downstream signaling pathway in the rat spleen.

## 2. Methods

### 2.1. Animals

Animal experiments were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University with an approval number – 1021226. Male Sprague–Dawley rats employed in all experiments were purchased from the Laboratory Animal Center of National Yang-Ming University, and were housed at 22 ± 1 °C under a 14-h light/dark (05:00–19:00) cycle. All animals were fed *ad libitum* with standard chow and drinking water.

### 2.2. Animal grouping, orchidectomy, and testosterone replacement

Male rats were divided into three groups. Untreated intact rats were employed as the control group. Surgical castration was applied in the orchidectomized (Orch) group and in the orchidectomized (Orch) combined with testosterone propionate (TP) replacement (Orch + TP) group. Before surgical procedures, the rats were anesthetized with pentobarbital (30 mg/ml/kg). Orchidectomy was performed through ventral midline scrotal incision, and testes were isolated and removed bilaterally. All blood vessels and deferential ducts were returned to the ventral cavity. The incision was then closed and sutured. One weeks after castration of the Orch and Orch + TP groups, rats of the intact and Orch groups were subcutaneously injected with sesame oil (Sigma, St Louis, MO, USA) once per day for 7 days. Then, 2 mg/kg body weight of testosterone propionate (Fluka, Buchs, Switzerland) dissolved in sesame oil was administrated to the rats in the Orch + TP group [24].

### 2.3. Testosterone and corticosterone radioimmunoassay (RIA)

Plasma testosterone levels were determined by specific RIA as previously described [25,26]. Briefly, plasma samples were extracted with 10-fold volume of diethyl ether. The extracts were collected and evaporated in a fume hood and stored at −20 °C until assayed by RIA with an anti-testosterone anti-serum (No. W8). The sensitivity of testosterone RIA was 2 pg/tube, and the intra- and inter-assay coefficients of variation were 4.1% ($n = 6$) and 4.7% ($n = 10$), respectively.

The plasma corticosterone concentration was quantified by RIA as described elsewhere [24,27] with an anti-corticosterone anti-serum PSW#4-9. Briefly, plasma samples were extracted as described for testosterone. The steroid range of the assay was 4.69–4800 pg, and the intra- and inter-assay coefficients of variation were 3.3% ($n = 5$) and 8.2% ($n = 4$), respectively.

### 2.4. Preparation of rat splenocytes

The rats were sacrificed by decapitation. Spleens were isolated and quickly washed with phosphate buffered saline (PBS, pH 7.4) in separate 6 cm Petri culture dishes. Samples were then minced and pressed through a 0.70-μm sterile wire mesh nylon screen with a 3-ml rubber syringe plunger, and cells were collected by centrifugation at 500*g* for 7 min at 4 °C. Erythrocytes were lysed by adding ACK lysis buffer (0.15 M $NH_4Cl$, 1 mM $KHCO_3$, and 0.1 mM $Na_2EDTA$, pH 7.4), following which the splenocytes were extensively washed with PBS. Before cell counting, splenocytes were re-suspended in appropriate volumes of RPMI-1640 medium (Sigma) supplemented with 2 mM L-glutamate, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, and 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS, Sigma). Subsequently, cell count exhibited >95% viability by trypan blue exclusion.

### 2.5. In vitro experiments

To determine the effects of testosterone withdrawal on basal (unstimulated) or lipopolysaccharide (LPS)-induced cytokine secretion, splenocytes were adjusted to a concentration of $4 \times l0^6$ cells/ml and loaded into 24-well culture plates at $1 \times 10^6$ cells/well. The cells were then incubated with or without LPS (10 μg/ml) at 37 °C in a humid atmosphere of 5% $CO_2$ for 4 h. To investigate the effects of testosterone on basal (unstimulated) or LPS-evoked TNF-α secretion, splenocytes were incubated with complete RPMI-1640 medium (vehicle) or with LPS (10 μg/ml) in the presence or absence of testosterone ($10^{-10}$–$10^{-7}$ M) for 4 h. The supernatant was harvested after centrifugation for determination of TNF-α concentrations by enzyme-linked immunosorbent assay (ELISA).

### 2.6. TNF-α assays

The TNF-α levels secreted by cultured splenocytes in the supernatants and plasma were measured using commercial ELISA kits specific for rat cytokines (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The optical densities (O.D.) obtained were normalized to per million cells/well in the *in vitro* experiment.

### 2.7. MTT assays

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (Sigma) was dissolved in PBS at 5 mg/ml concentration. To evaluate the effect of testosterone depletion on basal (unstimulated) or LPS-stimulated cell proliferation, the splenocytes in a final

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