# Sex steroids induce apoptosis of CD8$^+$CD4$^+$ double-positive thymocytes via TNF-α

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T cell production by the thymus, thymic size, cellularity and output all decrease drastically after puberty. Among the candidates that may mediate this decrease are the sex steroids: hypersecretion or pharmacological administration of these hormones has long been known to induce thymic hypocellularity, and their depletion yields thymic hypercellularity. Here we show that a typical sex steroid, testosterone, specifically targets CD8$^+$CD4$^+$ double-positive (DP) thymocytes for apoptosis via TNF-α. Anti-TNF-α monoclonal antibodies abrogated testosterone-induced DP apoptosis, and TNF-α$^{-/-}$ DP thymocytes were largely resistant to testosterone-mediated apoptosis *in vivo*. Testosterone accomplished this effect by up-regulating TNF-α production and by simultaneously sensitizing DP thymocytes to TNF-α. Thus, TNF-α is the critical mediator of sex steroid-induced apoptosis in thymocytes, and its manipulation should provide a point of intervention to modulate T cell production in sex hormone disorders.

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## 1 Introduction

Apoptosis plays a critical role in modulating T cell production and output from the thymus [1, 2]. This process is induced in both useless (*i.e.* those that failed pre-TCR selection or TCR-mediated intrathymic positive selection) and harmful (*i.e.* autoaggressive) thymocytes during intrathymic selection. Apoptosis is also frequently induced in thymocytes by physiological stimuli of neuronal and hormonal origin and by stimulation with pathogens (*i.e.* HIV) or their products (*e.e.* microbial toxins).

A particularly striking example of a loss of thymic cellularity and weight occurs with aging. Thymic involution effectively begins in early childhood, but becomes dramatically manifest from the time of puberty [3–5]. The molecular mechanisms underlying age-associated thymic involution are still not completely understood. One of the suspects that may induce and/or accelerate age-associated thymic involution are the sex steroids, whose peak of secretion correlates precisely with the decline in thymic output. Several lines of evidence are consistent with this view: (i) sex steroid receptors are expressed on thymocytes [6–9]; (ii) sex steroids have long been known to negatively modulate thymic cellularity and T cell production/output, as documented by transient thymic involution in physiological (*e.g.* pregnancy and menstrual cycle/oestrus) and pharmacological (sex hormone therapy) or experimental situations (reviewed) in [7]; [10–13]; and (iii) surgical or pharmacological ablation of sex steroid secretion is known to lead to hypercellularity of the thymus [7, 10–13]. These processes affect immature, cortical thymocytes, and have relatively little impact upon mature T cells [7, 9].

The studies presented here were aimed at investigating how the sex steroids affect thymic cellularity and output. Our results clearly demonstrate that TNF-α plays an essential role in sex steroid-mediated thymocyte apoptosis.

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**Abbreviation: DP:** CD8$^+$CD4$^+$ double positive **DT:** Depo testosterone **PNO:** Peanut oil

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## 2 Results

### 2.1 Testosterone stimulation induces CD4⁺CD8⁺ thymocyte apoptosis *in vitro* and *in vivo*

To investigate whether sex steroids induce thymocyte apoptosis, we incubated enriched $CD4^+CD8^+$ double-positive (DP) thymocytes with increasing doses of depo-testosterone (DT) *in vitro*. Apoptosis was assessed in overnight-cultured DP thymocytes by detecting hypodiploid nuclear content in permeabilized cells stained with PI (Fig. 1). (This time point was elected because it allows an accurate assesment of apoptosis with acceptable background values. At later time points, in both normal and knockout mice used in this study, spontaneous apoptosis usually increased to $>40$ %, disallowing observation of the specific effects of stimulation). Under these conditions, DT induced DP thymocyte apoptosis in a dose-dependent manner (Fig. 1 A). Levels of apoptosis achieved with $>50$ μg/ml DT were comparable with those induced by one of the strongest apoptotic stimulators, dexamethasone (Fig. 1 A; see also [14]). Furthermore, *in vivo* treatment of mice with DT in peanut oil (PNO) induced a pronounced drop in DP percentage and numbers (Fig. 1 B), while treatment with PNO alone had no effect. These results are consistent with the previous data [9, 12] showing that both testosterone and estrogen, another typical sex steroid, induce profound cell loss and apoptosis in rodent DP thymocytes.

