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The influence of aging and estradiol to progesterone ratio on rat macrophage phenotypic profile and NO and TNF-α production

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ABSTRACT

The phenotype and function of tissue macrophages substantially depend on the cellular milieu and biological effector molecules, such as steroid hormones, to which they are exposed. Furthermore, in female rats, aging is associated with the altered macrophage functioning and the increased estrogen level is followed by a decrease in that of progesterone. Therefore, the present study aimed to investigate the influence of estradiol/progesterone balance on rat macrophage function and phenotype throughout whole adult lifespan. We ovariectomized rats at the late prepubertal age or at the very end of reproductive lifespan, and examined the expression of ED2 (CD163, a marker of mature resident macrophages related to secretion of inflammatory mediators) on peritoneal macrophages and their ability to produce TNF-α and NO upon LPS-stimulation at different age points. In addition, to delineate direct and indirect effects of estrogen, we assessed the in vitro influence of different concentrations of 17β-estradiol on LPS-induced macrophage TNF-α and NO production. Results showed that: (a) the low frequency of $ED2^{high}$ cells amongst peritoneal macrophages of aged rats was accompanied with the reduced TNF-α, but not NO production; (b) estradiol level gradually increased following ovariectomy; (c) macrophage ED2 expression and TNF-α production were dependent on estradiol/progesterone balance and they changed in the same direction; (d) changes in estradiol/progesterone balance differentially affected macrophages TNF-α and NO production; and (e) estradiol exerted pro-inflammatory and anti-inflammatory effects on macrophages in vivo and in vitro, respectively. Overall, our study discloses that estradiol/progesterone balance contributes to the fine-tuning of rat macrophage secretory capacity, and adds to a better understanding of the ovarian steroid hormone role in the regulation of macrophage function, and its significance for the age-associated changes in innate immunity.

1. Introduction

Gender differences in the susceptibility to infectious, malignant and autoimmune diseases suggest that sex steroid hormones, together with other factors, play an important role in the regulation of the immune/inflammatory response (Cutolo et al., 2004; Klein, 2000; Tanriverdi et al., 2003; Verthelyi, 2001; Whitacre et al., 1999). Macrophages are key cells in innate and adaptive immune function. These cells may act directly, by destroying bacteria, parasites, viruses and tumour cells, or indirectly, by releasing an array of mediators (IL-1β, TNF-α, ROS, NO, etc.) with cytotoxic, pro- and anti-inflammatory, angiogenic, fibrogenic and mitogenic activities, which function to fight infections, limit tissue injury and promote wound healing. However, uncontrolled or deregulated release of these mediators by hyperresponsive macrophages can exacerbate acute tissue injury and/or promote the development of chronic diseases such as fibrosis and cancer. The responsiveness of tissue macrophages is extremely dependent on their cellular and molecular surroundings, which encompass various cytokines, chemokines and other bioregulatory molecules (Stout and Suttles, 2004). Among the latter are female sex steroids, that is, estrogen (reviewed in Straub, 2007) and progesterone (Chao et al., 1995; Gomez et al., 1998; Miller and Hunt, 1998; Routley and Ashcroft, 2009).

In female rats, there is a normal variation of estrogen and progesterone levels during the estrus cycle. The beginning of the estrous cycle is characterized by low estrogen levels followed by a gradual increase, with peak levels reached at proestrus, whereas the highest and lowest progesterone levels are measured at metaestrus/diestrus and estrus/proestrus, respectively (Larsen et al., 2006). Considering the previous data and findings indicating cell-cycle dependent changes

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in macrophage phenotype (Frazier-Jessen and Kovacs, 1995; Schultz et al., 1993) and function (Houdeau et al., 2007), it may be hypothesized that the estrogen/progesterone level ratio influences macrophage phenotype and function.

In the rat, where the onset of reproductive senescence is frequently characterized by a period of persistent estrous/diestrus (LeFevre and McClintock, 1988), high estrogen levels continue to be maintained until advanced age, while progesterone level declines with aging (Maffucci and Gore, 2006; Miller and Riegle, 1980). Therefore, it may be also hypothesized that in the rat the age-related loss of cycling combined with the changes in serum concentration of the main ovarian steroids, and likely, consequently, in their receptor expression (Chakraborty and Gore, 2004; Jezierski and Sohrabji, 2001; Maffucci and Gore, 2006; Uotinen et al., 1999; Wynne et al., 2004), could affect macrophage phenotype and function. It has been shown that macrophages from aged rats exhibit lower surface density of ED2 (CD163, also designated as M130, a macrophage-associated antigen that is a member of the scavenger receptor cysteine-rich superfamily) (Polfliet et al., 2006), which is accompanied by reduced capacity of macrophages to secrete proinflammatory IL-1 and IL-6 cytokines (Kizaki et al., 2000). Considering that ED2 cell surface density expression correlates with nuclear factor κB (NF-κB) activity in macrophages upon stimulation with LPS (Kizaki et al., 2000), and that during monocyte/macrophage activation this transcription factor coordinately controls expression of multiple genes, including genes encoding NO synthase and TNF-α (Baeuerle and Henkel, 1994), which expression is also influenced by sex steroids (Calippe et al., 2010; Chao et al., 1995; Miller et al., 1996; Miller and Hunt, 1998), we examined rat peritoneal macrophages for ED2 expression and LPS-stimulated NO and TNF-α production. Given that in the rat, unlike other rodents, the serum estradiol level increases gradually following ovariectomy (Zhao et al., 2005a, 2005b), to assess the influence of changes in the estradiol/progesterone level ratio on the macrophage surface and secretory phenotype, and consequently their putative contribution to the age-related macrophage remodelling, we decided to use a model of surgical gonadal hormone deprivation of different duration. Furthermore, to get more accurate insight into the influence of the estradiol/progesterone balance on peritoneal macrophage remodelling over the whole adult lifespan, we ovariectomized rats at the late prepubertal period (at the age of 1 month) and at the very end of the rat reproductive lifespan (at the age of 10 months) (Maffucci and Gore, 2006), and their macrophages were examined at different time points (from 1 to 10 months) following the surgery. Finally, as changes in estradiol level can influence macrophage functions directly, but also indirectly, through changes in secretion of some other hormones, e.g., growth hormone, prolactin and progesterone (Couse and Korach, 1999), to delineate direct from indirect estrogen effects we assessed 17β-estradiol influence on TNF-α and NO production upon LPS stimulation in vitro.

2. Methods

2.1. Animals

Female Albino Oxford (AO) rats, used in the present study, were born and bred in the animal housing facility at the Immunology Research Centre "Branislav Janković" in Belgrade. The animals were housed in standard macrolone cages in a controlled environment (22 ± 1 °C, 12-h light/dark cycle) with free access to food pellets and tap water. The experimental protocols and all procedures with animals and their care were approved by the Experimental Animal Committee of the Immunology Research Center "Branislav Janković", and were in accordance with the 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).

2.2. Experimental design

2.2.1. Experiment 1

Virgin (naïve) rats were ovariectomized or sham-ovariectomized (control rats) at the age of 1 month (at late prepubertal period) and sacrificed when they reached 2, 7 or 11 months. The 2-, 7- and 11-month-old rats were assigned as young adult, adult and middle-aged rats, respectively. In sham-ovariectomized rats, estrous cyclicity was assessed by daily microscopic analysis of vaginal lavages (between 08:30 and 10:30 h) for at least ten days before experimentation. Due to practical constraints, at autopsy young adult and adult animals were in different stages of the estrous cycle, while middle-aged rats entered an acyclic state of persistent estrous. Each experimental group consisted of at least six animals.

2.2.2. Experiment 2

Rats were ovariectomized or sham-ovariectomized (control rats) at the age of 10 months (at the very end of rat reproductive lifespan) and sacrificed when they reached 11 or 20 months. Age-matched groups of rats were sacrificed at the same time in order to be used as controls. The 11- and 20-month-old rats were assigned as middle-aged and aged rats, respectively. Each experimental group consisted of at least six animals.

