Neonatal castration affects intrathymic kinetics of T-cell differentiation and the spleen T-cell level

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Abstract

To test putative interdependence in the ontogenesis of the hypothalamic–pituitary–gonadal and thymic–lymphatic axes, thymocyte differentiation and maturation was examined in neonatally castrated (Cx) adult rats. In the hypercellular thymi of Cx rats, the proportion of the least mature $CD4^-CD8^-TCR\alpha\beta^-$ triple negative (TN) thymocytes was reduced, while the proportions of all downstream double positive (DP) subsets ($TCR\alpha\beta^-$, $TCR\alpha\beta^{low}$ and $TCR\alpha\beta^{high}$) were increased when compared with neonatally sham-castrated (Sx) adult rats. This suggested an accelerated thymocyte transition from the TN to DP $TCR\alpha\beta^{low}$ developmental stage accompanied by an increased positive/reduced negative thymocyte selection. The increased thymocyte surface density of Thy-1, which is implicated in thymocyte hyposensitivity to negative selection, in Cx rats further supports the previous assumption. The finding that the proportions of both single positive (SP) $TCR\alpha\beta^{high}$ thymocyte subsets were reduced, while their numbers were increased ($CD4^+CD8^-$) or unaltered ($CD4^-CD8^+$), coupled with results demonstrating an increased level of $CD4^-CD8^+$ cells without changes in that of $CD4^+8^-$ cells in the spleen indicate: (i) accelerated differentiation and maturation of the positively selected DP $TCR\alpha\beta^{high}$ thymocytes towards $CD4^-8^+$ $TCR\alpha\beta^{high}$ cells followed by increased emigration of the mature cells and (ii) decelerated differentiation and maturation towards $CD4^+8^-$ $TCR\alpha\beta^{high}$ cells in Cx rats. Furthermore, the unaltered proportion of intrathymically developing $CD4^+CD25^+Foxp3^+$ regulatory cells in Cx rats, in light of putative hyposensitivity of thymocytes to negative selection suggesting reduced elimination of autoreactive cells, may provide a firm basis for understanding the reasons behind increased susceptibility of Cx rats to autoimmune disease induction.

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Introduction

In its simplest form, effective T-cell-mediated immunity emanates from the expansion of specific T-cells activated in response to antigen. Therefore, any manipulation that changes the levels of peripheral T-cells and/or affects their activation may interfere with the host's ability to efficiently respond to antigen challenge.

In establishing and maintaining the peripheral T-cell pool, the thymus plays a critical role providing a microenvironment within which the T-cell precursors proliferate, differentiate and undergo selection processes to create a fully functional population of major histocompatibility complex (MHC)-restricted, self-tolerant T-cells (Shortman *et al.* 1991, Zamoyska & Lovatt 2004). The intrathymic differentiation of conventional T-cell receptor (TCR) αβ cells proceeds via a series of distinct maturation steps, each characterised by a unique pattern of gene expression and a specific array of cell surface proteins, including the TCRαβ–CD3 complex, CD4 and CD8. Upon entry into the thymus,

T-cell precursors express neither the TCRαβ–CD3 complex nor the accessory molecules (CD4 and CD8), i.e. they are triple negative (TN). As differentiation proceeds, they acquire both accessory molecules becoming $CD4^+CD8^+$ double positive (DP). At this stage, TCRαβ–CD3 is expressed at a low level on their cell membrane and as a consequence the cells become eligible for both positive and negative selection (Zamoyska & Lovatt 2004). Thymocytes expressing functional TCRαβ–CD3 interact with peptides presented by MHC on the membranes of non-lymphoid cells, and if the resulting avidity of MHC/peptide–TCR interactions reaches threshold level, the thymocytes receive survival signals and further increase TCRαβ–CD3 expression (positive selection). However, if the avidity is too high or too low, the thymocytes are functionally inactivated or deleted by apoptosis (negative selection) or die by neglect respectively. The cells that survive thymic selection further differentiate to either $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) mature cells and leave the thymus (Zamoyska & Lovatt 2004).

It has been suggested that androgens generally blunt the T-cell-dependent immune response. In the absence of androgens, the opposite effect is observed (Olsen & Kovacs 1996, Verthelyi 2001). Although androgens have been implicated as negative modulators of host immunity, the precise mechanism(s) responsible for the bolstering effect of androgen deprivation on host immunity remain(s) to be fully determined. It is assumed that androgens most likely exert their effects on thymocytes, rather than on mature T-effector cells, because androgen receptors (ARs) have not been detected in peripheral organs of the immune system (Kovacs & Olsen 1987, Takeda *et al.* 1990), but are present in rodent and human thymi (Sasson & Mayer 1981, Kovacs & Olsen 1987, Viselli *et al.* 1995). Furthermore, it has been suggested that androgens, besides their direct effects on the thymus via ARs, may exert indirect effects on the thymus via negative feedback action on gonadotrophin-releasing hormone (GnRH) and pituitary gonadotrophin secretion (Rouabhia *et al.* 1989).

Androgen removal by castration before puberty increases thymic weight throughout puberty in rodents, while androgen administration prevents castration-induced thymic hypertrophy (Utsuyama & Hirokawa 1989, Windmill *et al.* 1993, Leposavić *et al.* 1995). Furthermore, changes in thymus weight and its cellularity produced by alterations in the circulating androgen level are followed by alterations in the distribution of thymocyte subsets (Aboudkhil *et al.* 1991, Olsen *et al.* 1991, Windmill *et al.* 1993, Leposavić *et al.* 1995) which differ depending on their maturational stage at the point of hormone deprivation and its duration (Utsuyama *et al.* 1995).

However, despite the facts indicating that: (i) in rodents, the sexually dimorphic organisation of hypothalamic centres controlling gonadotrophin secretion arise as a result of the presence or the absence of androgens during the first 5 postnatal days (Beyer 1987) and (ii) gonadal hormones may be essential, timely and critical agents which in a sexually dimorphic manner, render immune cells capable of modifying their intrinsic potential, especially by their ability to differentiate (Pierpaoli *et al.* 1977, Morale *et al.* 1991), data within the literature concerning the effects of neonatal androgen ablation on the thymus and the T-cell-dependent immune response are extremely limited. We found that neonatal castration produced an increase in both thymic weight and cellularity in adult rats and hypothesised that the androgen status during the early postnatal period may be critical for programming postnatal thymic development (Leposavić *et al.* 1995). To test this hypothesis, the thymocyte subset composition was examined in neonatally castrated (Cx) rats to: (i) ascertain whether thymic hyperplasia in neonatally Cx rats (Leposavić *et al.* 1995) involved a global increase across T-cell development or targeted-specific enhancing points leading to depletion of cells at one particular stage and accumulation of others beyond that stage and (ii) visualise effects of early postnatal gonadal ablation on the levels of T-cell subsets within the periphery.

Materials and Methods

Animals

Ten pregnant AO rats, born and maintained in the vivarium belonging to the Immunology Research Centre 'Branislav Janković' in Belgrade, were selected for the present study. The date of birth was designated as 'postnatal day 0'. The litter size was equated to six animals by cross-fostering pups. The litters and their mothers were raised in a temperature- and light-controlled animal room. Laboratory chow and tap water were available *ad libitum*. The rats were weaned at postpartal day 21. Male offspring were assigned randomly for castration (Cx rats) or sham castration (Sx rats).

Experimental protocol

Rats were castrated at postnatal day 3 using refrigerating anaesthesia. Bilateral castration was performed via a transverse scrotal incision allowing exposure of the testicles and transection of spermatic cords. Sham castration followed the same procedure except that pericordal fat was resected *in lieu* of the testicles. Three months after surgery, both Cx and Sx rats were killed, their thymi and spleens were carefully removed and freed from extraneous tissue, weighed and appropriately processed for flow cytometric analysis (FCA). All animal experimentation was conducted under local ethical guidelines and approved by the Institutional Animal Care and Use Committee.