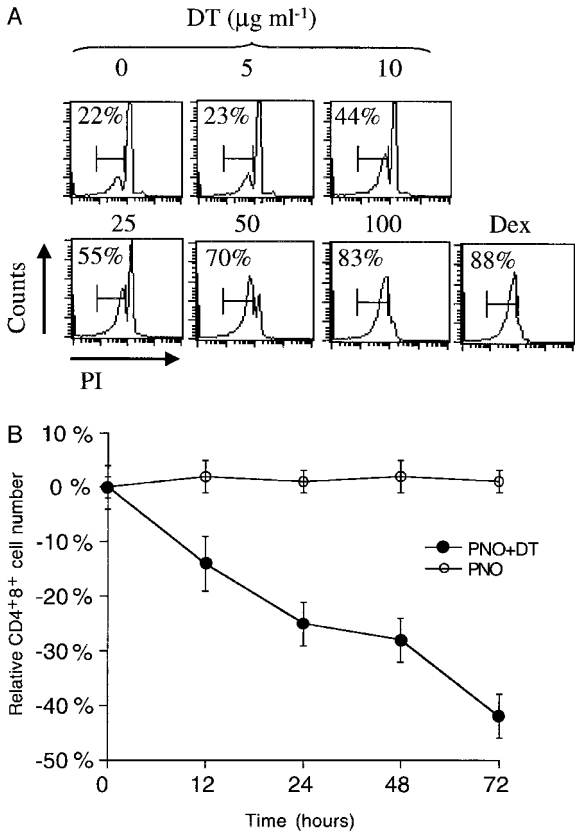


*Fig. 1. In vitro* and *in vivo* DP thymocyte apoptotis in response to DT stimulation. (A) DP thymocytes from B6 mice, prepared as described in methods, were treated *in vitro* for 18 h with indicated doses of DT and Dex ($10^{-7}$M). Apoptosis was determined by the permeabilization/PI method [29], and the % of cells with hypodiploid DNA, diagnostic of apoptosis, shown as determined by marker M1. (B) B6 mice were injected with DT in PNO at 0.5 mg/100 g body weight, or with an equivalent volume of PNO alone. % change in DP cell number was determined by multiplying absolute trypan blue counts by the percentage of DP thymocytes obtained by FCM analysis (in this experiment, absolute number of DP thymocytes 72 h after treatment was $50.9 \times 10^6$ in control and $29.1 \times 10^6$ in DT-treated mice). Cell values of unmanipulated mice were assigned the 100 % value at the beginning of the experiment. Results are representative of four and three experiments, for A and B, respectively.

### 2.2 Testosterone induces DP thymocyte apoptosis via TNF-α

TNF-α and TNFR are both expressed in the thymus [15, 16], and our recent data indicate that this pair is involved in thymocyte death by cAMP activators [17]. Sex steroids are known to activate cAMP, prompting us to investigate the role of TNF-α in testosterone-induced apoptosis. We initially used a neutralizing anti-TNF-α mAb (MP6-XT22), to treat DP thymocytes simultaneously with DT. Fig. 2 A shows that this antibody abrogated, in a dose-dependent manner, DT-mediated apoptosis in DP thymocytes, while the control rat $IgG_1$ had no effect (not shown). By contrast, TNF-α neutralization had no effect on DP apoptosis induced by dexamethasone; 61 % of DP cells underwent DNA fragmentation in the presence and 63 % in the absence of anti-TNF mAb (not shown). The above data were confirmed in three separate experiments using two different anti-TNF-α antibodies (MP6-XT22, Fig. 2 and 2E2, not shown). These results strongly suggest that TNF-α may be the key mediator of DT-mediated apoptosis.

