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# Peritoneal exudate cells from long-lived rats exhibit increased IL-10/IL-1β expression ratio and preserved NO/urea ratio following LPS-stimulation in vitro

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**Abstract** In humans, usual aging, differently from successful aging, is associated with deregulation of proinflammatory/anti-inflammatory cytokine balance. The corresponding data from rat studies are limited. Therefore, we examined (i) cytokine messenger RNA (mRNA) profile of fresh peritoneal cells from 6- (adult), 24- (old), and 31-month-old (long-lived) AO rats and (ii) proinflammatory (IL-1β and IL-6) and anti-inflammatory (IL-10) cytokine, NO, and urea production in their LPS-stimulated cultures. Comparing with adult rats, cells from old ones expressed lower amount of TNF-α and IL-6 mRNAs, but greater amount of IL-1β mRNA. On the other hand, cells from long-lived rats exhibited a dramatic increase in IL-10 mRNA expression followed by diminished TNF-α and IL-6 mRNA expression, and comparable expression of IL-1β mRNA relative to adult rats. Consequently, IL-10/IL-1β mRNA ratio was greater in cells from long-lived rats than in adult and old rats. In LPS-stimulated peritoneal cell cultures (contained ≥95 % macrophages) from old rats, concentration of common proinflammatory cytokines was higher than in those from adult rats. Comparing with adult and old rats, in LPS-stimulated macrophage cultures from long-lived rats, TNF-α and IL-6 concentrations were lower; IL-1β concentration was comparable or greater (in respect to adult rats), whereas that of IL-10 was strikingly higher. Consistently, in macrophage cultures from long-lived rats, NO (iNOS activity marker)/urea (arginase activity marker) ratio was less and not different from that in old and adult rats, respectively. The study suggests that macrophages from long-lived rats, differently from those of old ones, have substantial ability to limit proinflammatory mediator production, which may contribute to their longevity.

**Keywords** Aging · Macrophages · Proinflammatory cytokines · Anti-inflammatory cytokines · NO/urea balance · TLR4

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## Introduction

Although adverse effects of aging on immune responsiveness have mainly been attributed to alterations in T

lymphocyte compartment (Nikolich-Zugich 2005), perturbations in macrophages have also been suggested (reviewed in Lloberas and Celada 2002; Mahbub et al. 2011). The age-related macrophage changes have been related to a state of low-grade chronic inflammation, i.e., phenomenon called "inflammaging." This state promotes or exacerbates important age-associated diseases such as atherosclerosis, Alzheimer's disease, arthrosis and arthritis, sarcopenia, and diabetes type II (reviewed in Salvioli et al. 2006). The inflammatory response, which involves action of various mediators, is designed to limit tissue damage induced by various insults and to promote its repair. Among inflammatory mediators, a leading role is played by cytokines, which are shown to exhibit not only proinflammatory (e.g., TNF-α, IL-1β, and IL-6), but also anti-inflammatory action (e.g., IL-10 and TGF-β). The age-related loss of physiological control of the inflammatory response is mirrored in an imbalance of the finely tuned equilibrium between the levels of proinflammatory/anti-inflammatory mediators, or in the incapability to restore the equilibrium once the inflammatory stimulus has been relieved (Salvioli et al. 2006). These deregulations lead to a strong inflammatory response upon action of various insults, which, most likely, has a nontrivial outcome, i.e., it substantially affects human longevity (Salvioli et al. 2006).

Macrophages represent dynamic and phenotypically and functionally heterogeneous cell population with a crucial role in inflammation. These cells are broadly classified into two subsets (M1 and M2 cells), which differ in terms of cytokine, chemokine, and inducible NO synthase (iNOS) expression and effector functions (reviewed in Benoit et al. 2008; Mosser 2003). Differently from microbicidal and proinflammatory M1 macrophages, M2 macrophages (also called as anti-inflammatory macrophages), expressing the enzyme arginase, are poorly microbicidal and have pronounced immunomodulatory capacity. Classically activated M1 macrophages are induced through gram-negative bacteria lipopolysaccharide (LPS) and/or microbial product stimulation. In contrast, alternative activation of macrophages by IL-4/IL-13, immune complexes, toll-like receptor (TLR) agonists, including LPS (Sonoki et al. 1997), and glucocorticoids results in their polarization into M2 cells (reviewed in Benoit et al. 2008; Gordon and Martinez 2010; Mosser 2003). It should be pointed out that (i) macrophage activation has been shown to exhibit significant plasticity, as well as rapid, and full reversibility, and (ii) macrophage population is dynamic and may first take part in inflammation and then participate in its resolution (Porcheray et al. 2005).

The ability of human macrophages to polarize into M1 and M2 cells changes with aging (Mahbub et al. 2012). These changes are related to inflammatory response deregulation and pathology in the elderly (Mahbub et al. 2012). The production of proinflammatory/anti-inflammatory mediators substantially differ in centenarians, i.e., in those showing so-called successful or healthy aging (Rowe and Kahn 1997), from the corresponding production in younger but old subjects (reviewed in Salvioli et al. 2006), and these differences are often taken as indicators of longevity.