Each thymus was aseptically isolated, trimmed of all excess body fat and gently blotted onto sterile gauze to remove excess blood. The thymus and spleen tissues were dissociated by gently grinding the tissue on a sterile 60 µm sieve screen in complete RPMI-1640 medium. Erythrocytes were lysed by the addition of an isotonic solution of ammonium chloride (warmed to room temperature) to spleen cell samples in a volume ratio of 1:5. The resulting cell suspensions were washed thrice in ice-cold complete RPMI-1640 medium. The cells were then counted in an enhanced Neubauer haemocytometer and the number of cells per thymus (spleen) was estimated. The viability of such cell preparations, determined by Trypan blue exclusion, was routinely >95%.

Serum testosterone levels

Blood was taken from 3-day-old non-castrated rat pups and 3-month-old Sx rats by cardiac puncture. Thereafter, the serum was separated and stored at −20 °C until the RIA was performed. Serum testosterone was quantitated (in duplicate) using a commercial testosterone kit (INEP-DIJAGNOS-TIKA, Zemun, Serbia). The RIA procedures were carried out according to the guidelines provided by the kit producer. Testosterone levels of 0.3 ± 0.01 ng/ml (mean $\pm$ S.E.M.) and 2.60 ± 0.5 ng/ml (mean $\pm$ S.E.M.) were detected in sera from 3-day-old non-castrated rat pups and 3-month-old Sx rats

respectively. However, in 3-month-old Cx rats testosterone levels were below the level of detectability (0·2 ng/ml).

Chemicals, antibodies and immunoconjugates

Sodium azide, merocyanine (MC) 540 and concanavalin A (ConA) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Dexamethasone (Dex) was obtained from Galenika (Zemun, Serbia).

RPMI-1640 powdered medium (Sigma–Aldrich) was dissolved in redistilled water according to the manufacturer's instructions. To prepare complete RPMI medium, 2 mM L-glutamine (Serva, Heidelberg, Germany), 1 mM sodium pyruvate (Serva), 100 units/ml penicillin (ICN, Costa Mesa, CA, USA), 100 µg/ml streptomycin (ICN) and 10% foetal calf serum (FCS; Gibco) were added. FCS was previously inactivated by heating the serum at 56 °C for 30 min.

For staining, the following first-step monoclonal antibodies (mAbs) were used: phycoerythrin (PE)-conjugated anti-CD4 (clone W3/25, Serotec, Oxford, UK), fluorescein-isothiocyanate (FITC)-conjugated anti-CD8 (clone MRC OX-8, Serotec), peridinin chlorophyll protein (PerCP)-conjugated anti-TCRαβ (clone R73, BD Biosciences Pharmingen, Mountain View, CA, USA), biotin-conjugated anti-CD25 (clone MRC OX-39, Serotec), PE-conjugated anti-CD90 (Thy-1.1; clone HIS 51, BD Biosciences Pharmingen) and biotin-conjugated anti-CD3 (clone G4.18, BD Biosciences Pharmingen). The following second-step reagents were used: streptavidin–PerCP and streptavidin–PE. Appropriate IgG isotype controls were obtained from BD Biosciences Pharmingen.

Phenotyping lymphocytes by FCA

Detection of surface markers Staining of thymocytes and splenocytes was performed as previously described (Leposavić *et al*. 2005). Briefly, aliquots of 1×10^6 lymphoid cells (thymocytes or splenocytes rendered erythrocyte free) in 100 µl RPMI-1640 medium were centrifuged at 350 ***g*** for 5 min at 4 °C to yield a pellet. The cells were incubated for 30 min on ice in the dark with fluorochrome-conjugated mAbs (direct staining) or with biotin-conjugated mAbs and fluorochrome-conjugated streptavidin (indirect staining) before being washed thrice using RPMI-1640 medium. When biotin-conjugated mAbs were used, the cells were incubated again for 30 min on ice with the appropriate fluorochrome-conjugated streptavidin. After staining, the cells were washed twice using RPMI-1640 medium and then in ice-cold PBS (pH 7·4) containing 0·09% sodium azide. Twenty thousand cells per sample were analysed on the same day using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Non-specific IgG isotype-matched controls were used for each fluorochrome type to define background staining, while dead cells and debris were excluded from analysis by selective gating based on anterior and right-angle scatter. The percentage of positive cells for

each staining was determined using CellQuest Software (Becton Dickinson). Final estimates of marker-positive lymphocytes within each lymphoid organ were calculated according to the following formula:

$$[(\text{cells in suspension/ml} \times \text{total suspension volume})$$
$$\times \text{percentage of marker} - \text{positive lymphocytes}] \times \frac{1}{100}$$

In addition, CD90 expression on thymocytes was analysed by estimation of the mean channel number that represents the density of the surface marker expression. The mean channel number was determined for thymocytes from both Cx and Sx rats. The relative change in the mean fluorescence intensity (MFI) was calculated according to the following formula:

$$\frac{(\text{MFI of the histogram of Cx rats} - \text{MFI of the histogram of Sx rats}) \times 100}{\text{MFI of the histogram of Sx rats}}$$

(Kamath *et al*. 1998).

Detection of intracellular markers After CD4 and CD25 staining, which was performed according to the surface staining protocol, thymocytes were subjected to staining for Foxp3 using a commercial Foxp3 staining kit (eBioscience, San Diego, CA, USA) containing FITC-conjugated anti-Foxp3 mAbs (clone FJK-16s) according to the manufacturer's instructions. Lymphocytes from inguinal lymph nodes were used as positive controls to validate Foxp3 reactivity. Twenty thousand cells per sample were analysed using the FACScan flow cytometer and CellQuest Software.

Detection of apoptotic thymocytes

Since apoptotic thymocytes are normally rapidly eliminated by phagocytes *in vivo*, the relative number of apoptotic cells was quantified after 18 h of cultivation. This time point was elected because it allows an accurate assessment of apoptosis with acceptable background values (Kamath *et al*. 1998). Aliquots of 100 µl freshly isolated thymocytes in complete RPMI-1640 medium (5×10^6 cells/ml) were plated in 96-well flat-bottom plates (Nunc A/S, Roskilde, Denmark). To these cells, 100 µl complete medium containing Dex were added to attain a final concentration of 100 nM Dex, a dose known to induce apoptosis of rat thymocytes *in vitro* (Brown *et al*. 1993). In control cultures, 100 µl complete medium were added. After 18 h of culture, the cells were harvested and apoptotic thymocytes were detected using MC540 lypophilic dye. Similar to annexin-V, MC 540 labels a decreased packing order of phospholipids in the outer leaflet of the apoptotic cell plasma membrane. The percentage of apoptotic cells labelled with MC540 has been shown to be equivalent to that obtained by annexin-V labelling (Laakko *et al*. 2002).

The staining was performed according to the procedure described by Mower *et al*. (1994). Briefly, just before FCA 5 µl of 1 mg/ml MC540 solution in double-distilled H_2O

was added to 1 ml thymocyte suspension containing 10^6–10^7 cells. All samples were analysed using a FACScan flow cytometer and CellQuest Software. According to the intensity of MC540 fluorescence and forward scatter (Cohen 1991), two subsets of apoptotic cells can be distinguished: (1) cells in early apoptosis and (2) cells in advanced/late apoptosis. Cells in early apoptosis exhibit high level of MC540 staining, whereas those in advanced/late apoptosis show lower levels of MC540 staining and lower forward scatter.