To test the relevance of these observations by a genetic approach, we used TNF-$\alpha^{-/-}$ knockout mice that have normal thymocyte numbers and phenotype, and are otherwise indistinguishable from normal counterparts [18]. DP thymocytes of these animals did not undergo apoptosis in response to DT stimulation *in vitro*, while dexamethasone-mediated apoptosis was not impeded (Fig. 2 B). These results definitively demonstrate an essential role for TNF-α in the induction of DT-mediated DP thymocyte death.

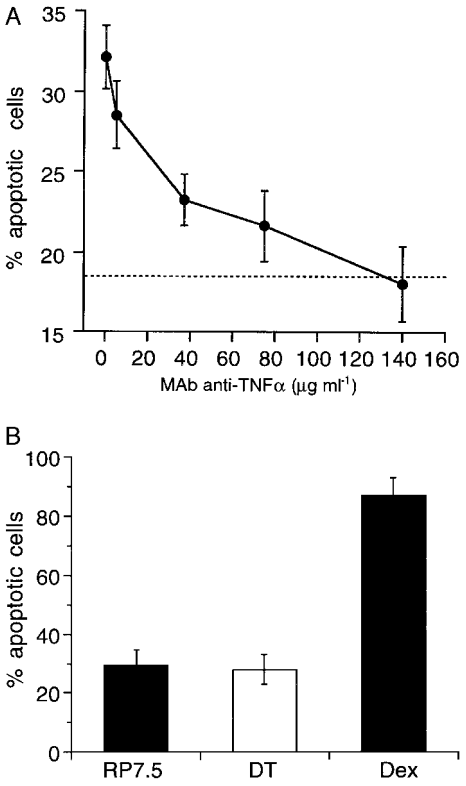


*Fig. 2.* The role of TNF-α in DT-induced thymocyte apoptosis. (A) TNF-α neutralization abrogates DT-induced thymocyte apoptosis. Assays were performed and results displayed as in Fig. 1 B, except that 15 μg/ml DT was added and cells then incubated in the absence or the presence of increasing concentrations of the anti-TNF-α mAb MP6. Dotted line indicates spontaneous apoptosis. Results are representative of four independent experiments. (B) TNF-$\alpha^{-/-}$ DP thymocytes are resistant to DT-induced apoptosis *in vitro*. Experiments were preformed and results reported exactly as in (A).