A number of studies in mice demonstrated age-related changes in ability of macrophages to produce proinflammatory/anti-inflammatory mediators (Arranz et al. 2010b; Boehmer et al. 2004; Boyd et al. 2012; Cecilio et al. 2011; Chen et al. 1996; Gomez et al. 2010; Kohut et al. 2004; Renshaw et al. 2002; Shaik-Dasthagirisaheb et al. 2010). We have recently reported diminished TNF-α production in response to LPS in macrophages from 11- to 20-month-old female rats of Albino Oxford (AO) strain (Dimitrijević et al. 2013). In our animal facility, the average and maximum life span of AO rats is longer, whereas the frequency of apparent illness in aged animals is lower compared to other rat strains (Wistar and Dark Agouti). Therefore, female AO rats have been imposed as candidates for studying markers of rat longevity.

The data on the relationship between changes in macrophage capacity to produce inflammatory mediators and rodent longevity are extremely limited. Therefore, in the present study, in order to distinguish putative indictors of rat longevity, we concurrently examined the ability of macrophages from long-lived (31-month-old rats, which almost reached the maximum life span of 32 months in our animal facility), old (24-month-old), and adult (6-month-old) AO female rats to produce various proinflammatory and anti-inflammatory mediators ex vivo and in vitro. More specifically, fresh peritoneal exudate cells from these animals were examined for expression of mRNAs for anti-inflammatory (IL-10) and proinflammatory (TNF-α, IL-6, and IL-1β) cytokines. In addition, following stimulation with LPS in vitro, peritoneal macrophages were examined for (i) production of both anti-inflammatory and proinflammatory cytokines and (ii) activity of iNOS and arginase by measuring NO and urea production, respectively.

## Methods

### Animals

In this study, we used 6- (adult, $n=8$), 24- (old, $n=8$), and 31-month-old (long-lived, $n=5$) female AO rats from the breeding colony at Immunology Research Center "Branislav Janković," Belgrade (Serbia). In our animal facility, the female rats of this strain exhibit a longer average life span (29 months) and longer average healthy life span (without severe pathologies, such as obesity, diabetes, and tumors) than Wistar (26 months) and Dark Agouti (24 months) rats of the same sex. In our animal facility, only 20 % of AO female rats assigned for aging studies reach the age of 31 months, and one third of them must be excluded from the experiments because of clinically and macropathologically overt signs of illness. It is noteworthy that in a pilot experiment, fresh peritoneal exudate cells from adult rats differed significantly in none of the examined phenotypic parameters from the corresponding cells from young 2- to 3-month-old rats. 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. All animals, which were included in the study, were apparently healthy and free of clinical signs of short- or long-term illness. Additionally, at necropsy, each animal underwent a physical examination and was determined to be free of gross tumor or any other signs of overt pathology. Animals were euthanized using increasing dose of $CO_2$. The experimental protocol and all procedures with animals and their care were approved by experimental animal committee of the Immunology Research Center "Branislav Janković," 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).

### Isolation of peritoneal exudate cells

Resident peritoneal cells were isolated by washing the peritoneal cavity with 10 ml of ice-cold phosphate-buffered saline (PBS) pH 7.4 supplemented with 2 % fetal calf serum (FCS, Gibco, Grand Island, NY, USA). Individual cell suspensions were washed two times with PBS/2 % FCS. Irrespective of age, this cell population, according to flow cytometric analysis, consisted of approximately 75 % macrophages ($ED1^+$ cells). The nonmacrophage cell population, apart from small percentage (≤3 %) of granulocytes, includes lymphocytes (Dimitrijević et al. 2009; Stanojević et al. 2013).

### PCR analyses

Total RNA was isolated from freshly isolated peritoneal macrophage cells using an ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA) and Total RNA Chemistry (Applied Biosystems). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and 5 μl of complementary DNA (cDNA) was used for real-time PCR. Triplicate 25 μl reactions were run under Applied Biosystems 7500 universal cycling conditions. Gene Expression Master Mix and commercial TaqMan Gene Expression Assays for rat TNF-α (Rn99999017_m1), IL-6 (Rn99999011_m1), IL-1β (Rn99999009_m1), and IL-10 (Rn00563409_m1) were obtained from Applied Biosystems. All procedures were performed according to the manufacturer's recommendations. β-Actin was the internal standard to normalize for input cDNA variations, as it displayed an optimal stability among various samples tested. Quantitative differences in gene expression levels were assessed using Applied Biosystems SDS software (v 1.4.0.) and the $2-\Delta\Delta Ct$ method.

### Flow cytometric analysis

For immunophenotyping single-cell suspensions of peritoneal cells were adjusted to $1\times10^7$ 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 $1\times10^6$ cells were centrifuged at 350×g for 5 min at 4 °C to yield a pellet. For analyses of peritoneal exudate cell surface phenotype, cells were incubated with one of the following primary antibodies: PE-conjugated mouse monoclonal anti-rat ED2 (clone HIS36, BD Pharmingen, San Diego, CA, USA), polyclonal rabbit anti-rat TLR4 (Abcam, Cambridge, MA, USA), and polyclonal goat anti-rat CD14 (Santa Cruz Biotechnology, Heidelberg, Germany) antibody. When primary antibody was unconjugated, as secondary antibody, either FITC-conjugated goat anti-rabbit IgG (BD Pharmingen, San Diego, CA, USA) or FITC-conjugated rabbit anti-

goat IgG (Sigma-Aldrich Chemie, Taufkirchen, Germany) was used. All incubations were performed at 4 °C in the dark, 30 min, and were followed by thorough washings.

