Unopposed Estrogen Supplementation/Progesterone Deficiency in Post-Reproductive Age Affects the Secretory Profile of Resident Macrophages in a Tissue-Specific Manner in the Rat

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Keywords
Aged AO rats, cytokine production, iNOS/arginase activity, ovariectomy, peritoneal macrophages, splenic macrophages

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Problem
The influence of unopposed estrogen replacement/isolated progesterone deficiency on macrophage production of pro-inflammatory/anti-inflammatory mediators in the post-reproductive age was studied.

Method of study
Considering that in the rats post-ovariectomy the circulating estradiol, but not progesterone level rises to the values in sham-operated controls, 20-month-old rats ovariectomized at the age of 10 months served as an experimental model. Estrogen and progesterone receptor expression, secretion of pro- and anti-inflammatory cytokines, and arginine metabolism end-products were examined in splenic and peritoneal macrophages under basal conditions and following lipopolysaccharide (LPS) stimulation *in vitro*.

Results
Almost all peritoneal and a subset of splenic macrophages expressed the intracellular progesterone receptor. Ovariectomy diminished cytokine production by splenic (IL-1β) and peritoneal (TNF-α, IL-1β, IL-10) macrophages and increased the production of IL-10 by splenic and TGF-β by peritoneal cells under basal conditions. Following LPS stimulation, splenic macrophages from ovariectomized rats produced less TNF-α and more IL-10, whereas peritoneal macrophages produced less IL-1β and TGF-β than the corresponding cells from sham-operated rats. Ovariectomy diminished urea production in both subpopulations of LPS-stimulated macrophages.

Conclusion
Although long-lasting isolated progesterone deficiency in the post-reproductive age differentially affects cytokine production in the macrophages from distinct tissue compartments, in both subpopulations, it impairs the pro-inflammatory/anti-inflammatory cytokine secretory balance.

Introduction

Aging affects both the immune and the endocrine systems. Age-associated changes in the innate and adaptive arms of the immune system are referred to as 'immunosenescence'.[1–4] Age-related changes in macrophages are shown to affect their ability to appropriately balance pro- and anti-inflammatory

cytokine secretion.[5] They are often linked to age-related alterations in gonadal steroid hormone secretion.[6,7] Unlike mice, the age-related loss of estrous cyclicity in rats is associated with relatively high levels of circulating estradiol and decreased levels of circulating progesterone.[8–10] In agreement with data showing that, in adult rats, the blood estradiol concentration increases gradually over time post-ovariectomy,[11] we have recently shown that in ovariectomized rats at the very end of their reproductive age (10 months), the circulating estradiol level reaches that of intact control rats 10 months after the surgery, remaining stable until the age of 20 months, whereas the circulating progesterone level decreases after ovariectomy and remains lower than in controls until the age of 20 months.[8] Thus, rats ovariectomized at the very end of the reproductive age may serve as a model for studying the effects of long-term unopposed estrogen replacement on immune and inflammatory responses. The latter seem to be particularly relevant, as ovarian steroid hormone replacement treatments, including unopposed estrogen replacement, are broadly used to protect post-menopausal women from elevated risk of cardiovascular diseases and osteoporosis; however, the data on the effect of these treatments on immune cells are limited.[12]

Macrophages display diverse functions that depend on the tissue in which they reside.[13,14] Based on their morphology and surface molecular characteristics, the peritoneal macrophages appear to be more mature than splenic macrophages.[15] In addition, splenic macrophages require prolonged stimulation for their activation and produce less NO than peritoneal macrophages.[16,17] Splenic macrophages are tissue-fixed cells crucial for the efficient trapping of blood-borne pathogens and antigens,[18,19] physiological iron recycling,[20] and the regulation of both humoral and cellular immune responses.[21]

Peritoneal macrophages provide immune surveillance of the peritoneal microenvironment by dynamic changes that involve cell influx, emigration, and adhesion to the mesentery.[22] Anatomical localization of splenic versus peritoneal macrophages (the latter being in direct communication with the periovarial space[23]) may offer an additional rationale for including the cells from these locations in studies aiming to examine the putative influence of ovarian steroid hormone disbalance on macrophage populations.

With the aforementioned in mind, splenic and peritoneal macrophages from 20-month-old rats ovariectomized at the very end of the reproductive life span, and age-matched sham-ovariectomized control animals were examined for (i) their ability to produce prototypic inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, anti-inflammatory/regulatory cytokines IL-10 and transforming growth factor (TGF)-β, and (ii) the activities of the inducible isoform of nitric oxide synthase (iNOS) and arginase under basal conditions and after lipopolysaccharide (LPS) stimulation, *in vitro*. iNOS and arginase activity were estimated by nitric oxide (NO) and urea release, respectively. In addition, freshly isolated splenic and peritoneal macrophages from ovariectomized and control rats were investigated for the expression of intracellular estrogen receptors (ER) α and β, and the progesterone receptor (PR).

Materials and methods

Animals

Female Albino Oxford (AO) rats were derived from the breeding colony at Immunology Research Center 'Branislav Janković', Belgrade (Serbia). The animals were housed in standard polycarbonate cages, three rats per cage, with free access to food pellets and tap water. The rooms were maintained at 20–23°C at humidity ranging 40–70% and under 12:12-hr light: dark cycle. The experimental protocol and all procedures with animals and their care were approved by Experimental Animal Committee of the Institute of virology, vaccines and sera 'Torlak' and were in accordance with principles declared in Directive 2010/63/EU of the European Parliament and of the Council from 22 September 2010 on the protection of animals used for scientific purposes (revising Directive 86/609/EEC).