Detection of bromodeoxyuridine (BrdU)-positive (BrdU$^+$) cells

BrdU incorporation was employed to identify DNA-synthesising cells *in vitro*. A total of 2×10^5 thymocytes/well (100 μl) were dispersed into plastic 96-well plates (Nunc A/S) and cultured for 48 h at 37 °C in a 5% CO_2 humified air atmosphere without (ConA-) or with 2·5 μg/ml ConA (ConA+) in a total volume of 200 μl complete RPMI-1640 culture medium. Since it has been shown that in thymocyte cultures stimulated with ConA, there is significant interleukin (IL)-2 production and upregulation of the IL-2 Rα providing the second proliferative signal (Čolić *et al.* 2000) ConA was added alone. All cultures were performed in triplicate. During the last 18 h of culture, the cells were pulsed using 1 μM BrdU. BrdU incorporation into cells was detected using the BrdU/7-AAD flow kit (BD Biosciences Pharmingen). The 7-AAD staining enabled the detection of the late apoptotic/necrotic hypodiploid cells and subsequently their exclusion from the analysis. To determine the phenotypic characteristics of the BrdU$^+$ cells, staining with FITC-conjugated anti-BrdU Abs and 7-AAD was combined with staining with biotin-conjugated anti-CD3 mAbs as a first-step reagent and streptavidin–PE as a second-step reagent. The staining was performed according to the BrdU/7-AAD flow kit producer's manual. All samples were analysed using a FACScan flow cytometer and CellQuest Software.

Statistical analysis

To assess the significance of differences between groups, the Mann–Whitney U test was applied using the programme SPSS 10.0 for Windows. $P \leq 0.05$ was considered significant.

Results

Neonatal castration increases thymic weight and cellularity in adult rats

In adult Cx rats, both absolute and relative (ratio to body weight) thymic weights were significantly increased compared with those in age-matched Sx controls (Table 1). Furthermore, thymi from Cx rats contained a significantly greater number of thymocytes than those from Sx rats and the relative cellularity of these thymi (ratio to body weight) was significantly augmented compared with those from Sx rats (Table 1).

Neonatal castration diminishes the percentage of apoptotic cells in thymocyte cultures from adult rats

To assess the putative contribution of alteration in thymocyte apoptosis to thymic hypercellularity in Cx rats, the percentage of apoptotic cells in thymocyte cultures was measured. Figure 1 indicates that Dex significantly augmented the percentage of apoptotic cells in thymocyte cultures from both Cx and Sx rats. However, regardless of the presence of Dex, the percentage of apoptotic cells was lower in thymocyte cultures from Cx rats compared with those from Sx rats. In both Dex+ and Dex− thymocyte cultures, the decrease in the percentage of apoptotic cells reflected a drop in the relative number of cells in the early phase of apoptosis. Therefore, our results clearly indicated that androgen hormone deprivation rendered thymocytes less prone to induction of apoptosis.

Neonatal castration increases the percentage of BrdU$^+$ cells in the absence but not in the presence of ConA in thymocyte cultures from adult rats

To further clarify the mechanism(s) responsible for the increased cellularity of thymi from Cx rats, the overall frequency of BrdU$^+$ thymocytes and the frequency of BrdU$^+$ cells within thymocyte subsets defined by surface density of CD3 (CD3$^-$, CD3^{low} and CD3^{high}) were examined in ConA− and ConA+ thymocyte cultures. In the absence of ConA, the percentage of BrdU$^+$ cells was significantly greater in thymocyte cultures from Cx rats than in cultures from Sx rats (Fig. 2A). ConA significantly

Table 1 Increased absolute and relative thymic weight as well as total and relative thymocyte number in 3-month-old neonatally castrated rats (Cx) compared with age-matched sham-castrated rats (Sx)

	Absolute thymus weight (g); median (25th–75th percentiles)	Relative thymus weight (g/100 g bw); median (25th–75th percentiles)	Total thymocyte number ($\times 10^7$)/thymus; median (25th–75th percentiles)	Relative thymocyte number ($\times 10^7$)/100 g bw; median (25th–75th percentiles)
Treatment				
Sx ($n=8$)	0·38 (0·34–0·44)	0·18 (0·16–0·19)	41·00 (36·00–49·50)	18·13 (16·78–22·25)
Cx ($n=6$)	0·70 (0·58–0·74)*	0·34 (0·27–0·36)*	78·00 (65·50–108·00)*	35·74 (32·40–52·11)*

The table indicates data from a single experiment. Very similar data were obtained in an identically performed independent experiment. *$P<0.001$; bw, body weight.

increased the percentage of BrdU$^+$ cells in thymocyte cultures from both Cx and Sx rats (Fig. 2A) in such a way that the percentage of BrdU$^+$ cells did not significantly differ between thymocyte cultures from Cx and Sx rats.
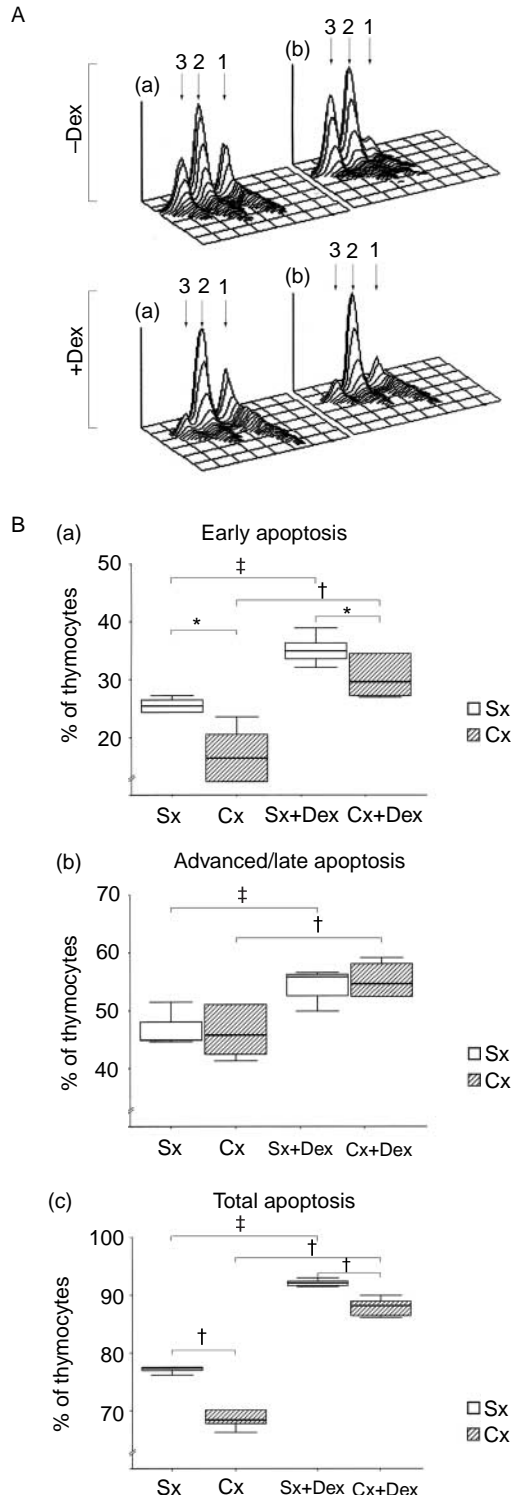

Irrespective of the presence of ConA in thymocyte cultures from both Cx and Sx rats, BrdU$^+$ cells were found in all subsets of cells defined by CD3 expression. As expected, since ConA has been shown to act on immature and mature T-cells by TCR cross-linking (Pongracz *et al.* 2003) causing survival and further maturation of the former and proliferation of the latter, in thymocyte cultures from both Cx and Sx rats, a significant increase in the percentage of BrdU$^+$ cells within CD3^{low} and CD3^{high} cell subset was found (Fig. 2B). Irrespective of the ConA presence, the percentage of BrdU$^+$ within none of the cell subsets defined by CD3 expression significantly differ between Cx and Sx rats (Fig. 2B).

Neonatal castration affects distribution of thymocyte subsets delineated by expression of CD4/8/TCRαβ

Figure 3 indicates that thymocyte suspensions from both Cx and Sx rats displayed three distinct surface levels of the TCRαβ–CD3 complex by fluorescent staining with R73 mAbs: (i) high (TCRαβ^{high}), (ii) low (TCRαβ^{low}) and (iii) an undetectable level (TCRαβ$^-$). With respect to the expression of CD4 and CD8 in combination with the level of TCRαβ expression, 12 subsets of thymocytes were delineated (Leposavić *et al.* 2005) and the relative and absolute numbers of the thymocytes belonging to each of them were quantified.