For the analysis of intracellular CD68 antigen expression, upon incubation with anti-rat ED2 antibody, cells were washed in FACS buffer and then fixed with 0.25 % paraformaldehyde and permeabilized by 0.2 % Tween 20 (15 min, 4 °C) and then incubated with FITC-conjugated mouse anti-rat CD68 (clone ED1; Serotec, Oxford, UK) antibody. After immunolabeling, 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 analyzed using FlowJo software version 7.8. (TreeStar Inc., Ashland, OR, USA). Non-specific IgG isotype-matched controls were used for each fluorochrome 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).

## Macrophage culture

Aliquots of cells were adjusted to $1\times10^6$/ml in RPMI 1640 medium (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) and plated to 24-well flat-bottomed tissue culture plates (NUNC, Roskilde, Denmark) for PGE2 and cytokine assays. For NO and arginase assays, cells ($1\times10^5$ per well in RPMI 1640 medium) were plated to 96-well flat-bottomed tissue culture plates (NUNC, Roskilde, Denmark). Cells were cultured to adhere for 2 h at 37 °C in a moist atmosphere of 5 % $CO_2$ in air. The nonadherent 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 further incubated at 37 °C and 5 % $CO_2$ in RPMI with or without 1 μg/ml LPS (Sigma-Aldrich Chemie, Taufkirchen, Germany). Culture supernatants collected following 4 and 24 h of incubation in 24-well plates were frozen at −70 °C until assayed for PGE2 and cytokines, respectively, whereas culture supernatants collected after 48 h 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 800 rpm for each wash/rinse step. Cells were lysed 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. Culture supernatants collected following 4 and 24 h of incubation in 24-well plates were frozen at −70 °C until assayed for PGE2 and cytokines, respectively.

## Cytokine and PGE2 assays

The following commercially available ELISA kits were used: TNF-α and IL-6 (Biolegend Inc., San Diego, CA, USA), IL-1β (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA), and PGE2 and IL-10 (R&D Systems, Inc., Minneapolis, MN, USA). Prior to ELISA performing, the thawed culture supernatants were centrifuged on 250×*g*, 15 min, + 4 °C. All assays were carried out according to the instructions provided by the manufacturer. The absorbencies were measured on Multiskan Ascent (Labsystems, Helsinki, Finland). A standard curve was generated for each assay with the limit of detection for IL-10<10 pg/ml, TNF-α=5 pg/ml, IL-6=5.3 pg/ml, IL-1β≤12 pg/ml, and PGE2=27.5 pg/ml.

## 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 in the samples was calculated using a $NaNO_2$ (1–80 μM) as a standard.

## Arginase assay

Arginase activity was indirectly determined by measuring the metabolite urea, a byproduct of arginine degradation from cells cultured in vitro (Corraliza et al. 1994; Wynn et al. 2011). 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 in 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 h. 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 urea concentration was measured at 540 nm (Multiskan Ascent, Labsystems, Helsinki, Finland) after the addition of 25 μl of α-isonitrosopropiophenone dissolved in 100 % ethanol followed by heating at 95 °C for 45 min. The concentration of urea in the samples was calculated according to the standard curve obtained with known concentrations of a urea (50–1,600 μM).

Statistical analysis

Data were analyzed by one-way ANOVA followed by Bonferroni test for post hoc comparisons. Data are presented as mean+SEM. Differences are regarded as statistically significant if $p<0.05$. All the analyses were performed using the SPSS 20.0 for Windows.

## Results

The frequency of macrophages within fresh peritoneal exudate cells was comparable across all age groups

Despite age-related differences in the body weight and the total number of recovered peritoneal exudate cells, the proportions of macrophages within these cells were similar in all age groups (Fig. 1). Namely, old rats were heavier than adult ($p<0.01$) and long-lived ($p<0.05$) rats (Fig. 1a), and exhibited a greater number of peritoneal cells ($p<0.05$) relative to adult, but not long-lived, rats (Fig. 1b). However, there were no age-related differences in the percentages of macrophages, estimated by immunolabeling with ED1 antibody, within peritoneal exudate cells (Fig. 1c).

Fresh peritoneal exudate cells from long-lived rats express higher level of IL-10 mRNA but lower of IL-1β than those cells from old rats

Freshly isolated peritoneal exudate cells were analyzed for the expression of mRNA for anti-inflammatory IL-10 cytokine and common proinflammatory cytokines. IL-10 mRNA expression was strikingly increased ($p<0.001$) only in peritoneal exudate cells from long-lived rats (Fig. 2a). However, results showed progressive age-related decrease in the expression of mRNAs for TNF-α (Fig. 2b) and IL-6 (Fig. 2c) in peritoneal exudate cells. Thus, the amount of these cytokines was less ($p<0.01$) in peritoneal exudate cells from long-lived