Surgical Procedure

Virgin (naïve) rats were ovariectomized (Ox) or sham-ovariectomized (Sham) at the age of 10 months (at the very end of rat reproductive life span) and killed when they reached 20 months. Animals were subjected to bilateral ovariectomy or sham-ovariectomy under anesthesia. Anesthetic solution (800 μL/100 g body weight, i.p.) was comprised of ketamine (100 mg/mL Ketamidor; Richter Pharma AG, Wels, Austria), xylasine (20 mg/mL Xylased; Bioveta, Ivanovice na Hané, Czech

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Republic) and saline, mixed in a 1:0.5:8.5 ratio. Ovaries were removed through small dorsal incisions in the skin and the muscle wall on each side of the lumbal backbone. Sham controls were also anesthetised, the skin and muscle layers were opened, and ovaries were manipulated but not excised. The completeness of ovariectomy was verified at the day of kill. Rats were euthanized by the exposure to increasing doses of CO_2, all visceral organs were autopsied for evidence of gross pathologies, and spleens were dissected for the study. Animals showing overt signs of illness, including low body weight, visible lesions, tumors, or splenomegaly, were removed from the study, and their tissues were excluded from analyses. Finally, experimental groups consisted of 7–9 animals.

Serum Concentration of Estradiol and Progesterone

Serum estradiol and progesterone levels were determined using the IMMULITE solid-phase competitive chemiluminescent enzyme immunoassay (EIA) on an IMMULITE 1000 analyzer (Euro/DPC, Caernarfon, UK), according to the guidelines provided by the manufacturer.

Isolation of Peritoneal Macrophages

Abdomen of each rat is soaked with 70% alcohol and the abdominal skin with fur retracted to expose the intact peritoneal wall. Small incision was made along the midline with sterile scissors, and 10 mL of sterile ice-cold phosphate-buffered saline (PBS) pH 7.4 supplemented with 2% fetal calf serum (FCS; Gibco, Grand Island, NY, USA) was injected. By lifting the abdominal wall with forceps, entire body was gently shaked for 10 s. After exposing the peritoneal cavity, the fluid was gently aspirated.[24] Individual cell suspensions were washed twice in PBS/2% FCS (250 × *g*) at 4°C. The purity of peritoneal macrophage population obtained by this method was 95%, according to staining with FITC-conjugated anti-CD68 antibodies (clone ED1; AbD Serotec, Oxford, UK).

Isolation and Immunomagnetic Cell Separation of Splenic Macrophages

Spleens were aseptically removed and minced in cold sterile PBS/2% FCS. Cell suspensions were passed through a 40-μm mesh screen and washed twice in PBS/2% FCS. Following final centrifugation (250 × *g*) at 4°C, supernatants were aspirated and cell pellets resuspended in ice-cold sterile hypotonic solution of NH_4Cl for red blood cell lysis. Splenic macrophages were separated by an immunomagnetic cell separation technique with anti-rat anti-PanT, anti-CD45RA, and anti-OX62 microbeads (Miltenyi Biotec, Teterow, Germany) used for the elimination of T and B lymphocytes and dendritic cells, respectively. The separation procedure was conducted according to the manufacturer's instructions (Miltenyi Biotec). Briefly, following the cell incubation with microbeads (4°C, 15 min), a negative selection was performed on a magnetic cell separation column using a QuadroMACS magnet (Miltenyi Biotec). Viability was determined by trypan blue dye exclusion and was routinely more than 95%. The purity of splenic macrophages obtained in this manner was up to 88% (as shown by staining with FITC-conjugated anti-CD68 antibodies).

Flow Cytometric Analysis

Single-cell suspensions were adjusted to 1×10^7 cells/mL in ice-cold PBS/2% FCS pH 7.4 supplemented with 0.01% NaN_3. Aliquots of 1×10^6 cells were centrifuged at 350 × *g* for 5 min at 4°C to yield a pellet. For the analysis of intracellular expression of hormone receptors, cells were fixed with 0.25% paraformaldehyde for 1 hr at room temperature in the dark and then washed once in PBS. Further fixation was achieved by 1-hr incubation with ice-cold 70% methanol at 4°C. After centrifugation and washing in PBS, cells were permeabilized by two 10-min incubations with 0.05% Triton X-100 in PBS at 4°C. After each incubation, samples were centrifuged for 10 min at 300 × *g* at 4°C. Following permeabilization, cells were incubated overnight with unconjugated polyclonal rabbit anti-estrogen receptor α (ERα), polyclonal rabbit anti-estrogen receptor β (ERβ), or monoclonal mouse antiprogesterone receptor (PR) antibody, all supplied by Abcam, Cambridge, UK. The appropriate isotype controls were purchased from BD Biosciences Pharmingen. Cells were then washed twice with PBS containing 0.05% Triton X-100 and incubated with PE-conjugated polyclonal goat anti-rabbit IgG antibody (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) or PE-conjugated polyclonal donkey F(ab')2 anti-mouse IgG-H&L antibody (Abcam) as second step antibodies. After two washes in PBS,

cells were resuspended for flow cytometric analysis. Data were collected using a FACSVerse™ flow cytometer and analyzed by BD FACSuite™ software (Becton Dickinson, Mountain View, CA, USA).