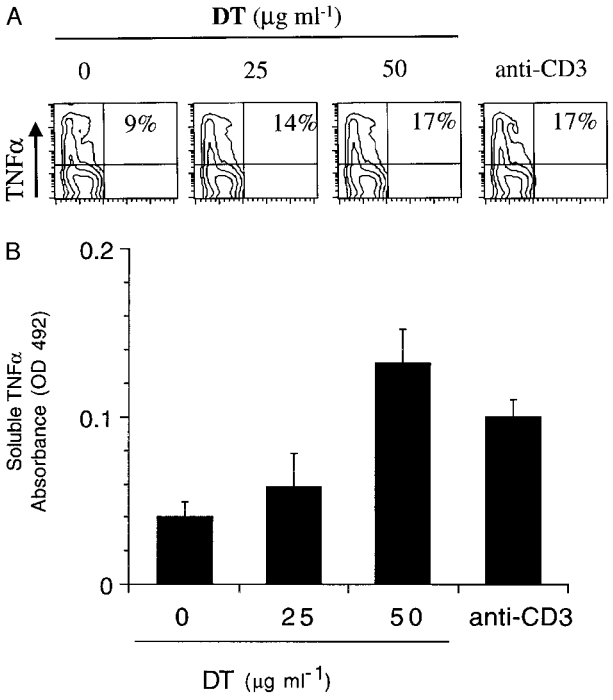


*Fig. 3.* Induction of membrane and soluble TNF-α by DT. (A) Surface staining of DP thymocytes treated with indicated stimuli overnight and stained with the biotinylated mAb 1C6 [38] followed by streptavidin-PE. Numbers represent percentage of surface TNF-$\alpha^+$ cells and were similar in three other experiments. (B) TNF-α capture ELISA shows the 18 h accumulation of soluble TNF-α following incubation of DP cells in the presence of RP 7.5, DT (25 μg/ml), LPS (2.5 μg/ml) or plate-bound anti-CD3 mAb (2C11, 2.5 μg/ml). All results are representative of a minimum of two experiments.

### 2.3 Mechanism of apoptosis induced by sex steroids

The simplest explanation for the effect of DT would be that it stimulated DP thymocytes to produce TNF-α, which then kills them by fratricide our suicide. To test this hypothesis, we investigated expression of membrane and soluble forms of TNF-α in control and stimulated DP cells. Several reports have shown that TNF-α is processed from the membrane-bound precursors into a soluble effector form by a metalloproteinase TACE [19, 20]. However, other evidence has indicated that the membrane form of TNF-α can also have marked effects *in vivo* [21]. We found that both forms were induced by DT. Cell surface staining indicated that DT was at least as potent as anti-CD3 mAb, a typical inducer of TNF-α. Another known TNF-α inducer, LPS, was slightly less effective at inducing this TNF-α form (not shown). Furthermore, DT induced a threefold increase in soluble TNF-α secretion, which is again equal or superior to other perennial TNF-α inducers (Fig. 3 B and data not shown). Therefore, TNF-α induction is likely to contribute to the apoptotic effect of DT.

TNF-α can induce only low levels of apoptosis in otherwise unstimulated thymocytes [22]. Furthermore, anti-TCR-induced DP thymocyte apoptosis is not TNF dependent, despite a large induction of TNF-α [18, 23, 24]. It was, therefore, plausible [17] that DT might also facilitate DP apoptosis by other means. To address this possibility directly, recombinant murine TNF-α (rTNF-α) was added to DP thymocytes from TNF-$\alpha^{-/-}$ mice in the presence or absence of DT. DP cells from TNF-$\alpha^{-/-}$ mice did not undergo apoptosis in the presence of either DT or rTNF-α alone. Remarkably, TNF-$\alpha^{-/-}$ DP thymocytes underwent significant apoptosis in the presence of rTNF-α when incubated simultaneously with DT (Fig. 4). These data indicate that DT induces DP apoptosis by

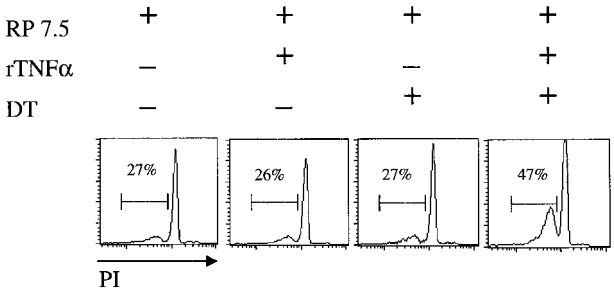


*Fig. 4.* DT sensitizes DP thymocytes to TNF-α. DP thymocytes from TNF-$\alpha^{-/-}$ mice were incubated either individually or simultaneously with 10 ng/ml rTNF-α and 25 μg/ml DT. Dexamethasone was used as positive control. Apoptotis was assessed as in Fig. 1 A. Results are representative of two experiments.

two simultaneous effects: by inducing TNF-α secretion and by sensitizing DP thymocytes to the apoptotic effect of TNF-α.