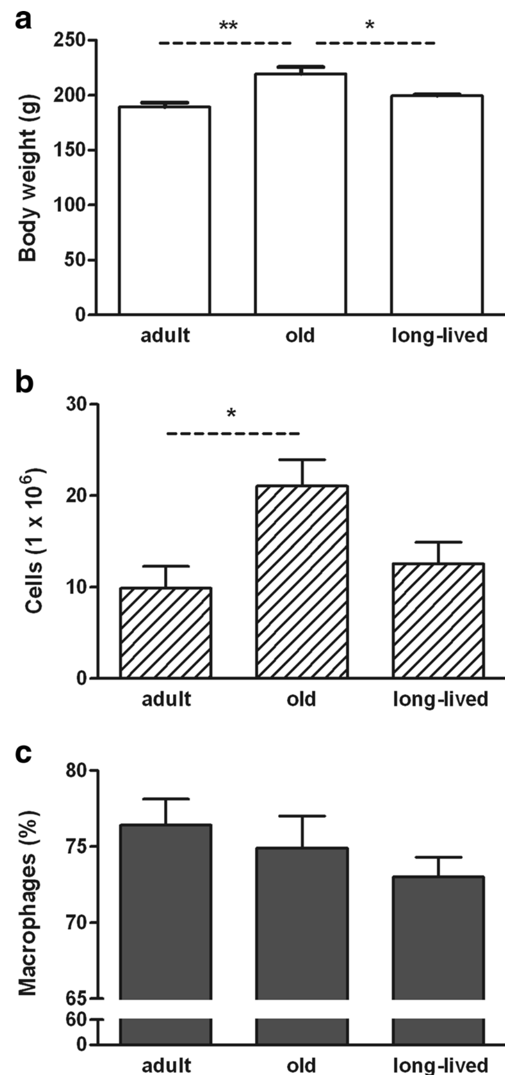
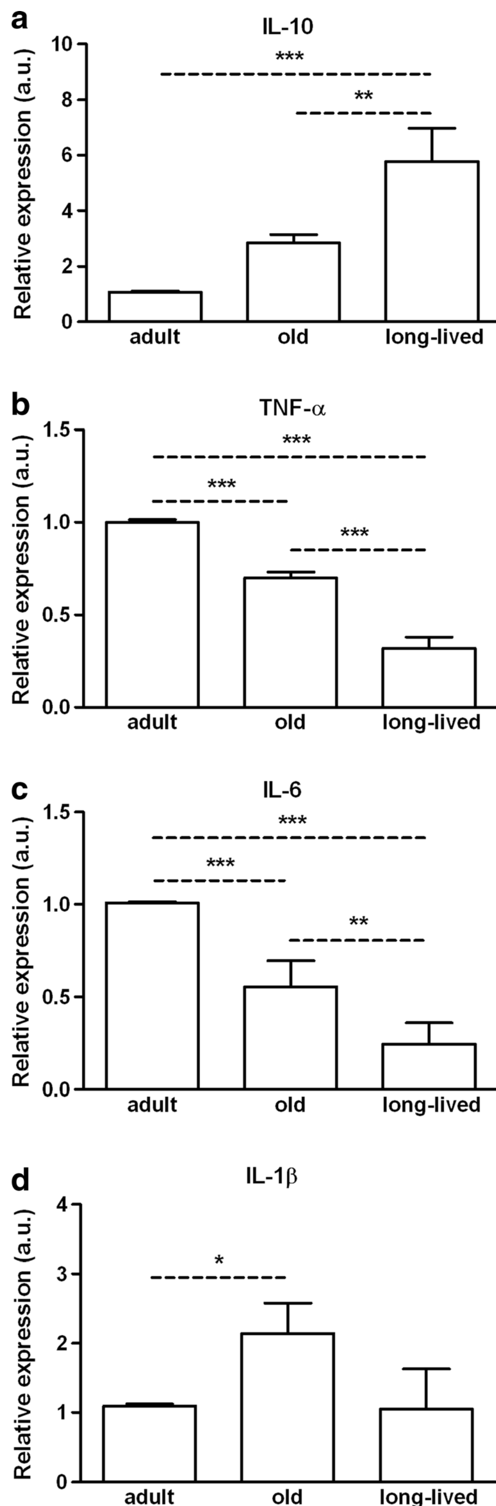



**Fig. 1** Aging does not alter the proportion of macrophages within peritoneal exudates cells. Body weight (**a**), total number of recovered peritoneal exudate cells (**b**), and the percentages of macrophages (ED1$^{+}$ cells) within peritoneal exudate cells (**c**) from adult, old, and long-lived rats. Results are presented as mean+SEM. Statistically significant differences: *$p<0.05$ and **$p<0.01$

rats than in those cells from old and adult ones. Comparing with adult rats, in peritoneal cells from long-lived rats, the expression of IL-1β was not significantly different, whereas it was augmented ($p<0.05$) in the corresponding cells from old rats (Fig. 2d).

Thus, peritoneal exudate cells from long-lived rats expressed strikingly higher amounts of IL-10 mRNA and comparable levels of IL-1β in respect to those cells from adult rats, whereas peritoneal exudate cells from old rats expressed greater amount of IL-β compared to those cells from adult rats. Consequently, IL-10/IL-1β




◀**Fig. 2** Age-related changes in cytokine mRNA expression in peritoneal cells. Fold change in mRNA expression for IL-10 (**a**), TNF-α (**b**), IL-6 (**c**), and IL-1β (**d**) in freshly isolated peritoneal cells of old and long-lived rats relative to those from adult rats. Results are presented as mean+SEM. Statistically significant differences: *$p<0.05$, **$p<0.01$, and ***$p<0.001$

mRNA expression ratio was markedly greater ($p<0.05$) in peritoneal exudate cells from long-lived (5.5±0.6) than in those from old (1.3±0.3) and adult (1.02±0.1) rats.