Cell Culture

In all cell cultures, phenol red-free RPMI 1640 medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine (Serva, Heidelberg, Germany), 100 units/mL penicillin (ICN, Costa Mesa, CA, USA), 100 μg/mL streptomycin (ICN) and 5% charcoal-stripped fetal calf serum (FCS; Gibco) were used. Aliquots of cells (splenic and peritoneal macrophages) were adjusted to 1×10^6/mL in RPMI and plated to 24-well flat-bottomed tissue culture plates (NUNC, Roskilde, Denmark) for cytokine assays. For NO and arginase assays, cells (1×10^5 per well in RPMI 1640 medium) were plated to 96-well flat-bottomed tissue culture plates (NUNC). Cells were cultured to adhere for 2 hr at 37°C in a moist atmosphere of 5% CO_2 in air. The non-adherent cells were removed by washing the plates twice with warm RPMI. The remaining adherent cells, highly enriched for macrophages (≥95%),[25] were further incubated at 37°C and 5% CO_2 in RPMI with or without 1 μg/mL LPS (Sigma-Aldrich Chemie). Culture supernatants collected following 24 hr of incubation in 24-well plates were frozen at −70°C until assayed for TNF-α, IL-6, IL-1β, IL-10, and TGF-β. Culture supernatants collected after 48 hr of incubation in 96-well plates were immediately analyzed for NO. Subsequently, 96-well plates were rinsed twice with warm PBS and centrifuged for 5 min at 100 × *g* for each wash/rinse step. Cells were lyzed with 50 μL of 0.1% Triton X-100 containing 2 mM phenylmethylsulfonyl fluoride per well and plates were shaken (200 cycles/min) for 30 min at room temperature. The 96-well culture plates with the lysis buffer were sealed with parafilm and frozen at −20°C until assayed for arginase activity.

Cytokine Assays

Commercially available ELISA kits for TNF-α and IL-6 (Biolegend Inc., San Diego, CA, USA), IL-10 and TGF-β (R&D Systems, Inc., Minneapolis, MN, USA), and IL-1β (Thermo Scientific, Rockford, IL, USA) were used. Prior to ELISA performing, the thawed culture supernatants were centrifuged on 250 × *g*, 15 min, at +4°C. All assays were carried out according to the instructions provided by the manufacturer. The absorbencies were measured on Multiscan Ascent (Labsystems, Helsinki, Finland). Specifically, interference of TGF-β content of RPMI in the culture medium with TGF-β secreted by cells was avoided by subtraction of values obtained from wells without cells (filled only with RPMI) from the values obtained by wells in which the cells were incubated with RPMI or LPS. Cytokine content in the supernatant was expressed as pg/ml, whereas the magnitude of cell response to LPS was expressed as stimulation index (SI, LPS-stimulated/basal production).

NO Assay

The concentration of nitrite, a stable breakdown product of NO, was measured in the culture supernatants by a method based on the Griess reaction.[26] Briefly, sample supernatants were transferred to microtiter plates and incubated with Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% orthophosphoric acid) for 10 min. The absorbencies were measured at 545 nm (Multiscan Ascent; Labsystems), and concentrations of nitrite in the samples were calculated according to the standard curve obtained with known concentrations of $NaNO_2$ (1–80 μM). The magnitude of cell response to LPS was expressed as SI.

Arginase Assay

Arginase activity was indirectly determined by measuring the metabolite urea, a by-product of arginine degradation from cells cultured *in vitro*.[27,28] Once the 96-well tissue culture plates were thawed at 37°C, arginase was activated by 50 μL per well of 10 mM $MnCl_2$/25 mM Tris–HCl, pH 7.5, for 10 min at 56°C. Aliquots of the activated lysate (25 μL) were transferred into Eppendorf tubes, and arginine hydrolysis was initiated by the addition of 25 μL of 0.5 M arginine, pH 9.7 and carried out at 37°C for 22 hr. The reaction was stopped by the addition of 400 μL of an acid mixture containing H_2SO_4, H_3PO_4, and H_2O (1:3:7). The absorbencies were measured at 540 nm (Multiscan Ascent; Labsystems) after the addition of 25 μL of α-isonitrosopropiophenone dissolved in 100% ethanol followed by heating at 95–100°C for 45 min. The concentration of urea in the samples

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was calculated according to the standard curve obtained with known concentrations of urea (50–1600 μM). The magnitude of cell response to LPS was expressed as SI.

Statistical Analysis

As the experimental design included variations in two factors: surgery (sham-ovariectomy and ovariectomy) and cell type (splenic and peritoneal macrophages), data were analyzed by two-way ANOVA with Bonferroni's multiple comparison *post hoc* test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Serum steroid hormone concentration and cell steroid hormone receptor expression were analyzed using Student's *t*-test. Differences were regarded as statistically significant if $P < 0.05$.

Results

Ovariectomy Differentially Affected Serum Concentrations of Estradiol and Progesterone in Aged Rats

In agreement with our previous study,[8] the 10 months post-ovariectomy serum concentrations of estradiol in ovariectomized female rats were comparable to that in control sham-operated rats, whereas the concentrations of progesterone remained significantly lower than in the controls (Table I).