TCRαβ$^-$ subsets Neonatal castration led to profound changes in the relative numbers of TCRαβ$^-$ thymocyte subsets delineated by CD4/CD8 expression. The relative number of the least mature DN and that of CD4$^+$CD8$^-$ SP cells were significantly decreased. In contrast, the relative number of DP cells and that of CD4$^-$CD8$^+$ SP cells were significantly increased in Cx rats compared with Sx rats (Figs 3 and 4A).

Figure 1 Neonatal castration decreases the percentage of apoptotic cells in thymocyte cultures from 3-month-old adult rats castrated at postnatal day 3 (Cx). In 18-h cultured thymocytes from Cx rats and age-matched sham-castrated control rats (Sx), the percentage of apoptotic cells, in the presence of dexamethasone (+Dex) and in the absence of Dex (−Dex), was assessed using MC540 and flow cytometric analysis. (A) Three-dimensional plots of MC540-stained thymocytes in +Dex and −Dex cultures from Sx rats (a) and Cx rats (b). According to the intensity of MC540 thymocyte staining and forward scatter (FSC), two subsets of cells at distinct phases of apoptosis (early apoptosis and advanced/late apoptosis) were delineated. 1, early apoptosis; 2, advanced/late apoptosis and 3, alive cells. (B) Overall percentage of apoptotic cells in early (a) and advanced/late apoptosis (b) as well as total percentage of apoptotic cells (c) in thymocyte cultures from Cx and Sx rats. The results are presented as box plots with the central line representing the sample median and lower and upper borders of the box representing the 25th and 75th percentiles respectively. The lower and the upper lines indicate minimal and maximal values of the sample respectively. The figure indicates data from a single experiment performed with six Cx rats and eight Sx rats. Very similar data were obtained in an identically performed independent experiment. *$P<0.05$; †$P<0.01$, ‡$P<0.001$.
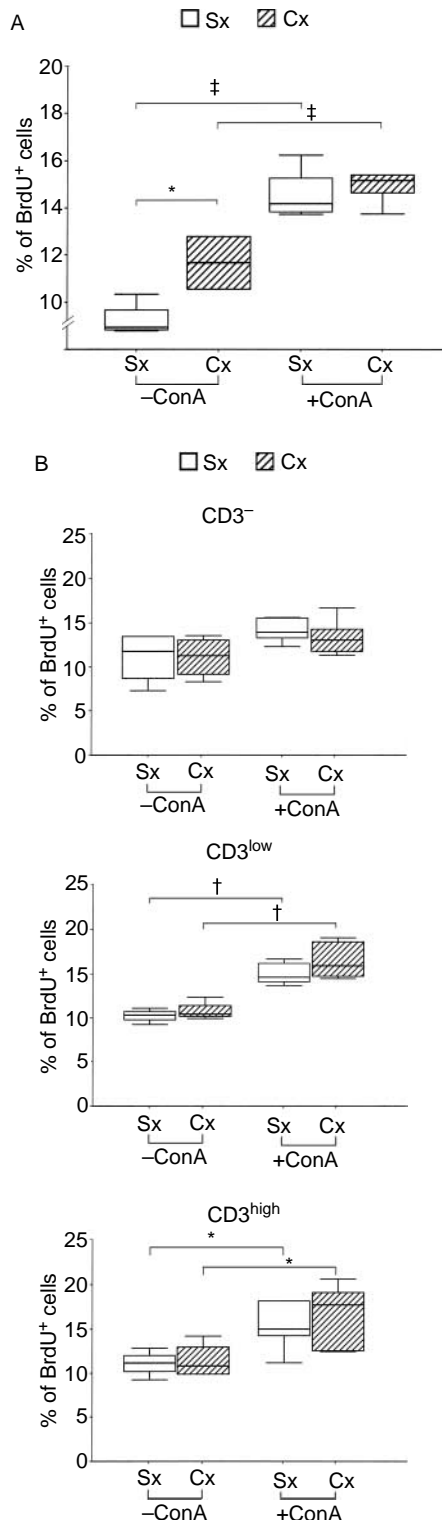
In Cx rats, due to a significant increase in thymocyte yield, the absolute cell number was increased across all TCRαβ⁻ subsets, except for the CD4⁺CD8⁻ subset, which was significantly decreased (Fig. 4B).

TCRαβ^low subsets In Cx rats, the percentage of DP cells, which are believed to represent the thymocyte subset entering the selection processes (Zamoyska & Lovatt 2004), was significantly increased in comparison with Sx rats (Figs 3 and 4A). The percentage of CD4⁻CD8⁺ SP cells, which as SP TCRαβ⁻ cells are believed to emerge along the developmental route from the DN TCRαβ⁻ to the DP TCRαβ^low stage (Petrie *et al*. 1990, Leposavić *et al*. 2005), was significantly increased, whereas that of CD4⁺CD8⁻ SP cells was significantly reduced (Figs 3 and 4A). The relative number of DN cells remained unaffected by neonatal castration (Figs 3 and 4A).

In Cx rats, numerical increases were found within all thymic subsets, except for the CD4⁺CD8⁻ subset which indicated a reduction (Fig. 4B).

TCR αβ^high subsets In Cx rats, the percentage of DP cells, which are believed to be at the intermediate stage of development between the DP TCRαβ^low and the most mature SP TCRαβ^high stage (Shortman *et al*. 1991, Zamoyska & Lovatt 2004), was significantly increased. However, the percentage of DP TCRαβ^high cell descendents CD4⁺CD8⁻ and CD4⁻CD8⁺ SP cells was significantly reduced in the thymus of Cx rats (Figs 3 and 4A). The percentage of cells belonging to the small subset of DN thymocytes did not differ between Cx and Sx rats (Figs 3 and 4A).

The absolute number of DP cells was significantly greater in Cx rats than in Sx rats (Fig. 4B). However, although the percentage of both CD4⁺CD8⁻ and CD4⁻CD8⁺ cells of SP thymocytes was significantly reduced, the absolute number of CD4⁺CD8⁻ SP cells was significantly increased, while that of CD4⁻CD8⁺ SP cells remained unaltered (Fig. 4B). The absolute number of DN cells did not significantly differ between Cx rats and Sx rats (Fig. 4B).

