Original Research

Ovarian hormone withdrawal in prepubertal developmental stage does not prevent thymic involution in rats

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Abstract

The study was undertaken to assess the effects of ovarian hormone withdrawal in prepubertal age on thymopoiesis in 2- (young) and 11-month-old (middle-aged) rats. In ovariectomized (Ox) rats, irrespective of age, thymic weight and cellularity were greater than in age-matched controls, but the values of both parameters exhibited the age-related decline. In addition, although thymopoietic efficiency was increased in both groups of Ox rats when compared with age-matched controls, thymopoiesis exhibited the age-related decline mirrored in the lower numbers of both CD4+ and CD8+ recent thymic emigrants in peripheral blood. This reflected the prethymic changes affecting bone marrow progenitor generation/entry and the thymic alterations encompassing the impaired progenitor progression through early pre-T-cell receptor developmental stages (defined by CD45RC/CD2 expression) and, possibly, a more pronounced decrease in the proliferation of the most mature thymocytes. Apart from the changes at thymocyte level, in Ox rats the age-related alterations in thymic stroma (substantiated in a prominent loss of thymic epithelial cells) were registered. Ovariectomy-induced changes in thymic lymphoid and epithelial component, most probably, influenced each other leading to the increase in thymic expression of interleukin-6 and interleukin-7 mRNAs along with time after ovariectomy. Collectively, the study showed that the withdrawal of ovarian hormones in prepubertal age increases the efficiency of thymopoiesis in young adult rats, but does not prevent decline in thymopoiesis occurring with age.

Keywords: ovarian hormones, thymic epithelial cells, thymic cytokine expression, thymopoiesis, Thymic involution

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Introduction

The thymus is the principal site of T-cell development. The earliest T-cell progenitors in this organ are CD4–CD8– T-cell receptor (TCR)- triple negative (TN) cells, and in rats they progress through a series of developmental stages defined by CD45RC and CD2 expression.[1,2] The most immature CD45RC+CD2− cells differentiate to become CD45RC+ CD2+ and then CD45RC−CD2+ cells. Further progression to CD4+CD8+ double positive (DP) stage hinges on the successful rearrangement of functional TCR β-chain (β-selection). At the DP developmental stage, the cells rearrange the TCR α-chain to become DP TCR$\alpha\beta^{low}$ cells. These cells are tested for the avidity of TCRαβ for self-peptide/major histocompatibilty complex (MHC) on thymic non-lymphoid cells.[3] Most DP cells die through activation-induced apoptosis (negative selection) or by neglect because their TCRs exhibit either too much or no avidity for the self-peptide/MHC complexes. Only DP cells with TCR exhibiting low avidity for the self-peptide/MHC receive signals for survival (positive selection), upregulate TCRαβ expression (TCR$\alpha\beta^{high}$) and further differentiate into CD4+CD8− or CD4−CD8+ single positive (SP) cells. The mature SP TCR$\alpha\beta^{high}$ cells then migrate to secondary lymphoid organs and replenish the naive T-cell population.[3]

It is well documented that the thymus involutes with age, which is characterized by a reduction in the thymic mass and cellularity coupled with disruption of structure and function of organ, culminating in a decrease of T cells exiting the thymus.[4–6] The reduction in naïve T-cell output from the thymus is related to the reduced response to vaccination and infections, and increased incidence of malignant and autoimmune diseases observed in the elderly.[7–9]

Many studies have implicated sex steroid hormones in the regulation of thymic growth and involution.[10] In female

rodent, the initiation of thymus involution and maintenance/progression of involutive changes with age are linked with the peripubertal rise and postpubertal high levels of ovarian hormones, respectively.[11–13] This view is supported by numerous studies showing that (i) rise in circulating levels of ovarian steroids, which is induced by the hormone administration, in female rodents causes thymic atrophy similar to that associated with ageing,[14–18] and (ii) surgical ovariectomy before puberty postpones thymic involution, whereas later in life this treatment leads to the reversal of age-related thymic changes.[2,16,19,20] It is noteworthy that in none of these studies the effects of ovariectomy were followed for more than 60 days postsurgery. However, Min et al.[21] showed that the effects of ovariectomy performed in 4-week-old mice are long-lasting but transitory, so that 20 weeks postsurgery there was no difference in the thymocyte number between ovariectomized (Ox) and control animals. These findings have challenged the role of ovarian hormones, at least, in the maintenance of thymic involution. Given that the kinetics of age-related changes in ovarian hormone level substantially differs between mice and rats, so that relatively high oestrogen levels are sustained until very old age in rats, but not in mice,[22] we designed this study in order to re-evaluate the role of ovarian hormones in the induction and, particularly, in the maintenance/progression of thymic involution starting in rodents around the puberty. This seems to be particularly important in light of numerous experimental attempts to prevent or diminish the undesirable effect of thymic involution on immune system.[23] Therefore, in this study, we examined the influence of ovarian hormones on rat thymus size and, in particular, T-cell differentiation/maturation reshaping from peripubertal to middle-age, in detail. The study included hormonally replete 1-month-old (immature), immunologically and sexually mature 2-month-old (young adult)[24] and 11-month-old (middle-aged) rats showing clear signs of reproductive ageing,[22] as well as adult age-matched rats Ox at the age of 1 month. In these animals, we examined (i) the thymic weight and cellularity and (ii) relative abundance of thymic epithelial cytokeratin-positive cells in thymic cortex and medulla. Considering that thymic epithelial cell (TEC)-derived cytokines, such as interleukin (IL)-6 and IL-7[25–29] may affect thymocyte and/or TEC survival/proliferation and consequently thymocyte and TEC abundance,[26,30] we measured IL-6 and IL-7 mRNA expressions in thymi from both Ox and control rats. Furthermore, we estimated thymocyte differentiation/maturation in these rats by examining (i) the relationships among the main thymocyte subsets (identifiable by characteristic expression of surface CD4, CD8 and TCRαβ), (ii) the expression of CD69, classical positive selection marker[31,32] and surface density of Thy-1, molecule which was implicated in TCRαβ signalling and thymocyte selection threshold regulation[33] and (iii) thymocyte sensitivity to apoptotic and proliferative signalling. Finally, considering that, besides conventional T cells, cells with immunoregulatory properties, i.e. CD4+CD25+FoxP3+ (Tregs) and TCRαβ+CD161+ natural killer T (NKT) cells also mature in the thymus,[34–36] we also explored their relative and absolute abundance in thymi from control and Ox rats.

Materials and methods

Animals

In this study, female Albino Oxford rats, born and bred in the animal housing facility at the Immunology Research Centre 'Branislav Janković' in Belgrade were used. All animals were housed in polyurethane boxes at constant temperature ($22 \pm 1^{\circ}C$) on a 12-h light/dark cycle with lights starting at 07:00 h. They had free access to standard laboratory chow and tap water.

The animals used in this study were handled in accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (revising Directive 86/609/EEC), and the experimental protocol was approved by the Experimental Animal Committee of the Immunology Research Centre 'Branislav Janković'.

Experimental protocol

One-month-old prepubertal rats without signs of vaginal opening were randomly assigned to ovariectomy, sham-ovariectomy and intact control groups. Sham-Ox and Ox rats were sacrificed at the age of 2 and 11 months. Two-month-old adult animals showed 4- to 5-day oestrous cycles, whereas 11-month-old rats entered acyclic state of persistent oestrous. Oestrous cyclicity was assessed in adult non-Ox rats by daily microscopic analysis of vaginal lavages (between 08:30 and 10:30 h). Due to practical constraints, at autopsy, young animals were in different stages of the oestrous cycle.

Surgical procedure

The animals were anaesthetized with intraperitoneal injection (0.08 mL/100 g/body weight [b.w.]) of solution containing ketamine (100 mg/mL, Ketamidor®, Richter Pharma AG, Austria), xylazine (20 mg/mL, Xylased®, Bioveta, Czech Republic) and saline, in a 1:0.5:8.5 ratio and subjected to bilateral ovariectomy. Ovaries were removed through small dorsal incisions in the skin and the muscle wall on each side of the lumbal backbone. Sham-Ox rats were anaesthetized, the skin and muscle layers were opened; ovaries were manipulated but not excised. Completeness of ovariectomy was confirmed by histological examination of removed tissue and careful inspection at postmortem. In addition, given that oestrogens play a predominant role in determining adult uterine gain,[37] the efficiency of ovariectomy was also confirmed at autopsy by measuring uterine wet weight. All animals were euthanized by exposure to increasing doses of CO_2 followed by cardiac puncture exsanguinations (between 09:00 and 10:00 h).

The thymi were dissected out, weighed and processed for immunocytochemical analyses of apoptotic and epithelial cells, cell phenotyping by flow cytometric analysis, as well as analysis of IL-6 and IL-7 mRNA expressions by real-time quantitative polymerase chain reaction (qPCR).

Chemicals, antibodies and immunoconjugates

Sodium azide (NaN_3) and 7-amino-actinomycin D (7-AAD) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and BD Biosciences Pharmingen (Erembodegem, Belgium), respectively. Ethylenediamine-tetraacetic acid (EDTA) and bovine serum albumin (BSA) were obtained from Fluka AG (Chemie) GmbH (BuchsSG, Switzerland).

Phenol red-free RPMI-1640 medium (with L-glutamine) was obtained from Gibco (Invitrogen Corporation, Carlsbad, CA). To prepare complete RPMI medium, 25 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Sigma-Aldrich), 1 mmol/L sodium pyruvate (Serva, Heidelberg, Germany), 100 units/mL penicillin (ICN, Costa Mesa, CA), 100 mg/mL streptomycin (ICN) and 10% inactivated fetal calf serum ([FCS] Gibco, Grand Island, NY) were added.

Liquid diaminobenzidine (DAB)+Substrate-Chromogen System, LSAB+ System, Antibody Diluent, Faramount Aqueous Mounting Medium and Target Retrieval Solution were obtained from DakoCytomation (Glostrup, Denmark).

The following monoclonal antibodies (mAbs) were used: fluorescein-isothiocyanate (FITC)-conjugated anti-CD2 (reactive with LFA-2; clone OX-34), phycoerythrin (PE)-conjugated anti-CD45RC (clone OX-22), PE-conjugated anti-CD4 (clone OX-38), FITC-conjugated anti-CD8 (clone OX-8), peridinin chlorophyll protein (PerCP)-conjugated anti-TCRαβ (clone R73), PE-conjugated anti-CD90 (Thy-1.1, clone OX-7) and biotin-conjugated anti-CD25 (clone OX-39). All these mAbs were purchased from BD Biosciences Pharmingen (Mountain View, CA). FITC-conjugated anti-CD161 (clone 10/78) was obtained from Serotec (Oxford, UK). Purified goat anti-CD69 antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Streptavidin-PerCP, FITC-conjugated goat anti-mouse IgG, FITC-conjugated rabbit anti-goat IgG and isotype IgG controls were purchased from BD Biosciences Pharmingen.

Immunocytochemical staining and quantification

Blocks of thymic tissue were fixed in 10% buffered-formalin and embedded in paraffin. Five-micrometre sections were cut from this paraffin blocks, mounted on adhesion slides, dewaxed and hydrated for immunohistochemistry.

Detection of apoptotic cells. Apoptotic cells were identified in thymic sections by the TdT-mediated dUTP-biotin nick end labelling (TUNEL) method using ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (S7100; Chemicon International, Temecula, CA). The apoptotic cell labelling was performed according to the manufacturer's protocol, and the sections were counterstained with 0.5% methyl green. TUNEL+ thymocytes were counted using an Olympus BH2-RFL microscope (Olympus Optical Co., Ltd, Tokyo, Japan) and ×40 objective lens by an observer with no previous knowledge of which slide was being analysed. The average of 10 randomly placed microscopic fields on cortical and medullary areas of thymus was recorded in five thymic sections from each animal, and the number of TUNEL+ thymocytes was expressed per mm^2 of thymic cortex and medulla.