Ovariectomy Differentially Affected the Expression of Estrogen and Progesterone Receptors in Aged Rat Splenic and Peritoneal Macrophages

Intracellular estrogen receptors (ER) were expressed in almost all macrophages from the spleen (ERα 94.8 ± 6.3%; Fig. 1a and ERβ 98.1 ± 3.1%; Fig. 1b, left panels) and peritoneum (ERα 91.2 ± 4.4%; Fig. 1a and ERβ 99.6 ± 8.1%; Fig. 1b, right panels) of 20-month-old (aged) sham-ovariectomized (control) rats. Ovariectomy did not affect ERα and ERβ expression in splenic and peritoneal macrophages (Fig. 1a,b).

On the other hand, intracellular progesterone receptor (PR) was detected only in a subset of splenic macrophages (27.2 ± 1.4%, Fig. 1c, left panels) and in the majority of peritoneal macrophages (94.1 ± 1.9%, Fig. 1c, right panels) from control rats. Ovariectomy at the very end of the rat reproductive age significantly increased the percentage of splenic macrophages expressing PR (41.1 ± 2.1%, $P < 0.001$ versus sham-operated; Fig. 1c, left panels), but did not affect the proportion of peritoneal macrophages (89.1 ± 4.8; Fig. 1c, right panels) carrying PR.

Table I Ovariectomy at the Very End of Rat Reproductive Age (at the Age of 10 months) Differentially Altered Concentration of Estradiol and Progesterone in Serum of 20-month-old Rats. The Mean Values ± S.E.M. for Serum Levels of Estradiol and Progesterone (n = 7–9/group)

	Estradiol (pM)	Progesterone (nM)
Sham-ovariectomized (Sham)	237.1 ± 25.5	63.2 ± 8.9
Ovariectomized (Ox)	212.2 ± 12.3	22.3 ± 6.9*

Statistically significant differences: *$P < 0.05$ Ox versus Sham.

Different Effects of Ovariectomy on Aged Rat Splenic and Peritoneal Macrophage Cytokine Production *In Vitro*

In vitro, under basal conditions, the cytokine profiles of splenic and peritoneal macrophages isolated from both ovariectomized and control rats differed significantly. The basal production of all examined pro-inflammatory and anti-inflammatory cytokines was lower in the splenic than in the peritoneal macrophages from control rats (Fig. 2a). In splenic macrophages, ovariectomy decreased the basal production of IL-1β and increased that of IL-10. On the other hand, in peritoneal macrophages, it decreased the basal production of TNF-α, IL-1β, and IL-10 and increased that of TGF-β. Splenic macrophages from ovariectomized rats, like those from the controls, produced less TNF-α, IL-6, and TGF-β compared with peritoneal macrophages from the same animals (Fig. 2a). However, these cells released more IL-1β and IL-10 than the peritoneal ones (Fig. 2a).

The stimulation index (SI), which is the parameter showing the magnitude of cell response to LPS, was greater for TNF-α, IL-10, and TGF-β in splenic macrophages than in peritoneal macrophages from control rats (Fig. 2b). Ovariectomy diminished the magnitude of the response to LPS with respect to the production of these cytokines and increased the magnitude of IL-6 production in splenic macrophages. On the other hand, it increased the SI for TNF-α, IL-1β, and IL-10 in peritoneal macrophages.