## Differential ED2 expression on fresh peritoneal macrophages from long-lived and old rats

We also examined phenotypic profile of peritoneal macrophages. Irrespective of age, as expected (Dijkstra et al. 1985), almost all cells (adult 96.5±0.2 %, old 94.5±0.5 %, and long-lived 93.2±2.0 %) within this macrophage population, which was gated by plotting FSC versus SSC, expressed rat homolog of human CD68 ($ED1^+$ cells; Fig. 3a). In addition, we examined the surface expression of CD163 (recognized by ED2 mAb), a receptor molecule for various endogenous and exogenous ligands, which participate in the initiation and/or perpetuation of the inflammatory response (reviewed in Kowal et al. 2011). The expression of this receptor is regulated in humans by proinflammatory/anti-inflammatory cytokine balance and, depending of ligand, it could mediate the release of both anti-inflammatory and proinflammatory mediators (reviewed in Kowal et al. 2011). The frequency of $ED1+ED2^+$ cells (Fig. 3a), and that of cells expressing ED2 at high level ($ED2^{high}$) within $ED1^+$ macrophage population (Fig. 3b, c), was diminished in old ($p<0.05$), but not in long-lived rats relative to adult rats.

## The expression of LPS signaling complex differs on fresh peritoneal macrophages from long-lived and old rats

The expression of TLR4 (an essential signaling receptor for LPS) and CD14 (a molecule that binds to LPS and facilitates its signaling) was also examined on cells within macrophage gate (da Silva et al. 2001). The proportion of both $TLR4^+$ cells and $CD14^+$ cells was increased in old ($p<0.001$) and long-lived rats ($p<0.05$) relative to those cells from adult rats (Fig. 4a, c). In addition, surface density of both TLR4 ($p<0.05$) and CD14 ($p<0.01$) on these cells from old rats, as shown

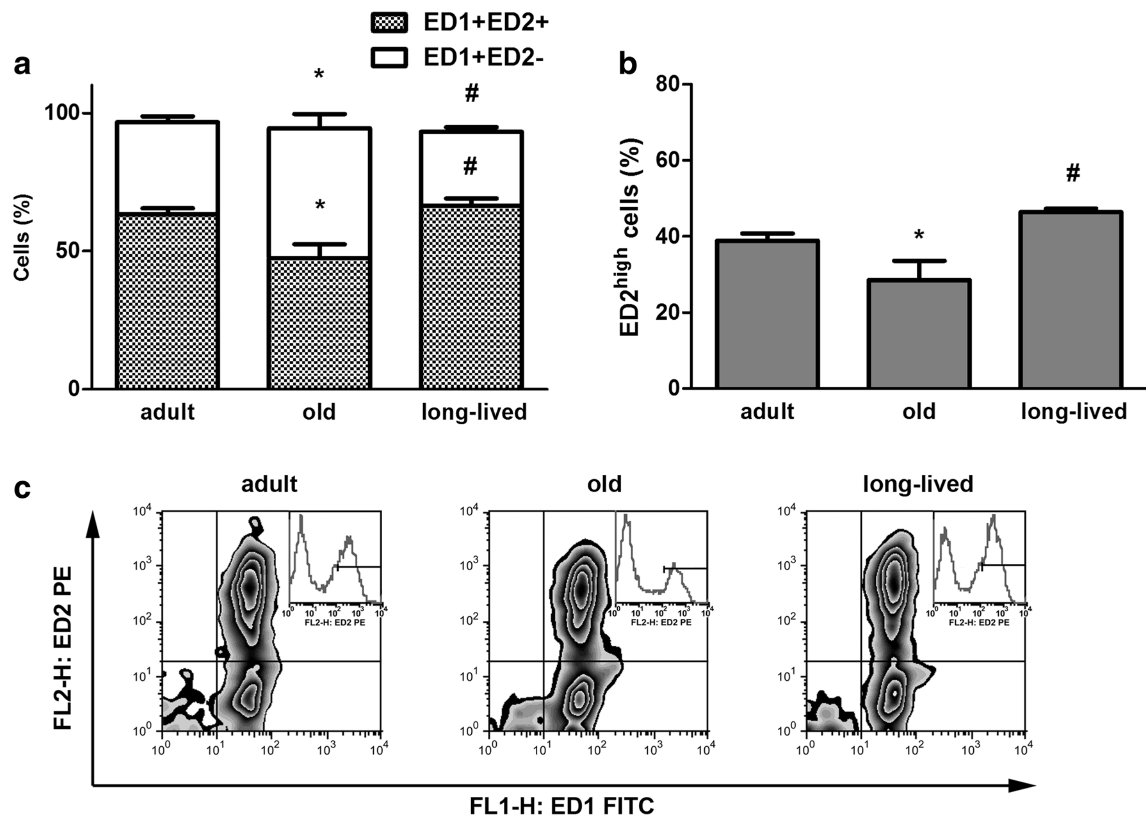



**Fig. 3** Age-related changes in the expression of ED2 on macrophages. Peritoneal cells were obtained from adult, old, and long-lived rats. **a** The percentages of ED1+ED2$^{+}$ and ED1+ED2$^{-}$ macrophages. **b** The percentages of ED2$^{high}$ macrophages within ED2$^{+}$ cells. Results are presented as mean+SEM. Statistically significant differences: *$p<0.05$ versus adult, and $^{\#}p<0.05$ versus old. **c** Representative plots of two-color flow-cytometric analysis of ED1 and ED2 expression in peritoneal cells

by mean fluorescence intensity (MFI), was greater than in the corresponding cells from adult rats (Fig. 4b, c). Furthermore, greater proportions of TLR4$^{+}$ cells ($p<0.01$) and CD14$^{+}$ cells ($p<0.05$) were detected within peritoneal macrophages from old rats relative to those from long-lived rats (Fig. 4a, c).