The experimental design is shown in Table 1.

2.3. Surgical procedure

Animals were subjected to bilateral ovariectomy or sham-ovariectomy under anaesthesia. Anaesthetic 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 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 anesthetized, the skin and muscle layers were opened, and ovaries were manipulated but not excised. The completeness of ovariectomy was verified on the day of sacrifice. All rats were euthanized by exposure to increasing doses of CO_2 followed by cardiac puncture exsanguination (between 09:00 and 10:00 h). At the time of sacrifice, all visceral organs were autopsied for evidence of gross pathologies. Animals showing overt signs of illness, including low body weight, visible lesions, tumours or splenomegaly, were removed from this study and their tissues were excluded from analyses.

2.4. 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 analyser (Euro/DPC, UK), according to the guidelines provided by the manufacturer.

Table 1
Experimental design.

	Ovariectomy or sham-ovariectomy	Mø phenotype &TNF-α and NO; Mø TNF-α and NO ± 17β-estradiol in vitro; serum estradiol and progesterone levels[a]
Experiment 1.	Late prepubertal age (1-month-old)	Young adult (2-month-old) Adult (7-month-old) Middle-age (11-month-old)
Experiment 2.	End of reproductive lifespan (10-month-old)	Middle-aged (11-month-old) Aged (20-month-old)

[a] In young adult, middle-aged and aged rats.

2.5. Macrophage harvest and culture

Resident peritoneal macrophages were isolated by washing the peritoneal cavity with 10 ml of ice-cold minimal essential medium without phenol red (MEM, Torlak, Belgrade, Serbia). Individual cell suspensions were washed three times in MEM and adjusted to 1×10^6/ml in RPMI 1640 medium without phenol red (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), supplemented with heat-inactivated 5% FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 μg/ml penicillin and 100 U/ml streptomycin (RPMI). Cells were plated (1×10^5 per well) to 96-well flat-bottomed tissue culture plates (NUNC, Roskilde, Denmark) and cultured to adhere for 2 h 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%) (Donnelly et al., 2005) were incubated at 37 °C and 5% CO_2 in RPMI with or without 1 μg/ml LPS (Sigma-Aldrich Chemie, Taufkirchen, Germany). The percentage of macrophages that adhered to the plates appeared equivalent by microscopic inspection regardless of whether the cells were derived from young or aged rats (data not shown). After 48 h of incubation the culture supernatants were collected and immediately analysed for NO. Extra aliquots of supernatants were frozen at −70 °C until assayed for TNF-α.

2.6. 17β-estradiol and LPS treatments

A stock solution of 17β-estradiol (Calbiochem) in ethanol was prepared immediately prior to treatment. Serial dilutions of 17β-estradiol were made in RPMI containing 1 μg/ml LPS. The final ethanol concentration was 0.1% or less. Cells were treated with 10^{-8} M–10^{-6} M 17β-estradiol or ethanol as a vehicle control in the presence of 1 μg/ml LPS for the 48 h. Culture supernatants were analysed for TNF-α and NO production.

2.7. TNF-α assay

TNF-α was measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (OptEIATM Set Rat TNF-α, BD Biosciences, San Diego, CA, USA). Prior to ELISA performing, the thawed culture supernatants were centrifuged on 250 ×g, 15 min, +4 °C. The assay was carried out according to the instructions provided by the manufacturer. The absorbances were measured on Multiscan Ascent (Labsystems) at 492/620 nm. The concentrations of TNF-α (pg/ml) in the samples were determined using recombinant rat TNF-α as a standard.

2.8. NO assay

The nitrite concentration in the culture medium was measured by a method based on the Griess reaction (Green et al., 1982). The concentration of nitrite (μM) in the samples was calculated using a $NaNO_2$ as a standard.

2.9. Flow cytometric analysis (FCA)

For immunophenotyping single-cell suspensions of peritoneal cells were adjusted to 1×10^7 cells/ml in ice-cold phosphate-buffered saline (PBS) pH 7.4 supplemented with 2% fetal calf serum (Gibco, Grand Island, NY, USA) and 0.01% NaN_3 (FACS buffer). Aliquots of 1×10^6 cells in 100 μl of FACS buffer were centrifuged at 350 ×g for 5 min at 4 °C to yield a pellet. All subsequent incubations were performed at 4 °C in the dark, for 30 min, and were followed by thorough washings. Direct membrane staining of cells was performed using the phycoerythrin (PE)-conjugated anti-rat ED2 (clone HIS36, BD Biosciences Pharmingen, Mountain View, CA, USA) or HIS48 (clone RP-1 BD Biosciences Pharmingen) antibody. For the intracellular antigen immunolabelling with fluoresceine isothiocyanate (FITC)-conjugated anti-rat CD68 (clone ED1; Serotec, Oxford, UK) antibody, the cells were fixed with 0.25% paraformaldehide and permeabilized by 0.2% Tween 20 (15 min, 4 °C) prior to addition of the antibody. After labelling, the cells were washed in FACS buffer and then in ice-cold PBS containing 0.01% NaN_3. Data were collected using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA), and analysed 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).

2.10. Statistical analysis

Statistics were calculated using the SPSS 20.0 for Windows. Student's t-test, one-way ANOVA followed by Fisher's PLSD test for post hoc comparisons, and Pearson correlation were applied where appropriate. Data are presented as mean ± S.E.M. Differences are regarded as statistically significant if $p < 0.05$.

3. Results

3.1. Age-associated alterations in ED2 expression on peritoneal macrophages

The majority of cells within peritoneal exudate cells gated by FSC vs. SSC (as shown in Fig. 1A) were ED1+ macrophages (85.2%–87.9%). Gated cells included low percentages of HIS48+ granulocytes (≤4.5%) and lymphoid cells (≤11%). The percentages of ED1+ macrophages did not vary with the aging (data not shown). According to ED2 surface expression amongst ED1+ cell population, three subsets of cells, i.e., $ED2^{high}$, $ED2^{low}$ and $ED2^{negative}$ cells, were easily distinguishable. ED2 MFI on $ED2^{high}$ cells was approximately 6-fold higher than that on $ED2^{low}$ cells. The proportions of ED2 subsets within ED1+ populations significantly changed with age (Fig. 1B). The percentage of $ED2^{high}$ cells markedly decreased with age, so that these cells were almost absent in aged rats. In contrast, the percentages of $ED2^{low}$ and $ED2^{negative}$ increased with advanced age. Statistical analysis revealed significant correlations between age (in months) and percentages of $ED2^{high}$, $ED2^{low}$ and $ED2^{negative}$ macrophages.

3.2. Age-associated decrease in LPS-elicited peritoneal macrophage TNF-α production

Peritoneal macrophage TNF-α production significantly decreased with age (Fig. 2A). More specifically, age-related decline in basal TNF-α production started as early as at the adult age ($p < 0.001$), so that both middle-aged and aged rats produced less TNF-α compared with young adult ($p < 0.001$) and adult rats ($p < 0.05$). Furthermore, in response to LPS, macrophages from middle-aged and aged rats produced a lower amount of TNF-α than macrophages from young adult and adult rats ($p < 0.01$). However, unlike with TNF-α secretion, due to high individual variations within each age group, we failed to detect significant age-related differences in either basal or LPS-stimulated macrophage NO production (Fig. 2B).