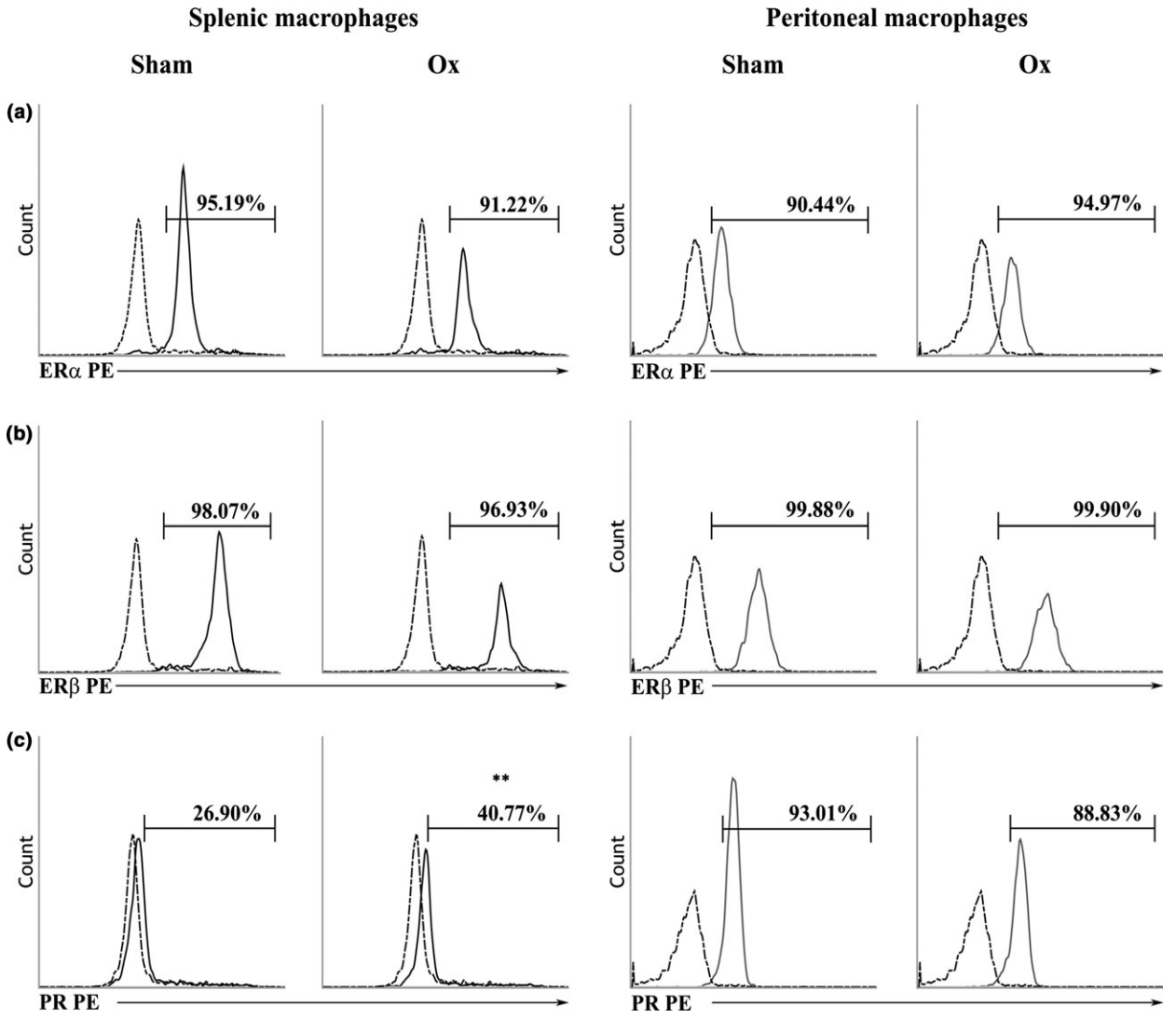

Fig. 1 Ovariectomy at the very end of rat reproductive age differentially affected ovarian steroid hormone receptor expression in splenic and peritoneal macrophages of aged rats. Splenic (left panels) and peritoneal macrophages (right panels) of ovariectomized (Ox) and sham-ovariectomized (Sham) rats were gated according to forward and side scatter for further analysis of ERα (a), ERβ (b), and PR (c) expression on macrophages. Dotted lines in representative histograms indicate secondary antibody control staining. The number of animals per group was 7–9. Statistically significant differences: $^{**}P < 0.001$ Ox versus Sham.

Fig. 2 Ovariectomy at the very end of rat reproductive age differentially altered cytokine production by splenic and peritoneal macrophages of aged rats. Splenic and peritoneal macrophages of ovariectomized (Ox) and sham-ovariectomized (Sham) rats were cultured in the absence (-LPS, a) or in the presence of LPS (+LPS, c). Cytokine levels (pg/ml) of TNF-α, IL-6, IL-1β, IL-10, and TGF-β were measured by ELISA in the culture supernatants. The magnitude of cell response to LPS (b) was expressed as stimulation index (SI, LPS-stimulated/basal production). The number of animals per group was 7–9. Two-way ANOVA revealed the significant interaction of surgery x cell type for unstimulated and LPS-stimulated production of TNF-α ($P < 0.001$), IL-1β ($P < 0.01$), IL-10 ($P < 0.01$), and TGF-β ($P < 0.05$) and for TNF-α ($P < 0.001$), IL-6 ($P < 0.001$), IL-1β ($P < 0.05$), and IL-10 ($P < 0.001$) stimulation index. Significant main effect of cell type ($P < 0.05$) was observed for both basal and LPS-stimulated release of IL-6, and a significant main effect of surgery ($P < 0.001$) for TGF-β stimulation index. Results are presented as mean + S.E.M. Statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ Ox versus Sham; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ Sham peritoneal versus Sham splenic; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ Ox peritoneal versus Ox splenic.