## Peritoneal cells from long-lived and old rats exhibit differential cytokine secretory profile following LPS stimulation in vitro

In the second step, we examined the production of anti-inflammatory (IL-10) and proinflammatory (TNF-α, IL-6, and IL-1β) cytokines in peritoneal exudate cell cultures (consisting of ≥95 % macrophages; macrophage cultures) in the presence and in the absence of LPS. In the absence of LPS, detectable levels of IL-10 was found in all the macrophage cultures, but they were higher ($p<0.001$) in cell cultures from long-lived rats compared to those from old and adult rats (Fig. 5a). Upon LPS stimulation, peritoneal macrophages from old ($p<0.01$) and long-lived ($p<0.001$) rats produced more IL-10 than those from adult rats.

In the absence of LPS in macrophage culture supernatants from both old ($p<0.05$) and long-lived ($p<0.001$) rats, we found lower levels of TNF-α, and this age-related decrease was particularly prominent in long-lived rats (Fig. 5b). Thus, the level of this cytokine was lower ($p<0.001$) in macrophage supernatants from long-lived rats than in those from old ones (Fig. 5b). On the other hand, following stimulation with LPS, the level of TNF-α in macrophage culture supernatants from old and long-lived rats increased ($p<0.01$) and decreased ($p<0.001$) relative to that from adult rats, respectively (Fig. 5b).

In the absence of LPS, the levels of IL-6 were undetectable in all the macrophage cultures. However, in the presence of LPS, macrophages from old rats produced more ($p<0.05$) IL-6 than macrophages from adult and long-lived rats (Fig. 5c).

