

## Ovarian hormone level alterations during rat post-reproductive life-span influence CD8 + T-cell homeostasis

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### Abstract

The study examined the putative role of ovarian hormones in shaping of rat peripheral T-cell compartment during post-reproductive period. In 20-month-old rats ovariectomized (Ox) at the very end of reproductive period, thymic output, cellularity and composition of major TCR $\alpha\beta$  + peripheral blood lymphocyte and splenocyte subsets were analyzed. Ovariectomy led to the enlargement of CD8 + peripheral blood lymphocyte and splenocyte subpopulations. This reflected: (i) a more efficient thymic generation of CD8 + cells as indicated by increased number of CD4+CD8 + double positive and the most mature CD4-CD8+TCR $\alpha\beta$ <sup>high</sup> thymocytes and CD8 + recent thymic emigrants (RTEs) in peripheral blood, but not in the spleen of Ox rats, and (ii) the expansion of CD8 + memory/activated peripheral blood lymphocytes and splenocytes. The latter was consistent with a greater frequency of proliferating cells among freshly isolated memory/activated CD8 + peripheral blood lymphocytes and splenocytes and increased proliferative response of CD8 + splenocytes to stimulation with plate-bound anti-CD3 antibody. The former could be related to the rise in splenic IL-7 and IL-15 mRNA expression. Although ovariectomy affected the overall number of CD4 + T cells in none of the examined compartments, it increased CD4+FoxP3 + peripheral blood lymphocyte and splenocyte counts by enhancing their generation in periphery. Collectively, the results suggest that ovariectomy-induced long-lasting disturbances in ovarian hormone levels (mirrored in diminished progesterone serum level in 20-month-old rats) affects both thymic CD8 + cell generation and peripheral homeostasis and leads to the expansion of CD4+FoxP3 + cells in the periphery, thereby enhancing autoreactive cell control on account of immune system efficacy to combat infections and tumors.

**Keywords:** Ovarian gland hormones, mature naïve T cells, memory/activated T cells, regulatory T cells  
T-cell proliferation/apoptosis

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### Introduction

Immunosenescence is characterized by a progressive decline in the functioning of the immune system. The disorders in immune response in elderly reflect intrinsic defects occurring at the level of lymphocytes, antigen presenting cells and other cells participating in immune response, and changes at the level of cell subpopulations. The latter results primarily from age-related disturbances in new immune cell generation, renewal and death, as well as cell subpopulation dynamics.<sup>1,2</sup> At clinical level, age-related immune changes lead to weakening of the immune response to infectious agents and tumors, less efficient response to vaccines and increased risk of autoimmunity in the elderly.<sup>3,4</sup>

Although it is clear that aging affects innate immune function, accumulating evidence indicate that the adaptive arm of the immune system, particularly the T-cell compartment, exhibits more profound and consistent changes than the innate arm.<sup>5</sup> They primarily rise from thymic involution, and consequent reduction in the thymic output. This cause age-related narrowing of T-cell repertoire diversity in the periphery, and consequently diminishes the efficacious defense against infection with new or re-emerging pathogens with advanced ages.<sup>1,2,6</sup> The age-related decline in the number of naïve T cells is partially compensated by their homeostatic expansion due to more extensive divisions and/or a longer lifespan. This requires weak stimulation of TCR and receptors for homeostatic IL-7 cytokine.<sup>7-9</sup>

In addition, cumulative exposure to foreign pathogens and environmental antigens promotes the accumulation of memory T cells with age.<sup>6,10</sup> Their survival is TCR-independent, but requires combination of IL-7 and IL-15 signals.<sup>11</sup>

Thymic involution in rodent has been linked with the peripubertal elevation of gonadal steroid hormone level.<sup>12-14</sup> In support of this notion are data that in rodent surgical castration before puberty and in early adulthood prevents thymic involution and reverses the early involutive changes, respectively.<sup>15-20</sup> However, differently from the role of ovarian steroids in the initiation of rodent thymic involution, their role in maintenance and progression of thymic involution is still a matter of dispute.<sup>21</sup> The latter seems to be particularly relevant for the rat as it has been shown in many studies that, despite of lack of cyclicity, estrogen concentration is maintained at relatively high level in many rat strains even in advanced age.<sup>22-24</sup> Our findings indicating that one-month long deprivation of ovarian hormones initiated at the very end of rat reproductive age leads to reversal of thymic involution and re-shaping of peripheral T-cell compartment corroborate the notion that ovarian hormones contribute to the maintenance/progression of thymic involution, and consequently remodeling of the peripheral T-cell compartment.<sup>25</sup> Specifically, we showed that in 11-month-old AO rats ovariectomized (Ox) at the age of 10 months: (i) thymopoiesis is more efficient as shown by increased absolute and relative numbers of CD4+ and CD8+ recent thymic emigrants (RTEs) in peripheral blood and spleen, (ii) CD4+:CD8+ cell ratio in the periphery is altered, and (iii) number of CD4+CD25+FoxP3+ cells in both thymus and peripheral blood is increased.<sup>25</sup> However, there are no data on the long-lasting effects of ovarian gland removal at that time point on the thymopoiesis and peripheral T-cell compartment. These data are needed to get the insight into the putative role of ovarian hormones in the age-related reshaping of peripheral T-cell compartment. Having all that in mind we undertook the present study. We firstly verified the influence of aging on the peripheral T-cell compartment by examining the relative proportions of the major T-cell subpopulations and their subsets defined by the expression of activation/differentiation antigens and regulatory cell markers in 10- and 20-month-old control AO rats. Next, to assess the putative contribution of ovarian hormones to the age-related changes in the peripheral T-cell compartment, T lymphocytes from peripheral blood and spleen of 20-month-old (aged) rats subjected to bilateral ovariectomy or sham-ovariectomy at the age of 10 months were examined for the composition of the main T-cell subpopulations in respect to proportion of mature naïve and memory/activated cells and regulatory T cells, that is CD4+FoxP3+ and CD161+TCR $\alpha\beta$ + natural killer (NKT) cells. To elucidate mechanisms underlying the age-related ovarian hormone-dependent changes, we evaluated the efficacy of thymopoiesis in Ox and sham-Ox rats by examining the commonly used indicators of this process as are numbers of RTEs, all thymocytes, CD4+CD8+ double positive and the most mature CD4+CD8- and CD4-CD8+ TCR $\alpha\beta$ <sup>high</sup> thymocytes. In addition, to assess contribution of the putative ovarian hormone-dependent extrathymic mechanisms maintaining

the peripheral T-cell homeostasis in aged rats, we explored: (i) the proliferation and apoptosis of mature naïve and memory/activated CD4+ and CD8+ peripheral blood lymphocyte (PBL) and splenocyte T subsets, (ii) the expression of receptors for the main T-cell survival factors, that is IL-7 and IL-15, on T lymphocytes and (iii) expression of mRNAs for the key homeostatic cytokines (IL-7 and IL-15) in both 20-month-old Ox and age-matched control rats.

## Materials and methods

### Animals

Female inbred Albino Oxford (AO) rats, born and bred in the animal housing facility at the Immunology Research Centre "Branislav Janković" in Belgrade were used. The animals 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

Ten-month-old rats were randomly assigned to ovariectomy or sham-ovariectomy or left without any treatment and euthanized 10 months post-ovariectomy. To assess the influence of aging on the analyzed parameters 10-month-old non-operated rats were also included in the study. The each experimental group consisted of at least 6 animals.

The animals anesthetized using solution (0.08 mL/100 g b.w.) containing ketamine (100 mg/mL, Ketamidol<sup>®</sup>, Richter Pharma AG, Austria), xylazine (20 mg/mL, Xylased<sup>®</sup>, Bioveta, Czech Republic) and saline in a 1:0.5:8.5 ratio were subjected to bilateral ovariectomy or sham-ovariectomy as previously described in details.<sup>25</sup> Completeness of ovariectomy was validated by histological examination of removed tissue and detailed inspection at post-mortem. Animals were euthanized by exposure to increasing doses of CO<sub>2</sub> followed by cardiac puncture exsanguinations.

All animals were examined for overt signs of illness, including low body weight, visible lesions, tumors or splenomegaly. According to these parameters, all animals included in the experiment described in this manuscript were healthy. To test reproducibility of results, the experiment was performed two more times. One sham-Ox and one non-operated 20-month-old rat from these experiments were excluded from the analyses due to urogenital tract associated tumors.

### Serum concentration of estrogen and progesterone

Serum estrogen and progesterone levels were determined using the IMMULITE solid-phase competitive chemiluminescent enzyme immunoassay (EIA) on an IMMULITE 1000 analyzer (Euro/DPC, UK), according to the guidelines provided by the manufacturer. There was no significant difference in either estradiol or progesterone levels between sham-Ox and non-operated age-matched control animals.