3.3. Responsiveness of macrophages from ovariectomized rats to LPS stimulation

3.3.1. Effects of ovariectomy at the late prepubertal age on macrophage ED2 expression and TNF-α and NO production

The serum levels of both estradiol and progesterone were dramatically diminished in ovariectomized young adult rats ($p < 0.001$) (Fig. 3A). In middle-aged rats the estradiol level was comparable to

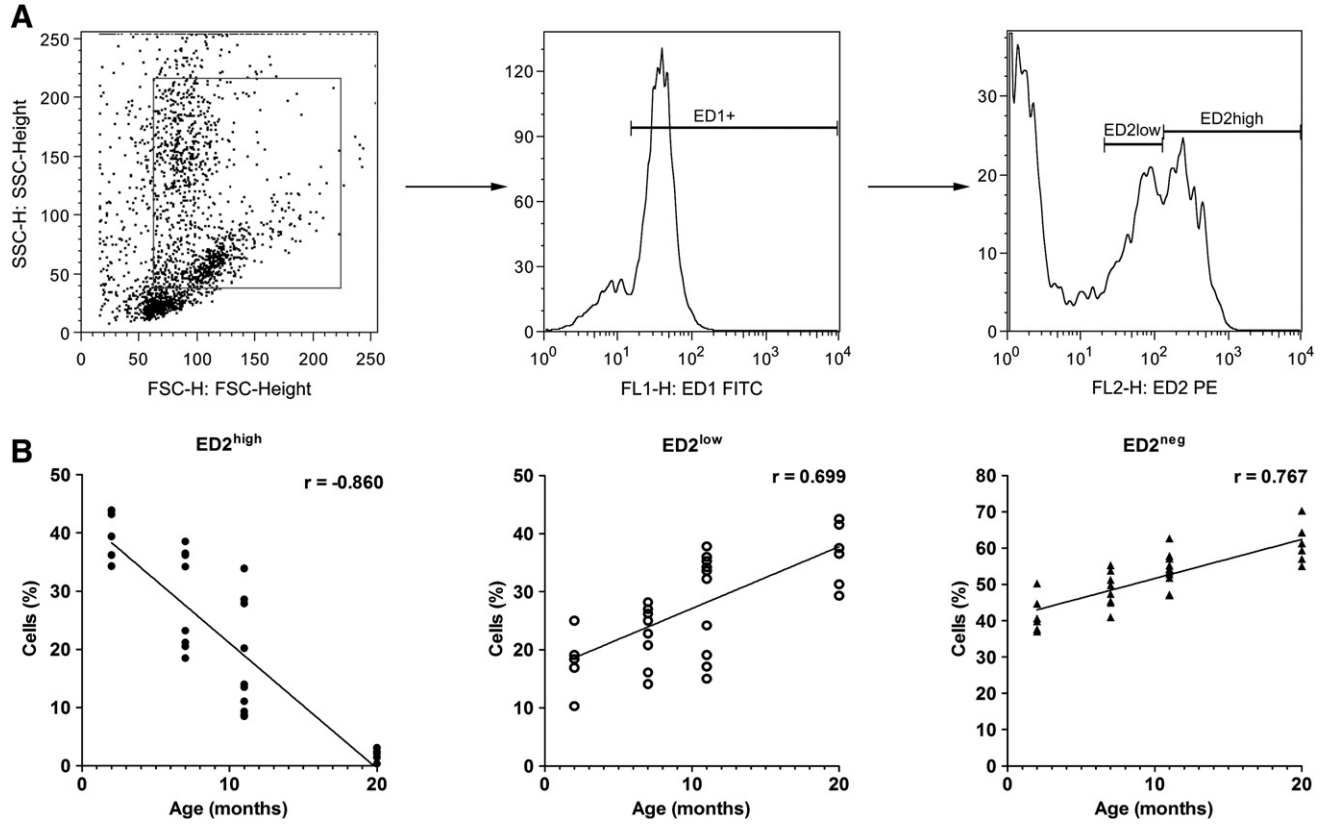

Fig. 1. Age-related changes in the expression of ED2 on macrophages. (A) Macrophage population was gated from peritoneal exudates cells by FSC vs SSC for further analysis of CD68 expression using ED1 monoclonal antibody, and consequently ED2 expression on ED1+ macrophages. (B) Correlation of age and percentages of $ED2^{high}$ (Pearson r = −0.860), $ED2^{low}$ (Pearson r = 0.699) and $ED2^{neg}$ (Pearson r = 0.767) cells, (n = 33), $p < 0.01$.

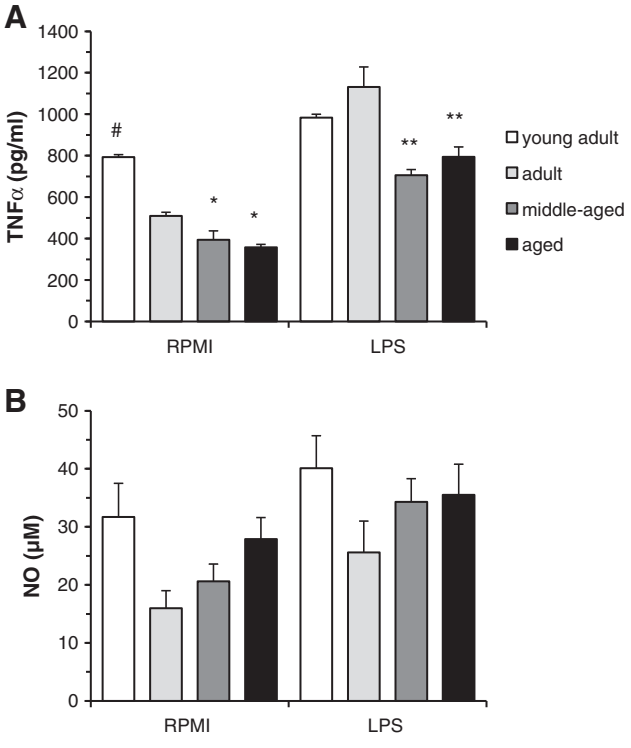

Fig. 2. Age-related decrease in macrophages' capacity to produce TNF-α. Levels of TNF-α (A) and NO (B) secreted from macrophages of young adult, adult, middle-aged and aged rats. Macrophages were cultured for 48 h in culture medium in the absence (RPMI) or in the presence of 1 μg/ml LPS (LPS). The data are expressed as mean + S.E.M. (n = 6–8). Statistically significant differences: #, $p < 0.001$ vs. all; and *, $p < 0.05$, and **, $p < 0.01$, vs young adult and adult.

that in age-matched controls ($p > 0.05$), whereas that of progesterone was diminished ($p < 0.001$) (Fig. 3A).

3.3.1.1. Effects of ovariectomy on TNF-α and NO production. Ovariectomy performed at the age of 1 month diminished ($p < 0.01$) both basal and LPS-stimulated macrophage TNF-α production when tested at the age considered as young adult and adult (Fig. 3B). This suppressive effect of ovariectomy was not detected in macrophage cultures from middle-aged rats. Quite the reverse, the LPS-stimulated TNF-α production was elevated ($p < 0.001$) in macrophage cultures from middle-aged ovariectomized rats.

In addition, ovariectomy induced similar changes in both basal and LPS-stimulated macrophage NO production, as those detected in TNF-α production, but only the increase of LPS-elicited NO production in macrophage cultures from middle-aged rats reached statistical significance ($p < 0.05$) (Fig. 3C).

3.3.1.2. Effects of ovariectomy on ED2 expression. Phenotyping of cells revealed decreased percentages of $ED2^{high}$ cells ($p < 0.05$) in peritoneal macrophages of rat ovariectomized at the age of 1 month in young adult and adult rats, whereas the percentages of $ED2^{low}$ cells appeared to be significantly elevated in these rats ($p < 0.05$, and $p < 0.01$, respectively) (Fig. 4A). Quite the reverse, ten-month-long ovarian hormone deprivation in middle-aged rats significantly elevated the proportion of $ED2^{high}$ cells and reduced that of $ED2^{neg}$ cells ($p < 0.01$). The representative histograms of ED2 expression on ED1+ cells are shown in Fig. 4B.