Analysis of TEC abundance. To identify TECs, thymic sections were subjected to heat-induced epitope retrieval in Target Retrieval Solution. Endogenous peroxidase activity was quenched using an aqueous 3% H_2O_2 solution. The sections were then incubated with anti-pan cytokeratin mAb (1:300) for 1 h at room temperature in a moist chamber and then sequentially rinsed in phosphate-buffered saline (PBS) and incubated with universal biotinylated link. Next, the sections were incubated with streptavidin-conjugated horseradish peroxidase for 30 min and then with the DAB+ substrate-chromogen system for 5 min. Upon completion of the reaction, sections were counterstained with haematoxylin and mounted. Omission of the primary antibodies with inclusion of the secondary antibody produced no labelling. To further confirm the specificity of immunolabelling, the primary antibody was replaced with IgG isotype-matched antibodies. No apparent immunostaining was observed.

The size of TEC (cytokeratin+) area in thymic sections was quantified using a modification of method described by Boehm et al.[38] Briefly, high-resolution images (10 per animal) selected at random from cortical and medullary area of thymic sections were obtained with a digital colour camera (Color View III, Olympus Soft Imaging Solutions, Münster, Germany) mounted on an Olympus BH2-RFL microscope, and pan-cytokeratin stained brown pixels were counted using Image J software. The surface area of TECs was expressed as the percentage of pan-cytokeratin stained brown pixels relative to all number of pixels per image.

Flow cytometric analysis

Single-cell suspension preparation. For single-cell suspensions, thymi were dissociated by grinding on a sterile 60-μm sieve screen submerged in ice-cold PBS (pH 7.4) supplemented with 2% FCS and 0.01% NaN_3 (FACS buffer). The cells in suspension were enumerated using an improved Neubauer haemacytometer and trypan blue dye to exclude non-viable cells.

Isolation of CD4–CD8− double negative (DN) thymocytes. To obtain an enriched population of DN cells, thymocyte suspensions were depleted of cells expressing CD4+ and/or CD8+ by magnetic-activated cell sorting (MACS). Briefly, 10^7 thymocytes were resuspended in 60 μL MACS staining buffer (a degassed solution of PBS with 0.5% BSA and 2 mmol/L EDTA) and incubated with 20 μL of anti-CD4 MicroBeads (clone W3/25, Miltenyi Biotec, Gladbach, Germany) and 20 μL anti-CD8α MicroBeads (clone G28, Miltenyi Biotec) for 15 min at 4°C. The cells were then washed with 1 mL MACS staining buffer, pelleted at 300 × g and resuspended in 500 μL of the same buffer. After washing, cell suspensions were loaded onto MACS LS column (Miltenyi Biotec) placed in the magnetic field of Quadro MACS separator (Miltenyi Biotec) as described in the manufacturer's protocol. The unlabelled cells passing through the column were collected and subsequently labelled with fluorochrome-conjugated antibodies specific for CD2, CD45RC and TCRαβ.

Detection of antigens

Surface antigens. Aliquots of 1×10^6 thymocytes in 100 μL of FACS buffer were centrifuged at $350 \times g$ for 5 min at 4°C to yield a pellet. The pellet was subsequently resuspended in FACS buffer and incubated for 30 min on ice with saturating concentrations of fluorochrome/biotin-conjugated mAbs or polyclonal Abs and then washed in FACS buffer. When cells were incubated with biotin-conjugated mAbs in the first step, they were subsequently incubated with fluorochrome-conjugated streptavidin for an additional 30 min. After labelling, all cells were washed in FACS buffer and then in ice-cold PBS containing 0.01% NaN_3. Data acquisition was performed on the same day using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and the CELLQuest™ software (BD Biosciences).

Intracellular antigens. For analysis of FoxP3 expression in CD4+CD25+ cells, following the surface molecule (CD4 and CD25) immunolabelling, the thymocytes were subjected to FoxP3 immunostaining using staining set containing FITC-conjugated anti-FoxP3 mAb (clone FJK-16 s) from eBioscience (San Diego, CA). The immunostaining was performed according to the manufacturer's protocol. Data acquisition was also performed on the same day using a FACScan flow cytometer and the CELLQuest™ software.

Cell cycle analysis. Thymocyte cell cycle analysis using intracellular staining with the DNA dye 7-AAD and flow cytometry was performed as previously described.[39] Aliquots of freshly isolated thymocytes (0.5×10^6 cells/ml) were centrifuged at $350 \times g$ for 5 min at 4°C to yield a pellet. Subsequently, the pellet was resuspended in 150 μl 50% FCS in PBS and the cells were fixed/permeabilized by dropwise addition of 450 μl of cold 70% ethanol in double distilled H_2O. The fixed/permeabilized cells were incubated overnight, washed with PBS to remove ethanol and precipitated protein, and incubated with 7-AAD at 4°C for 30 min before the analysis on FACScan flow cytometer. Doublets were excluded by analysing correlated area against width signals of the 7-AAD fluorescence on doublet discrimination module dot plot. DNA analysis was performed using Watson (pragmatic) model and FlowJo proliferation platform software version 7.8.[40]

Analysis of thymocyte apoptosis in vitro. Thymocytes (2×10^5 cells/well) were cultivated in 96-well flat-bottom plates (Nunc A/S, Roskilde, Denmark) in RPMI 1640 for 18 h. The cells were harvested from culture and incubated with the DNA-binding dye 7-AAD (20 μL) at 4°C for 20 min, and subsequently analysed on a FACScan flow cytometer using CELLQuest™ software. Staining of cells with 7-AAD allows the clear delineation of living lymphocytes with a normal morphology (unmodified FSC/SSC) and a normal membrane integrity (7-AAD− cells) from apoptotic cells exhibiting altered morphology (lower FSC and higher SSC) and membrane alterations leading to increased 7-AAD incorporation (7-AAD+ cells).[41]

Tissue RNA extraction and real-time PCR

Total RNA was isolated from 20 mg of spleen tissue using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA) and Total RNA Chemistry (Applied Biosystems). Reverse transcription was performed with high capacity cDNA reverse transcription kit (Applied Biosystems), and 5 μL of cDNA was used for real-time PCR. Triplicate 25 μL reactions were ran under Applied Biosystems 7500 universal cycling conditions. Gene Expression Master Mix and commercial TaqMan Gene Expression Assays for rat IL-7 (Rn00681900_m1), IL-6 (Rn99999011_m1) and β-actin (Rn00667869_m1) were obtained from Applied Biosystems. All procedures were performed according to manufacturer's instructions. β-Actin was the internal standard to normalize input cDNA variations as it displayed an optimal stability among various samples tested. Quantitative differences in gene expression levels were assessed using Applied Biosystems SDS software (v 1.4.0.) and $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance of age-related differences was evaluated by the one-way analysis of variance (ANOVA) followed by the Tukey test for *post hoc* comparisons. Statistical significance of the differences in ovariectomy-induced effects between 2- and 11-month-old rats (ovariectomy × age interaction) was determined by two-way ANOVA followed by the Bonferroni test for *post hoc* comparisons. All statistical analyses were performed using GraphPad Prism 5.0.

Results

Ovariectomy-induced increase in thymic weight and cellularity exhibited age-dependent decline

In 2-month-old rats, the absolute thymic weight was greater ($P < 0.001$) than in 1-month-old rats, but it declined ($P < 0.001$) by the age of 11 months (Table 1). The absolute number of thymocytes exhibited a similar pattern of the age-related changes. On the other hand, the relative (normalized to 100 g b.w.) thymic weight and thymocyte number decreased ($P < 0.001$) between the ages of 1 and 2 months, and continued to decrease ($P < 0.001$) with age, so that the values of these parameters were lower ($P < 0.001$) in 11- than in 2-month-old rats (Table 1). Given that there was no significant difference in value of any of the analysed parameters of thymic structure and thymopoiesis between sham-Ox and intact rats of the same age in all analyses and graphs, these two groups were treated as one control group.

In Ox rats of both ages, the absolute thymic weight and thymocyte numbers were greater than in age-matched controls, but these effects of ovariectomy were more pronounced in 11- than in 2-month-old rats ($F_{1,20} = 51.25$, $P < 0.001$ for thymic weight; $F_{1,20} = 68.84$, $P < 0.001$ for thymocyte number; Table 1). The values of both parameters exhibited age-dependent ($P < 0.001$) decrease. In addition, in these rats, the relative thymic weight and thymocyte were increased over the corresponding values in age-matched controls, but to the greater extent in

11-month-old rats than in younger animals ($F_{1,20} = 48.00$, $P < 0.001$ for relative thymic weight; $F_{1,20} = 57.70$, $P < 0.001$ for relative thymocyte number; Table 1). The values of these thymic indices also exhibited age-related decrease ($P < 0.001$; Table 1). Furthermore, the age-related decreases in the thymic absolute (approximately 2.74 fold in Ox versus 2.85 fold in controls) and relative weight (approximately 3.96 fold in Ox versus 3.67 fold in controls) did not differ between Ox and control rats, whereas those in absolute (approximately 4.32 fold in Ox versus 7 fold in controls) and relative thymocyte number (approximately 6.37 fold in Ox versus 9.19 fold in controls) were less pronounced ($P < 0.001$) in Ox rats than in controls. This suggested that in Ox rats the age-associated loss in the thymic non-lymphoid component was more pronounced than that in lymphoid component.

Ovariectomy-induced increase in TEC abundance exhibited age-dependent decline

Next, we quantified the size of cytokeratin+ area in thymic sections from both Ox and control rats (Figure 1). The effects of ovariectomy on the relative proportions of cytokeratin+ area in both the thymic cortex ($F_{1,236} = 24.85$, $P < 0.001$) and medulla ($F_{1,236} = 5.72$, $P < 0.05$) were dependent on duration of ovarian hormone deprivation. In 2-month-old Ox rats, the relative proportion of cytokeratin+ area in both thymic cortex ($P < 0.001$) and medulla ($P < 0.001$) was greater than in age-matched controls, whereas in 11-month-old Ox rats, in none of the two major thymic compartments it significantly differed from the corresponding in age-matched controls (Figure 1). As expected from the changes in the thymic weight and total number of thymocytes, the age-related decrease in the relative proportion of cytokeratin+ area was more pronounced in both thymic cortex (approximately 50% in Ox versus 30% in controls) and medulla (approximately 50% in Ox versus 37% in controls) of Ox rats.

Ovariectomy exerted differential effects on thymic IL-6 and IL-7 mRNA expression

IL-6. The IL-6 mRNA level increased ($P < 0.05$) between ages of 1 and 2 months (Figure 2a), and it continued to rise ($P < 0.01$) between the ages of 2 and 11 months (Figure 2a). Two-way ANOVA showed that the effect of ovariectomy on IL-6 mRNA level was dependent on duration of deprivation ($F_{1,20} = 7.91$, $P < 0.05$). Ovariectomy reduced ($P < 0.01$) IL-6 mRNA level in 2-month-old rats, but this effect disappeared by age of 11 months (Figure 2a). Thus, the age-related increase in thymic IL-6 mRNA expression was more pronounced in Ox rats (almost two-fold) than in controls (by approximately 30%; Figure 2a).

IL-7. As shown in Figure 2(b), in control rats, thymic IL-7 mRNA level increased with age. Compared with age-matched controls, thymic IL-7 mRNA expression was not affected by ovariectomy in 2-month-old Ox rats, whereas it was lower ($P < 0.001$) in 11-month-old Ox rats than in age-matched controls ($F_{1,20} = 50.23$, $P < 0.001$; Figure 2b). Furthermore, the amount of IL-7 mRNA was greater ($P < 0.05$) in thymi from 11-month-old Ox rats than in those from 2-month-old Ox rats (Figure 2b).