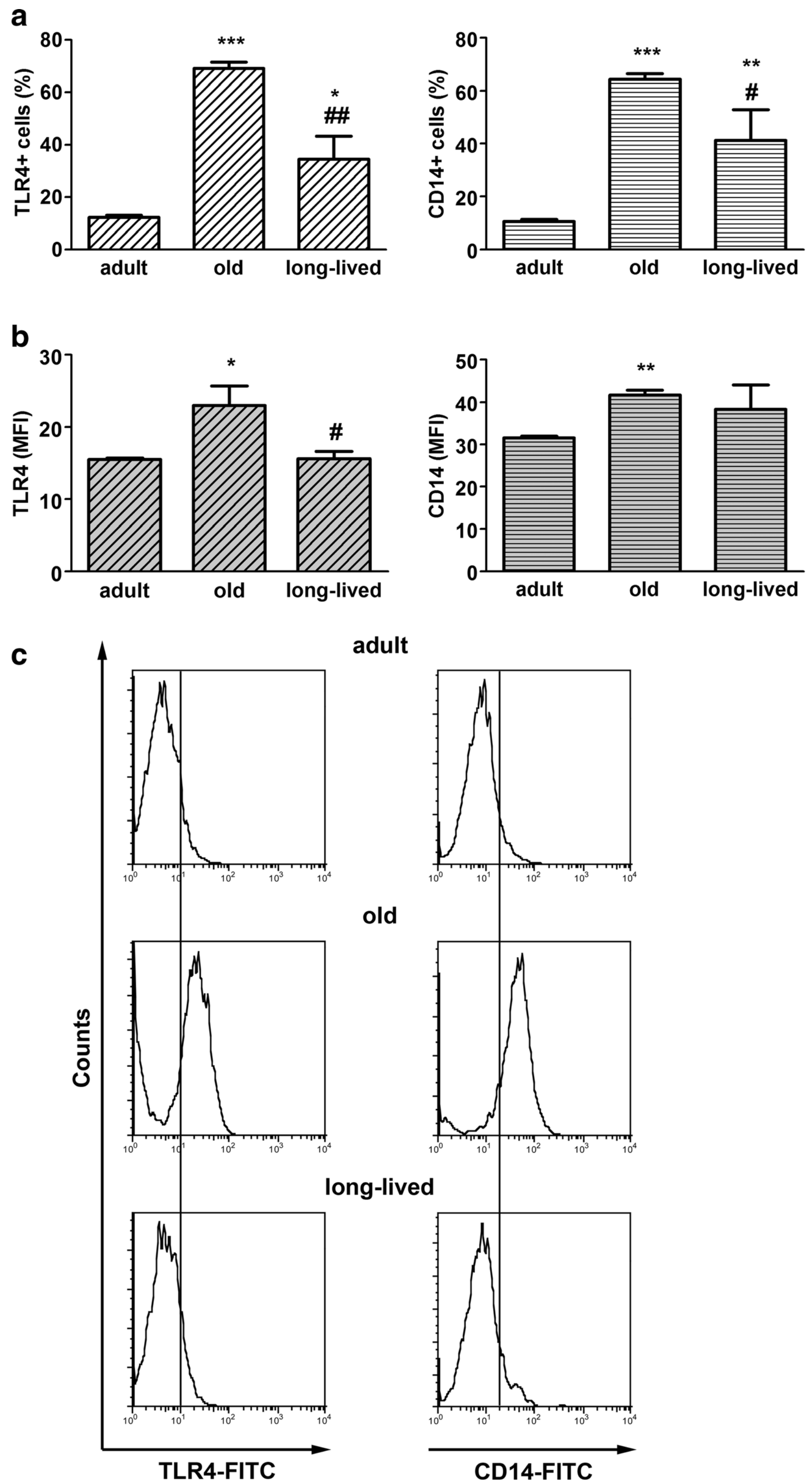



**Fig. 4** Age-related changes in the expression of CD14 and TLR4 on peritoneal macrophages. Peritoneal cells were obtained from adult, old, and long-lived rats. **a** The percentages of $CD14^+$ and $TLR4^+$ cells. **b** Mean fluorescence intensity (*MFI*) of CD14 and TLR4 on macrophages. The percentages of $TLR4^+$ and $CD14^+$ cells were determined by using appropriate staining controls. Results are presented as mean+SEM. Statistically significant differences: $*p<0.05$, $**p<0.01$, and $***p<0.001$ versus adult; and $^{\#}p<0.05$ and $^{\#\#}p<0.01$ versus old. **c** Representative histograms show CD14 and TLR4 expression on peritoneal macrophages

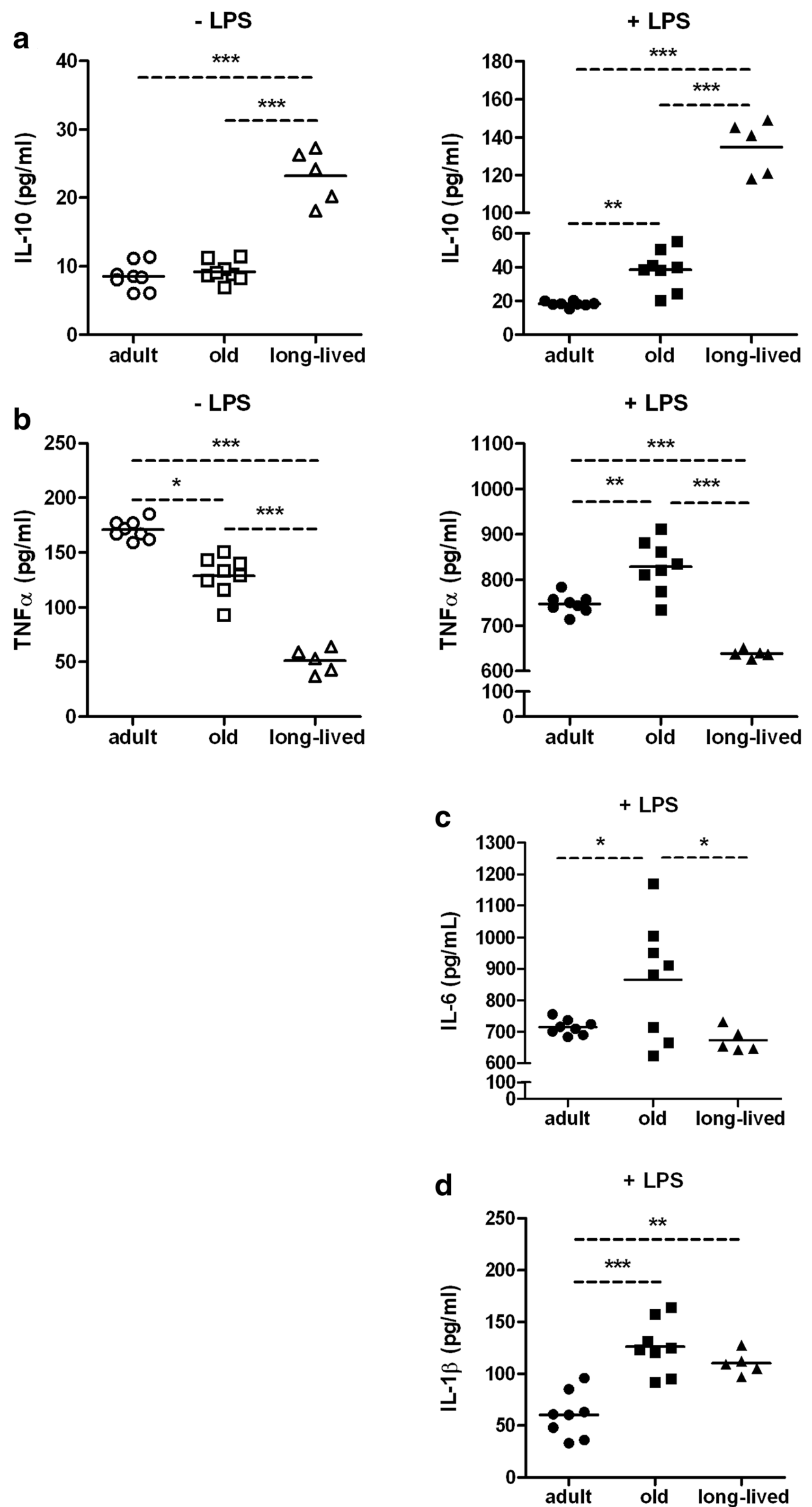



**Fig. 5** Aging alters cytokine production by macrophages. Peritoneal macrophages of adult, old, and long-lived rats were cultured in the absence (−LPS) or in the presence of LPS (+LPS). Levels (pg/ml) of IL-10 (**a**), TNF-α (**b**), IL-6 (**c**), and IL-1β (**d**) in the culture supernatants of peritoneal macrophages measured by ELISA. Results are presented as individual values. *Horizontal full line* indicates mean value for each age group. Statistically significant differences: *$p<0.05$, **$p<0.01$, and ***$p<0.001$