Therefore, in all subsequent analyses and graphs these two groups were considered as unique control group. In control rats, serum levels of estradiol ( $145.79 \pm 14.95$  pmol/L in 10-month-old vs  $153.25 \pm 7.15$  pmol/L in 20-month-old rats) and progesterone ( $67.40 \pm 7.30$  nmol/L vs  $44.02 \pm 6.53$  nmol/L in 10- and 20-month-old rats, respectively) remained stable between the ages of 10 and 20 months. Ten months post-ovariectomy, only progesterone concentration was lower ( $p \leq 0.05$ ) in Ox ( $19.10 \pm 1.32$  nmol/L) than in control ( $44.02 \pm 6.53$  nmol/L) rats.

### Chemicals, antibodies and immunoconjugates

Sodium azide ( $\text{NaN}_3$ ) was purchased from Sigma-Aldrich (Taufkirchen, Germany), fetal calf serum (FCS) was purchased from Gibco (Grand Island, NY, USA), while 7-Amino-actinomycin D (7-AAD) and recombinant rat IL-2 were obtained from BD Biosciences Pharmingen (Mountain View, CA, USA). Phenol red-free RPMI-1640 medium (with L-glutamine) was obtained from Gibco (Invitrogen Corporation, Carlsbad, CA, USA). To prepare complete RPMI medium 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma-Aldrich), 1 mM sodium pyruvate (Serva, Heidelberg, Germany), 100 units/mL penicillin (ICN, Costa Mesa, CA, USA), 100 mg/mL streptomycin (ICN) and 10% FCS (Gibco) were added. FCS was previously inactivated by heating the serum at  $56^\circ\text{C}$  for 30 min.

The antibodies (Abs) namely Phycoerythrin (PE)-conjugated anti-CD45RC (clone OX-22), fluorescein isothiocyanate (FITC)/PE-conjugated anti-CD4 (clone OX-38), FITC/PE-conjugated anti-CD8 (clone OX-8), peridinin chlorophyll protein (PerCP)-conjugated/purified anti-TCR $\alpha\beta$  (clone R73), PerCP/biotin-conjugated anti-CD90 (Thy-1.1, clone OX-7), FITC-conjugated anti-CD161a (clone 10/78) and biotin-conjugated anti-CD25 (clone OX-39) were purchased from BD Biosciences Pharmingen. Polyclonal PE-conjugated anti-IL-7R $\alpha$ /CD127 Ab was purchased from R & D Systems (Abingdon, UK), whereas polyclonal FITC-conjugated IL-15R $\alpha$  Ab was obtained from Antibodies-online Inc. (Atlanta, GA, USA). Streptavidin-PerCP and isotype IgG controls were obtained from BD Biosciences Pharmingen.

### Single-cell suspension preparation

Spleens and thymuses were carefully removed, weighed, and single-cell suspensions were prepared by grinding respective tissue on a sterile 60- $\mu\text{m}$  sieve screen submerged in ice-cold phosphate-buffered saline (PBS) supplemented with 2% FCS and 0.01%  $\text{NaN}_3$  (FACS buffer). Blood samples and single-cell splenocyte suspensions were subjected to  $\text{NH}_4\text{Cl}$  lyses to remove red blood cells. The resulting cell suspensions were washed in ice-cold FACS buffer. The cells in suspension were enumerated using an improved Neubauer hemacytometer and trypan blue dye to exclude non-viable cells and adjusted to  $1 \times 10^7$  cells/mL.

### Isolation of cells by magnetic-activated cell sorting (MACS) system

For analyses of proportions and numbers of cells at distinct stages of maturation/activation within CD4+ and CD8+ T-lymphocyte subpopulations, TCR $\alpha\beta$ +PBLs and splenocytes were isolated using magnetic-activated cell sorting (MACS), as previously described.<sup>26</sup> Briefly, cells were firstly labeled with mouse anti-rat TCR $\alpha\beta$  specific antibody and anti-mouse IgG microbeads, and then TCR $\alpha\beta$ + cells were isolated using LS column and Quadro MACS separator (Miltenyi Biotec, Gladbach, Germany) and collected for further staining.

In addition, for analysis of proliferation and apoptosis of mature naïve and memory/activated cells, TCR $\alpha\beta$ + cells were positively selected following the depletion of cells expressing CD90 by incubation with biotin-conjugated mouse anti-rat CD90 antibody and anti-biotin microbeads.

### Flow cytometric analysis (FCA)

Following immunostaining, all samples were acquired on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) using CELLQuest software (BD Biosciences), whereas data analyses were performed using FlowJo software version 7.8. (TreeStar Inc, Ashland, OR, 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 forward scatter (FSC) and side scatter (SSC). The absolute number of T lymphocytes and cells within the major subpopulations of T lymphocytes and their subsets were calculated by multiplying the absolute number of cells in "lymphocyte gate" (calculated from the absolute number of all splenic cells or white blood cells/mL blood following erythrocyte lysis and relative proportions of cells in "lymphocyte gate" defined by FSC vs SSC) by the relative proportion of cells in "lymphocyte gate" displaying specific phenotype.

**Surface antigen expression.** Aliquots of  $1 \times 10^6$  cells were incubated for 30 min on ice with saturating concentrations of fluorochrome-conjugated mAbs (direct labeling) or with biotin-conjugated anti-CD25 mAb (indirect labeling) and then washed in FACS buffer. When biotin-conjugated anti-CD25 mAb was applied, cells were incubated with streptavidin-PerCP for an additional 30 min. After labeling the cells were washed in FACS buffer, followed by PBS containing 0.01% sodium azide.

**Intracellular antigen expression.** After CD4/CD25 surface immunolabeling, cells were subjected to immunostaining for FoxP3 using a commercial Foxp3 staining set containing a FITC-conjugated anti-FoxP3 mAb (clone FJK-16s) according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

### T-cell apoptosis

Briefly, aliquots of  $0.5 \times 10^6$  TCR $\alpha\beta$ +CD90- PBLs and splenocytes were incubated with mAb cocktail containing FITC-conjugated anti-CD8 and PE-conjugated

anti-CD45RC mAbs for 30 min. After washing in cold PBS, cells were incubated with 5  $\mu$ L of DNA binding dye 7-AAD at 4°C for additional 20 min. 7-AAD staining allows delineation of living cells with a normal morphology (unchanged FSC/SSC) and a normal membrane integrity (7-AAD<sup>dull</sup>) from apoptotic cells with a modified morphology (lower FSC and higher SSC) and membrane alteration (7-AAD<sup>dim/bright</sup>).<sup>27</sup> The previous studies have shown that 7-AAD analysis closely matches with annexin V-FITC analysis.<sup>28,29</sup>

### T-cell proliferation

The proliferation of TCR $\alpha\beta$ +CD8+CD90- PBLs and splenocytes isolated using MACS, and CD8+ splenocytes following in vitro stimulation with plate-bound anti-CD3 antibody was examined.

Briefly, as previously described,<sup>30</sup> for analysis of CD8+ splenocyte proliferation capacity, 96-well round-bottom plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 100  $\mu$ L of anti-rat CD3 mAb (NA/LE, Clone G4.18, BD Pharmingen) diluted in PBS to 1  $\mu$ g/mL. Subsequently, plates were washed three times with PBS, and  $2 \times 10^5$  splenocytes in 200  $\mu$ L phenol red-free RPMI-1640 culture medium supplemented with recombinant rat IL-2 (0.4 ng/mL) was added to each well. Following 48 h cultivation the cells were analyzed for proliferation.

To determine proportion of proliferating cells among CD8+TCR $\alpha\beta$ +CD90- PBLs and splenocytes exhibiting mature naïve (CD45RC+) and memory (CD45RC-) phenotypes, and among cultivated CD8+ splenocytes, cell cycle analysis was performed simultaneously with two-color surface immunofluorescence staining.<sup>31</sup> Briefly, following appropriate cell surface antigen staining and washing, cells were resuspended in 150  $\mu$ L of 50% FCS in PBS and fixed/permeabilized by incubation with 450  $\mu$ L of cold 70% ethanol in double distilled H<sub>2</sub>O overnight. Following the incubation, cells were washed with cold PBS and incubated with 10  $\mu$ L of 7-AAD at 4°C for additional 30 min. For DNA analysis Watson (pragmatic) model<sup>32</sup> and proliferation platform generated by FlowJo software version 7.8. (TreeStar Inc.) was used.