3.3.2. Effects of ovariectomy at the end of the reproductive lifespan on macrophage ED2 expression and TNF-α and NO production

Compared to age-matched control rats, both estradiol and progesterone serum levels were decreased in middle-aged and aged

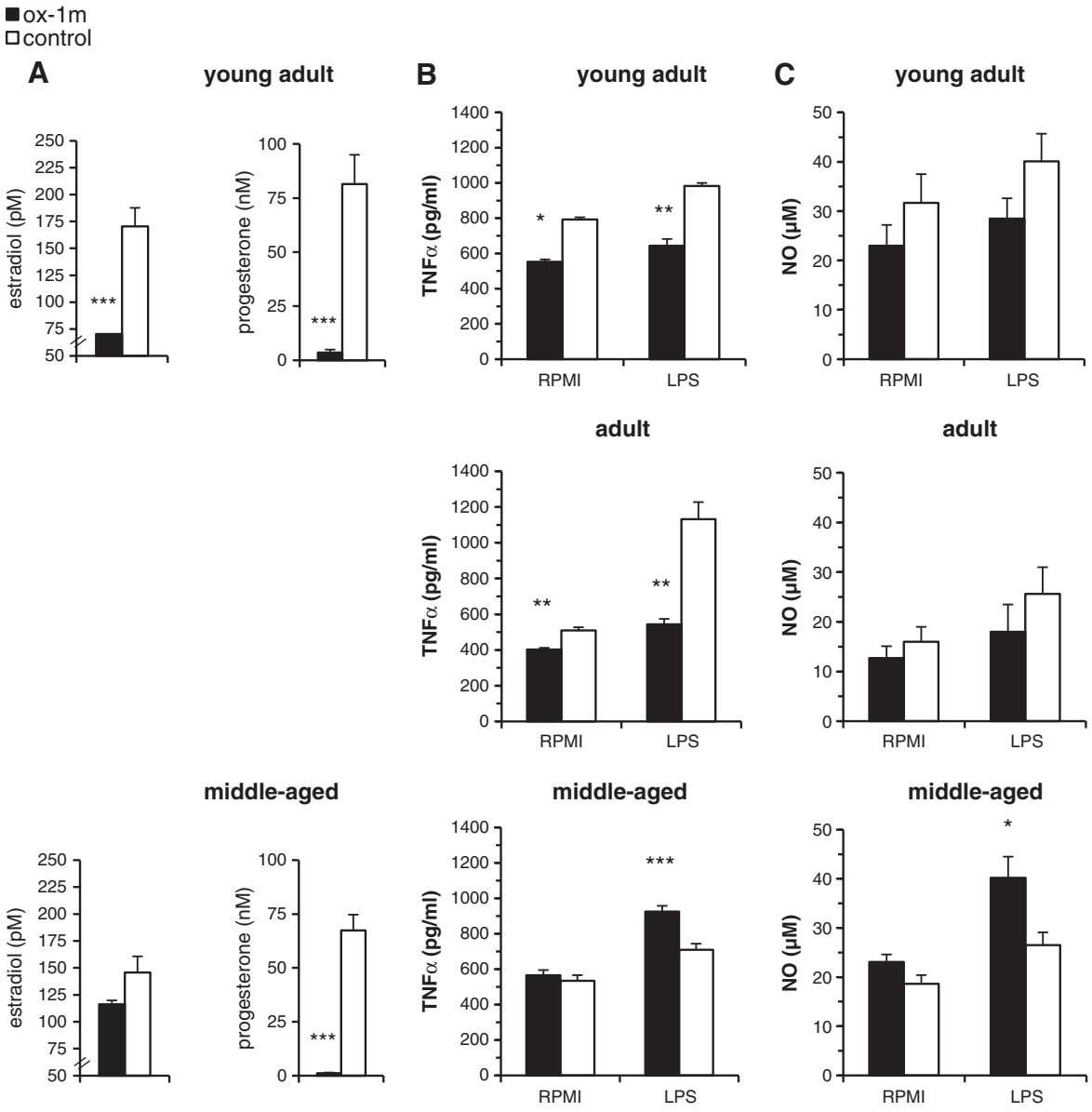

Fig. 3. Ovarian hormone deprivation initiated at the late prepubertal age influences macrophages secretory functions. Serum levels of estradiol and progesterone in rats ovariectomized at the age of 1 month (ox-1 m) and age-matched controls (A). Levels of TNF-α (B) and NO (C) secreted from macrophages of rats ovariectomized at the age of 1 month (ox-1 m) and age-matched controls. Macrophages were cultured for 48 hours in culture medium in the absence (RPMI) or in the presence of 1 μg/ml LPS (LPS). The data are expressed as mean + S.E.M. (n = 6–8). Statistically significant differences: *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$, vs control.

ovariectomized rats, but only the decrease in progesterone levels reached statistical significance ($p < 0.05$) (Fig. 5A).

3.3.2.1. Effects of ovariectomy on TNF-α and NO production. Ovariectomy performed at the age of 10 months reduced basal and LPS-stimulated TNF-α production in macrophages from both middle-aged and aged rats ($p < 0.01$) (Fig. 5B).

However, ovariectomy did not significantly affect NO production in macrophages from middle-aged rats, and reduced NO concentration in macrophage cultures from aged rats ($p < 0.05$) (Fig. 5C).

3.3.2.2. Effects of ovariectomy on ED2 expression. Regarding ED2 expression on peritoneal macrophages of rats ovariectomized at 10 months of age, the percentage of $ED2^{high}$ cells was decreased ($p < 0.01$) in middle-aged, but not in aged rats (Fig. 6A). In addition, the percentages of $ED2^{low}$ cells in ovariectomized middle-aged and aged rats were elevated in comparison with their respective controls ($p < 0.05$). The representative histograms of ED2 expression on ED1+ cells are shown in Fig. 6B.

3.4. Influence of 17β-estradiol on peritoneal macrophages' response to LPS stimulation in vitro

3.4.1. Ovariectomy at the late prepubertal age

Next, we attested influence of increasing concentrations (10^{-8} M–10^{-6} M) of 17β-estradiol on macrophage TNF-α and NO production upon LPS stimulation. Addition of 17β-estradiol (10^{-7} M–10^{-6} M) to cultures markedly reduced TNF-α production ($p < 0.001$) in peritoneal macrophages from ovariectomized young adult rats and age-matched control rats (Fig. 7A). This suppressive effect of estradiol was not detected in middle-aged ovariectomized or middle-aged control rats.

When NO production was examined, decreased levels were observed in the presence of 17β-estradiol in macrophages from young