The frequency of apoptotic thymocytes was diminished one month postovariectomy and remained stable with age

To assess the putative contribution of alteration in thymocyte apoptosis to thymic hypercellularity in Ox rats, the frequency of apoptotic cells was assessed in thymocyte cultures and *in situ* in the thymus. The overall frequency of apoptotic cells did not differ between cultures of thymocytes isolated from 1- and 2-month-old rats (Figure 3A). However, compared with 2-month-old rats, the frequency of these cells was increased ($P < 0.01$) in 11-month-old rats (Figure 3A). The frequency of apoptotic cells was diminished in thymocyte cultures from both 2- ($P < 0.05$) and

Table 1 Absolute and relative thymic weight and absolute and relative number of thymocytes in 1-, 2-, 11-month-old control rats (controls) and 2- and 11-month-old rats Ox at the age of 1 month, and the age-related relative changes in each of these parameters in control and Ox rats[a]

	Thymic weight, g	Relative thymic weight, g/100 g b.w.	Absolute number of thymocytes, $\times 10^7$/thymus	Relative number of thymocytes, $\times 10^7$/100 g b.w.
1-month-old controls	0.23 ± 0.01	0.43 ± 0.01	53.78 ± 1.30	100.28 ± 2.63
2-month-old controls	$0.34 \pm 0.01^{***b}$	$0.22 \pm 0.01^{***b}$	$104.03 \pm 3.43^{***b}$	$67.40 \pm 2.31^{***b}$
2-month-old Ox	$0.74 \pm 0.03^{***c}$	$0.38 \pm 0.01^{***c}$	$213.93 \pm 8.03^{***c}$	$110.77 \pm 3.62^{***c}$
11-month-old controls	$0.12 \pm 0.01^{***d}$	$0.06 \pm 0.01^{***d}$	$14.85 \pm 1.68^{***d}$	$7.33 \pm 0.72^{***d}$
	$35.43 \pm 3.90\%$	$27.33 \pm 3.04\%^{f}$	$14.56 \pm 1.90\%^{f}$	$11.03 \pm 0.30\%^{f}$
11-month-old Ox	$0.27 \pm 0.01^{***c,e}$	$0.10 \pm 0.01^{**c,***e}$	$49.41 \pm 1.84^{***c,e}$	$17.38 \pm 0.53^{**c,***e}$
	$37.34 \pm 2.16\%^{g}$	$25.29 \pm 1.13\%^{g}$	$23.29 \pm 1.36\%^{g,**h}$	$15.97 \pm 0.51\%^{g,**h}$

b.w.: body weight.
[a]The data are expressed as mean ± SEM ($n \geq 6$). The Student's *t*-test was used to assess the significance in age-related relative changes in controls and Ox rats
[b]2-month-old controls vs.1-month old controls.
[d]11-month-old controls vs. 2-month-old controls.
[c]Ox rats vs. age-matched controls.
[e]11-month-old Ox vs. 2-month-old Ox.
[f]The values represent % of corresponding value in 2-month-old controls.
[g]The values represent % of corresponding value in 2-month-old Ox rats.
[h]Percentage of change in controls vs. percentage of change in Ox rats.
$^{**}P < 0.01$; $^{***}P < 0.001$.

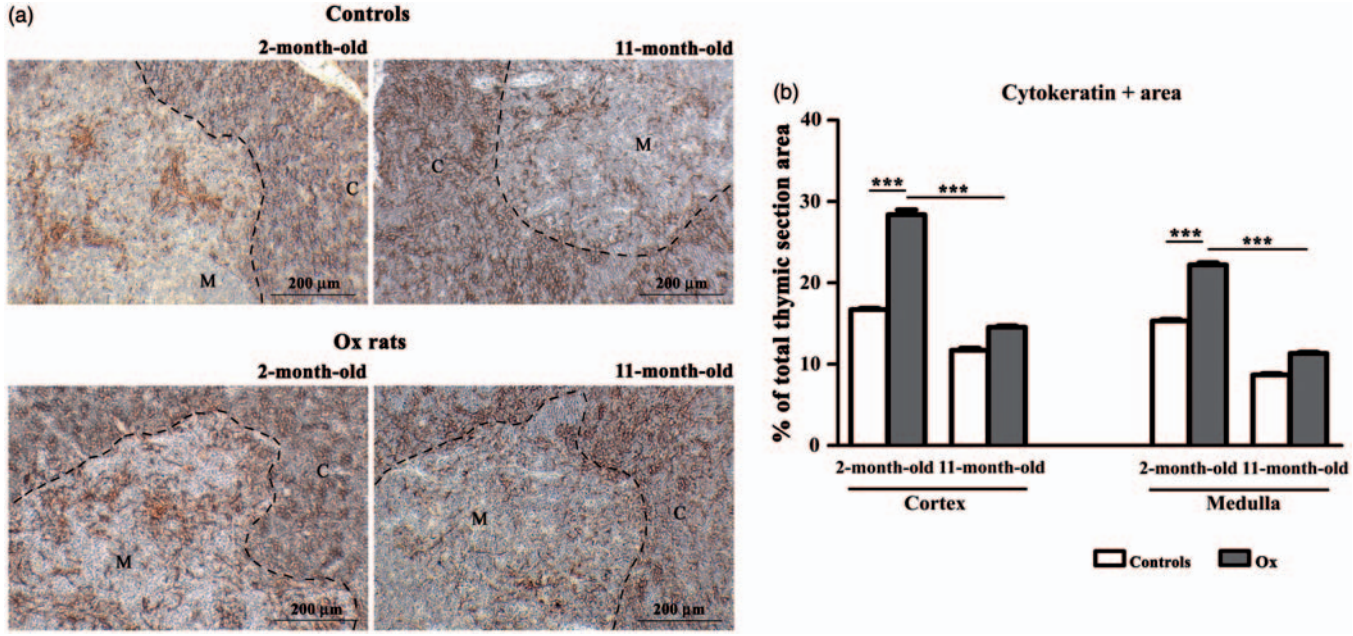

Figure 1 Ovariectomy at age of one month caused a transitory enlargement of cytokeratin+ area in thymic sections
(Panel a) Representative photomicrographs of anti-pan-cytokeratin antibody-labelled thymic sections from (lower photomicrographs) 2- and 11-month-old Ox rats and (upper photomicrographs) age-matched control rats (controls). Dashed lines are used to delineate thymic cortex (C) and medulla (M). Original magnification: ×10. (b) The bar graph represents the relative proportions of cytokeratin+ area in thymic cortex and medulla from 2- and 11-month-old Ox and age-matched controls. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 6$); ***$P < 0.001$. (A color version of this figure is available in the online journal)

11-month-old ($P < 0.001$) Ox rats to the same level, but this decrease was more pronounced in the thymocyte cultures from 11-month-old Ox rats ($F_{1,20} = 24.15$, $P < 0.001$; Figure 3A).

In agreement with the previous findings, two-way ANOVA showed that ovariectomy diminished the number of TUNEL+ thymocytes in both 2- ($P < 0.01$) and 11-month-old ($P < 0.001$) rats, but to a greater extent in older animals ($F_{1,20} = 5.8$, $P < 0.05$; Figure 3B). In both Ox and control rats, TUNEL+ cells were observed in the thymic cortex (Figure 3Ba). Only rare TUNEL+ cells were noticed in the thymic medulla (Figure 3Ba).

The frequency of proliferating thymocytes was diminished in Ox rats of both ages, but to a greater extent in 11-month-old rats

The frequency of proliferating thymocytes decreased ($P < 0.001$) between the ages of 1 and 2 months and continued to decrease ($P < 0.05$) by the age of 11 months (Figure 4). Ovariectomy produced differential effects on frequency of proliferating cells in thymocyte suspensions from 2- and 11-month-old rats ($F_{1,20} = 5.17$, $P < 0.05$). Namely, ovariectomy decreased the frequency of proliferating thymocytes in rats of both ages ($P < 0.05$ and $P < 0.001$ for 2- and 11-month-old rats, respectively), but this effect was more pronounced in 11-month-old rats (Figure 4).

Ovariectomy affected differentiation/maturation of T cells in both 2- and 11-month-old rats

To define the influence of ovarian hormones on T-cell differentiation/maturation, the relationship between thymocyte subsets at distinct stages of T-cell development delineated by CD4/CD8 expression and TCRαβ surface density was analysed (Supplementary Figure 1).

Ovarian hormone deprivation diminished relative number of the most immature TN cells and affected their phenotypic profile defined by CD2/CD45RC expression. The relative and absolute number of CD4–CD8−TCRαβ TN cells decreased ($P < 0.05$) between the ages of one and two months. The relative number of these cells increased ($P < 0.001$) between ages of 2 and 11 months, whereas the absolute number remained stable (Figure 5A, Table 2).

In Ox rats of both ages, the frequency of TN cells was diminished compared with age-matched controls (Figure 5A), but this decrease was more pronounced in 11-month-old rats ($F_{1,24} = 13.98$, $P < 0.01$). However, due to greater thymic cellularity in Ox rats, the number of TN thymocytes was increased ($P < 0.001$) in both 2- and 11-month-old rats (Table 2).

Ovariectomy altered phenotypic profile of TN thymocytes. Given that TN thymocytes encompass cells at distinct phenotypically distinguishable developmental stages, we examined the effect of ovariectomy on the relationship between the thymocyte fractions within TN subpopulation using flow cytometry (Supplementary Figure 2). In rats, the sequential appearance of CD2 and CD45RC on early TN thymocytes, in conjunction with pronounced regenerative capacity of both CD45RC+ subsets (CD45RC+CD2− and CD45RC+CD2+), suggests that

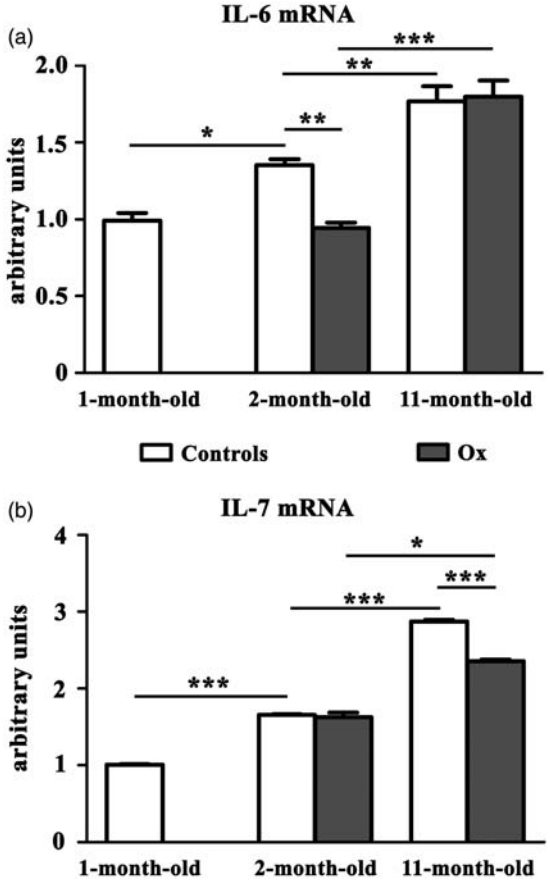

Figure 2 Ovariectomy at age of one month exerted differential effects on thymic IL-6 and IL-7 mRNA expression in adult rats. The bar graphs represent changes in mRNA expression for (a) IL-6 and (b) IL-7 in whole thymic tissue from 2- and 11-month-old Ox rats and age-matched control rats (controls) relative to 1-month-old baseline controls. β-Actin was selected as the housekeeping gene to normalize for input cDNA variations as it displayed optimal stability in our experimental system. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 6$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

early thymocyte development proceeds in the order CD45RC+CD2−, CD45RC+CD2+, CD45RC-CD2+.[1,2]

The frequency of the most immature CD45RC+CD2− cells among TN thymocytes was lower ($P < 0.05$) in 2-month-old than in 1-month-old rats. However, it increased with age and achieved a markedly ($P < 0.001$) higher value in 11-month-old rats (Figure 5Ba). The frequencies of downstream CD45RC+CD2+ and CD45RC-CD2+ cells did not differ between one- and two-month-old rats (Figure 5Bb, c). Compared with 2-month-old rats, in 11-month-old ones, the relative proportion of CD45RC+CD2+ cells was decreased ($P < 0.001$), whereas that of CD45RC-CD2+ cells was increased ($P < 0.001$;Figure 5Bb, c). The absolute number of CD45RC+CD2− cells decreased ($P < 0.05$) between the ages of 1 and 2 months, and then it remained stable by the age of 11 months (Table 2). However, the cellularity of downstream CD45RC+CD2+ and CD45RC-CD2+ subsets did not differ between 1- and 2-month-old rats and remained unaltered by the age of 11 months (Table 2).