Figure 2 Neonatal castration increases the percentage of BrdU⁺ cells only in ConA-free thymocyte cultures. (A) Percentages of all BrdU⁺ cells in thymocyte cultures from 3-month-old adult rats castrated at postnatal day 3 (Cx) and age-matched sham-castrated control rats (Sx) and (B) percentages of BrdU⁺ cells within different cell subsets delineated according to the intensity of CD3 staining (CD3⁻, CD3^low and CD3^high) determined by flow cytometric analysis. The thymocytes were cultured in medium without ConA (−ConA) or with ConA (+ConA). The results are presented as box plots with the central line representing the sample median and lower and upper borders of the box representing the 25th and 75th percentiles respectively. The lower and the upper lines indicate minimal and maximal values of the sample respectively. The figure indicates data from a single experiment performed with six Cx rats and eight Sx rats. Very similar data were obtained in an identically performed independent experiment. *$P<0.05$, †$P<0.01$, ‡$P<0.001$.
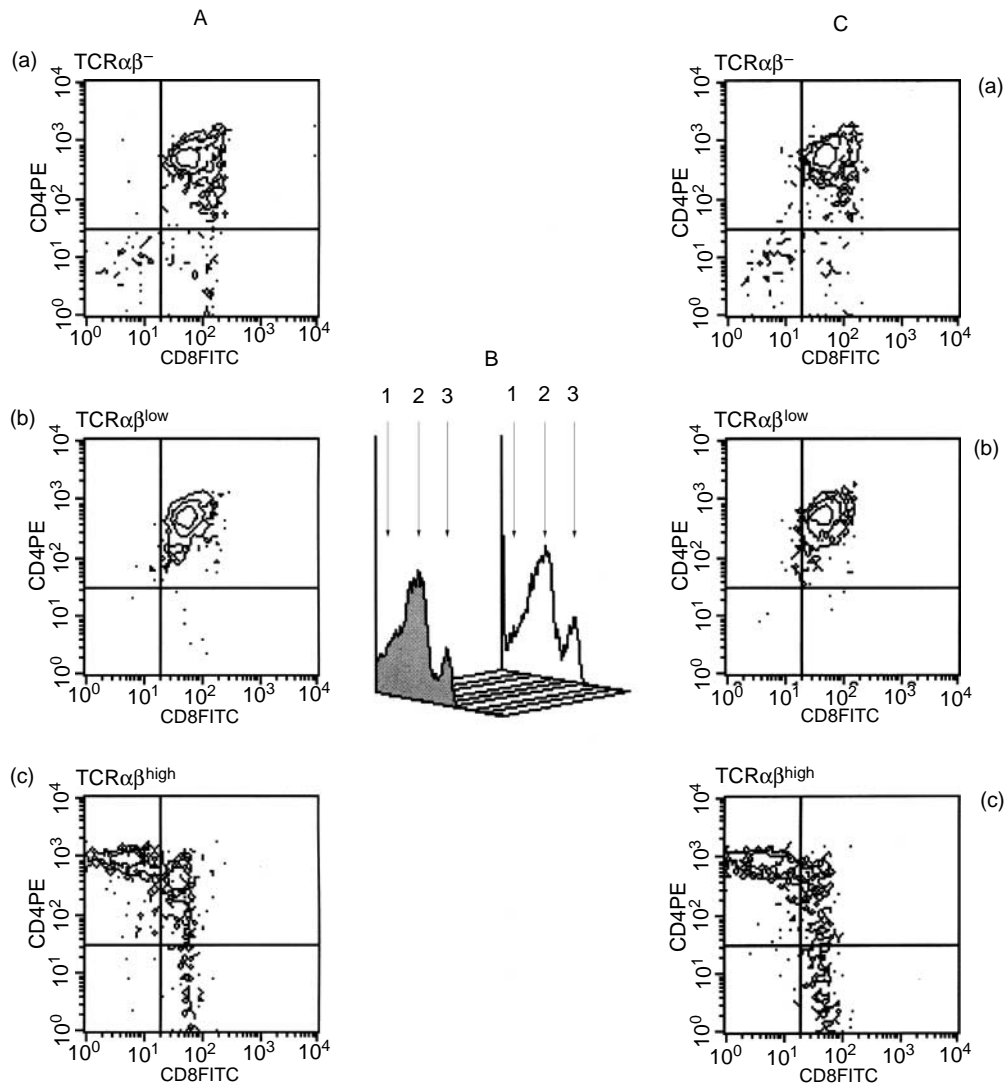
Figure 3 Three-colour flow cytometric analysis of thymocyte staining with anti-CD4PE, anti-CD8FITC and anti-TCRαβPerCP mAbs from 3-month-old adult rats castrated at postnatal day 3 (Cx) (A) and age-matched sham-castrated control rats (Sx) (C). In each panel, contour plots represent CD4 and CD8 expression on gated (a) TCRαβ⁻ (b) TCRαβ^{low} and (c) TCRαβ^{high} thymocytes. The histogram plot (B) represents flow cytometric profiles of TCRαβ staining of thymocytes from Cx rats (grey histogram) and Sx rats (white histogram). 1, TCRαβ⁻ cells; 2, TCRαβ^{low} cells and 3, TCRαβ^{high} cells. Representative plots (from repeated experiments) are shown in the figure.

Neonatal castration produces an increase in both the percentage of CD90⁺ thymocytes and density of thymocyte surface CD90 expression in adult rats

The expression of CD90 (Thy-1), which is believed to be a molecule involved in the negative regulation of TCRαβ signalling and thereby TCR–dependent selection thresholds (Hueber *et al.* 1997), on thymocytes was also examined. The percentage of CD90⁺ cells was significantly greater in Cx rats than in Sx rats. In addition, the MFI for CD90 was markedly elevated (196 ± 7%) in Cx rats compared with Sx rats (Fig. 5).

MFI indicates the density of antigen expression on a per cell basis and it has been shown that a 100% greater MFI represents a twofold greater surface antigen density (Kamath *et al.* 1998). Accordingly, the average density of CD90 expression was almost threefold greater in Cx rats compared with Sx rats.

We next analysed the expression of CD90 on TCRαβ^{low} thymocytes (encompassing mainly cortical DP cells) and on TCRαβ^{high} thymocytes. Although in Cx rats the relative number of both CD90⁺TCRαβ^{low} and CD90⁺TCRαβ^{high} cells remained unaltered (data not shown), the MFI for CD90 was strikingly greater on both TCRαβ^{low} ($199 \pm 7 \cdot 5$%) and
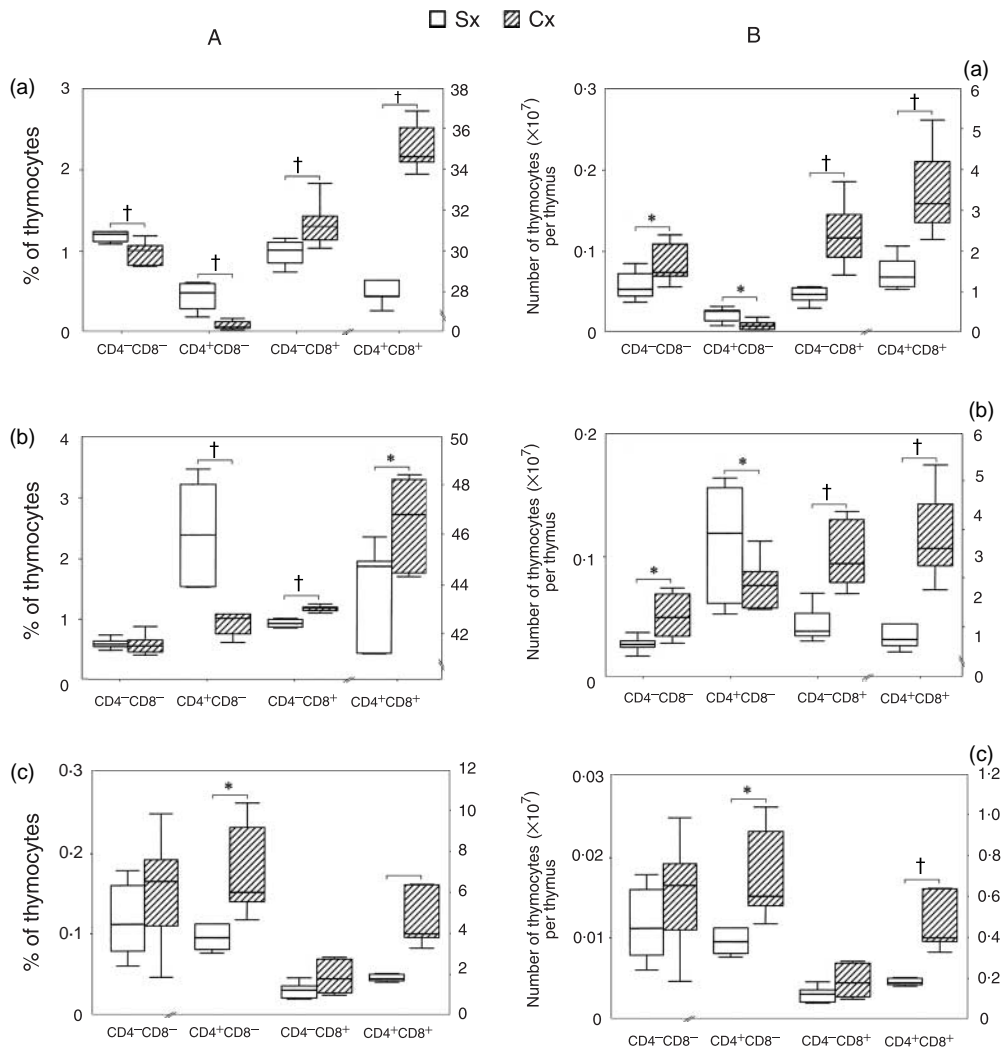
Figure 4 Neonatal castration affects the composition of thymocyte subsets in 3-month-old rats. Percentages (A) and absolute numbers (B) of TCRαβ$^-$ (a) TCRαβ^{low} (b) and TCRαβ^{high} (c) thymocyte subsets delineated by expression of CD4 and CD8 in 3-month-old adult rats castrated at postnatal day 3 (Cx) and age-matched sham-castrated control rats (Sx). The results are presented as box plots with the central line representing the sample median and lower and upper borders of the box representing the 25th and 75th percentiles respectively. The lower and the upper lines indicate minimal and maximal values of the sample respectively. The figure indicates data from a single experiment performed with six Cx rats and eight Sx rats. Very similar data were obtained in an identically performed independent experiment. *$P < 0.05$; †$P < 0.01$.