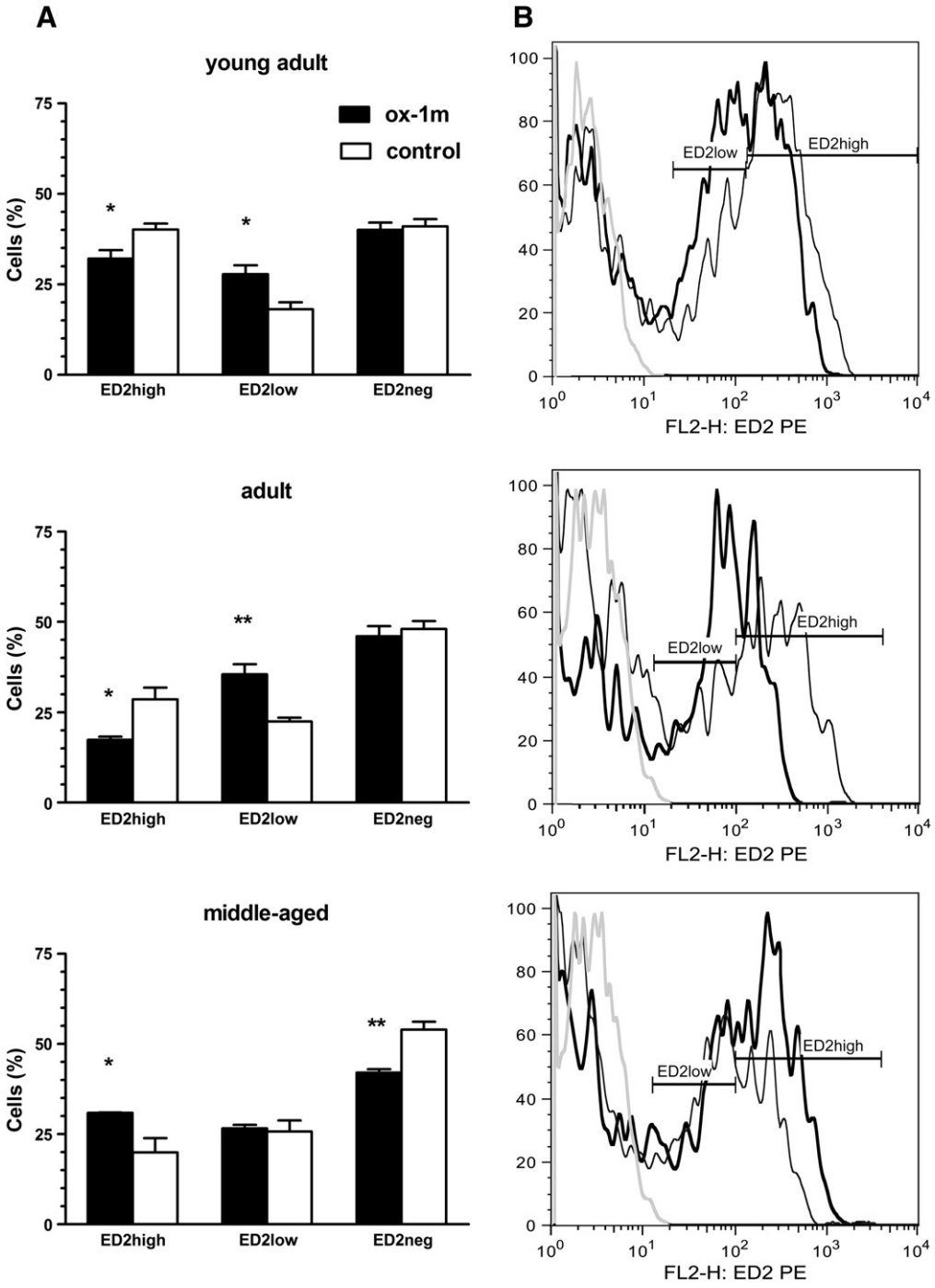

Fig. 4. Ovarian hormone deprivation initiated at the late prepubertal age influences ED2 expression on macrophages. Percentages (A) and representative histograms (B) of ED2^{high}, ED2^{low} and ED2^{neg} cells from rats ovariectomized at the age of 1 month (ox-1 m; thick line) and age-matched controls (thin line). The data are expressed as mean + S.E.M. (n = 6–8). Statistically significant differences: *, $p < 0.05$, and **, $p < 0.01$, vs control.

adult (at concentration of 10^{-6} M) and middle-aged (10^{-7} M–10^{-6} M) control rats ($p < 0.05$), but not in macrophages from ovarian hormone-deprived rats (Fig. 7B).

3.4.2. Ovariectomy at the end of the reproductive lifespan

We also found that 17-β estradiol at concentration of 10^{-6} M decreased ($p < 0.01$) TNF-α production in macrophages from middle-aged and aged rats ovariectomized at the age of 10 months (Fig. 8A). However, irrespective of concentration, 17β-estradiol did not influence TNF-α production in macrophages from age-matched controls (Fig. 8A).

In addition, 17β-estradiol (at concentrations 10^{-7} M–10^{-6} M) reduced ($p < 0.05$) NO levels in ovariectomized and control middle-aged rats (Fig. 8B). In macrophages from control aged rats, 17β-estradiol reduced NO production at a concentration of 10^{-6} M. On the other hand, none of the examined concentrations affected NO production in macrophages from ovarian hormone-deprived aged rats.

4. Discussion

Aging is accompanied by significant adaptation of macrophages, evidenced in alterations in their phenotypic and functional

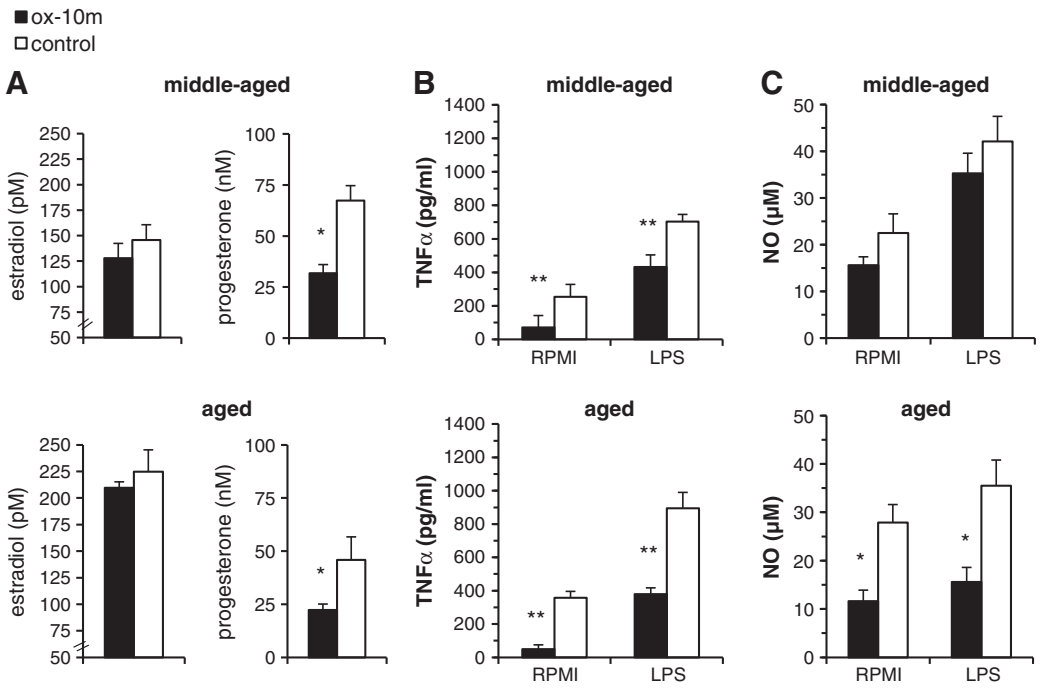

Fig. 5. Ovarian hormone deprivation initiated at the very end of reproductive lifespan influences macrophages' secretory functions. Serum levels of estradiol and progesterone in rats ovariectomized at the age of 10 months (ox-10 m) and age-matched controls (A). Levels of TNF-α (B) and NO (C) secreted from macrophages of rats ovariectomized at the age of 10 months (ox-10 m) and age-matched controls. Macrophages were cultured for 48 h in culture medium in the absence (RPMI) or in the presence of 1 μg/ml LPS (LPS). The data are expressed as mean + S.E.M. (n = 6–8). Statistically significant differences: *, $p < 0.05$, and **, $p < 0.01$, vs control.