Two-way ANOVA showed that the effects of ovariectomy on the frequency of CD45RC+CD2− and downstream CD45RC+CD2+ cells within TN thymocyte subset exhibited alterations with age ($F_{1,24} = 78.80$, $P < 0.001$ for CD45RC+CD2−; $F_{1,24} = 35.30$, $P < 0.001$ for CD45RC+CD2+). Compared with age-matched controls, the frequencies of both CD45RC+CD2− ($P < 0.001$) and CD45RC+CD2+ cells ($P < 0.05$) were decreased in 2-month-old Ox rats, whereas they were increased ($P < 0.001$) in 11-month-old rats (Figure 5Ba, b). In contrast, the frequency of CD45RC-CD2+ subsets was increased ($P < 0.001$) and decreased ($P < 0.001$) in 2- and 11-month-old Ox rats, respectively ($F_{1,24} = 73.10$, $P < 0.001$), when compared with age-matched controls (Figure 5Bc). Two-way ANOVA showed that the effects of ovariectomy on the number of CD45RC+CD2− cells changed with age ($F_{1,24} = 37.97$, $P < 0.001$). Ovariectomy did not affect the number of CD45RC+CD2− cells in 2-month-old rats, whereas it increased ($P < 0.001$) their number in 11-month-old rats (Table 2). Furthermore, the number of CD45RC+CD2+ cells was not affected by ovariectomy, whereas the number of CD45RC-CD2+ cells was increased in Ox rats of both ages ($P < 0.001$ and $P < 0.01$ for 2- and 11-month-old Ox rats, respectively, Table 2).

Ovariectomy altered the relative and absolute numbers of the CD4+CD8+ (DP) thymocytes. In control rats, the frequencies of none of three DP subsets (DP TCRαβ−, DP TCRαβ^{low} and DP TCRαβ^{high}) significantly differed between one- and two-month-old rats (Figure 6A). However, compared with 2-month-old rats, in 11-month-old ones there was no significant change in the frequency of DP TCRαβ-cells, whereas the frequency of DP TCRαβ^{low} and DP TCRαβ^{high} cells was decreased ($P < 0.05$) and increased ($P < 0.001$), respectively (Figure 6A). The numbers of DP TCRαβ- and DP TCRαβ^{low} cells were greater ($P < 0.001$) in two- than in one-month-old rats, whereas that of DP TCRα^{high} cells remained unaltered. In 11-month-old rats, the cellularity of all DP subsets was diminished ($P < 0.01$) compared with 2-month-old rats (Table 2).

Ovariectomy did not affect the frequency of DP TCRαβ- thymocytes (Figure 6A). On the other hand, compared with age-matched controls, in Ox rats, irrespective of age, the frequency of DP TCRαβ^{low} thymocytes was increased ($P < 0.001$), whereas that of DP TCRαβ^{high} cells was diminished ($P < 0.01$ and $P < 0.001$ in 2- and 11-month-old rats, respectively, Figure 6A). The effects of ovariectomy on the absolute number of DP TCRαβ- ($F_{1,24} = 29.21$, $P < 0.001$) and DP TCRαβ^{low} cells ($F_{1,24} = 111.6$, $P < 0.001$) were age-dependent (Table 2). Ovariectomy increased the numbers of DP TCRαβ- and DP TCRαβ^{low} cells in both 2- and 11-month-old rats, but its effects were more pronounced in older rats. Ovariectomy increased ($P < 0.01$) absolute number of DP TCRαβ^{high} cells only in two-month-old rats ($F_{1,24} = 6.31$, $P < 0.05$; Table 2).

Ovariectomy-induced increase in the numbers of the most mature CD4+CD8− and CD4−CD8+ TCRαβ^{high} SP thymocytes diminished with age. The frequency of SP CD4+CD8−TCRαβ^{high} cells increased ($P < 0.01$), whereas that of CD4−CD8+ SP TCRαβ^{high} cells did not

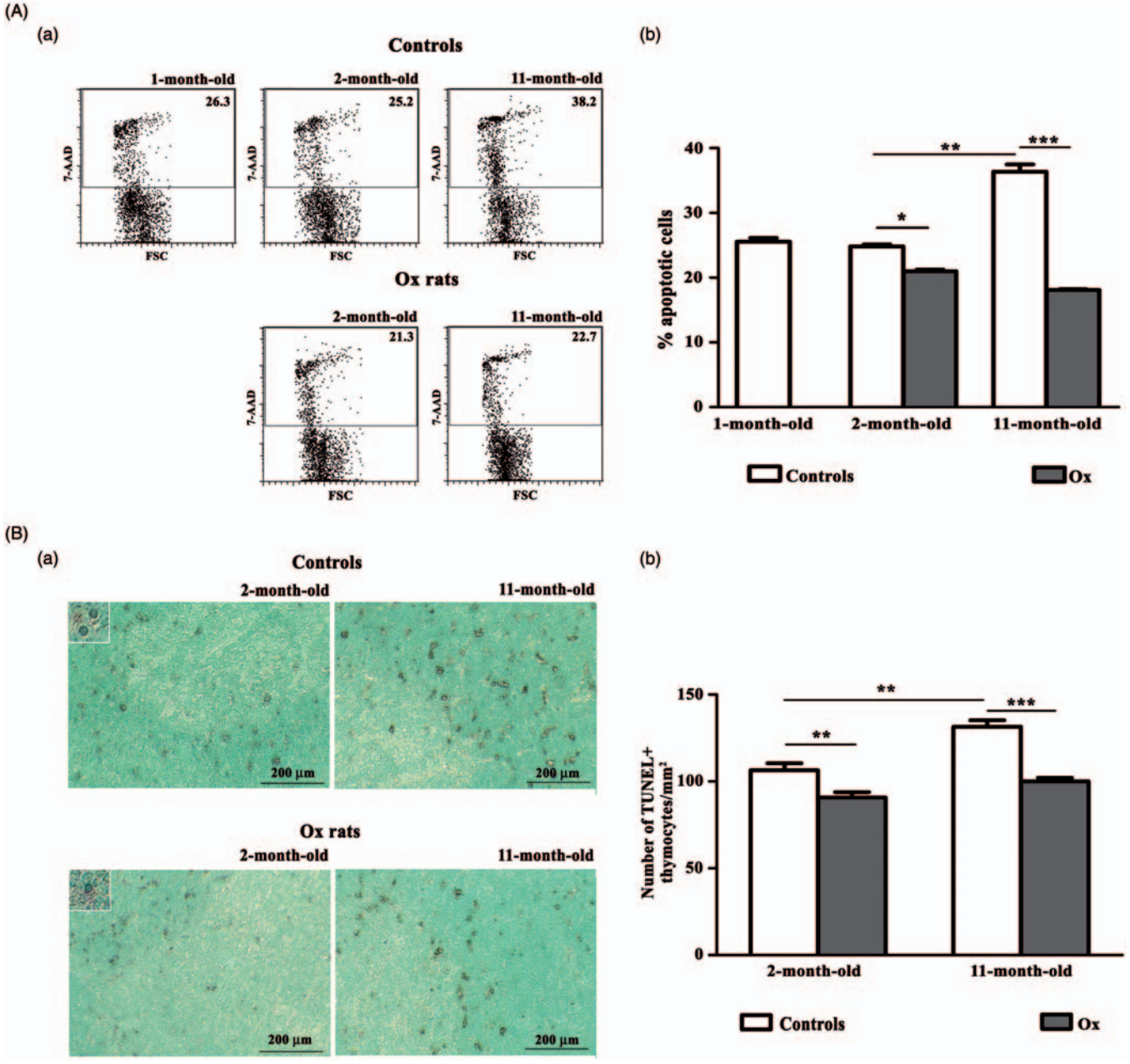

Figure 3 Ovariectomy diminished the percentage of apoptotic thymocytes in 2-month-old rats and this effect remained by age of 11 months
(Panel A) Flow cytometric dot plots show **(a)** 7-amino-actinomycin D (7-AAD) staining of thymocytes from cultures of **(**upper dot plots**)** 1-, 2- and 11-month-old control rats (controls) and (lower dot plots**)** 2- and 11-month-old Ox rats. The number in upper right corner indicates the percentage of apoptotic cells in representative sample. The bar graph shows **(b)** the overall percentage of apoptotic cells from Ox and control rats. **(**Panel B**)** Representative photomicrographs of **(a)** TUNEL staining in thymic sections from (lower photomicrographs) 2- and 11-month-old Ox rats and (upper photomicrographs) age-matched controls. The inserts in photomicrographs represent TUNEL-positive (+) cells at high power magnification. Original magnification: ×10 (photomicrographs), ×40 (inserts). The bar graph represents **(b)** the number of TUNEL+ thymocytes per mm^2 of thymic section from 2- and 11-month-old Ox rats and age-matched controls. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (A color version of this figure is available in the online journal)

change between the ages of one and two months (Figure 6B). However, the frequencies of both CD4+CD8− SP TCRαβ^{high} ($P < 0.001$) and CD4−CD8+ SP TCRαβ^{high} cells ($P < 0.01$) increased between the ages of 2 and 11 months (Figure 6B). The numbers of cells belonging to both SP subsets increased ($P < 0.001$) between the ages of 1 and 2 months, and then dramatically ($P < 0.001$) decreased by the age of 11 months (Table 2).

In both 2- and 11-month Ox old rats, the frequency of the most mature CD4+CD8−TCRαβ^{high} ($P < 0.001$) as well as CD4−CD8+TCRαβ^{high} thymocytes ($P < 0.05$ and $P < 0.001$ in 2- and 11-month-old Ox rats, respectively) was decreased compared with the corresponding values in age-matched controls (Figure 6B). Two-way ANOVA showed no significant ovariectomy × age interaction ($F_{1,24} = 0.96$, $P > 0.05$ for CD4+CD8−TCRαβ^{high} cell frequency; $F_{1,24} = 1.79$, $P > 0.05$