### RNA extraction and real-time PCR

Considering that: (i) T-cell homeostatic regulation requires contact of these cells with specialized cells in defined regions of the lymphoid tissues<sup>11,33</sup> and interaction with soluble molecules (homeostatic cytokines) produced by various lymphoid tissue cells and (ii) homeostatic cytokine (IL-7, IL-15, TGF- $\beta$ ) proteins are not easily detectable in tissue homogenates, their expression was quantified at mRNA level.<sup>34-36</sup> Total RNA was isolated from splenic tissue samples of 20-month-old Ox and control rats using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA) and Total RNA Chemistry (Applied Biosystems). Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and 5  $\mu$ L of cDNA was used for real-time PCR. Triplicate 25  $\mu$ L reactions were run under Applied Biosystems 7500 universal cycling

conditions. Gene Expression Master Mix and commercial TaqMan Gene Expression Assays for rat IL-7 (Rn00681900\_m1), IL-15 (Rn00689964\_m1), TGF- $\beta$ 1 (Rn00572010\_m1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Rn99999916\_s1), were obtained from Applied Biosystems. All procedures were performed according to manufacturer's instructions. Input cDNA was normalized to housekeeping gene GAPDH 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

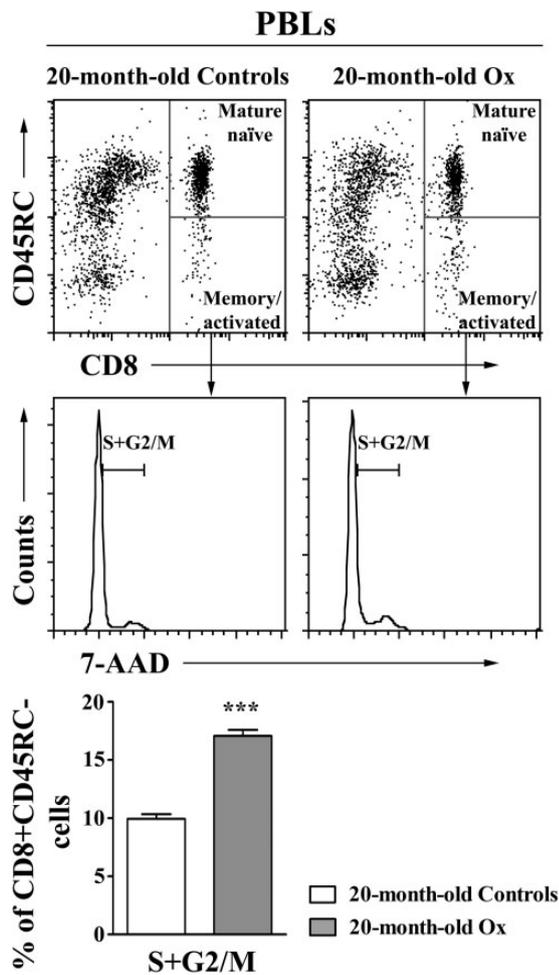
To assess influence of ovariectomy on T-cell homeostasis three independent experiments were performed. The data from these three experiments were combined into a single analysis (mega-analysis). Statistical significance of influence of aging and ovariectomy on T cells was tested using mixed model two-way ANOVA [aging and ovariectomy ("controlled factor", i.e. "fixed factor")  $\times$  experimental day ("random factor")] following by Bonferroni test for post hoc comparisons using PASW Statistic 19 statistical software. Differences were considered to be significant when  $p \leq 0.05$ .

## Results

### Long-lasting ovarian gland deprivation increases the number of CD8+TCR $\alpha\beta$ + cells in peripheral blood and spleen

**Peripheral blood.** The absolute numbers of TCR $\alpha\beta$  + PBLs and the cells within their major subpopulations (CD4 + and CD8 + cells) were determined using FCA (Supplementary Figure 1A). In control rats the count of all TCR $\alpha\beta$  + PBLs and those of CD4 + and CD8 + PBLs were comparable in 10- and 20-month-old rats (Table 1). However, at the age of 20 months the count of TCR $\alpha\beta$  + cells was greater ( $p \leq 0.05$ ) in Ox than in control rats (Table 1). This reflected a rise ( $p \leq 0.001$ ) in the number of CD8 + cells, whereas that of CD4 + cells was comparable in Ox and control rats (Table 1). Consequently, CD4+:CD8 + PBL ratio ( $1.29 \pm 0.12$ ), i.e. immunoregulatory index, in Ox rats was lower ( $p \leq 0.01$ ) than in age-matched control ( $1.67 \pm 0.02$ ) rats.

**Spleen.** In control rats, the count of TCR $\alpha\beta$  + splenocytes decreased ( $p \leq 0.001$ ) with aging (Table 1). This decrease reflected a decline ( $p \leq 0.001$ ) in the number of CD4 + cells (Table 1). At the age of 20 months the count of TCR $\alpha\beta$  + splenocytes in Ox rats exceeded ( $p \leq 0.001$ ) that in age-matched controls (Table 1). Similar to peripheral blood, this was a consequence of the increase ( $p \leq 0.001$ ) in the count of CD8 + splenocytes, whereas that of CD4 + cells remained unaltered (Table 1). Therefore, CD4+:CD8 + splenocyte ratio in Ox rats was shifted towards CD8 + cells ( $1.31 \pm 0.07$  in 20-month-old controls vs  $1.01 \pm 0.02$  in Ox rats).



**Figure 1** Effects of long-lasting ovarian gland deprivation on the proportion of proliferating cells within CD8 + memory/activated peripheral blood lymphocytes (PBLs). Flow cytometry histogram profiles illustrate 7-AAD staining of CD8 + memory/activated PBLs (CD8 + CD45RC<sup>-</sup>) gated as indicated in two-color dot plots showing CD8 versus CD45RC staining of TCR $\alpha\beta$ +CD90<sup>-</sup> PBLs from 20-month-old control rats (Controls) and 20-month-old rats ovariectomized (Ox) at the age of 10 months. TCR $\alpha\beta$ +CD90<sup>-</sup>PBLs were isolated by magnetic-activated cell sorting (MACS). The bar graph represents the percentage of proliferating cells (cells in S+G2/M phases of cell cycle) within CD8 + memory/activated PBLs from 20-month-old Ox and control rats. The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n = 18$  rats in 20-month-old Ox group,  $n = 34$  rats in 20-month-old Controls) \*\*\* $p \leq 0.001$

### Long-lasting ovarian gland deprivation increases the number of CD4+FoxP3+ cells in peripheral blood and spleen but it does not affect NKT cell counts

**CD4+FoxP3+ cells.** In the rat, under physiological conditions, there is unidirectional differentiation of thymic-derived immunoregulatory CD25+FoxP3+ cells (Tregs) to effector CD25-FoxP3+ cells.<sup>37</sup> Thus, apart from CD4+CD25+FoxP3+ cells encompassing undifferentiated thymic-derived Tregs and the cells arising in the periphery by conversion of naïve Foxp3-CD4+T conventional cells into “induced” cells of this phenotype,<sup>38</sup> there is also a pool of differentiated CD4+CD25-FoxP3+ cells of thymic origin.<sup>37</sup> Accordingly, we examined in rats of both ages absolute number of these cells using FCA (Supplementary Figure. 1B). In control rats, the number of both

CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+ PBLs increased ( $p \leq 0.001$ ) between the ages of 10 and 20 months (Table 1). Ovariectomy enhanced this effect of aging, so that the number of CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+ PBLs exceeded ( $p \leq 0.001$ ) those in age-matched controls (Table 1). Given that the number of CD4+CD25+FoxP3+ thymocytes was comparable in 20-month-old Ox ( $0.053 \times 10^7 \pm 0.007 \times 10^7$ ) and control ( $0.079 \times 10^7 \pm 0.011 \times 10^7$ ) rats, the increase in CD4+CD25+FoxP3+ PBLs, most likely, did not reflect a more efficient thymic production.

Differently from peripheral blood, in spleen from control rats the number of CD4+CD25+FoxP3+ splenocytes decreased ( $p \leq 0.001$ ) between the ages of 10 and 20 months (Table 1). However, the number of CD4+CD25-FoxP3+ splenocytes remained stable between these two age points (Table 1).

In Ox rats both the numbers of CD4+CD25+FoxP3+ ( $p \leq 0.001$ ) and CD4+CD25-FoxP3+ ( $p \leq 0.01$ ) splenocytes were greater than in age-matched controls (Table 1).

**NKT cells.** We also analyzed the numbers of NKT cells, which in rat exhibit mainly CD8+CD161+TCR $\alpha\beta$ + phenotype<sup>39</sup> in peripheral blood and spleen from control and Ox rats. The number of NKT PBLs, was comparable in 10- and 20-month-old control rats (Table 1). Ovariectomy did not influence the number of NKT cells (Table 1).

Given that NKT cells encompass both thymus-dependent and thymus-independent cells arising in the periphery,<sup>40</sup> we also examined the number of CD161+TCR $\alpha\beta$ + cells in thymi from both Ox and control rats. The number of these cells was comparable in thymi from Ox rats ( $0.047 \times 10^7 \pm 0.003 \times 10^7$ ) and age-matched controls ( $0.039 \times 10^7 \pm 0.004 \times 10^7$ ).

Analysis of splenocytes revealed that aging also did not influence the number of NKT splenocytes (Table 1). Ovariectomy also did not significantly alter the number of NKT splenocytes (Table 1).

### Long-lasting ovarian gland deprivation increases the number of CD8+ RTEs in blood and number of activated/memory CD8+ TCR $\alpha\beta$ + PBLs

The absolute number of cells at distinct stages of activation/maturation within CD4+ and CD8+ PBL pools was quantified using FCA (Supplementary Figure. 1C).