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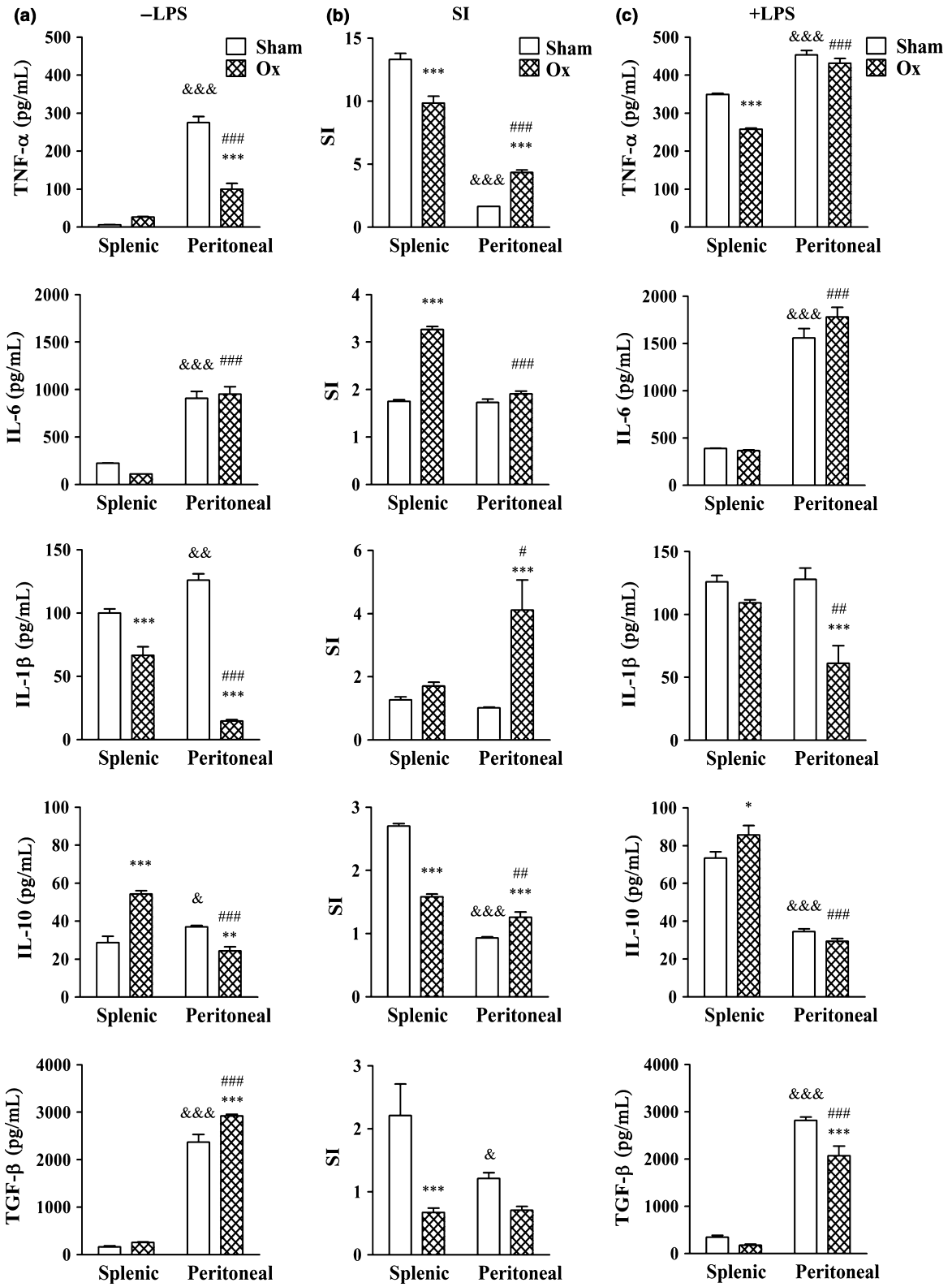

These changes led to a higher magnitude in the response to LPS with regard to TNF-α, IL-6, and IL-10 production, but a response of lower magnitude in the case of IL-1β production in splenic as compared with peritoneal macrophages from ovariectomized rats (Fig. 2b).

In LPS-stimulated splenic macrophages from aged control rats, the levels of TNF-α, IL-6, and TGF-β were lower, whereas that of IL-10 was higher as compared with LPS-stimulated peritoneal macrophages (Fig. 2c). Ovariectomy decreased the production of the pro-inflammatory cytokine TNF-α and increased that of the anti-inflammatory cytokine IL-10 in LPS-stimulated splenic macrophages (Fig. 2c). On the other hand, in peritoneal macrophages from ovariectomized rats, the LPS-stimulated production of both pro-inflammatory IL-1β and anti-inflammatory TGF-β cytokines was lower than in peritoneal macrophages from the sham-operated controls (Fig. 2c). Consequently, compared with LPS-stimulated peritoneal macrophages from ovariectomized rats, in the corresponding splenic cells, in addition to a reduced production of TNF-α, IL-6, and TGF-β, an augmented production of IL-1β and IL-10 was detected (Fig. 2c).

Different Effects of Ovariectomy on Aged Rat Splenic and Peritoneal Macrophage iNOS and Arginase Activity *In Vitro*

In general, basal iNOS and arginase activity (measured by NO and urea production, respectively) were lower in splenic macrophages from 20-month-old female sham-operated and ovariectomized rats than in peritoneal macrophages (Fig. 3a). Ovariectomy did not affect basal NO release by either splenic or peritoneal macrophages from 20-month-old rats, but it decreased basal urea release in peritoneal macrophages (Fig. 3a).

Although ovariectomy selectively diminished the SI in macrophages from the spleen, the responses of NO and urea to LPS stimulation were more pronounced in splenic than in peritoneal macrophages in both control and ovariectomized rats (Fig. 3b).