### 2.4 Resistance of TNF-$\alpha^{-/-}$ DP cells to testosterone treatment *in vivo*

To evaluate the physiological relevance of these findings *in vivo*, we treated normal and TNF-$\alpha^{-/-}$ mice with DT. Cell numbers and subset make-up were evaluated under two treatment regimens. In the acute injection protocol, a single injection of DT induced progressive loss of wild-type DP thymocytes over the course of the experiment (72 h; Fig. 5 A). Strikingly, no such loss was evident among thymocytes from TNF-$\alpha^{-/-}$ mice (Fig. 5 A). We also treated both types of mice chronically by injecting them with DT every other day for 2 weeks and then evaluating the resistance of DP thymocytes to such treatment. Upon such chronic treatment, testosterone induced profound depletion of normal DP thymocytes, reducing their percentage to 9.5 % of the control levels (Fig. 5 B). By contrast, DP thymocytes from TNF-$\alpha^{-/-}$ mice exhibited significantly ($p \geq 0.05$ by a two-tailed Student's *t*-test) increased survival when challenged with testosterone (34.7 % cells survived testosterone treatment, a 3.6-fold increase over the survival of normal thymocytes). Both the compensatory mechanisms that frequently develop in K.O. mice and the prolonged nature of treatment may have contributed to the fact that this resistance was not as absolute as the resistance of the DP cells from TNF-$\alpha^{-/-}$ mice to acute DT stimulation *in vitro* and *in vivo* (Figs. 2 and 5 A). Alternatively, it is possible that the TNF-α-mediated signaling is only one of the several apoptotic pathways induced by sex steroids. However, our results indicate that this is the key pathway under physiological conditions in an unmanipulated thymus. Based on these results, we conclude that TNF-α plays a prominent, if not critical, role in the apoptosis of DP thymocytes induced by sex steroids *in vivo.*

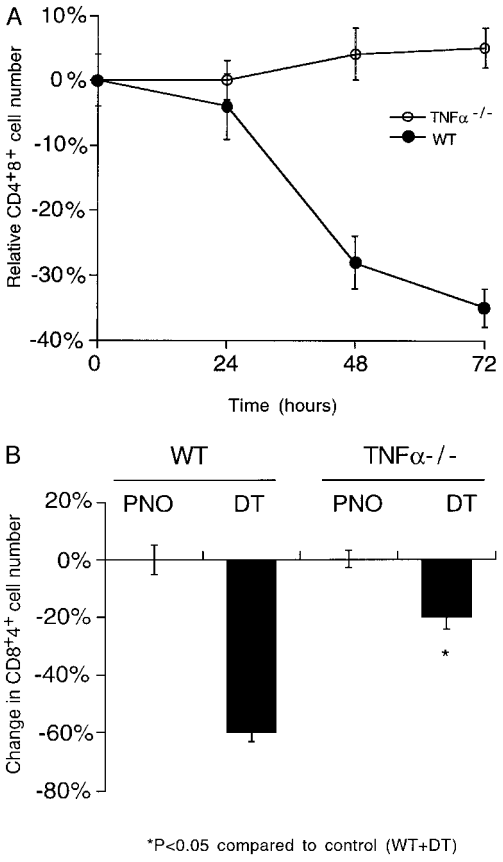


*Fig. 5.* DP thymocytes of TNF-$\alpha^{-/-}$ mice display increased resistance to both acute and chronic testosterone stimulation *in vivo*. (A) Control and TNF-$\alpha^{-/-}$ mice were injected with a single dose of 0.5 mg/100 g body weight DT/PNO or PNO alone, and thymocytes examined at indicated times for the reduction of the DP compartment as described in Fig. 1 A. (B) Three mice/group were injected with PNO or with DT s.c., every other day. After 14 days, thymi were harvested, counted and analyzed for the presence of DP cells. Data are presented as in Fig. 1 B. Absolute mean numbers of DP thymocytes in this experiment were $53.2 \times 10^6$ and $5.1 \times 10^6$ for the normal mice, and $39.8 \times 10^6$ and $13.9 \times 10^6$ for the TNF-$\alpha^{-/-}$ mice, for the PNO- (cntrol) and DT-treated groups, respectively. * $p \geq 0.05$ by a two-tailed Student's *t*-test.