**CD4+ PBLs.** In control rats, the number of CD4+ RTEs, which in the rat exhibit CD90+CD45RC<sup>-</sup> phenotype,<sup>41</sup> decreased ( $p \leq 0.001$ ) between the ages of 10 and 20 months (Table 2). Ovariectomy did not influence the number of CD4+ RTEs (Table 2). Furthermore, in control rats the count of CD4+CD45RC<sup>-</sup> cells, presumably mature naïve cells,<sup>42</sup> and all naïve (RTEs+mature naïve cells) cells decreased ( $p \leq 0.01$  and  $p \leq 0.001$ , respectively), whereas that of CD4+CD90-CD4RC<sup>-</sup> cells, presumably memory/activated<sup>43</sup> cells increased ( $p \leq 0.05$ ) with aging (Table 2). The numbers of mature naïve and all naïve

**Table 1** The number of TCR $\alpha\beta$ +, CD4+TCR $\alpha\beta$ +, CD8+TCR $\alpha\beta$ +, CD8+CD161+TCR $\alpha\beta$ +, CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+ peripheral blood lymphocytes (PBLs) and splenocytes from 10- and 20-month-old control rats (Controls) and 20-month-old rats ovariectomized (Ox) at the age of 10 months

	10-month-old Controls	20-month-old Controls	20-month-old Ox
<b>PBLs</b> ( $\times 10^6$ /mL)			
TCR $\alpha\beta$ +	2.747 $\pm$ 0.170	2.473 $\pm$ 0.122	3.078 $\pm$ 0.176 <sup>ab</sup>
CD4+TCR $\alpha\beta$ +	1.647 $\pm$ 0.099	1.507 $\pm$ 0.071	1.763 $\pm$ 0.102
CD8+TCR $\alpha\beta$ +	0.979 $\pm$ 0.061	0.862 $\pm$ 0.044	1.374 $\pm$ 0.063 <sup>***b</sup>
CD8+CD161+TCR $\alpha\beta$ +	0.044 $\pm$ 0.004	0.035 $\pm$ 0.003	0.046 $\pm$ 0.004
CD4+CD25+FoxP3+	0.023 $\pm$ 0.007	0.082 $\pm$ 0.005 <sup>****a</sup>	0.167 $\pm$ 0.007 <sup>****b</sup>
CD4+CD25-FoxP3+	0.069 $\pm$ 0.002	0.158 $\pm$ 0.004 <sup>****a</sup>	0.278 $\pm$ 0.008 <sup>****b</sup>
<b>Splenocytes</b> ( $\times 10^6$ /spleen)			
TCR $\alpha\beta$ +	78.555 $\pm$ 0.780	53.099 $\pm$ 0.560 <sup>****a</sup>	70.558 $\pm$ 0.805 <sup>****b</sup>
CD4+TCR $\alpha\beta$ +	49.703 $\pm$ 2.271	28.904 $\pm$ 1.630 <sup>****a</sup>	35.668 $\pm$ 2.345
CD8+TCR $\alpha\beta$ +	23.985 $\pm$ 0.496	22.900 $\pm$ 0.356	31.743 $\pm$ 0.513 <sup>****b</sup>
CD8+CD161+TCR $\alpha\beta$ +	1.192 $\pm$ 0.142	0.774 $\pm$ 0.102	1.166 $\pm$ 0.146
CD4+CD25+FoxP3+	4.799 $\pm$ 0.104	1.692 $\pm$ 0.075 <sup>****a</sup>	3.218 $\pm$ 0.108 <sup>****b</sup>
CD4+CD25-FoxP3+	4.128 $\pm$ 0.332	3.289 $\pm$ 0.238	4.852 $\pm$ 0.343 <sup>***b</sup>

The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n = 18$  rats in 10-month-old Controls,  $n = 34$  rats in 20-month-old Controls,  $n = 18$  rats in 20-month-old Ox group).

\* $p \leq 0.05$ ,

\*\* $p \leq 0.01$ ,

\*\*\* $p \leq 0.001$ .

<sup>a</sup>20-month-old control versus 10-month-old control rats.

<sup>b</sup>20-month-old Ox versus 20-month-old control rats.

**Table 2** The number of CD4+ and CD8+ recent thymic emigrants, RTEs (CD45RC-CD90+), mature naïve (CD45RC+), all naïve (mature naïve + RTE) and memory/activated (CD45RC-CD90-) TCR $\alpha\beta$ + peripheral blood lymphocytes (PBLs) and splenocytes from 10- and 20-month-old control rats (Controls) and 20-month-old rats ovariectomized (Ox) at the age of 10 months

	10-month-old Controls	20-month-old Controls	20-month-old Ox
<b>PBLs</b> ( $\times 10^6$ /mL)			
CD4+			
RTE (CD45RC-CD90+)	0.081 $\pm$ 0.004	0.043 $\pm$ 0.005 <sup>****a</sup>	0.062 $\pm$ 0.007
Mature naïve (CD45RC+)	1.078 $\pm$ 0.061	0.795 $\pm$ 0.044 <sup>***a</sup>	0.796 $\pm$ 0.063
All naïve (Mature naïve + RTE)	1.160 $\pm$ 0.062	0.838 $\pm$ 0.045 <sup>****a</sup>	0.869 $\pm$ 0.066
Memory/activated (CD45RC-CD90-)	0.500 $\pm$ 0.058	0.878 $\pm$ 0.060 <sup>****a</sup>	1.050 $\pm$ 0.060
CD8+			
RTE (CD45RC-CD90+)	0.031 $\pm$ 0.002	0.013 $\pm$ 0.001 <sup>****a</sup>	0.063 $\pm$ 0.002 <sup>***b</sup>
Mature naïve (CD45RC+)	0.909 $\pm$ 0.033	0.793 $\pm$ 0.028 <sup>***a</sup>	0.781 $\pm$ 0.041
All naïve (Mature naïve + RTE)	0.940 $\pm$ 0.034	0.806 $\pm$ 0.028 <sup>***a</sup>	0.844 $\pm$ 0.041
Memory/activated (CD45RC-CD90-)	0.054 $\pm$ 0.012	0.096 $\pm$ 0.009 <sup>****a</sup>	0.534 $\pm$ 0.013 <sup>***b</sup>
<b>Splenocytes</b> ( $\times 10^6$ /spleen)			
CD4+			
RTE (CD45RC-CD90+)	2.294 $\pm$ 0.184	1.020 $\pm$ 0.150 <sup>****a</sup>	1.659 $\pm$ 0.255
Mature naïve (CD45RC+)	25.931 $\pm$ 1.280	7.071 $\pm$ 0.344 <sup>****a</sup>	6.655 $\pm$ 0.496
All naïve (Mature naïve + RTE)	28.225 $\pm$ 1.233	8.090 $\pm$ 0.472 <sup>****a</sup>	8.314 $\pm$ 0.651
Memory/activated (CD45RC-CD90-)	20.153 $\pm$ 0.603	20.754 $\pm$ 0.620	23.987 $\pm$ 1.873
CD8+			
RTE (CD45RC-CD90+)	1.236 $\pm$ 0.121	0.882 $\pm$ 0.095	1.279 $\pm$ 0.135
Mature naïve (CD45RC+)	21.579 $\pm$ 0.975	15.536 $\pm$ 0.781 <sup>****a</sup>	18.631 $\pm$ 0.983
All naïve (Mature naïve + RTE)	22.815 $\pm$ 1.252	16.418 $\pm$ 0.873 <sup>****a</sup>	19.910 $\pm$ 1.131
Memory/activated (CD45RC-CD90-)	3.462 $\pm$ 0.343	6.484 $\pm$ 0.246 <sup>****a</sup>	13.159 $\pm$ 0.354 <sup>****b</sup>

The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n = 18$  rats in 10-month-old Controls,  $n = 34$  rats in 20-month-old Controls,  $n = 18$  rats in 20-month-old Ox group).

\* $p \leq 0.05$ ,

\*\* $p \leq 0.01$ ,

\*\*\* $p \leq 0.001$ .

<sup>a</sup>20-month-old control versus 10-month-old control rats.

<sup>b</sup>20-month-old Ox versus 20-month-old control rats.

**Table 3** Number of all thymocytes, CD4+CD8+, CD4+CD8–TCR $\alpha\beta$ <sup>high</sup>, CD4–CD8+TCR $\alpha\beta$ <sup>high</sup> and CD4+CD25+FoxP3+ thymocytes from 20-month-old control rats (Controls) and 20-month-old rats ovariectomized (Ox) at the age of 10 months

	20-month-old Controls	20-month-old Ox
All thymocytes ( $\times 10^7$ /thymus)	9.87 $\pm$ 0.30	14.06 $\pm$ 0.44***
CD4+CD8+ ( $\times 10^7$ /thymus)	8.38 $\pm$ 0.26	11.62 $\pm$ 0.37***
CD4+CD8–TCR $\alpha\beta$ <sup>high</sup> ( $\times 10^7$ /thymus)	0.87 $\pm$ 0.04	1.10 $\pm$ 0.06
CD4–CD8+TCR $\alpha\beta$ <sup>high</sup> ( $\times 10^7$ /thymus)	0.25 $\pm$ 0.01	0.41 $\pm$ 0.02***
CD4+CD25+FoxP3+ ( $\times 10^7$ /thymus)	0.06 $\pm$ 0.01	0.08 $\pm$ 0.01

The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n=18$  rats in 10-month-old Controls,  $n=34$  rats in 20-month-old Controls,  $n=18$  rats in 20-month-old Ox group).