Ovariectomy ultimately did not affect LPS-stimulated NO production either in splenic or in peri-

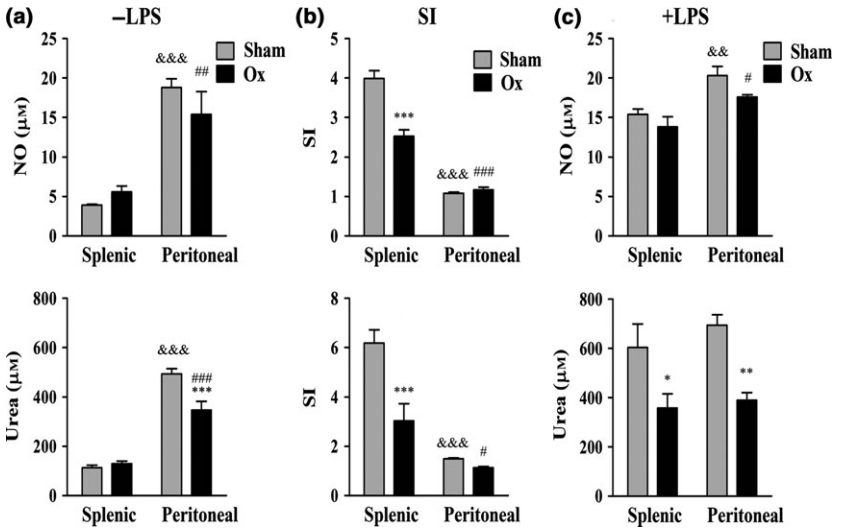

Fig. 3 Ovariectomy at the very end of rat reproductive age differentially altered iNOS and arginase activity by splenic and peritoneal macrophages of aged rats. Splenic and peritoneal macrophages of ovariectomized (Ox) and sham-ovariectomized (Sham) rats were cultured in the absence (-LPS, a) or in the presence of LPS (+LPS, c). NO production (μM) was measured by the Griess reaction and urea (μM) secretion from lyzed macrophages was measured by a colorimetric assay. The magnitude of cell response to LPS (b) was expressed as stimulation index (SI, LPS-stimulated/basal production). The number of animals per group was 7–9. Two-way ANOVA revealed significant main effect of cell type ($P < 0.0001$) for basal and LPS-stimulated production of NO and significant interaction of surgery x cell type for NO stimulation index ($P < 0.0001$). There was significant interaction of factors regarding basal urea production ($P < 0.01$) and urea stimulation index ($P < 0.0001$), whereas significant main effect of surgery ($P < 0.001$) was identified for LPS-stimulated urea production. Results are presented as mean + S.E.M. Statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ Ox versus Sham; $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ Sham peritoneal versus Sham splenic; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ Ox peritoneal versus Ox splenic.

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toneal macrophage cultures, but it substantially diminished LPS-stimulated urea release from both types of cells (Fig. 3c). Consequently, in LPS-stimulated splenic macrophages from aged control and ovariectomized rats, the level of NO was reduced in comparison with the respective peritoneal macrophages (Fig. 3c). In contrast, the levels of urea production were comparable in LPS-stimulated splenic and peritoneal macrophages from both control and ovariectomized rats.

Discussion

Our results, showing that the basal and LPS-stimulated cytokine secretory profiles of splenic and peritoneal macrophages prepared from aged sham-operated female rats substantially differ, add extra weight to the data indicating that the overall functional capacity of macrophages is contingent on their specific microenvironment.[2,29–31] In addition, the results confirm that macrophage extra-microenvironmental factors may alter the function of macrophages from aged animals.[2] To the best of our knowledge, our results, for the first time, indicate that a long-lasting isolated progesterone deficiency affects both basal and LPS-elicited cytokine production in macrophages from aged rats in a tissue-compartment-specific manner. This tissue-specific effect of progesterone deficiency on macrophages is corroborated by the findings indicating that macrophages isolated from different tissue sites exhibit different PR expression profiles.

Under basal conditions, the production of all the examined pro-inflammatory (TNF-α, IL-1β, IL-6) and anti-inflammatory/immunomodulatory (IL-10 and TGF-β) cytokines was lower in splenic than in peritoneal macrophages from aged sham-operated female rats. This is consistent with data indicating that the production of pro-inflammatory and anti-inflammatory/immunomodulatory cytokines in mouse peritoneal macrophages increases with aging,[31,32] whereas their production in splenic macrophages decreases.[29,30] The tissue-specific difference in the secretory profile of macrophages from aged rats may be related not only to their different functional characteristics and physiological role at distinct sites, but also to their distinct age-related intrinsic changes and specific changes in their microenvironment during aging.[33,34]

On the other hand, splenic macrophages from aged sham-operated rats exhibited a more vigorous cytokine secretory response to LPS, a Toll-like receptor (TLR) 4 agonist, than peritoneal macrophages, judging by the fold changes in their production of both pro-inflammatory (TNF-α) and anti-inflammatory (IL-10, TGF-β) cytokines. However, in spite of this, only the IL-10 level in LPS-stimulated macrophage cultures from splenic macrophages exceeded that in the corresponding peritoneal macrophage cultures. The markedly higher magnitude of the IL-10 response in splenic macrophages to LPS stimulation as compared with that in peritoneal macrophages could be associated with findings indicating that the early (4 hr) increase in LPS-stimulated TNF-α production leads to an increase in IL-10 production at later time points, which, in turn, exerts an autoinhibitory effect on IL-10 production and downregulates TNF-α expression.[35,36] Given that the frequency of cells expressing PR was markedly lower among splenic as compared with peritoneal macrophages from sham-operated rats, the greater fold changes in TNF-α and TGF-β in splenic macrophages could be associated with the data suggesting that progesterone inhibits the expression of both of these cytokines.[37,38] Suppressive effect of progesterone on macrophage TNF-α production was shown to reflect (i) diminishing progesterone action on the expression of TLR4,[39] (ii) its enhancing effect on I-kappaBα mRNA expression,[37,40] and consequently, (iii) the repression of NF-kappaB-mediated cytokine transcription.[41]