TCRαβ^{high} (94 ± 6·8%) cells (Fig. 5), suggesting that in Cx rats the average surface density of CD90 per thymocyte of TCRαβ^{low} and TCRαβ^{high} phenotype was almost three- and twofold greater respectively.

Neonatal castration has no effect on the percentage of CD4+ CD25+ Foxp3+ thymocytes

Bearing in mind that the thymus generates CD4$^+$ CD25$^+$Foxp3$^+$ regulatory T-cells (T reg), which have been shown to play a critical role in suppressing aberrant responses to self and consequently in preventing autoimmunity (Stephens & Ignatowicz 2003, Maggi et al. 2005), we quantified the relative and absolute numbers of CD4$^+$CD25$^+$Foxp3$^+$ cells in thymi isolated from Cx and Sx rats. The percentage of these cells did not significantly differ between Cx and Sx rats. However, the absolute number of these cells was significantly greater in Cx rats than in Sx rats due to organ hypercellularity (Fig. 6).

Neonatal castration increases spleen weight and cellularity in adult rats

Neonatal castration produced a significant increase in both the absolute and the relative spleen weight (ratio to body weight;
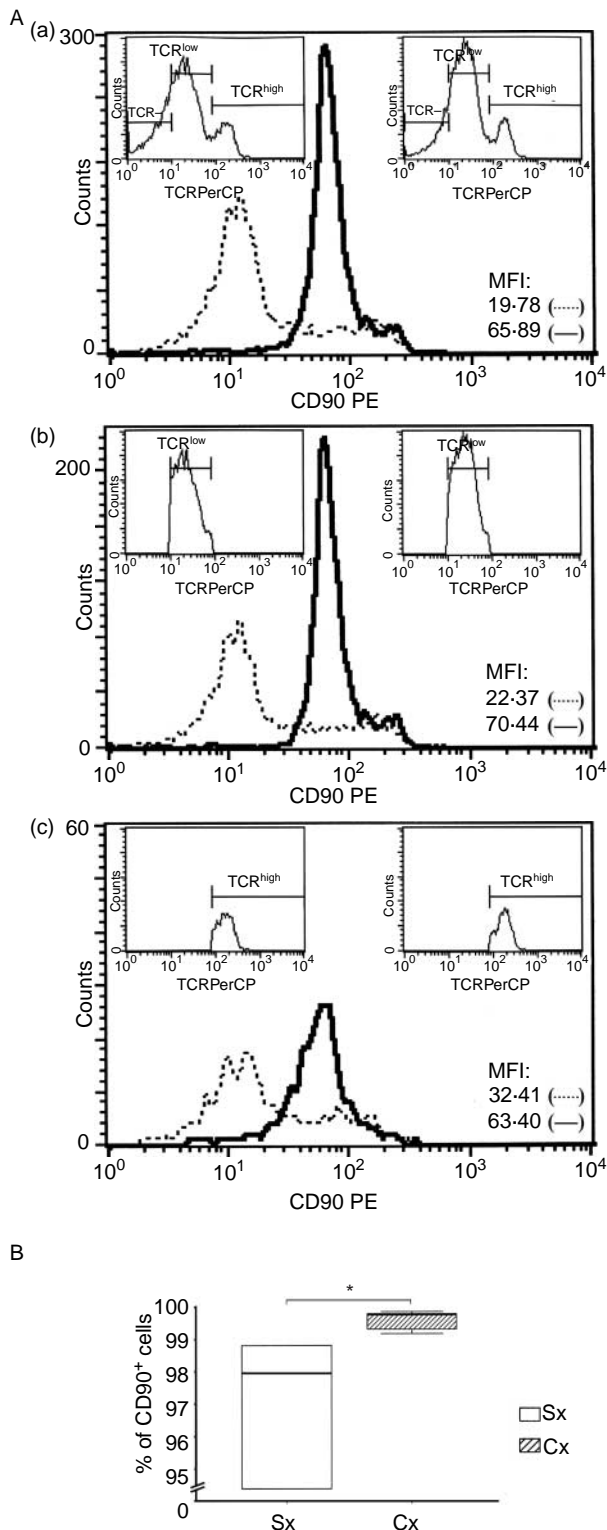
Figure 5 Neonatal castration increases the density of CD90 surface expression on thymocytes. (A) Representative overlaid histograms of the expression of CD90 on whole thymocyte population (a), gated TCRαβ^{low} thymocytes (b) and TCRαβ^{high} thymocytes (c) from neonatally castrated rats (Cx; solid line) and age-matched sham-castrated control rats (Sx; dotted line). Representative histograms of TCRαβ expression on thymocytes from Sx and Cx rats are inserted in the upper left and right corners of (a) respectively. Gates for TCRαβ^{low} (b) and TCRαβ^{high} (c) cells were set as shown in histograms inserted in the upper left (Sx) and upper right corner (Cx) respectively. (B) The box plot represents the percentage of thymocytes expressing CD90 on Sx and Cx rats. The central box line represents the sample median, while lower and upper borders of the box representing the 25th and 75th percentiles respectively. The lower and the upper lines indicate minimal and maximal values of the sample respectively. The figure shows data from a single experiment in which six Cx and eight Sx rats were used. Two further identical experiments gave very similar results. *$P<0·01$.

Table 2). The spleens from Cx rats contained a significantly greater number of lymphoid cells (Table 2). Moreover, the relative splenocyte number (relative to body weight) was also significantly ($P<0·05$) augmented in Cx rats (Table 2).

Neonatal castration increases total T-cell number and level of CD4$^-$ CD8$^+$ lymphoid cells within the spleen of adult rats

FCA of CD3 expression revealed a significant decrease in the percentage of CD3+ cells in the spleen of Cx rats compared with Sx rats (Table 3).

Since the total number of splenocytes was significantly increased in Cx rats, the spleens of these animals were found to contain significantly more CD3$^+$ cells.

FCA of CD4/CD8 expression demonstrated that the decrease in the percentage of CD3$^+$ cells mainly reflected a significantly diminished percentage of CD4$^+$CD8$^-$ cells. The percentage of CD4$^-$CD8$^+$ cells was not significantly affected by castration. However, due to a significant rise in the total number of splenocytes, the absolute number of CD4$^-$CD8$^+$ cells was significantly greater in Cx rats (Table 3), whereas that of CD4$^+$CD8$^-$ cells was increased, but this latter increase did not reach statistical significance (Table 3).

Discussion

Our study describes several novel observations pertaining to the role of gonadal hormones in regulating the development of the immune system. First, gonadal hormone ablation in neonatal rats affects thymic ontogenesis leading to: (i) a substantial increase in thymic cellularity, (ii) changes in the distribution of the major thymocyte subsets reflecting altered kinetics of the multi-step thymocyte differentiation process and (iii) increased Thy-1 surface density on thymocytes expressing TCRαβ implying altered thymocyte selection thresholds. Secondly, neonatal castration increases spleen weight and its lymphoid cell content in adult rats partly due to a numerical expansion in the CD8$^+$T subset.