\*\*\* $p \leq 0.001$ .

cells, and memory/activated cells in Ox rats did not differ from those in age-matched control rats (Table 2).

**CD8 + PBLs.** In control rats, the number of CD8 + RTEs also declined ( $p \leq 0.001$ ) between the ages of 10 and 20 months (Table 2). However, ten months post-ovariectomy the number of CD8 + RTEs was greater ( $p \leq 0.001$ ) in Ox rats than in age-matched control rats (Table 2). Given that the overall thymic cellularity, the numbers of all CD4+CD8+ double positive and the most mature CD4–CD8+TCR $\alpha\beta$ <sup>high</sup> thymocytes were greater ( $p \leq 0.001$ ) in Ox rats than in age-matched control rats (Table 3), it seems likely that the increase in the number of CD8 + RTEs reflected a more efficient thymopoiesis in Ox rats.

Further, between the ages of 10 and 20 months, in control rats the number of CD8 + mature naïve and all naïve PBLs decreased ( $p \leq 0.05$ ), while the count of CD8 + memory/activated PBL pool increased ( $p \leq 0.001$ ) (Table 2). Ovariectomy did not affect the number of mature naïve and all naïve cells, but it increased ( $p \leq 0.001$ ) the number of memory/activated cells (Table 2).

#### Long-lasting ovarian gland deprivation increases the number of memory/activated cells only in CD8+TCR $\alpha\beta$ + splenocyte pool

The number of CD4+ and CD8+ splenocytes at distinct stages of activation/maturation was determined using FCA. The similar gating strategy to that used for delineation of CD4+ and CD8+ PBLs at distinct stages of activation/maturation was used (Supplementary Figure. 1C).

**CD4 + splenocytes.** In control rats the number of CD4 + RTEs within CD4 + splenocytes ( $p \leq 0.001$ ) decreased between the ages of 10 and 20 months (Table 2). Ten months post-ovariectomy the number of CD4 + RTEs

did not differ between Ox and age-matched control rats (Table 2). Furthermore, in control rats the number of CD4 + mature naïve and all naïve splenocytes decreased ( $p \leq 0.001$ ) between the examined age-points (Table 2). On the other hand, the number of CD4 + memory/activated splenocytes was comparable in 10- and 20-month-old controls (Table 2). Ovariectomy influenced the number of neither CD4 + mature naïve and all naïve nor memory/activated splenocytes (Table 2).

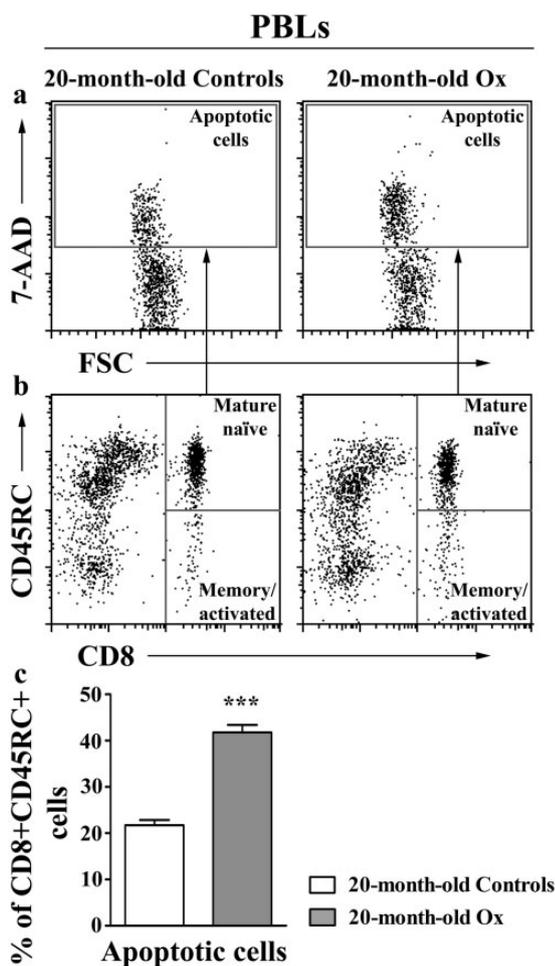
**CD8 + splenocytes.** In control rats, the number of RTEs within CD8 + splenocytes was comparable in 10- and 20-month-old rats (Table 2). Ovariectomy did not influence on the number of CD8 + RTEs (Table 2). Further, in control rats, the number of mature naïve and all naïve CD8 + splenocytes decreased ( $p \leq 0.001$ ) between the ages of 10 to 20 months, whereas the number of memory/activated CD8 + splenocytes increased ( $p \leq 0.001$ ) (Table 2). Ten months post-ovariectomy the number of mature naïve and all naïve CD8 + splenocytes remained unaltered, while that of memory/activated CD8 + splenocytes exceeded ( $p \leq 0.001$ ) that in age-matched control rats (Table 2).

#### Long-lasting ovarian gland deprivation increases the frequency of proliferating cells within CD8 + memory/activated PBL and splenocyte subsets and the frequency of apoptotic cells among CD8 + mature naïve PBL subset

Next, considering that long-lasting ovarian gland deprivation in post-reproductive ages led to the expansion of CD8 + cell pool in both peripheral blood and spleen, we attempted to elucidate mechanisms underlying these changes. For this purpose, we examined the main peripheral homeostatic mechanisms maintaining the size of this cell pool, i.e. the frequency of proliferating (cells in S+G2/M phases of cell cycle) and apoptotic cells within distinct CD8 + cell subsets from peripheral blood and spleen, and relevant data are displayed.

**Peripheral blood.** In agreement with the greater number of memory/activated CD8 + PBLs in Ox rats, we found the greater ( $p \leq 0.001$ ) percentage of proliferating cells (cells in S+G2/M phases of cell cycle) in this T-lymphocyte subset from Ox rats compared with age-matched controls (Figure 1). The frequency of apoptotic cells was comparable in memory/activated CD8 + PBL subset from Ox and age-matched control rats (data not shown).

Furthermore, to explain stable number of mature naïve CD8 + cells in the presence of the greater number of RTEs in Ox rats, we examined the frequency of proliferating and apoptotic cells among mature naïve CD8 + PBLs, as well. We found a greater ( $p \leq 0.001$ ) frequency of apoptotic cells among mature naïve CD8 + PBLs from Ox rats when compared with age-matched controls (Figure 2). The frequency of proliferating cells within this CD8 + PBL subset was not affected by ovariectomy (data not shown).

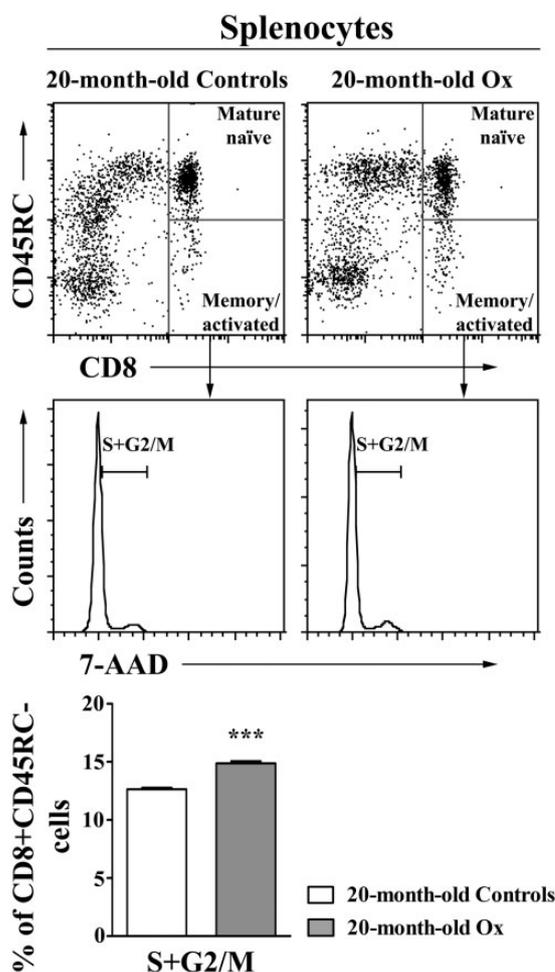


**Figure 2** Effects of long-lasting ovarian gland deprivation on the proportion of apoptotic cells within CD8 + mature naïve peripheral blood lymphocytes (PBLs). (a) Flow cytometry dot plots represent 7-AAD staining of CD8 + mature naïve PBLs (CD8+CD45RC+) gated as indicated in (b) two-color dot plots showing CD8 versus CD45RC staining of TCR $\alpha\beta$ +CD90- PBLs from 20-month-old control rats (Controls) and 20-month-old rats ovariectomized (Ox) at the age of 10 months. Dot plot regions (a) delineate apoptotic (7-AAD+) cells. (c) The bar graph represents the percentage of apoptotic cells within CD8 + mature naïve PBLs from 20-month-old Ox and control rats. The data are estimated marginal means adjusted for experimental day  $\pm$  SEM. ( $n = 18$  rats in 20-month-old Ox group,  $n = 34$  rats in 20-month-old Controls) \*\*\* $p \leq 0.001$

**Spleen.** Next, given that ovariectomy-induced increase in the number of CD8 + splenocytes reflected expansion of memory/activated CD8 + splenocyte subset, we analyzed this subset for the percentage of proliferating and apoptotic cells. The frequency of proliferating cells within CD8 + memory/activated splenocytes from Ox rats was greater ( $p \leq 0.001$ ) than in aged-matched controls (Figure 3). However, the frequency of apoptotic cells among memory/activated CD8 + splenocytes was comparable in Ox and control rats (data not shown).