## 3 Discussion

Developing thymocytes readily undergo apoptosis in response to not only TCR stimulation, but also when stimulated via β-adrenergic/cAMP, corticosteroid and other pathways. These pathways likely converge at a downstream point(s) at or close to the irreversible cas-

pase activation [1]. The precise signaling mechanism(s) have not been dissected for any of the thymocyte apoptosis pathways. This failure can be blamed in part on the redundancy of apoptosis pathways in the thymus. For example, a general redundancy in pathways signaling TCR-mediated negative intrathymic selection was recently documented [25, 26], with FasR-FasL being one of the dispensable backup pathways operating at high self-antigen doses [27].

Here, we conclusively linked testosterone-induced apoptosis to the TNF-α effector cascade. The major conclusion from this study is that TNF-α acts as one of the main mediators of sex steroid-induced apoptosis in normal DP thymocytes. Treatment of normal and TNF-$\alpha^{-/-}$ mice with testosterone (Fig. 5) confirmed the relevance of our findings *in vivo*. Furthermore, the doses of DT used in our study were well within the range of DT doses used in substitutive pharmacotherapy of sex steroid-deficiency disorders. The fact that TNF-$\alpha^{-/-}$ mice do not have enlarged thymi and that many of their DP cells die when stimulated with testosterone for prolonged periods of time (albeit at a strikingly lower rate than DP cells of normal mice) most likely reflects the action of compensatory mechanisms that are frequently revealed in K.O. animals. In normal mice or in TNF-$\alpha^{-/-}$ mice acutely treated with DT, where such mechanisms are not operative, antibody-mediated or genetic TNF-α neutralization completely abrogates DT-mediated apoptosis (Fig. 2). An alternative explanation is that testosterone stimulation of normal thymocytes also triggers other signaling pathways that induce apoptosis in a TNF-independent manner. Regardless of the existence and the importance of these pathways, the main conclusion of this work remains that TNF-α plays a very prominent, and probably critical, role in mediating DT-induced apoptosis of DP cells *in vivo*. Previously, Screpanti et al. [9] showed that another typical sex steroid, estrogen (17-β-estradiol), induced apoptosis among immature thymocytes [9]. In the context of the extensive sympathic innervation of the thymus, the presence of neurohumoral receptors and high thymic cAMP cellular content [28–32] and the recent findings on the effects of cAMP on thymocyte development [17, 33, 34], these results elucidate an important molecular mechanism that explains the negative influence of the neurohumoral axis on thymocyte production/output. Our recent findings [17] on the mechanisms of cAMP-induced thymocyte apoptosis further suggest that in inducing DP thymocyte apoptosis, sex steroids may act primarily via cAMP, and that this signaling axis promotes TNF-α susceptibility in DP thymocytes, likely by degrading cellular pools of the anti-apoptotic molecule TRAF-2. Thus, TNF-α is an important regulator of not only peripheral [35, 36] but also thymic homeostasis.

The most dramatic manifestations of sex steroid influence on thymic cellularity include the well-known phenomena of thymocyte depletion following stimulation with the sex steroids in pregnancy and menstrual cycle/estrus and of thymocyte hypercellularity following experimental and pharmacological castration (reviewed in [7]). Our results clearly point toward possible routes of intervention against such immunological disturbances.

## 4 Materials and methods

### 4.1 Mice

C57BL/6 (B6) mice were obtained from the NCI Breeding Program (Frederick, MD). TNF-$\alpha^{-/-}$ mice were described previously [18]. All mice were used at 6–10 weeks of age.