A

Figure 6 Neonatal castration does not affect the percentage of CD4$^+$CD25$^+$Foxp3$^+$ thymocytes in adult rats. (A) Flow cytometric analysis of CD25 and Foxp3 expression (right side) on CD4$^+$ gated cells (left side) from 3-month-old sham-castrated control rats (Sx) (a) and castrated rats (Cx) (b). (B) The box plots represent the percentage (left) and absolute number (right) of the CD4$^+$CD25$^+$Foxp3$^+$ thymocytes in Cx and Sx rats. The central box line represents the sample median, while lower and upper borders of the box representing the 25th and 75th percentiles respectively. The lower and the upper lines indicate minimal and maximal values of the sample respectively. The figure shows data from a single experiment in which six Cx and six Sx rats were used. One further identical experiment gave very similar results. *$P<0\cdot01$.

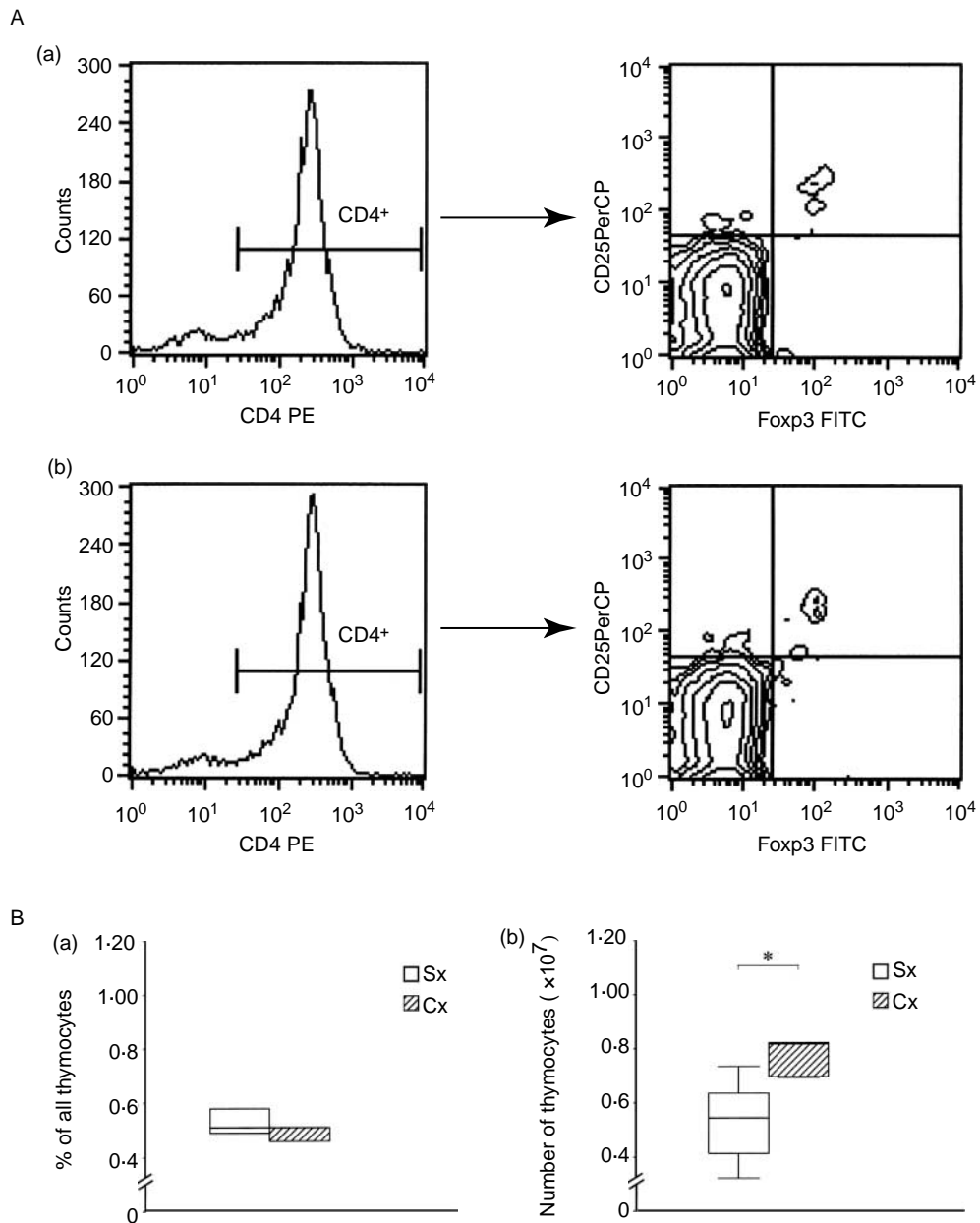Thymus enlargement and increased cellularity have already been reported in: (i) both mature and immature neonatally castrated rats (Leposavić *et al.* 1995, Shioya *et al.* 2000) and (ii) adult androgen-resistant (Tfm/Y) mice (Olsen & Kovacs 1989).

It is postulated that hypercellularity in Cx rats is produced by one or more of the following mechanisms: (1) reduced thymocyte death, (2) augmented thymocyte proliferation and (3) increased entry of thymocyte precursors. The

Table 2 Increased absolute and relative spleen weight as well as total and relative splenocyte number in 3-month-old neonatally castrated rats (Cx) compared with age-matched sham-castrated rats (Sx)

	Absolute spleen weight (g); median (25th–75th percentiles)	Relative spleen weight (g/100 g bw); median (25th–75th percentiles)	Total splenocyte number ($\times 10^7$)/spleen; median (25th–75th percentiles)	Total splenocyte number ($\times 10^7$)/100 g bw Median (25th–75th percentiles)
Treatment				
Sx ($n=8$)	0·37 (0·30–0·40)	0·174 (0·170–0·183)	8·25 (5·92–10·00)	4·09 (2·80–4·66)
Cx ($n=6$)	0·42 (0·41–0·45)*	0·20 (0·19–0·22)*	13·17 (12·02–14·75)*	6·26 (5·44–7·30)*

The table indicates data from a single experiment. Very similar data were obtained in an identically performed independent experiment. *$P<0.05$; bw, body weight.

attenuated vulnerability of thymocytes from Cx rats to unidentified apoptotic signals in culture as well as to specific Dex-induced apoptosis supports the first option. Further-more, since testosterone has been shown to: (i) accelerate thymocyte elimination via apoptosis and (ii) specifically target DP thymocytes for apoptosis (Guevara Patiño *et al.* 2000) our observed increase in the overall number and proportion of DP thymocytes in Cx rats further supports the same option. However, as an increased frequency of proliferating cells was demonstrated in ConA–thymocyte cultures from Cx rats, the second candidate mechanism also seems plausible. In keeping with this option are data that thymic enlargement in adult castrated mice occurs due to an accelerated rate of overall thymocyte proliferation (Olsen *et al.* 1994). In agreement with these findings, the percentage of BrdU$^+$ cells within none of CD3 subsets was shown to be significantly different between thymocyte cultures from Cx and Sx rats, suggesting that androgen deprivation affected most cell subsets that would be expected to be cycling in the normal thymus, to a quantitatively similar extent. However, neither the overall thymocyte responsiveness to ConA nor the responsiveness of any of the thymocyte subsets was altered by gonadal hormone deprivation. Although we do not have a fully satisfactory explanation for these findings, they are in line with previous data that, while some intrinsic factors that control thymocyte proliferation are obviously regulated by gonadal hormones, thymocyte mitogenic responsiveness to mitogenic ConA concentrations is rela–tively refractory to manipulations in the level of gonadal

hormone (Windmill *et al.* 1993, Utsuyama *et al.* 1995). Finally, the third option also cannot be completely ruled out as it has been suggested that stem cell migration from the bone marrow to the thymus is an androgen-sensitive thymic epithelial cell-dependent step on the route of T-cell development (Olsen *et al.* 2001).