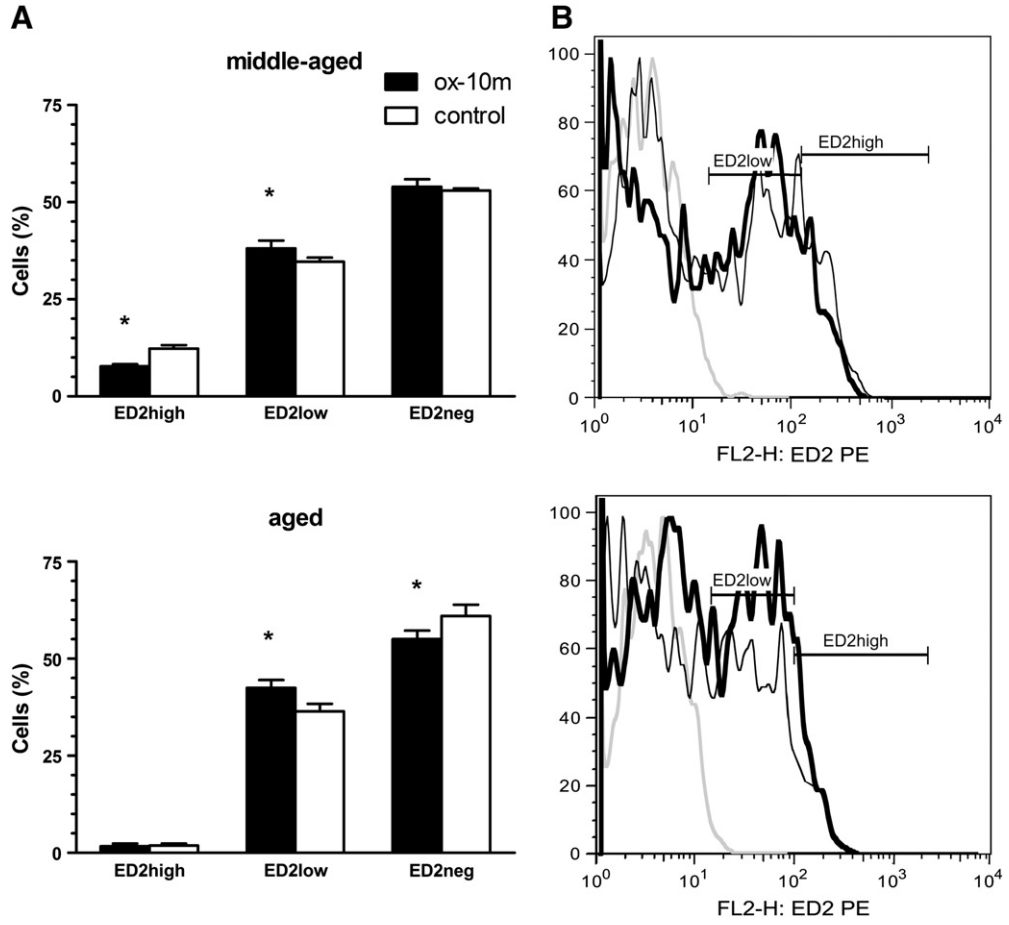

Fig. 6. Ovarian hormone deprivation initiated at the very end of reproductive lifespan influences ED2 expression on macrophages. Percentages (A) and representative histograms (B) of $ED2^{high}$, $ED2^{low}$ and $ED2^{neg}$ cells from rats ovariectomized at the age of 10 months (ox-10 m; thick line) and age-matched controls (thin line). The data are expressed as mean + S.E.M. (n = 6–8). Statistically significant differences: *, $p < 0.05$, vs control.

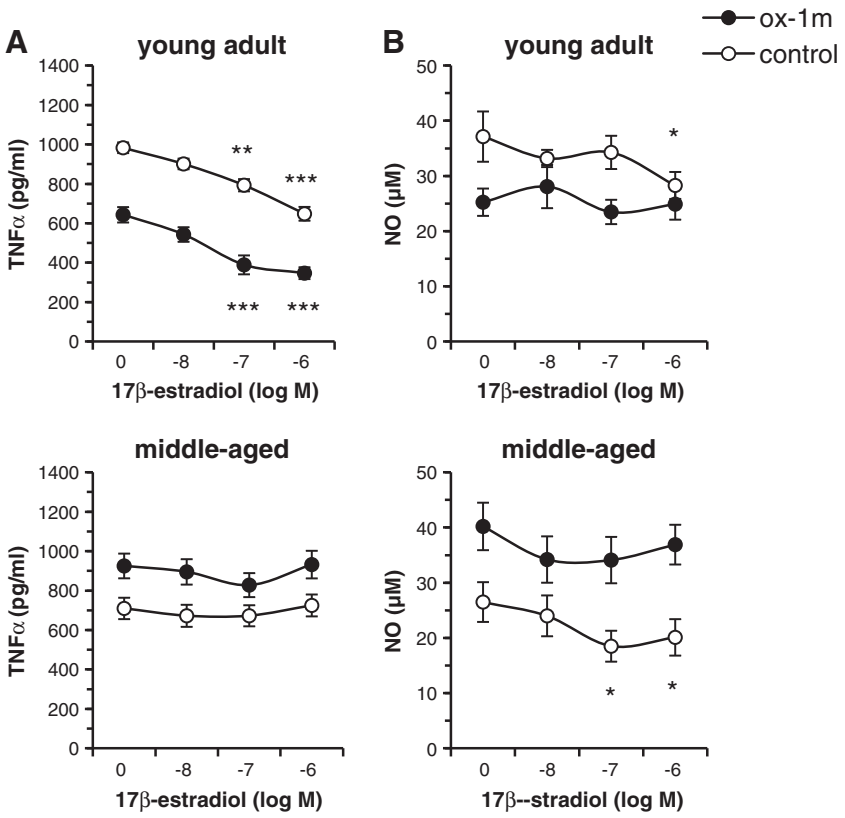

Fig. 7. Ovarian hormone deprivation at the late prepubertal age influences direct effects of estradiol on macrophages' secretory activity. Effects of estradiol in vitro on levels of TNF-α (A) and NO (B) secreted from macrophages of rats ovariectomized at the age of 1 month (ox-1 m) and age-matched controls. Macrophages were cultured for 48 h with or without estradiol (10^{-8} M–10^{-6} M) in the presence of 1 μg/ml LPS. The data are expressed as mean ± S.E.M. (n = 6–8). Statistically significant differences: *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$, vs LPS only.

characteristics (Gomez et al., 2005). Having that in mind, we firstly examined peritoneal macrophages for the expression of ED2, which is considered to be a marker of differentiated resident macrophages (Barbe et al., 1990). Confirming the previous findings (Kizaki et al., 2000), we found the age-related decrease in the proportion of cells expressing high surface density of ED2 within the peritoneal macrophage population. Given that ovarian steroids are shown to influence macrophage differentiation and ED2 expression, so that substantial changes in their differentiation were observed in young cycling and noncycling hypophysectomized rats (Frazier-Jessen and Kovacs, 1995; Ishida et al., 1996), the previous finding could be ascribed to the loss of cycling and substantial changes in ovarian hormone levels with aging. It was previously demonstrated that NF-κB activity in rat $ED2^{high}$ macrophages upon stimulation with LPS was markedly higher than that in $ED2^{low}$ ones (Kizaki et al., 2000). This transcription factor is shown to play an important role in coordinately controlling gene expressions during monocyte/macrophage activation (Baeuerle and Henkel, 1994). At least ten genes are candidates for the coordinate induction by NF-κB in monocytes/macrophages, including genes encoding IL-1, IL-6, TNF-α and NO synthase. The expression of IL-1β and IL-6 in $ED2^{high}$ cells was demonstrated to be markedly higher than in $ED2^{low}$ cells (Kizaki et al., 2000). Considering the previous findings, we compared LPS-stimulated TNF-α and NO production in macrophages from adult rats of different ages. Consistent with the age-related decline in the proportion of $ED2^{high}$ cells among peritoneal macrophages, LPS-elicited TNF-α production in macrophages from aged rats was diminished when compared with young adult and adult rats. Considering macrophages are the major producers of TNF-α, the age-related decrease of TNF-α production by peritoneal LPS-stimulated macrophages in vitro, which is shown in the present study and several other reports (Corsini et al., 1999; Davila et al., 1990), contrasts with earlier findings of elevated circulating TNF-α following LPS administration in aged rats (Foster et al., 1992). There are several explanations for this discrepancy. Firstly, this discrepancy could be related to the functional plasticity of macrophages depending on their microenvironment, viz., their in vivo settings vs in vitro conditions (Stout and Suttles, 2004). Secondly, it is noteworthy that besides macrophages, a variety of cell types, such as CD4+ lymphocytes, NK cells, microglia and endothelial cells, could contribute to circulating levels of TNF-α. In favour of the previous option are findings indicating increased production of TNF-α by microglia in aged mice following intracerebroventricular LPS administration (Kalehua et al., 2000). Finally, the data showing accumulation of ED1+ macrophages (Kullberg et al., 2001), and increased TNF-α mRNA levels in the murine brain (Vegeto et al., 2006) following intracerebroventricular LPS application in aged animals, indirectly suggest that distinct subtypes of macrophages exhibit different TNF-α secretory response to LPS.