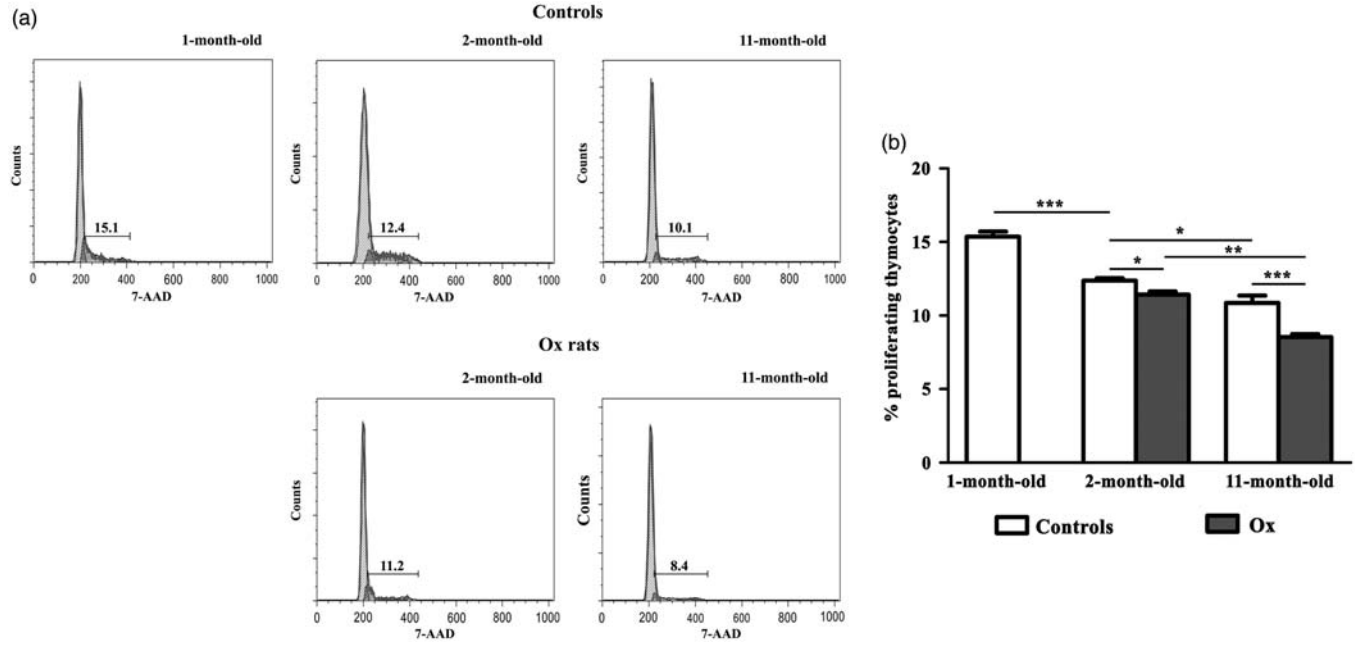

Figure 4 Ovariectomy diminished the frequency of proliferating cells in Ox rats of both ages, but to a greater extent in 11-month-old rats
(Panel a) Histogram plots show cell cycle analysis using 7-amino-actinomycin D (7-AAD) staining in freshly isolated thymocytes from (upper histograms) 1-, 2- and 11-month-old control rats (controls) and (lower histograms) 2- and 11-month-old rats Ox at age of 1 month, which were generated by FlowJo proliferation platform software version 7.8. The percentage of cells in S+G2/M phases of cell cycle (proliferating cells) was calculated using Watson (pragmatic) model. The number above the marker in each histogram plot indicates the percentage of proliferating cells in representative sample. (b) The bar graph represents the percentage of proliferating cells in freshly isolated thymocytes from 1-, 2- and 11-month-old controls and 2- and 11-month-old rats Ox at age of 1 month determined by 7-AAD staining. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 6$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

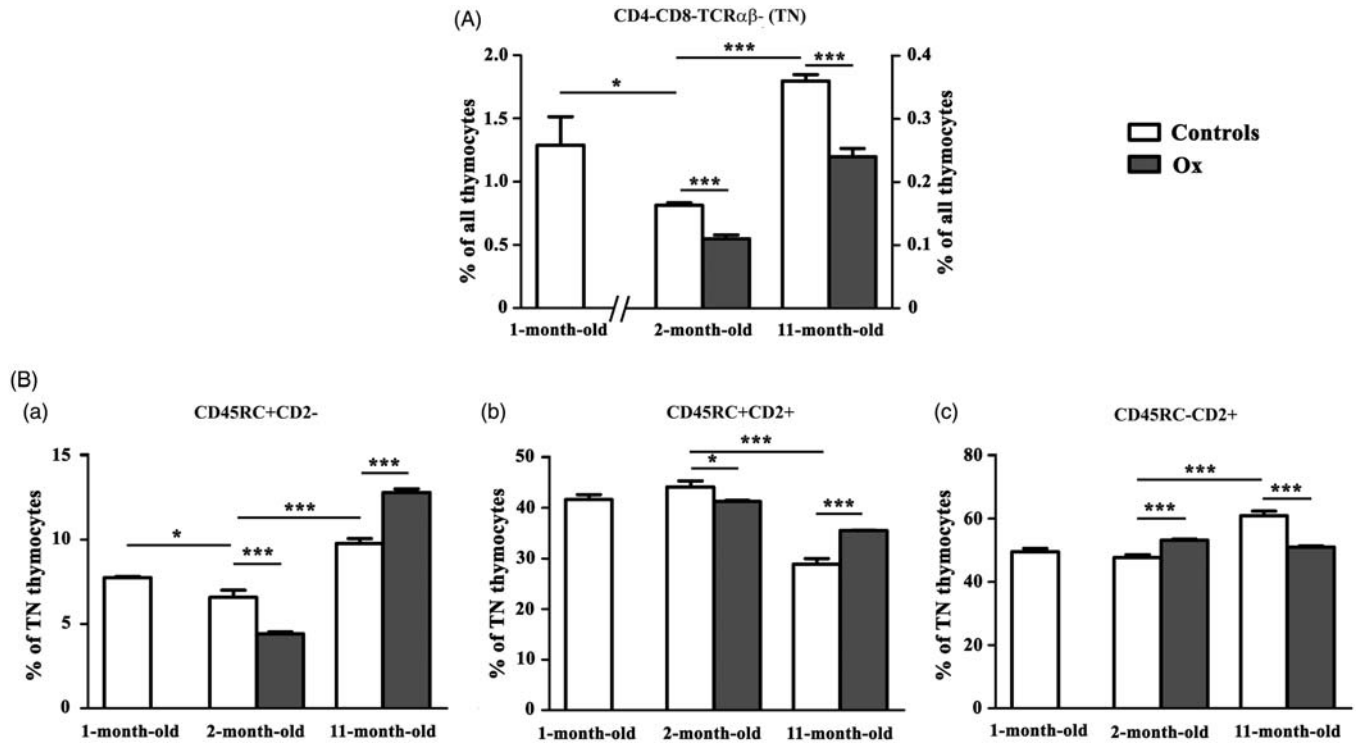

Figure 5 Ovariectomy decreased the frequency of the most immature CD4–CD8−TCRαβ- (TN) thymocytes and affected their phenotypic profile defined by CD2 and CD45RC expression
(Panel A) Bar graph shows the relative proportions of CD4–CD8−TCRαβ-(TN) cells in freshly isolated thymocyte suspensions from 1-, 2- and 11-month-old control rats (controls) and 2- and 11-month-old rats Ox at age of 1 month. **(**Panel B**)** Bar graphs indicate the relative proportions of **(a)** CD45RC+CD2−, **(b)** CD45RC+CD2+ and (c) CD45RC-CD2+ cells in TN thymocyte subpopulation from 1-, 2- and 11-month-old controls and 2- and 11-month-old Ox rats which were isolated by magnetic-activated cell sorting (MACS). Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 7$); $^{*}P < 0.05$, $^{***}P < 0.001$

Table 2 Absolute numbers of the most immature CD4–CD8− TCRαβ- (TN) thymocytes; the cells belonging to distinct subsets of TN and CD4+CD8+ (DP) thymocytes; CD4+CD8−TCRαβ^{high} and CD4-+CD8+TCRαβ^{high} single positive (SP) thymocytes and thymocytes expressing regulatory phenotype (CD4+CD25+FoxP3+ and CD161+TCRαβ+) in 1-, 2-, 11-month-old control rats (controls) and 2- and 11-month-old rats Ox at the age of 1 month (Ox rats)[a]

		1-month-old controls	2-month-old controls	2-month-old Ox rats	11-month-old controls	11-month-old Ox rats
TN ($\times 10^7$/thymus)	CD4–CD8− TCRαβ-	0.72 ± 0.29	0.17 ± 0.01*[b]	0.24 ± 0.02***[c]	0.05 ± 0.01	0.12 ± 0.01***[c]
	CD45RC+CD2−	0.056 ± 0.020	0.011 ± 0.001*[b]	0.011 ± 0.001	0.005 ± 0.001	0.015 ± 0.001***[c]
	CD45RC+CD2+	0.300 ± 0.120	0.075 ± 0.004	0.100 ± 0.014	0.016 ± 0.002	0.040 ± 0.003
	CD45RC-CD2+	0.360 ± 0.150	0.081 ± 0.005	0.130 ± 0.009***[c]	0.030 ± 0.005	0.060 ± 0.004**[c]
DP ($\times 10^7$/thymus)	CD4+CD8+ TCRαβ-	21.24 ± 0.87	46.02 ± 2.09***[b]	94.73 ± 5.67***[c]	6.63 ± 0.83***[d]	21.75 ± 1.19**[c]
	CD4+CD8+TCRαβ^{low}	21.52 ± 1.09	43.40 ± 1.78***[b]	99.76 ± 3.87***[c]	5.39 ± 0.53***[d]	19.67 ± 0.49***[c]
	CD4+CD8+TCRαβ^{high}	1.68 ± 0.11	2.91 ± 0.70	4.91 ± 0.17**[c]	0.71 ± 0.09**[d]	1.87 ± 0.10
SP ($\times 10^7$/thymus)	CD4+CD8−TCRαβ^{high}	2.54 ± 0.29	5.99 ± 0.13***[b]	10.08 ± 0.41***[c]	1.16 ± 0.13***[d]	3.21 ± 0.13***[c]
	CD4−CD8+TCRαβ^{high}	0.81 ± 0.05	1.74 ± 0.08***[b]	2.85 ± 0.24***[c]	0.32 ± 0.04***[d]	0.78 ± 0.04*[c]
CD4+CD25+FoxP3+ ($\times 10^7$/thymus)	CD4+CD25+FoxP3+	0.28 ± 0.01	0.58 ± 0.03***[b]	1.15 ± 0.07***[c]	0.14 ± 0.02***[d]	0.29 ± 0.09
CD161+TCRαβ+ ($\times 10^7$/thymus)	CD161+TCRαβ+	0.26 ± 0.04	0.51 ± 0.04***[b]	0.99 ± 0.10***[c]	0.11 ± 0.02***[d]	0.33 ± 0.01*[c]

[a]The table indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n \geq 6$).
[b]2-month-old controls vs. 1-month-old controls.
[c]Ox rats vs. age-matched controls.
[d]11-month-old controls vs. 2-month-old controls.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