Thymic hypercellularity in Cx rats was accompanied by substantial alterations in the composition of thymocyte subsets. Despite the fact that in Cx rats a numerical increase in the TN subset was found, the proportion of these cells was reduced while the proportion and number of downstream DP cells (TCR$\alpha\beta^-$, TCR$\alpha\beta^{low}$ and TCR$\alpha\beta^{high}$) was increased. These findings are fully consistent with the data that dihydrotestosterone treatment of adult Cx C57BL/6 mice produces an increase in the fraction of DN cells by nearly 35% accompanied by a decrease in the DP cell population (Olsen *et al.* 1991). It may be assumed that the increase in cell number and proportion of DP subsets was derived from accelerated thymocyte transition from the TN subset, the latter being maintained by recruitment of precursor cells. Our present data indicate that even if precursor immigration into the thymi of Cx rats was increased, as has been suggested (Olsen *et al.* 1991), it would not be sufficient to provide complete repopulation of this rapidly differentiating cell pool. Alternatively, the increase in cell number and proportions of downstream DP subsets may be due to augmented proliferation within these subsets, independent of the TN subset. However, the results obtained in thymocyte culture do not support this option.

Table 3 Absolute number and proportion of splenic lymphocytes defined by the expression of CD3, CD4 and CD8 in 3-month-old neonatally castrated rats (Cx) and age-matched sham-castrated rats (Sx)

	Percentage of cells; Median (25th–75th percentiles)		Absolute number of cells ($\times 10^7$)/spleen; median (25th–75th percentiles)	
	Sx ($n=8$)	Cx ($n=6$)	Sx ($n=8$)	Cx ($n=6$)
Splenocyte subset				
CD3$^+$	66·64 (64·31–69·83)	56·44 (50·52–59·73)*	5·95 (4·33–6·62)	7·07 (6·20–8·81)*
CD3$^-$	33·33 (30·14–35·62)	43·51 (40·23–49·28)*	2·77 (2·34–3·09)	5·86 (5·35–6·21)*
CD4$^-$CD8$^-$	29·44 (27·22–32·71)	41·13 (36·53–46·07)*	2·53 (2·04–2·97)	5·51 (4·84–5·85)*
CD4$^+$CD8$^-$	35·88 (35·09–36·97)	27·67 (25·77–29·27)*	3·18 (2·37–3·49)	3·51 (3·13–4·30)
CD4$^-$CD8$^+$	29·44 (27·22–30·26)	30·08 (27·15–33·16)	2·49 (1·98–2·90)	3·95 (3·33–4·67)*
CD4$^+$CD8$^+$	1·60 (1·28–1·95)	0·99 (0·79–1·38)*	0·14 (0·09–0·17)	0·13 (0·09–0·20)

The table indicates data from a single experiment. Very similar data were obtained in an identically performed independent experiment. *$P<0.05$.

The increase in the percentage of selected DP TCRαβ^{high} cells in Cx rats (∼20%), in light of the less-pronounced increase in the percentage of DP TCRαβ^{low} cells (∼7%), may suggest alterations in selection processes. Several findings support this notion. First, TCRαβ[+] thymocytes from Cx rats exhibit markedly increased surface density expression of CD90 (Thy-1). Since thymocytes from Thy-1^{−/−} mice have been shown to exhibit an exaggerated negative selection, most likely due to lack of Thy-1-mediated negative regulation of TCRαβ signalling (Hueber *et al.* 1997), it may be expected that the high density of CD90 on thymocytes from Cx rats induced hyposensitivity to negative selection. Secondly, the decreased percentage of apoptotic cells in thymocyte cultures from Cx rats is also consistent with the previous hypothesis. Since noradrenaline has been shown to decrease Thy-1 mRNA levels in murine thymocytes (Wajeman-Chao *et al.* 1998), the increase in Thy-1 surface density on thymocytes from Cx rats may be related to the decreased noradrenaline level in the thymus of these animals (data not shown).

Although neonatal castration produced an increase in the number of CD4^{+}CD8^{−} SP TCRαβ^{high} cells but did not affect that of CD4^{−}CD8^{+} SP TCRαβ^{high} cells, the relative proportion of both these subsets was decreased in Cx rats. Similar changes in the proportion of SP thymocyte subsets were found in castrated mice (Olsen *et al.* 1991, 1994). These findings together with: (i) the increase in both number and proportion of DP TCRαβ^{high} cells as the immediate source of all mature T-cells (Shortman *et al.* 1991) and (ii) alterations in the numbers of cells within spleen T-cell subsets (an increased number of CD4^{−}CD8^{+} but an unaltered number of CD4^{+}CD8^{−} cells) suggest changes in thymocyte terminal differentiation/maturation and in emigration of the mature cells. More precisely, our study clearly suggests accelerated differentiation/maturation of DP TCRαβ^{high} cells towards mature CD4^{−}CD8^{+} SP cells followed by their emigration and decelerated differentiation/maturation towards mature CD4^{+}CD8^{−} SP cells providing an increase in the number of CD4^{−}CD8^{+} SP cells and preservation of CD4^{+}CD8^{−} SP cell pool in the spleen of Cx rats respectively. The latter assumption is supported by the data that indicate: (i) androgen deprivation in mice stimulates thymic expansion and output culminating in increased levels of newly emigrated T-cells appearing within hyperplastic peripheral lymphoid tissues and (ii) this increase in peripheral T-cells is absent when thymectomy is performed 2 weeks before castration (Roden *et al.* 2004). A shifted CD4^{+}/CD8^{+} ratio in the spleen from Cx rats, due to a decreased frequency of CD4^{+} cells, is consistent with the findings in young testosterone-treated rats (Yao *et al.* 2003). However, to fully confirm the previous assumption the analysis of recent thymic emigrants in both Cx and Sx rats would have to be performed.

Finally, it should be emphasised that the assumed reduction in negative thymocyte selection may lead to an increased escape of potentially harmful autoreactive cells into the periphery of Cx rats. This proposal, coupled with data indicating that: (i) the relative number of thymic cells expressing the CD4^{+}CD25^{+}Foxp3^{+} regulatory phenotype is not affected by neonatal castration; (ii) the number of CD4^{+}CD25^{+} Foxp3^{+}T reg cells is unaltered, while their proportion is decreased, in the peripheral lymphoid tissues of Cx rats (data not shown) and (iii) in autoimmune disease models androgenic suppression of disease activity requires the presence of the thymus (Roubinian *et al.* 1978), suggests a misbalance in the thymic production of autoreactive and T reg cells in Cx rats that may be related to increased susceptibility of Cx animals to induction of autoimmune diseases (Olsen & Kovacs 1996).

Furthermore, the thymic changes in neonatally castrated rats cannot be explained simply and only by the lack of testicular androgens, serum levels of which during rat postnatal development have been previously reported (Leposavić & Mićić 1992). Several lines of evidence indicate that castration-induced deregulation of the hypothalamic–pituitary axis may also contribute to the development/maintenance of these changes. First, both gonadotrophins and prolactin are shown to influence T-cell differentiation (Rouabhia *et al.* 1989, Carreño *et al.* 2005). Secondly, it has been demonstrated that GnRH: (i) is synthesised locally in the thymus, (ii) its thymic level increases following castration (Azad *et al.* 1998) and (iii) it is implicated in regulation of T-cell development (Jacobson & Ansari 2004, Leposavić *et al.* 2005). Furthermore, in spite of the fact that rat T-cells are able to convert androgens to oestradiol (Samy *et al.* 2001) due to lack of adrenal gland contribution to plasma levels of androgen hormones in rats (van Weerden *et al.* 1992), a lack of regulation mediated via the oestradiol receptor (ER)α signalling system on postnatal thymus development (Yellayi *et al.* 2000) may also contribute to the observed changes in Cx rats.

In summary, our study demonstrates that neonatal castration affects thymus development and produces alterations in T-cell differentiation and selection (most likely by altering Thy-1 expression on the selected cells) leading to an increase in the level of CD8+T-cells within the spleen. Furthermore, it appears that neonatal ablation of gonadal hormone production may distort the production of auto-reactive and regulatory cells which could provide a fertile ground for the induction of autoimmune diseases.

Acknowledgements

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