In an attempt to elucidate mechanisms underlying the age-related changes in TNF-α production, we considered the data indicating that: a) estrogen (Calippe et al., 2010; Chao et al., 1995; Espinosa-Heidmann et al., 2005) and progesterone (Miller and Hunt, 1998) are shown to affect macrophage TNF-α production in vitro, acting in the opposing way, and b) both circulating estrogen and progesterone levels exhibit age-related changes. As previously shown (Maffucci and Gore, 2006), in aged rats we found the fall in circulating progesterone to the bottom levels of that seen in young cycling rats, whereas the level of circulating estradiol in these animals was sustained at a high level. Thus, we hypothesized that the age-related changes in the concentration of the main ovarian steroids combined with loss of cycling, and likely consecutive changes in the expression of their receptors (Chakraborty and Gore, 2004; Jezierski and Sohrabji, 2001; Maffucci and Gore, 2006; Uotinen

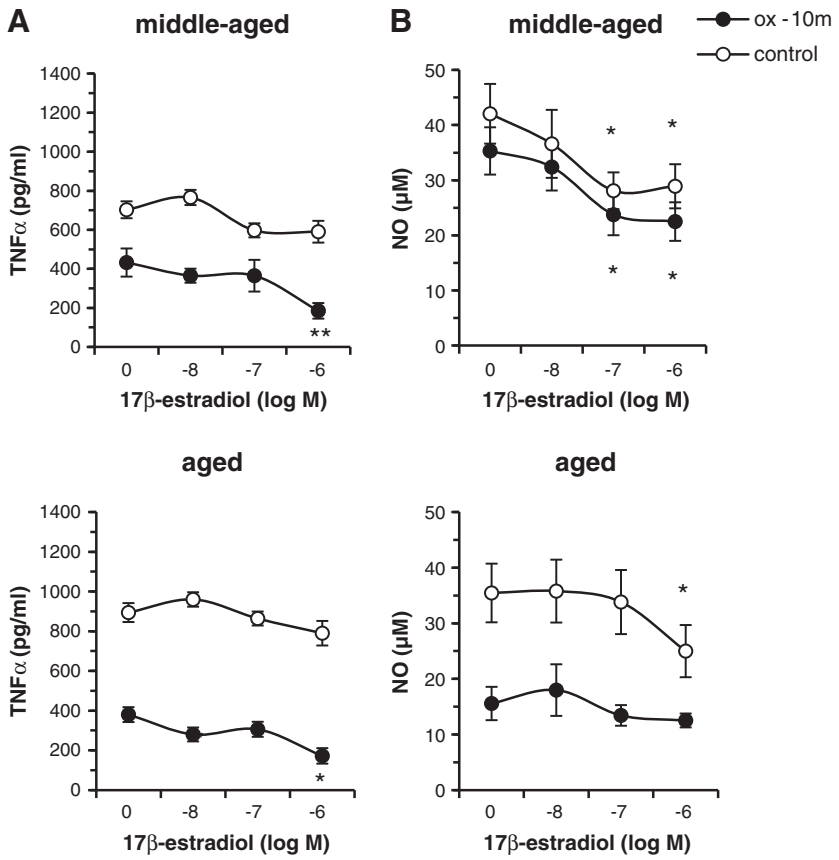

Fig. 8. Ovarian hormone deprivation initiated at the very end of reproductive lifespan influences direct effects of estradiol on macrophages' secretory activity. Effects of estradiol in vitro on levels of TNF-α (A) and NO (B) secreted from macrophages of rats ovariectomized at the age of 10 months (ox-10 m) and age-matched controls. Macrophages were cultured for 48 h with or without estradiol (10^{-8} M–10^{-6} M) in the presence of 1 μg/ml LPS. The data are expressed as mean ± S.E.M. (n = 6–8). Statistically significant differences: *, $p < 0.05$, and **, $p < 0.01$, vs LPS only.

et al., 1999; Wynne et al., 2004), contributed to the diminished ability of peritoneal macrophage to produce TNF-α in response to LPS. More specifically, we assumed that in aged rats, stimulatory action of estrogen on macrophage TNF-α production (Calippe et al., 2010; Chao et al., 1995; Espinosa-Heidmann et al., 2005) was overcome by the increased suppressive action of progesterone (Miller and Hunt, 1998). To further attest the previous hypothesis, we examined LPS-elicited TNF-α production in peritoneal macrophages isolated at various age points following ovarian gland ablation performed at the late prepubertal age or at the end of the rat reproductive lifespan. We decided on this model considering that in the rat, the circulating estrogen level gradually increases following ovariectomy (Zhao et al., 2005a, 2005b), thereby providing the possibility to test macrophage secretory capacity at various estrogen to progesterone ratios.

Unlike the results obtained by examining the influence of aging on TNF-α production, we failed to observe any significant effect of aging on LPS-induced macrophage NO secretion. The data on the influence of aging on NO production by rat peritoneal macrophages are inconclusive, as both decline and maintenance of NO secretory capacity have been reported (Alvarez et al., 1996; Tang et al., 2000). This discrepancy may have resulted from methodological variations and/or differences in the strain and sex of the animals studied. The differential effect of aging on LPS-elicited NO and TNF-α macrophage production could be related to data showing that TNF-α and NO production, in response to stimulation with LPS, is regulated by independent signalling pathways (Amura et al., 1997), so that upon LPS-stimulation, distinct phenotypic responses (i.e., preferential induction of TNF-α vs. NO) depending on the local settings can be readily elicited in these cells (Amura et al., 1997; Toshchakov et al., 2002). Considering that estrogen and progesterone could influence macrophage NO production, acting in the opposing manner (Calippe et al., 2010; Hong and Zhu, 2004; Miller et al., 1996), we also attested macrophage production of NO following ovariectomy.

In macrophage cultures from young and adult rats ovariectomized at the late peripubertal age, we measured lower LPS-stimulated TNF-α production than age-matched controls. Considering the fall in circulating estrogen and progesterone levels in these animals, the previous findings most likely indicated that the lack of estrogen stimulatory influence on macrophage TNF-α production (Calippe et al., 2010; Chao et al., 1995; Espinosa-Heidmann et al., 2005) was more pronounced than the lack of progesterone inhibitory action (Miller and Hunt, 1998). On the contrary, in macrophage cultures from middle-aged rats ovariectomized at the late peripubertal age, the LPS-stimulated TNF-α production was higher than in macrophage cultures from age-matched controls. Considering that in middle-aged rats, as expected from studies on estrogen level in rats ovariectomized at the end of reproductive age or in postestropousal ages (Jazbutyte et al., 2006; Keiler et al., 2013), the isolated progesterone deficiency was found, and the increased TNF-α production most likely could be associated with the unopposed lack of the progesterone inhibitory effect (Miller and Hunt, 1998). Furthermore, in accordance with previous (Kizaki et al., 2000) and herein reported data obtained by examining the age-dependent macrophage changes, the proportion of $ED2^{high}$ cells was decreased in the peritoneal macrophage population from ovariectomized rats exhibiting diminished TNF-α response to LPS stimulation. In contrast, amongst peritoneal macrophages from middle-aged rats ovariectomized at the late peripubertal age, showing enhanced TNF-α production, a greater frequency of $ED2^{high}$

cells was detected. Therefore, it seems obvious that low frequency of $ED2^{high}$ cells amongst rat peritoneal macrophages could be regarded as a predictor of their low ability to produce TNF-α in response to LPS, the prototype stimulus for triggering an inflammatory cascade in macrophages through Toll-like receptor 4.