### 4.2 Thymocyte preparation, activation and apoptosis detection

All experiments were performed in RPMI 1640 medium supplemented with pyruvate, 2-ME, L-glutamine, antibiotics and 7.5 % FBS (abbreviated RP 7.5 in the text). DP thymocytes were enriched from total thymocytes by "panning" using anti-CD8 mAb-coated petri dishes, as described previously [37]. DP thymocytes were treated in 24-well flat-bottom plates with DT (Sigma Chemical Co., St. Louis, MO) at indicated concentrations, or with immobilized anti-CD3 mAb 2C11 (Pharmingen, San Diego, CA) at an optimal, pretitrated concentration (2.5 μg/ml coating solution) or with $10^{-7}$ M dexamethasone (Upjohn, Kalamazoo, MI), and apoptosis detected by propidium iodide (PI) or Annexin V staining, as described below.

Neutralizing anti-mouse TNF-α mAb was obtained from Pharmingen (clone MP6-XT22) or from Dr. O. Stutman (MSKCC-mAb 2E2, [38]). Following overnight incubation, apoptosis was assessed by quantifying the percentage of hypodiploid nuclei undergoing DNA fragmentation using PI staining of permeabilized cells [39], or by monitoring the inversion of phosphatidylserine to the outer leaf of the plasma membrane by Annexin V staining in the presence of PI (performed according to the manufacturer's instructions; TACS Annexin V-FITC kit, Trevigen, Gaithersburg, MD). Flow cytometric analysis was performed using a FACScan flow cytometer and CellQuest 3.1 software (Becton Dickinson, Mountain View, CA), by analyzing at least $5 \times 10^3$ cells/sample, using wide scatter gates to include late apoptotic cells. For the sake of simplicity, results of Annexin V experiments are reported as a percentage of total Annexin$^+$ cells, of which 7.6–18.7 % was also PI$^+$ (*i.e.* late apoptotic or necrotic), while the remaining cells were early apoptotic. Mean values of at least three samples/group are reported ± SD, representative of at least two independent experiments.

### 4.3 ELISA for the detection of secreted TNF-α

A TNF-α capture ELISA was used to detect secreted TNF-α in cell culture supernatants from DP thymocytes incubated in the presence or the absence of 25 μg/ml DT or 2.5 μg/ml of plate-bound mAb 2C11 (18 h at 37 °C), or 2.5 μg/ml LPS (Sigma), respectively. Wells of polyvinyl chloride plates (Immulon 4; Fisher Scientific, Pittsburgh, PA) coated with 10 μg/ml of mAb anti-TNF-α (PharMingen, San Diego, CA) in carbonate coating buffer (pH 9.6) and blocked with 2 % FCS PBS, were incubated with 200 μl of cell culture supernatant. The plates were then washed and an optimal concentration of biotinylated anti-TNF-α polyclonal antibody (PharMingen, San Diego, CA) added and incubated for 1 h at 37 °C. Bound TNF-α was detected using avidin-conjugated horseradish peroxidase (Pierce, Rockford, IL), developed using the substrate σ-phenylediamine dihydrochloride. The developing reaction was stopped by adding 50 μl of 3 M $H_2SO_4$, and the absorbance at 490 nm was analyzed by a MCC/340 Multiskan microplate reader (Fisher Scientific). The assay was carried out in quadruplicates. Results are reported as mean values (± SD).

### 4.4 *In vivo* treatment with testosterone

Mice were treated with DT (testosterone 17β-cypionate, cat. no. T0141, Sigma) in sterile peanut oil (PNO) or with PNO alone, injected s.c. at 0.5 mg/100 g body weight, as described previously [13]. At indicated times, thymus cellularity and make-up of thymic subsets were analyzed by trypan blue exclusion counting and FCM, respectively. Results are shown as mean values (four mice/group) of DP thymocytes, expressed as percentage relative to control DP numbers from PNO-treated mice. These were obtained by calculating the absolute number of DP cells in control-treated and experimental group thymi (absolute number of thymocytes x % DP cells among them), with control values being taken as 100 %.

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