#### Long-lasting ovarian gland deprivation diminishes IL-7R surface density on CD8 + PBLs and splenocytes

Considering the role of IL-7 and IL-15 in the regulation of CD8 + naïve and memory cell homeostasis,<sup>44-47</sup> we also examined the surface expression of IL-7 receptor (IL-7R) and IL-15R on CD8+TCR $\alpha\beta$ + cells from blood and

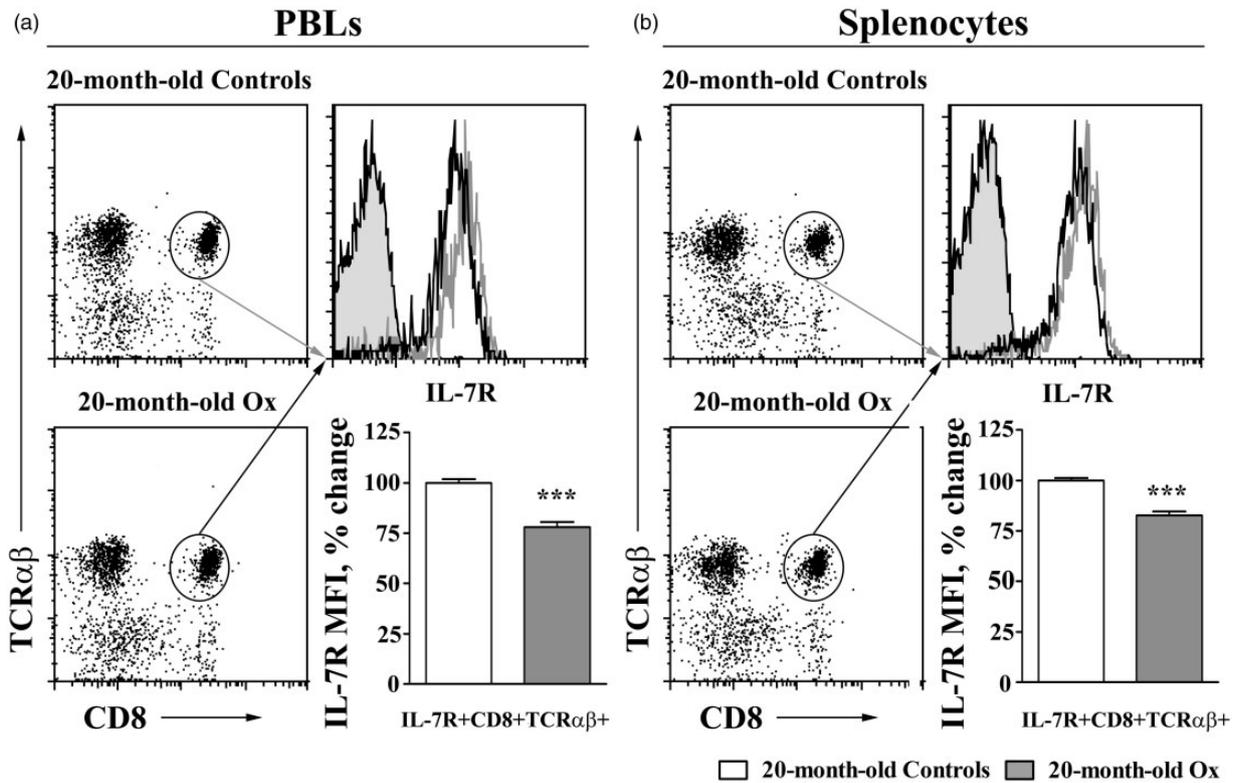


**Figure 3** Effects of long-lasting ovarian gland deprivation on the proportion of proliferating cells within CD8 + memory/activated splenocytes. Flow cytometry histogram profiles illustrate 7-AAD staining of CD8 + memory/activated splenocytes (CD8+CD45RC-) gated as indicated in two-color dot plots showing CD8 versus CD45RC staining of TCR $\alpha\beta$ +CD90- splenocytes from 20-month-old control rats (Controls) and 20-month-old rats ovariectomized (Ox) at the age of 10 months. TCR $\alpha\beta$ +CD90- splenocytes were isolated by magnetic-activated cell sorting (MACS). The bar graph represents the percentage of proliferating cells (cells in S+G2/M phases of cell cycle) within CD8 + memory/activated splenocytes from 20-month-old Ox and control rats. The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n = 18$  rats in 20-month-old Ox group,  $n = 34$  rats in 20-month-old Controls) \*\*\* $p \leq 0.001$

spleen. We found that IL-7R mean fluorescence intensity (MFI), as an indicator of the receptor surface density,<sup>48</sup> on IL-7R+CD8+TCR $\alpha\beta$ + PBLs and splenocytes was diminished ( $p \leq 0.001$ ) in Ox compared with control rats (Figure 4). On the other hand, mean IL-15R surface density (judging by IL-15R MFI) was comparable on CD8 + PBLs from Ox ( $20.20 \pm 0.46$ ) and control rats ( $19.23 \pm 0.30$ ). Similarly, the surface density of this receptor on CD8 + splenocytes from Ox rats ( $19.59 \pm 0.22$ ) did not differ from that ( $19.03 \pm 0.25$ ) on the corresponding cells from control rats.

#### Long-lasting ovarian gland deprivation increases splenic IL-7, IL-15 and TGF- $\beta$ mRNA expression

Given that increase in IL-7 production down-regulates IL-7R expression (so that changes in the expression of this



**Figure 4** Long-lasting ovarian gland deprivation diminishes IL-7R surface density on CD8+TCR $\alpha\beta$  + peripheral blood lymphocytes (PBLs) and splenocytes. Flow cytometry histograms show overlays of IL-7R expression on CD8+TCR $\alpha\beta$  + (Panel A) PBLs and (Panel B) splenocytes from 20-month-old control rats (Controls) (grey line) and 20-month-old rats ovariectomized (Ox) at the age of 10 months (black line) gated as indicated in two-color dot plots showing CD8 vs TCR $\alpha\beta$  staining. Grey shaded profile indicates negative control. Bar graphs represent IL-7R mean fluorescence intensity (MFI) on CD8+TCR $\alpha\beta$  + PBLs and splenocytes from 20-month-old Ox and control rats. The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n = 18$  rats in 20-month-old Ox group,  $n = 34$  rats in 20-month-old Controls) \*\*\* $p \leq 0.001$

cytokine were likely to occur in Ox rats),<sup>49,50</sup> we examined IL-7 mRNA expression in Ox and control rats. We analyzed the expression of this cytokine in spleen considering that homeostatic proliferation of naïve and central memory T cells in mature animals is spatially confined to the T-cell zones of secondary lymphoid organs, specifically the periarteriolar lymphocyte sheaths of the spleen and the paracortical regions of the lymph nodes.<sup>51,52</sup> The splenic expression of IL-7 mRNA was augmented ( $p \leq 0.001$ ) in Ox rats compared with age-matched controls (Figure 5).

In addition, to get insight into effects of ovariectomy on IL-15-mediated regulation of CD8 + cell homeostasis, we also quantified splenic IL-15 mRNA expression in Ox and control rats. Ovariectomy enhanced ( $p \leq 0.001$ ) splenic IL-15 mRNA expression (Figure 5).

Finally, given that an important role in regulation of CD8 + naïve and memory cell proliferation was ascribed to TGF- $\beta$ ,<sup>53,54</sup> we also examined splenic TGF- $\beta$  mRNA expression. The expression of TGF- $\beta$  mRNA was augmented ( $p \leq 0.001$ ) in Ox rats relative to age-matched controls (Figure 5).