In the next step, we examined the effects of long-lasting progesterone deficiency on macrophage ability to produce inflammatory mediators under basal conditions *in vitro*. Splenic macrophages from ovariectomized rats produced less IL-1β, but more IL-10 in comparison with the controls. On the other hand, ovariectomy downregulated basal IL-10, TNF-α, and in particular IL-1β production in rat peritoneal macrophages and exerted the opposite effect on their TGF-β production. Data indicating that splenic and peritoneal macrophages from adult mice exhibit distinct alterations after exposure to certain chemicals or stimulations corroborate previous findings.[42,43] As a consequence of the changes described above, under basal conditions, splenic macrophages from aged ovariectomized rats, contrary to age-matched non-ovariectomized rats, produced more IL-1β and IL-10 in respect to peritoneal macrophages; their production of the examined cytokines was (as in the control animals) lower than in the peritoneal macrophages.

The isolated progesterone deficiency, induced by ovariectomy at the age of 10 months, diminished the magnitude of LPS-elicited TNF-α, IL-10, and TGF-β responses from splenic macrophages, but increased that of IL-6. The diminished TNF-α and TGF-β splenic macrophage secretory response, in face of the reduced progesterone level in ovariectomized rats, could be explained by the decrease in the circulating progesterone level in ovariectomized rats, which was followed by an unproportional (of greater magnitude than the decrease in progesterone level) increase in the frequency of PR-expressing cells among splenic macrophages. As stated above, the diminished secretion of IL-10 could be linked to reduced TNF-α secretion.[35,36] A more pronounced IL-6 response to LPS in splenic macrophages could be explained by the data indicating that IL-10 is less efficient in controlling IL-6 than TNF-α secretion in LPS-stimulated macrophage cultures.[35,36] On the other hand, the greater LPS-induced TNF-α and IL-1β secretory response of peritoneal macrophages reflected both the slightly reduced frequency of cells expressing PR and the reduced circulating level of progesterone in ovariectomized rats.[37,39,41] The ultimate outcome of these changes was (i) reduced TNF-α, but increased IL-10 production in LPS-stimulated splenic macrophages from ovariectomized as compared with control rats, and (ii) diminished production of immunomodulatory TGF-β, and in particular the pro-inflammatory IL-1β cytokine in peritoneal macrophages from ovariectomized as compared with the same cytokines in control rat cultures. It should also be pointed out that ovariectomy at the very end of the reproductive life span, apart from progesterone, affects the circulating levels of other ovarian factors, such as activin,[44] which was shown to influence macrophage cytokine secretory capacity.[45,46] However, considering that activin is produced in a wide range of tissues, including the pituitary gland and macrophages themselves,[46,47] it does not seem likely that the lack of ovarian activin production significantly contributed to ovariectomy-induced alterations in cytokine secretion.

The macrophage enzymes arginase and iNOS compete for available arginine to skew its metabolism toward pro-inflammatory NO/citrulline mediator and ornithine/urea production, which stimulate cell proliferation and assist in repair processes, respectively.[48] Under basal conditions, irrespective of circulating steroid hormone levels, splenic macrophages from aged rats produced less NO and urea than peritoneal macrophages. Although LPS generally increases iNOS and arginase mRNA expression in macrophages from adult animals,[49,50] it increased both NO and urea production above basal levels only in the splenic macrophages of control aged rats. Splenic macrophages from ovariectomized rats retained the ability to respond to LPS with increased NO and urea production, but they exhibited a less pronounced increase in urea production in comparison with corresponding cells from control animals. However, peritoneal macrophages from the controls responded to LPS stimulation only by an increase in urea production. Ovariectomy abrogated this effect of LPS in peritoneal macrophages. The diminishing effect of ovariectomy on urea production observed in macrophages isolated from both sites is not easy to explain. Namely, data on the influence of progesterone on arginase activity and urea production in distinct cell types are conflicting.[51,52] This could be explained by the recent finding indicating that, apart from intracellular PR, many cell types, including macrophages, express membrane PR.[53] Specifically, transcripts for membrane PR isoforms α and β were shown to be expressed in bone marrow-derived macrophages and the RAW 264.7 macrophage cell line, whereas membrane PR isoform γ mRNA was found only in bone marrow-derived macrophages.[53] In other words, it is possible that progesterone, through different types of receptors in distinct cell types, exerts different effects.

Overall, our study shows that ovariectomy at the very end of reproductive age (by mimicking the effects of unopposed estrogen replacement therapy) affects the ability of both splenic and peritoneal macrophages to produce pro-inflammatory and anti-inflammatory mediators in a tissue-specific manner. Thus, it could have some adverse consequences. For example, the diminished production of pro-inflammatory mediators (e.g., TNF-α, IL-1β) may affect macrophage ability to eliminate efficiently various pathogens, whereas a diminished production of mediators involved in tissue repair (e.g., IL-10, TGF-β, and ornithine) may impair their ability to efficiently repair tissue damage. This seems to be particularly relevant as elderly subjects are plagued by poor responses to infectious challenges. Thus, unopposed estrogen replacement during post-reproductive age, apart from its well-known beneficial effect on the cardiovascular system and bone metabolism, might also cause some adverse effects by affecting macrophage function.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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