Next, to further clarify influence of estrogen on macrophage TNF-α production, we examined macrophage response to LPS in culture in the presence of the increasing concentrations of 17β-estradiol. In macrophage cultures from both young adult ovariectomized and control rats, 17β-estradiol at pharmacological concentrations, 10^{-7} M and 10^{-6} M, decreased TNF-α production by approximately 20% in control vs 40% in ovariectomized rats, and 34% in control vs 46% in ovariectomized rats, respectively. The previous findings were in agreement with observations suggesting that estradiol exerts anti-inflammatory properties on monocyte/macrophage cell lines or microglial cells following their activation with LPS in vitro (Bruce-Keller et al., 2000; Ghisletti et al., 2005; Vegeto et al., 2001), whereas in vivo it exerts opposite effects on the same cell populations (Calippe et al., 2010; Soucy et al., 2005). There are several plausible explanations for this phenomenon. First, it has been shown that this discrepancy mirrors differences in short-term estradiol treatment in vitro vs a long-term exposure to estrogens in vivo (Ghisletti et al., 2005). Second, the results obtained in vivo most likely reflect both the direct and the indirect effects of estrogen, through alterations in the secretion of some other hormones (Couse and Korach, 1999), whereas those in vitro reflect only direct effects of this hormone.

Dose-related suppressive effects of 17β-estradiol on TNF-α production were also observed in murine macrophage and the macrophage RAW 264.7 cell line cultures (Deshpande et al., 1997; Tomaszewska et al., 2003; Zhang et al., 2001). The more pronounced suppressive effect of estradiol on LPS-stimulated TNF-α response in cultures from young ovariectomized rats compared with age-matched control cultures could be linked with the data, indicating that long-term ovarian hormone deprivation increases estrogen receptor expression in many target tissues (Mohamed and Abdel-Rahman, 2000). Furthermore, 17β-estradiol in none of tested concentrations significantly affected LPS-stimulated TNF-α production by macrophages from middle-aged and aged control rats. This clearly suggested that macrophage sensitivity to the suppressive 17β-estradiol action in vitro decreases with aging. Moreover, in macrophage cultures from middle-aged and aged rats ovariectomized at the end-of-reproductive age 17β-estradiol exerted suppressive effect on TNF-α production only at a concentration of 10^{-6} M, whereas in middle-aged rats ovariectomized at the late prepubertal age it was ineffective in this respect, in all the examined concentrations. This also could be associated with the variability in the expression of estrogen receptor, in particular in the relative cellular expression of the two subtypes (α and β) of classical intracellular estrogen receptor (Frasor et al., 2003), depending on the steroid hormone concentration in their microenvironment in vivo (Jezierski and Sohrabji, 2001). To support this option are data indicating that estradiol may influence NF-κB activity, and therefore cytokine production via specific estrogen receptor subtypes (reviewed in Kassi and Moutsatsou, 2010).

Furthermore, we measured NO production in response to LPS in macrophage cultures from ovariectomized rats. Comparing with age-matched control cultures, in macrophage cultures from ovariectomized rats a similar pattern of changes in LPS-elicited NO production to that observed in their TNF-α production was found. However, statistically significant differences were found only in macrophage cultures from middle-aged rats ovariectomized at the late prepubertal age and in aged rats ovariectomized at the end of the reproductive lifespan. The dissimilarities in the ovariectomy-induced alterations in macrophage production of TNF-α and NO could be related to the aforementioned findings, indicating that upon LPS-stimulation distinct phenotypic responses, i.e., preferential induction of TNF-α vs NO, depending on the local microenvironment, can be readily elicited in macrophages (Amura et al., 1997; Toshchakov et al., 2002). In this respect, distinct efficiency of biological modulators, as are steroid hormones, in modulating production of these inflammatory mediators seems to be quite understandable. Furthermore, the increase in NO production in response to LPS in macrophage cultures from middle-aged rats ovariectomized at the late peripubertal age could be ascribed to isolated progesterone deficiency, given that progesterone is shown to inhibit inducible NO synthase gene expression and NO production in murine macrophages (Miller et al., 1996). On the other hand, the impaired NO response of macrophages from aged ovariectomized rats to LPS, coupled with substantially different constellation of sex hormone levels, most likely further supported a role for estrogens in the modulation of macrophage NO production (Calippe et al., 2008; Hong and Zhu, 2004).

Next, we examined 17β-estradiol influence on NO production in LPS-stimulated macrophages in culture. We found that 17β-estradiol at pharmacological concentrations diminished LPS-stimulated NO production in macrophage cultures from control rats. This was in accordance with the aforementioned data, indicating that 17β-estradiol exerts anti-inflammatory and pro-inflammatory properties on monocyte/macrophage cell lines or microglial cells following their activation with LPS in vitro (Bruce-Keller et al., 2000; Ghisletti et al., 2005; Vegeto et al., 2001) and in vivo (Calippe et al., 2008; Soucy et al., 2005), respectively. Similar results have been previously obtained in monocyte/macrophage and macrophage cell line cultures (Hayashi et al., 1998; Kim and Jeong, 2003; Tomaszewska et al., 2003). However, in macrophage cultures from ovariectomized rats, 17β-estradiol exhibited suppressive effect on NO production only in the cultures of these cells from middle-aged rats ovariectomized at the end of the reproductive lifespan. These findings, in conjunction with those related to the effects of the 17β-estradiol on the production of TNF-α in the same cultures further supports the above stated notion, that this hormone exerts differential influence on the production of these two inflammatory mediators. Alternatively, given that distinct subtypes of classical intracellular estrogen receptor detected on macrophages (Hildebrand et al., 2006), it may be hypothesized that estradiol, through distinct types of estrogen receptors, could exert differential effects on NO and TNF-α production upon LPS stimulation. Furthermore, it is noteworthy that estrogen may also act through nonclassic membrane receptors, leading to rapid intracellular responses (Ropero et al., 2002). The research aimed to address putative role of distinct types of estrogen receptors in the modulation of macrophage production of NO and TNF-α is in progress in our laboratory.

In conclusion, the present study pointed to low frequency of $ED2^{high}$ cells amongst peritoneal macrophages as a predictor of their decreased ability to produce TNF-α in response to LPS, the prototype stimulus for triggering an inflammatory cascade in macrophages. Additionally, the study showed age-related decline in the frequency of these cells, and consequently in peritoneal macrophage ability to produce TNF-α upon LPS stimulation, and suggested that the age-related changes in the circulating estrogen/progesterone ratio could contribute to this phenomenon. Moreover, it demonstrated that changes in the circulating estrogen/progesterone ratio most likely lead to alterations in the LPS-induced macrophage secretory profile, by affecting production of TNF-α and NO, key inflammatory mediators. Finally, it confirmed the previous findings, suggesting that estradiol exerts opposing effects on macrophages in vitro and in vivo, exhibiting anti-inflammatory properties on LPS-stimulated macrophages in vitro (Ghisletti et al., 2005). Overall, the study contributes a better understanding of the ovarian steroid hormone role in the regulation of macrophage function, and its significance for the age-associate changes in innate immunity.

Conflict of interest

The authors have no conflicts of interests.

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