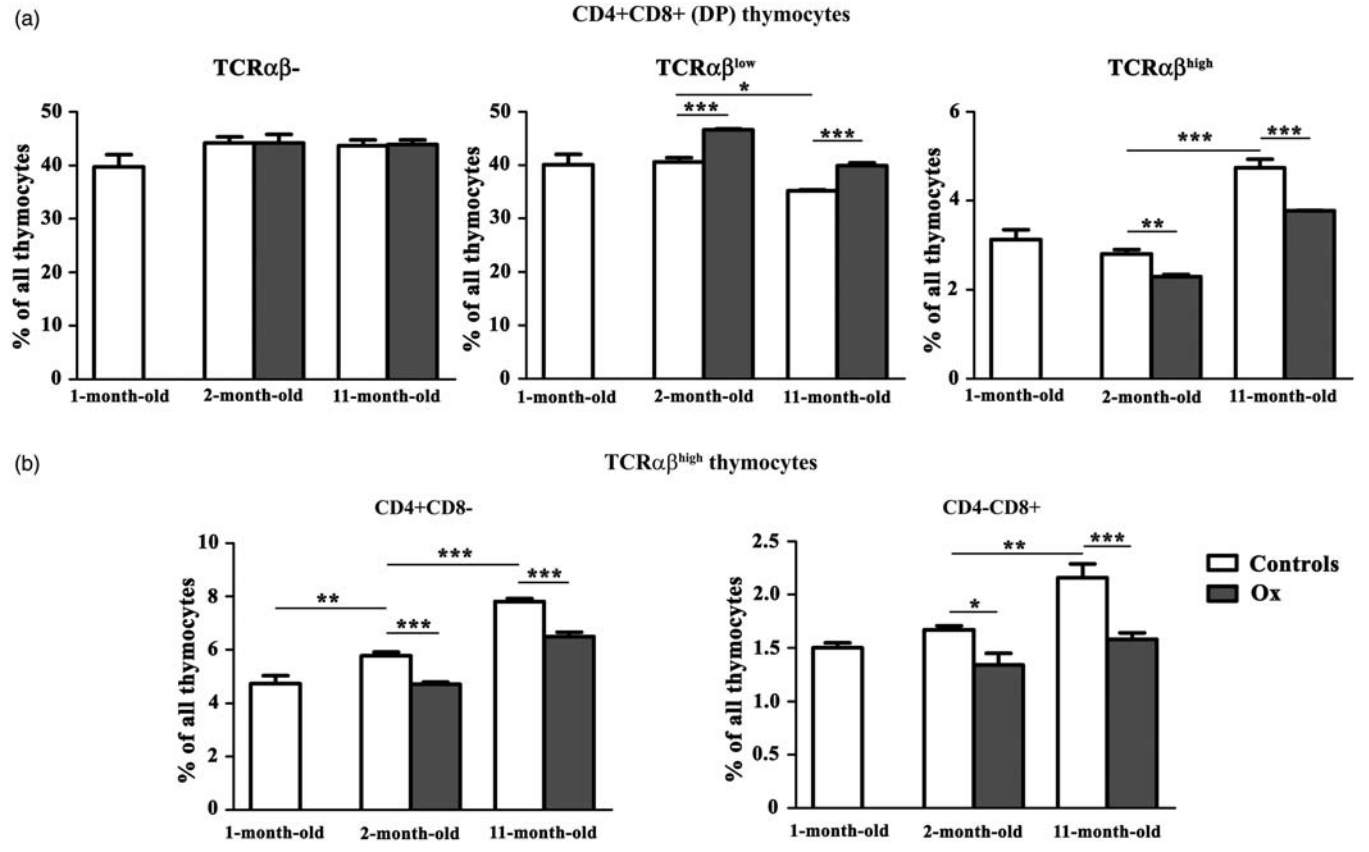

Figure 6 Ovariectomy influenced the relative proportions of CD4+CD8+ double positive (DP) and CD4+CD8− and CD4−CD8+ single positive (SP) TCRαβ^{high} thymocytes in both 2- and 11-month-old rats
(Panel a**)** The bar graphs show the relative proportions of DP thymocyte subsets delineated by the level of surface TCRαβ expression (TCRαβ-, TCRαβ^{low} and TCRαβ^{high}) and **(**Panel b**)** the most mature CD4 + CD8− and CD4−CD8+ (SP) TCRαβ^{high} thymocytes in 1-, 2- and 11-month-old control rats (controls) and 2- and 11-month-old rats Ox at age of 1 month. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 7$); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

for CD4−CD8+TCRαβ^{high} cell frequency). On the contrary, although the numbers of both CD4+CD8−TCRαβ^{high} and CD4−CD8+TCRαβ^{high} thymocytes achieved greater values in Ox rats than in age-matched controls, these effects of ovariectomy were more striking in 11-month-old rats ($F_{1,24} = 19.02$, $P < 0.001$ for CD4+CD8−TCRαβ^{high} thymocytes; $F_{1,24} = 6.28$, $P < 0.05$ for CD4−CD8+TCRαβ^{high} thymocytes). This suggested more efficient thymopoiesis in Ox rats (Table 2).

Ovariectomy-induced increase in the numbers of both CD4+ and CD8+ recent thymic emigrants (RTEs) in peripheral blood exhibited age-dependent decrease

To further confirm augmented efficiency of thymopoiesis in Ox rats, we measured the number of CD4+CD90+CD45RC− and CD8+CD90+CD45RC− RTEs in the peripheral blood. Indeed, we found greater ($P < 0.01$) numbers of CD4+ and CD8+ RTEs in peripheral blood from both 2- and 11-month-old Ox rats than in their age-matched controls (Table 3). However, the number of CD4+ and CD8+ RTEs markedly decreased ($P < 0.01$) in both Ox and control rats with age (Table 3).

Ovariectomy decreased the frequency of CD69+ thymocytes

Next, we quantified the thymocyte expression of CD69, classical marker of thymocyte positive selection.[31,32] Following ovariectomy, the percentage of all CD69+TCRαβ+ cells as well as that of CD69+TCRαβ^{high} cells was diminished in thymocyte suspensions freshly isolated from both 2- ($P < 0.01$ and $P < 0.001$ for CD69+TCRαβ+ and CD69+TCRαβ^{high}, respectively) and 11-month-old rats ($P < 0.05$ and $P < 0.01$ for CD69+TCRαβ+ and CD69+TCRαβ^{high}, respectively) to a similar extent ($F_{1,24} = 0.37$, $P > 0.05$ for CD69+ TCRαβ+ and $F_{1,24} = 0.47$, $P > 0.05$ for CD69+TCRαβ^{high}; Figure 7). Thus, a reduced positive thymocyte selection may be assumed in both 2- and 11-month-old Ox rats.

Ovariectomy increased Thy-1(CD90) thymocyte surface density

Considering the role of Thy-1 in modulation of TCRαβ signalling strength and, consequently, thymocyte selection threshold,[29] we also measured Thy-1 (CD90) mean fluorescence intensity (MFI) on TCRαβ+ thymocytes (undergoing selection processes).

Two-way ANOVA revealed that ovariectomy increased the MFI for CD90 on TCRαβ+ thymocytes in both 2- ($P < 0.001$) and 11-month-old ($P < 0.001$) rats, but its effect was more pronounced in former ($F_{1,24} = 27.94$, $P < 0.001$; Figure 8).

Influence of ovariectomy on the relative and absolute number of thymocytes exhibiting regulatory phenotype

CD4+CD25+FoxP3+ thymocytes. There was no difference in the frequency of CD4+CD25+FoxP3+ thymocytes between 1- and 2-month-old rats, whereas their frequency increased ($P < 0.001$) between the ages of 2 and 11 months (Figure 9A). The number of these cells increased ($P < 0.001$) between the age of 1 and 2 months, but it decreased ($P < 0.001$) between the age of 2 and 11 months (Table 2).

Irrespective of age, the frequency of CD4+CD25+FoxP3+ thymocytes in Ox rats did not significantly differ from that in age-matched controls (Figure 9A). However, the effects of ovariectomy on the absolute number of these cells changed with age ($F_{1,24} = 11.88$, $P < 0.01$). Namely, we found a higher ($P < 0.001$) number of CD4+CD25+FoxP3+ thymocytes in 2-month-old Ox rats than in their age-matched controls, whereas in 11-month-old rats their number was comparable to that in age-matched controls (Table 2).

CD161+TCRαβ+ thymocytes. The frequency of CD161+TCRαβ+ thymocytes remained stable between the ages of 1 and 2 months, but it increased ($P < 0.05$) between the ages of 2 and 11 months (Figure 9B). The number of CD161+TCRαβ+ cells increased ($P < 0.001$) between the age of 1 and 2 months, but it decreased ($P < 0.001$) between the ages of 2 and 11 months (Table 2).

In none of two examined time points, the frequency of CD161+TCRαβ+ thymocytes was affected by ovariectomy (Figure 9B). On the other hand, the absolute number of these cells was increased in both 2- (by approximately 60%, $P < 0.001$) and 11-month-old rats (by approximately 200%, $P < 0.05$), but to a greater extent in the latter ($F_{1,24} = 5.58$, $P < 0.05$; Table 2).

Discussion

The study confirmed that the thymocyte number achieves greater values in 2-month-old rats Ox at age of 1 month compared with age-matched controls,[20] and showed that it remains higher than in control animals by the age of

Table 3 Absolute numbers of CD4+ and CD8+ recent thymic emigrants (RTE) in peripheral blood (PB) from 1-, 2-, 11-month-old control rats and 2- and 11-month-old rats Ox at the age of 1 month[a]

	1-month-old controls	2-month-old controls	2-month-old Ox	11-month-old controls	11-month-old Ox
CD4+RTE in PB (μl^{-1})	210 ± 9	$188 \pm 5^{*b}$	$441 \pm 21^{**c}$	$69 \pm 5^{**d}$	$123 \pm 4^{**c}$
CD8+RTE in PB (μl^{-1})	129 ± 5	$101 \pm 3^{*b}$	$208 \pm 8^{**c}$	$37 \pm 4^{**d}$	$87 \pm 4^{**c}$

[a]The table indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n \geq 6$).
[b]2-month-old controls vs. 1-month-old controls.
[c]Ox rats vs. age-matched controls.
[d]11-month-old controls vs. 2-month-old controls.
*$P < 0.05$; **$P < 0.01$.

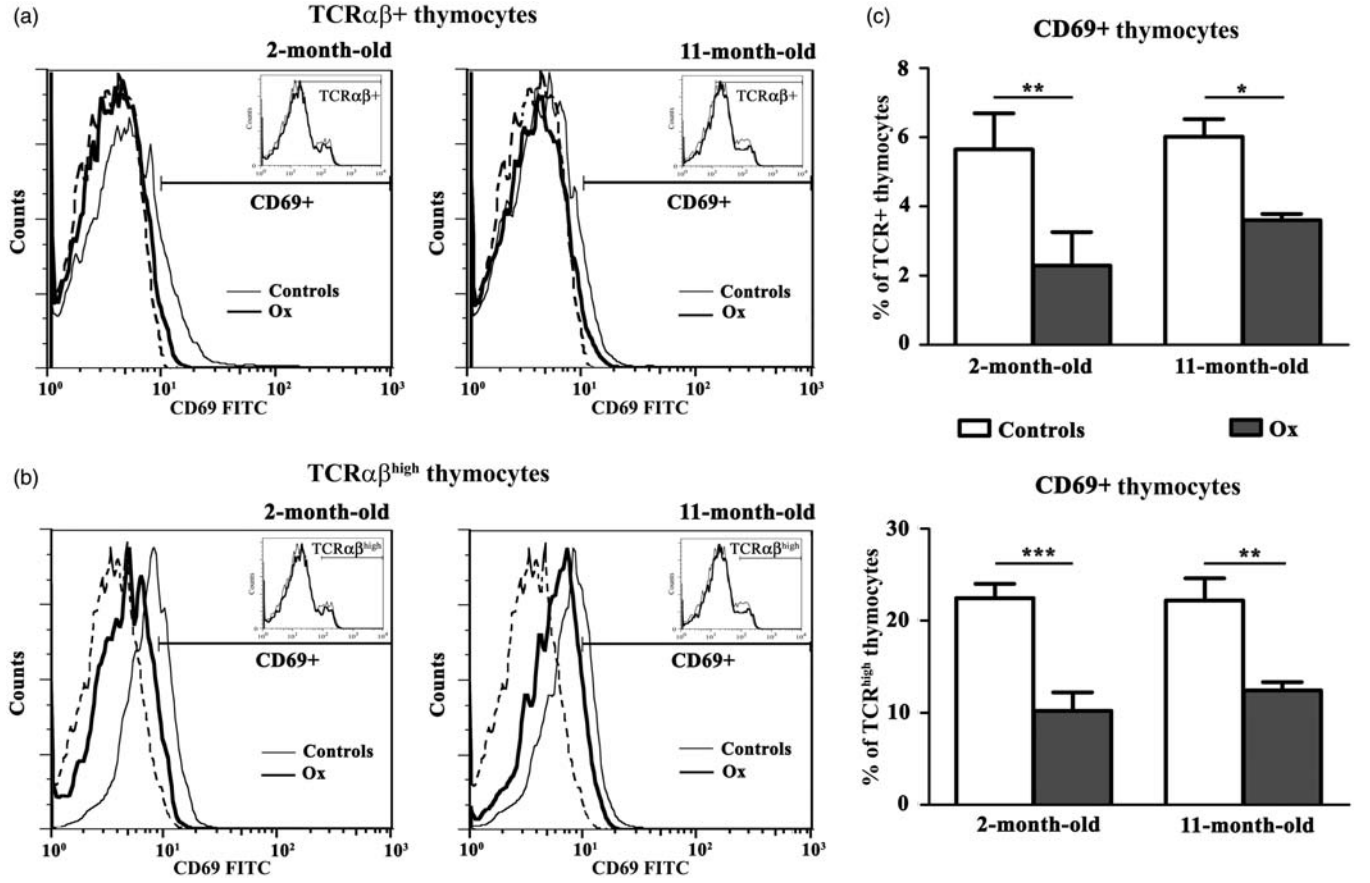

Figure 7 Ovariectomy decreased the frequency of CD69+ cells among thymocytes expressing TCRαβ in both 2- and 11-month-old rats
(Panel a) The overlayed histogram plots illustrate the expression of CD69 on TCRαβ+ (gated as shown in overlaid histogram plots inserted in upper right corner) and **(**Panel b**)** TCRαβ^{high} thymocytes (gated as shown in overlaid histogram plots inserted in upper right corner) from (left) 2- and (right) 11-month-old Ox rats and age-matched control rats (controls). Note that dashed line indicates binding of secondary antibody only. **(**Panel c**)** The bar graphs represent the percentage of CD69+ cells in **(**upper**)** TCRαβ+ and **(**lower**)** TCRαβ^{high} thymocyte subsets from 2- and 11-month-old Ox rats and age-matched controls. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 7$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