#### Long-lasting ovarian gland deprivation enhances CD8 + splenocytes proliferation *in vitro*

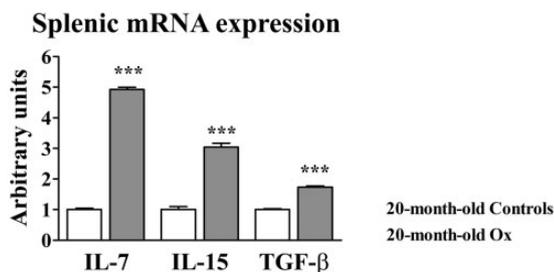
Finally, to estimate CD8 + cell proliferative capacity, we examined CD8 + splenocyte proliferative response to

plate-bound anti-CD3 mAb. We found a pronounced increase ( $p \leq 0.001$ ) in the frequency of proliferating cells (cells in S/G2M phases of cell cycle) within CD8 + splenocytes from Ox rats compared with age-matched controls (Figure 6).

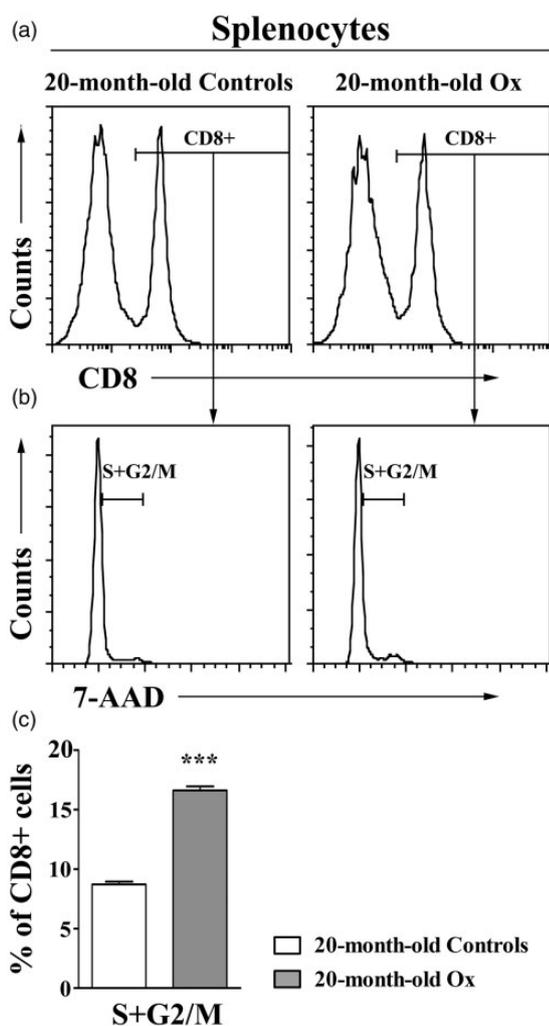
## Discussion

### Ovariectomy at the end of reproductive age increases memory/activated CD8 + cell number and shifts CD4+:CD8 + cell ratio towards CD8 + cells

To the best of our knowledge, this study represents the first attempt to elucidate the long-lasting effects of ovarian deprivation during the post-reproductive age on the levels of the major rat T-cell subpopulations and their subsets in blood and spleen. In peripheral blood and spleen from 20-month-old rats subjected to gonadal ablation at the very end of reproductive age, a shift in CD4+:CD8 + T-cell ratio towards CD8 + cells compared with age-matched controls occurred. The processes that regulate the ratio of the major T-cell subpopulations are still only dimly understood. In athymic nude mice CD4+:CD8 + cell ratio does not depend on the relative proportions of CD4 + and CD8 + cells used for reconstitution.<sup>55</sup> This is highly suggestive that this ratio is carefully regulated.<sup>55</sup> In control 10- and 20-month-old rats exhibiting



**Figure 5** Long-lasting ovarian gland deprivation increases splenic IL-7, IL-15 and TGF- $\beta$  mRNA expression. Bar graphs represent fold change in splenic expression of mRNAs for IL-7, IL-15 and TGF- $\beta$  in 20-month-old rats ovariectomized (Ox) at the age of 10 months relative to age-matched control rats. GAPDH was selected as the housekeeping gene to normalize for input cDNA variations as it displayed optimal stability in our experimental system. The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n = 18$  rats in 20-month-old Ox group,  $n = 34$  rats in 20-month-old Controls) \*\*\* $p \leq 0.001$



**Figure 6** Effects of long-lasting ovarian gland deprivation on splenocyte proliferative response *in vitro*. (b) Flow cytometry profiles show 7-AAD staining of CD8+ splenocytes from 20-month-old control rats (Controls) and 20-month-old rats ovariectomized (Ox) at the age of 10 months stimulated with 1  $\mu$ g/mL plate-bound anti-CD3 mAb and 0.4 ng/mL recombinant rat IL-2 for 48h in culture. CD8+ cells were gated as indicated in (a) corresponding flow cytometry histograms. (c) The bar graphs show the percentages of proliferating cells (cells in S+G2M phases of cell cycle) within CD8+ splenocyte subset from 20-month-old Ox and control rats following the stimulation with plate-bound anti-CD3 mAbs. The data are estimated marginal means adjusted for experimental day  $\pm$  SEM ( $n = 18$  rats in 20-month-old Ox group,  $n = 34$  rats in 20-month-old Controls) \*\*\* $p \leq 0.001$

comparable levels of sex steroid hormones the value of parameter was also comparable.

The shift in CD4+:CD8+ cell ratio in the T-cell compartments of 20-month-old Ox rats was related to the expansion of CD8+ cell pools. Considering that the absolute number of CD8+ phenotype,<sup>39</sup> was comparable in Ox and control rats, it is obvious that these cells were not responsible for the expansion of the peripheral CD8+ T-cell compartment in Ox rats. Furthermore, as estrogen level was comparable in Ox and age-matched controls, whereas that of progesterone was lower in Ox rats, it suggests that progesterone is involved in the control of the size of CD8+ peripheral lymphocyte pool during aging. The hereby reported hormonal changes conforms with data indicating that following ovariectomy the level of circulating estrogen gradually increases reflecting the augmented adrenal production of androgens and their peripheral aromatization.<sup>56,57</sup>

#### Ovariectomy at the end of reproductive age enhances thymic generation of CD8+ lymphocytes in 20-month-old rats

In accordance with the increase of the peripheral CD8+ lymphocyte number, in Ox rats (judging by the total thymocyte number, as well as numbers of CD4+CD8+ double positive, and especially the most mature CD8+CD4-TCR $\alpha\beta$ <sup>high</sup> thymocytes and their immediate descendants, viz. CD8+ RTEs in peripheral blood) the thymic generation of these cells was slightly enhanced. This enhancement most likely reflected progesterone deficiency. In favor of this are the data indicating that progesterone inhibits mainly CD8+ cell differentiation in thymocyte cultures.<sup>58</sup> In addition, it has been shown that: (i) ovariectomy-induced sustained progesterone deficiency in reproductive ages also augments thymic generation of CD8+ cells,<sup>26</sup> (ii) progesterone supplementation abrogates this effect of ovariectomy<sup>26</sup> and (iii) one month post-ovariectomy performed at the very end of reproductive age, when both estrogen and progesterone circulating levels were diminished, thymic generation of both CD8+ and CD4+ lymphocytes was augmented.<sup>25</sup>

#### Changes in peripheral homeostatic mechanisms lead to the expansion of the memory/activated CD8+ cell pool in aged Ox rats

Although thymopoiesis was slightly more effective in 20-month-old Ox rats than in age-matched control rats, the enlargement of CD8+ pool mainly reflected the expansion of CD8+ memory/activated cells. This is consistent with data indicating that the number of thymic emigrants, even in young adult mice, is too small to account for the rate of lymphocyte loss in the periphery, so that the most peripheral T cells are presumably produced in the peripheral lymphoid tissues.<sup>59</sup> Given that, in accordance with some previous findings,<sup>60-62</sup> we observed age-related expansion of activated/memory CD8+ cells with aging, it seems obvious that ovariectomy augments this effect of aging. The increase in cellularity of memory/activated CD8+ cell subsets in 20-month-old Ox rats most likely reflected the