11 months. In addition, irrespective of the presence of ovarian hormones, there was the age-related decrease in the thymic cellularity, but it was less pronounced in Ox rats. Generally, this was consistent with the findings obtained in prepubertally Ox C57BL/6J (B6) mice.[21] However, in these animals, the increase in thymic cellularity registered 2 weeks postovariectomy completely disappeared by the age of 24 weeks.[21] This suggested species-specific difference in kinetics of thymic changes following ovariectomy. The difference could reflect the fact that relatively high oestrogen levels are sustained until very old age in rats, but not in mice.[22]

Nevertheless there are data on acute thymic atrophic action of both oestrogen and progesterone, the age-related thymic involution is believed to be a progesterone-independent process.[17,42] Given that the extended exposure of adult female mice to supraphysiological levels of 17β-oestradiol leads to greater frequency of apoptotic thymocytes and increased thymic Fas/FasL mRNA level,[43–45] thymoatrophic action of oestrogen has been partly ascribed to its pro-apoptotic action on thymocytes.[10] In line with these findings, the diminished frequencies of apoptotic thymocytes in thymic sections and thymocyte suspensions from Ox rats were found. On the other hand, data on oestrogen action on thymocyte proliferation are contradictory. It has been described that the pregnancy-induced rises in circulating oestrogen level increase and decrease mouse thymocyte proliferation.[46,47] Considering data indicating that, depending on concentration, oestrogens through distinct types of oestrogen-specific receptors, could increase and decrease cell (e.g. TECs) proliferation,[48] the previously mentioned discrepancy could be related to the fact that thymocyte proliferation was measured at distinct points during gestation. Given that a gradual increase in circulating oestrogen concentration postovariectomy (due the augmented adrenal production and peripheral aromatization of adrenal androgens) was found,[49] a more pronounced decrease in the frequency of proliferating thymocytes in 11-month-old than in 2-month-old Ox rats further supports the notion that the effects of oestrogen on cell proliferation are dependent on circulating oestrogen concentration. On the other hand, the comparable frequency of apoptotic thymocytes in Ox rats of the both ages suggested that the gradual rise in circulating oestrogen concentration following ovariectomy was sufficient to further suppress thymocyte proliferation, but insufficient to significantly affect thymocyte apoptosis.

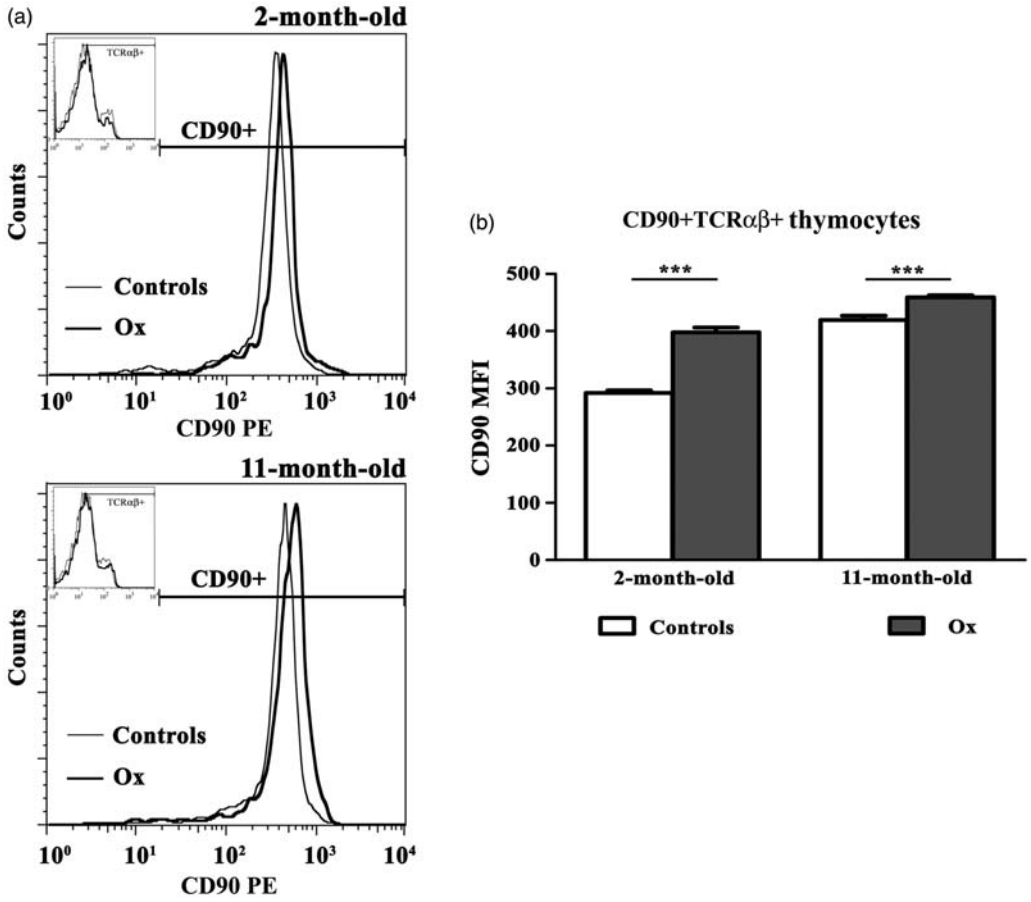

Figure 8 Ovariectomy increased Thy-1 (CD90) thymocyte surface density in both 2- and 11-month-old rats
(Panel a) The overlayed histogram plots illustrate CD90 expression on TCRαβ+ thymocytes (gated as shown in overlaid histogram plots in upper left corner) from (upper) 2- and (lower) 11-month-old Ox rats and their age-matched controls (controls). **(**Panel b**)** The bar graphs represent CD90 mean fluorescence intensity (MFI) on CD90+TCRαβ+ thymocytes from 2- and 11-month-old Ox rats and age-matched controls. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 7$); ***$P < 0.001$

The age-associated decrease in the frequency of proliferating thymocytes, in conjunction with findings indicating that the frequency of apoptotic thymocytes remained stable with age, could explain the age-related decline in thymocyte yield in Ox rats.

A comparable age-related decrease in the thymic weight in Ox and control rats, with a less pronounced decline of the thymocyte number in Ox rats, strongly suggested a more profound loss of stromal tissue in these rats. Considering that the thymic atrophy is initiated in the thymic stromal cells,[23] the previous observation may be taken as indicative of the age-related thymic involution. To further clarify the role of stromal changes in thymic involution, we measured pan-cytokeratin+ area in thymic sections of both Ox and control rats. We found an unproportional (relative to lymphoid population) increase in the size of TEC component one month postovariectomy. It is in keeping with data indicating that ovarian steroids at higher levels inhibit rat TEC proliferation promoting thymic involution.[48,50] Considering the gradual increase in circulating oestrogen concentration postovariectomy,[49] the age-related narrowing of TEC area in Ox rats due to a diminished TEC proliferation is likely.

Next, the expression of IL-6 and IL-7, which are suggested to be causally involved in the thymic involution,[51–53] was assessed. In the strikingly enlarged thymi from 2-month-old Ox rats, exhibiting the expanded pan-cytokeratin+ areas, IL-6 mRNA level was diminished. In light of the data indicating that (i) IL-6 regulates TEC survival/proliferation, acting in an autocrine manner[26] and (ii) TECs, most likely, have a negative feedback influence on the IL-6 signalling pathway to prevent the dysregulation of IL-6 production (so once IL-6 secreted by TECs reaches a plateau level, exogenous IL-6 will not increase the TEC proliferation or IL-6 production, whereas TEC survival is partially inhibited by higher doses of exogenous IL-6),[26] the diminished thymic IL-6 mRNA level in 2-month-old Ox rats was not surprising. More specifically, the decrease in thymic IL-6 mRNA in Ox rats in the absence of ovarian hormone-mediated inhibitory influence on TEC proliferation could, at least partly, reflected existence of a thymic regulatory mechanism,[26] which regulates thymic IL-6 production by controlling IL-6+ TEC abundance and consequently IL-6 stimulatory action on lymphocyte proliferation. On the other hand, the increased IL-6 mRNA expression in thymi from 11-month-old Ox rats, exhibiting the decline in

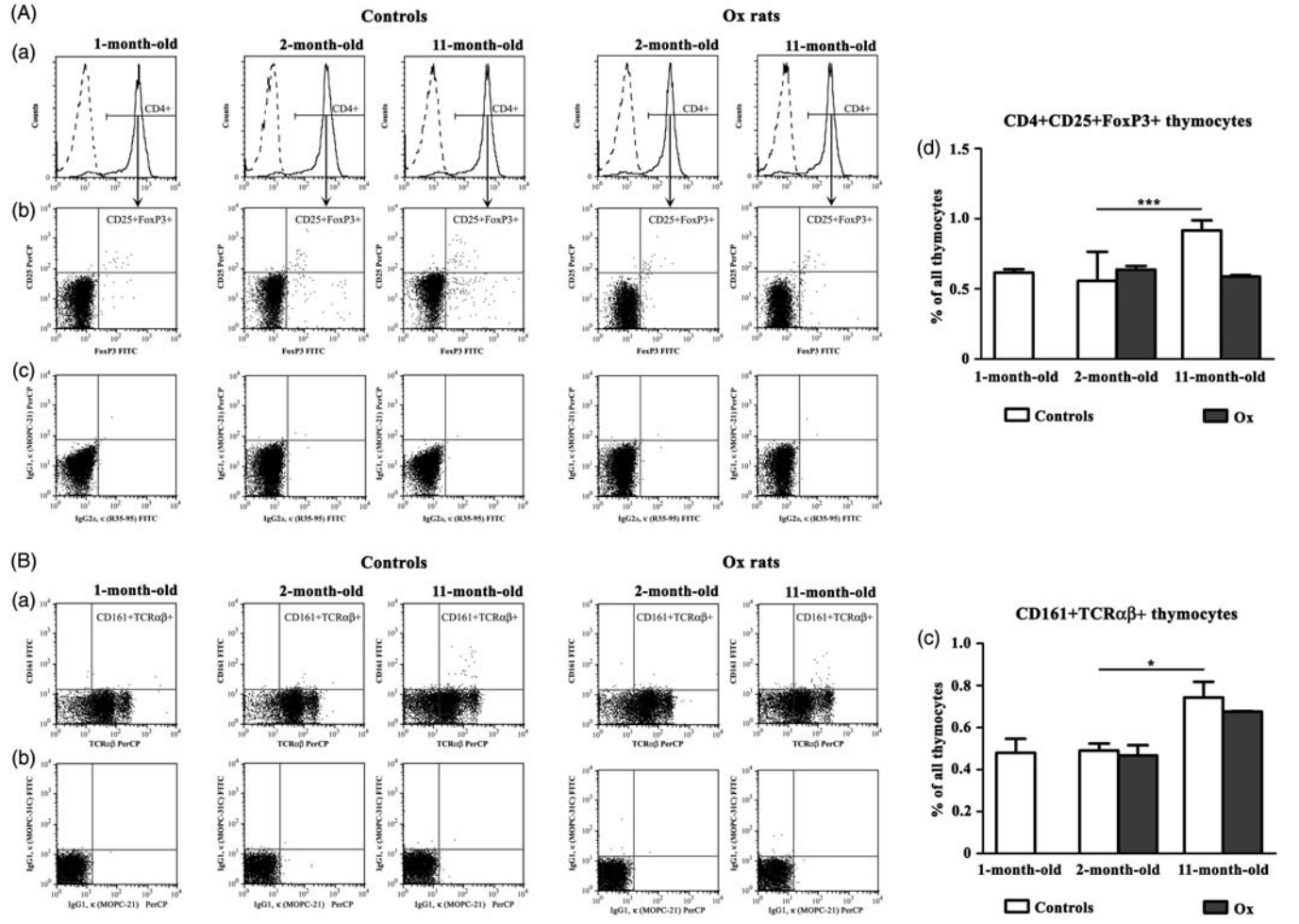

Figure 9 Ovariectomy did not affect the relative proportions of CD4+CD25+FoxP3+ and CD161+TCRαβ+ thymocytes
(Panel A) The representative histogram plots show **(a)** CD4 staining of thymocytes from 1-, 2- and 11-month-old control rats (controls) and 2- and 11-month-old Ox rats. Dashed line indicates isotype control. The flow cytometry dot plots show staining of gated (as shown in histogram plots) CD4+ cells from controls and Ox rats with **(b)** antibody to CD25 and FoxP3 or **(c)** isotype controls. **(d)** The bar graph represents the frequency of CD4+CD25+Foxp3+ thymocytes in Ox and control rats. **(**Panel B**)** The flow cytometry dot plots show staining of thymocytes from controls and Ox rats with (a) antibody to CD161 or (b) isotype controls. (c) The bar graph represents the frequency of CD161+TCRαβ+ thymocytes from controls and Ox rats. The figure indicates results from a single experiment. Similar results were obtained in three independent experiments. The data are expressed as mean ± SEM ($n = 7$); $^{*}P < 0.05$, $^{***}P < 0.001$

thymocyte number and narrowing of TEC area, compared with 2-month-old Ox rats was consistent with the data showing the dysregulated (augmented) local IL-6 production in thymi involuted by aging, stress, sepsis and cancer therapy.[51,52] In other words, the age-associated increase in thymic mRNA IL-6 expression in Ox rats could be also regarded as a sign of the thymic involution.

Furthermore, we found that (i) thymic IL-7 mRNA expression in 2-month-old Ox rats did not differ from that in age-matched controls and (ii) in Ox rats, the expression of this cytokine increased with age. The increased levels of thymic IL-7 mRNA at the time of its profound atrophy in both acute and chronic (oestradiol-administration induced) models were described.[54] It was also shown that the IL-7 expression inversely correlated with the kinetics of the thymus regeneration.[54] Considering the previous[54] and herein reported findings, one may speculate that (i) the maximum effect of ovariectomy on the thymus (and consequently the minimum level of IL-7 mRNA expression) was attained before the age of 2 months and (ii) in Ox rats, with gradual decrease in thymopoietic efficiency, IL-7 mRNA started to increase reaching by the age of 2 months the levels characteristic for age-matched controls, and even greater levels by the age of 11 months. The data obtained exploring the mechanisms underlying the changes in IL-7 mRNA expression following various experimental treatments causing thymic atrophy are in favour of this hypothesis.[54] They showed that IL-7 mRNA expression in the TECs from atrophic thymi upregulates in response to the thymocyte depletion, but not in response to the any individual treatment itself. Similar findings have been reported by some later studies.[55,56] More specifically, it has been demonstrated that there is a negative feedback mechanism in which signals derived from β-selected thymocytes (DP and SP thymocytes) modulate TEC-dependent IL-7 expression.[56] The age-related increase in IL-7 mRNA expression in both control and Ox rats is fully consistent with the previous findings. Moreover, in light of all the previously stated

findings, the age-related increase in thymic IL-7 mRNA expression in Ox rats may suggest a gradual thymic involution in the absence of ovarian hormones.

Moreover, in thymi from both 2- and 11-month-old Ox rats, substantial alterations in the frequency of the major thymocyte subsets were found. Compared with age-matched controls, in Ox rats, in spite of the reduced frequency of TN cells, the greater TN counts were found. However, the distribution of the distinct thymocyte fractions within this subset substantially differed between 2- and 11-month-old Ox rats. Compared with age-matched controls, in 2-month-old Ox rats, the frequencies of the most immature CD45RC+CD2− and CD45RC+CD2+ cells were diminished, whereas that of downstream CD45RC−CD2+ was increased. Given that the numbers of the most immature CD45RC+CD2− and CD45RC+CD2+ cells remained stable in Ox rats, whereas thymocyte apoptosis decreased with age, the previous findings suggested an accelerating transition from CD45RC+CD2− to CD45RC−CD2+ developmental stage, which is accompanied by a proportional increase in the progenitor entering rate. Data indicating that oestradiol in mice drastically reduces the lineage-negative, Flt3+Sca-1+c-Kit+ population that contains thymic homing progenitors in the bone marrow are in favour of this possibility.[55] The changes in the most immature thymocyte subpopulations from Ox rats are in keeping with the data indicating that, although thymic atrophy is initiated in thymic stromal cells, thymic regeneration requires some direct effects on thymocytes and/or thymocyte bone marrow precursors, most likely a positive feedback loop which requires expansion of progenitors and downstream thymocytes in order to expand stromal cells.[23] Moreover, considering changes in thymocyte apoptosis, it may be speculated that a diminished thymocyte apoptosis contributed to the unaltered numbers of the most mature thymocytes in two-month-old Ox rats. The unaltered frequency of TN cell descendants, i.e. DP TCRαβ- cells, further supports accelerated transition of the progenitors to CD45RC-CD2+ TN developmental stage in two-month-old Ox rats. Contrary to 2-month-old Ox rats, in 11-month-old Ox rats, the frequencies of CD45RC+CD2− and CD45RC+CD2+ cells among TN cells were increased, whereas that of CD45RC-CD2+ cells was decreased in relation to age-matched controls. Given that the frequencies of proliferating cells in freshly isolated thymocyte suspensions from 11-month-old rats were lower in age-matched controls, and that the circulating levels of ovarian steroids, acting as negative regulators of Flt3+Sca-1+c-Kit+ population in the bone marrow,[55] elevate gradually along with time after ovariectomy,[45] the previous findings, most likely, indicated a decelerating progression through the earliest thymocyte developmental steps in 11-month-old Ox rats. The unaltered frequency of descendant DP TCRαβ- cells suggested an unaltered or increased transition rate from TN to DP TCRαβ- developmental stage. Furthermore, given that in both 2- and 11-month-old Ox rats similar changes in the distribution of DP and SP thymocytes with respect to age-matched controls were found, it seems obvious that the age-related decrease in the thymopoietic efficiency (judging by the reduced number of the most mature SP thymocyte and RTEs) in Ox rats could be related to a decrease in bone marrow progenitor cell generation and their transition through early developmental steps.

The unaltered frequency of DP TCRαβ- cells harbouring mainly cells which surpassed β-selection,[3] in conjunction with the increased frequency of downstream DP TCRαβ^{low} cells, suggested a more efficient β-selection in Ox rats. This assumption was corroborated by the findings indicating the lower frequency of apoptotic cells in the thymic cortex and thymocyte cultures from both 2- and 11-month-old Ox rats compared with age-matched controls. The increased frequency of downstream DP TCRαβ^{low} cells entering positive selection[3] in Ox rats of both ages in respect to age-matched controls, in conjunction with the decreased frequencies of post-selected DP TCRαβ^{high} thymocytes,[57] and descendant CD4+CD8− and CD4−CD8+ TCRαβ^{high} SP cells was indicative of a less efficient positive selection. The reduced percentage of positively selected CD69+ TCR^{high} thymocytes in these animals further corroborated the previous assumption. However, it should be pointed that the reduced frequency of the most mature SP cells could also reflect an augmented negative selection. However, given that the thymocyte surface density of Thy-1 (CD90), which is shown to act as a negative regulator of thymocyte selection threshold and subsequently negative selection,[33] was increased in Ox rats, the previous option does not seem likely. The changes in Thy-1 expression could be, at least in 2-month-old Ox rats, related to the lower thymic noradrenaline concentration.[58] Namely, this neurotransmitter is shown to act as a negative regulator of Thy-1 mRNA availability.[59] The diminished frequency of apoptotic cells in the thymi of Ox rats also contrasted with more efficient thymocyte negative selection. In favour of a reduced thymocyte transition from DP to SP developmental stage in Ox rats are findings showing that oestrogen enhances the transition from DP to the SP thymocyte developmental stage in mice.[60] Moreover, a more pronounced decrease in pre-emigrational proliferation of SP cells could also contribute to the diminished frequency of SP cells. Considering the lower frequency of proliferating cells in thymocyte suspensions from 11-month-old Ox rats, the lower counts of SP thymocytes in these animals than in their younger counterparts could be related to the diminished thymocyte proliferation.

In Ox rats, the frequency of Tregs and NKT cells remained stable, whereas their numbers achieved greater values. The increased number of Tregs in thymi of Ox rats is consistent with data showing a reduced number of CD4+CD25+ in thymi from pregnant mice.[47] The unaltered frequency of Tregs, in light of the diminished that of all CD4+CD8− SP cells (data not shown) and CD4+CD8− TCRαβ^{high} cells in Ox rats, suggests that ovariectomy is more effective in modulating development of non-regulatory than regulatory CD4+CD8− SP thymocytes. Considering that naturally arising Treg cell lineage commitment is induced in response to self-reactive TCRs with an avidity range for self-peptide-MHC somewhere between that is required for positive and negative selection,[61]

enlargement of thymic Treg could be related to a less efficient thymic negative selection.

Although NKT cells develop in thymus alongside conventional T cells and share early stages of thymic development, they do not undergo the same manner of thymic positive selection to that shown for conventional T cells.[62] Therefore, the unaltered frequency of NKT cells expressing in rat CD8+ phenotype[63] in the face of the diminished frequency of all CD4−CD8+ SP cells (data not shown) and CD4−CD8+ TCR$\alpha\beta^{high}$ cells further corroborate diminished positive selection of conventional thymocytes in Ox rats.

In conclusion, although the withdrawal of ovarian hormones in prepubertal age increased the efficiency of thymopoiesis in two-month-old rats, it failed to prevent the age-related changes in thymic stromal and lymphoid component underlying thymic involution. Consequently, a significant decline in thymopoiesis with age was registered not only in control, but also in Ox rats. Furthermore, the study indicated that in Ox rats, most likely, changes at prethymic level as well as impaired early pre-TCR developmental stages, in addition to reduced proliferative capacity of the mature cells, contributed to the diminished thymopoietic efficiency in 11-month-old Ox rats. Moreover, it suggested that these changes in thymopoiesis could be partly underlined by the alterations in TECs and *vice versa*. These findings seem to be important not only for understanding basic mechanisms providing immunosenescence, but also for formulating strategy to prevent/minimize negative effect of aging on the immune system.

Author contributions: IP, MP, BB, ZSV and NAR carried out rat experiments and immunophenotyping analyses. DK participated in data acquisition. MP and NAR analysed the data. GL and NAR conceived, designed and coordinated the study. GL and NAR wrote the manuscript. ZSV provided expert consultation. All authors read and approved the final manuscript.

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