increased proliferation of these cells. This is consistent with data indicating that in the absence of antigen-specific signals the proportion of memory T cell is largely regulated by their proliferation induced by homeostatic cytokines.<sup>63</sup> Namely, given that chronic IL-7 administration markedly increases T-cell numbers mainly via a homeostatic proliferation,<sup>64</sup> and that this increase could occur even when IL-7 receptor surface expression is low,<sup>65</sup> the expansion of memory/activated CD8 + splenocyte pool in Ox rats, despite a small decrease in the surface density of IL-7R on these cells, could reflect a pronounced (four-fold) increase in the splenic expression of IL-7 mRNA. This finding is consistent with data indicating a rise in splenic IL-7 mRNA in adult Ox mice.<sup>66</sup> The very moderate decrease in the density of IL-7R expression on CD8 + splenocytes from Ox rats further corroborates the upregulation of IL-7 production, as it has been shown that increased level of IL-7 down-regulates IL-7R surface density.<sup>49,50</sup> To explain selective expansion of CD8 + subpopulation in Ox rats are data indicating that IL-7 generally has more potent effects on CD8 + than on CD4 + T cells, a feature of its biology that remains poorly understood.<sup>67</sup> Furthermore, in spleen from Ox rats the expression of IL-15 mRNA was also increased, whereas IL-15R lymphocyte surface expression remained unaltered. Given that among all T-cell subsets, memory CD8 + cell subset is the most responsive to IL-15 stimulatory action,<sup>44,45</sup> the increased splenic IL-15 production could also contribute to the expansion of memory/activated CD8 + cell subset from Ox rats. In favor of this finding are data indicating that progesterone affects the expression of IL-15 by decidual HLA-DR + cells.<sup>68</sup> Finally, in Ox rats splenic TGF- $\beta$  mRNA was moderately increased. Considering the alterations in CD8 + activated/memory splenocyte pool, it should be pointed that, despite the clear inhibitory effects of TGF- $\beta$  on T-cells, several studies also demonstrate that this cytokine in certain circumstances can enhance their growth.<sup>69,70</sup> However, it is more likely that ovariectomy-induced rise in TGF- $\beta$  expression was not sufficient to restrict CD8 + memory cell expansion in the presence of the increased IL-7 and IL-15 expression. Given that negative TGF- $\beta$ -mediated effect on CD8 + memory cell homeostasis is shown to be mediated, at least in part, by down regulation of IL-15R expression,<sup>69</sup> unchanged expression of this receptor on CD8 + cells further supports the previous option. Furthermore, it should be pointed that the absolute and relative numbers of naïve and memory T-cell subsets are under complex homeostatic control, which apart from output of thymic emigrants, cytokine-driven homeostatic proliferation, also involves antigen-stimulated expansion of specific naïve T cells to an activated compartment followed by loss of 90% or more of the activated cells and retention of 10% or fewer as antigen-specific memory T cells.<sup>71</sup> Given that progesterone is suggested to exert a dominant inhibitory effect on lymphocyte expansion by affecting signaling events downstream of the TCR $\alpha\beta$  complex,<sup>72,73</sup> the enhanced antigen-stimulated expansion of specific naïve T cells may be expected in Ox rats. The enhanced proliferative response of CD8 + splenocytes from Ox rats to plate-bound anti-CD3 antibody further corroborates the previous assumption. Finally, although it is

yet to be determined how the age-associated expansion of memory CD8 + T cells exactly affects the host immunity, such phenomenon could be harmful to hosts.<sup>74,75</sup> Namely, it is suggested that homeostatic expansion of memory CD8 + T cells impairs the ability of CD8 + T cells to properly develop immune responses to newly encountered microorganisms such as emerging strains of influenza virus, i.e. that this phenomenon could be contributing factor to the immunodeficiency in senescence.<sup>74,75</sup> Furthermore, there are data indicating that exaggerated homeostatic-type proliferation of T cells could generate autoimmunity.<sup>76</sup>

### Differential mechanisms provide numerical stability of mature naïve CD8 + lymphocyte pool in distinct T-cell compartments of 20-month-old Ox rats compared with age-matched controls

The total number of mature naïve CD8 + cells was comparable in 20-month-old Ox and age-matched control rats. It should be pointed out that, in agreement with the previous studies,<sup>5,73</sup> age-related decline in the number of mature naïve CD8 + cells was observed in AO rats. Therefore, our findings indicate that ovariectomy at the end of reproductive age does not influence age-associated alterations in this CD8 + cell pool.

Given that the number of CD8 + RTEs increased in the peripheral blood from Ox rats, the unaltered total number of naïve CD8 + cells in these animals was related to the greater frequency of apoptotic cells among mature naïve CD8 + cells from Ox compared with control rats. This is consistent with data indicating that the mature naïve cell longevity depends on the thymic export of newly generated cells, being shorter with their greater export.<sup>11,77,78</sup> Furthermore, it should be pointed that number of CD8 + RTEs in peripheral blood of 20-month-old Ox rats compared to that in age-matched controls could reflect, apart from the thymic generation, impaired migration of these cells into peripheral lymphoid organs. To corroborate this assumption was comparable count of CD8 + RTEs in spleens from 20-month-old Ox and age-matched control rats. Several findings in conjunction provide plausible support to the previous assumption. Firstly, triiodothyronine administration decreases RTE number in spleen by affecting the expression of extracellular matrix components, such as laminin and fibronectin.<sup>79</sup> Secondly, injection of progesterone dramatically increases the levels of fibronectin and laminin mRNA in rat uterus.<sup>80</sup> Therefore, it may be hypothesized that altered CD8 + RTE migration into lymphoid organs in the state of sustained progesterone deficiency contributed to the increase in CD8 + RTE number in peripheral blood of Ox rats. The selective effects on CD8 + T cells could be related to the fact that, differently from CD4 + lymphocytes, the expression of progesterone receptor is progesterone level dependent,<sup>81</sup> so that apart from lower progesterone level, the diminished expression of this receptor may contribute to the changes in CD8 + population from Ox rats.

The unaltered number of mature naïve cells within the CD8 + splenocytes from 20-month-old Ox rats was consistent with stable number of their immediate CD8 + RTE

precursors. The lack of expansion of CD8 + mature naïve splenocytes may be associated with data indicating that in nonlymphopenic settings, differently from memory cells, mature naïve CD8 + T cells have relatively low basal metabolic activity and are maintained in interphase for months to years while sustaining their naïve differentiation state.<sup>82,83</sup> Furthermore, although it is shown that in a nonlymphopenic setting, naïve CD8 + T cells under certain conditions (e.g. when IL-7 is overexpressed or administered) can proliferate in response to homeostatic factors (self-peptide/MHC and IL-7) acquiring phenotypic and functional characteristics of memory CD8 + T cells,<sup>80</sup> we failed to observe any significant difference in their proliferation in Ox rats compared with age-matched controls. This suggested that ovariectomy-induced increase in IL-7 production was not sufficient to significantly affect the capacity of naïve CD8 + cells to escape quiescence.<sup>83</sup> Alternatively, lack of significant changes in naïve CD8 + cell proliferation in the presence of enhanced IL-7 mRNA expression could be related to the increase in TGF- $\beta$  mRNA expression in 20-month-old Ox rats compared with age-matched control rats. Namely, it has been shown that the expression of TGF- $\beta$  increases late during CD8 + naïve cell homeostatic proliferation in order to restrain their expansion.<sup>53</sup>

#### **Ovariectomy does not affect the total number of CD4 + PBLs and splenocytes, but increases the number of CD4+CD25+FoxP3 + PBLs and splenocytes in aged rats**

In addition, although ovariectomy did not significantly affect the size of CD4 + cell subpopulation, the number of CD4+CD25+FoxP3 + cells, presumably newly thymus-derived and induced in the periphery,<sup>37,38</sup> achieved greater values in peripheral blood and spleen of 20-month-old Ox rats compared with age-matched control rats. This reflected, most likely, enhanced extrathymic generation of these cells, as the number of cells exhibiting this phenotype did not significantly change in the thymus from Ox rats compared with age-matched control rats. Considering crucial role of IL-7 in regulation of the inducible Treg subset size,<sup>84</sup> the expansion of this pool in Ox rats seems to be highly likely. This may represent a compensatory mechanisms to balance increased autoimmunity due to the expansion of CD8 + activated/memory cells.<sup>85</sup> On the other hand, the expansion of Tregs, besides favorable effects in the prevention of autoimmune diseases, could increase rat susceptibility to infectious diseases and cancer, which are shown to be the leading causes of morbidity and mortality in the elderly.<sup>86</sup> This seems to be particularly relevant if one considers that aging in rats, as in mice,<sup>87</sup> increases number of CD4+CD25 + cells in peripheral blood.

#### **Conclusions**

In conclusion, the study indicates that ovarian gland ablation at the very end of reproductive period exerts long-lasting effects on the T-cell compartment, through affecting thymic output of newly generated CD8 + cells, CD8 + RTE trafficking and homeostatic mechanisms maintaining the size of memory/activated CD8 + pool. Considering data

indicating that homeostatic expansion of memory CD8 + T cells (as it can be seen in Ox rats) impairs the ability of CD8+T cells to properly develop immune responses to newly encountered antigens, thereby becoming contributing factor to the immunodeficiency and autoimmune pathology in senescence,<sup>74-76</sup> it seems that, despite a weak enhancing effect on CD8 + thymic output, ovarian gland ablation augments negative effects of aging on CD8 + T-cell pool. Furthermore, the study showed enhanced peripheral generation of Tregs in Ox rats, which could also contribute to the increased rat susceptibility to infectious diseases and cancer. Collectively, the results suggest that long-lasting disturbances in ovarian hormone levels in post-reproductive age (substantiated in the loss of estrogen level cyclicity and reduced progesterone level) could enhance undesirable effects of aging on the immune system. Considered that this rat model mimics hormonal changes occurring in postmenopausal women subjected to unopposed estrogen hormone replacement therapy, the present findings, apart from fundamental, may have also clinical relevance.

**Author contributions:** GL and NAR designed, whereas NAR, DK, MNA, IP, ZSV, JDJ and BB conducted the experiments and immunophenotyping analyses. DK and NAR analyzed the data. NAR, DK and GL wrote the manuscript. All authors read and approved the final manuscript.

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