Similarly to IL-6, in the absence of LPS, IL-1β levels were undetectable across all the macrophage cultures. However, following LPS stimulation, IL-1β level increased ($p<0.001$) in cell cultures from old and long-lived ($p<0.01$) rats compared to those from adult ones (Fig. 5d).

### Peritoneal cells from long-lived rats exhibit diminished PGE2 production in respect to those from old rats

Next, considering that there is positive correlation between IL-6 and PGE2 secretion (Bouffi et al. 2012; Hinson et al. 1996), we investigated the efficacy of LPS-stimulated peritoneal macrophages from adult and aged rats to secrete PGE2. The production of PGE2 in LPS-stimulated peritoneal macrophages from long-lived rats was diminished ($p<0.05$) compared to both adult and old rats (Fig. 6).

### NO/urea ratio differs in LPS-stimulated peritoneal cell cultures from old and long-lived rats

In addition, we measured iNOS and arginase activity in the presence and in the absence of LPS. The activity of iNOS was estimated from nitrite accumulation in the culture supernatants, whereas arginase activity was

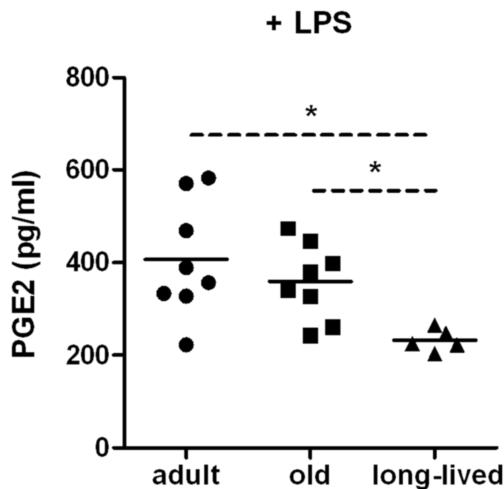



**Fig. 6** Aging alters PGE2 production by macrophages. Peritoneal macrophages of adult, old, and long-lived rats were cultured in the presence of LPS (+LPS). Levels of PGE2 (pg/ml) in the culture supernatants of peritoneal macrophages measured by ELISA. Results are presented as individual values. *Horizontal full line* indicates mean value for each age group. Statistically significant difference: *$p<0.05$

assessed by measuring arginine-derived urea in the cell extracts (Corraliza et al. 1994; Wynn et al. 2011).

In the absence of LPS macrophages, long-lived rats produced greater ($p<0.05$) NO amount than these cells from adult and old rats (Fig. 7a). Upon LPS stimulation, NO levels in cultures of macrophages from rats of all the examined ages were above their levels in the corresponding control cultures without LPS (Fig. 7a). However, in the presence of LPS, NO production was greater ($p<0.05$) in macrophages from both old and long-lived rats than in corresponding cells from adult rats (Fig. 7a).

In the absence of LPS, detectable levels of urea were found in all the cell extracts, but in those from old rats, they were lower ($p<0.01$) than in adult rats (Fig. 7b). In macrophages from all the experimental groups, LPS increased arginase activity judging by urea level (Fig. 7b). However, comparing with adult rats, the activity of arginase was less ($p<0.05$) in LPS-stimulated macrophages from old rats, but markedly greater ($p<0.01$) in these cells from long-lived rats (Fig. 7b). Consequently, arginase activity was considerably higher ($p<0.01$) in LPS-stimulated macrophages from long-lived rats than in those from old ones (Fig. 7b).

To estimate the influence of aging on macrophage M1/M2 polarization, we calculated NO (μM)/urea (μM) ratio. In the absence of LPS, in macrophage cultures from old rats, we found a shift ($p<0.05$) in NO/urea ratio towards NO (suggesting macrophage differentiation towards M1 cells) comparing with those from adult rat (Fig. 7c). However, this ratio was comparable in long-lived and adult rats (Fig. 7c). Following stimulation with LPS, considerably higher ($p<0.001$) NO/urea ratio was found in macrophages from old rats compared to that in macrophages from adult and long-lived rats (Fig. 7c).

## Discussion

The study showed that peritoneal exudate cells from long-lived rats and old rats substantially differ in respect of the expression/production of various proinflammatory and anti-inflammatory mediators when examined both immediately after harvesting and following culturing in the presence of LPS. Fresh peritoneal exudate cells from long-lived rats exhibited higher IL-10 mRNA/IL-1β ratio when compared to those cells from old rats. Moreover, peritoneal exudate cells from long-lived rats in response to LPS produced more IL-10 and

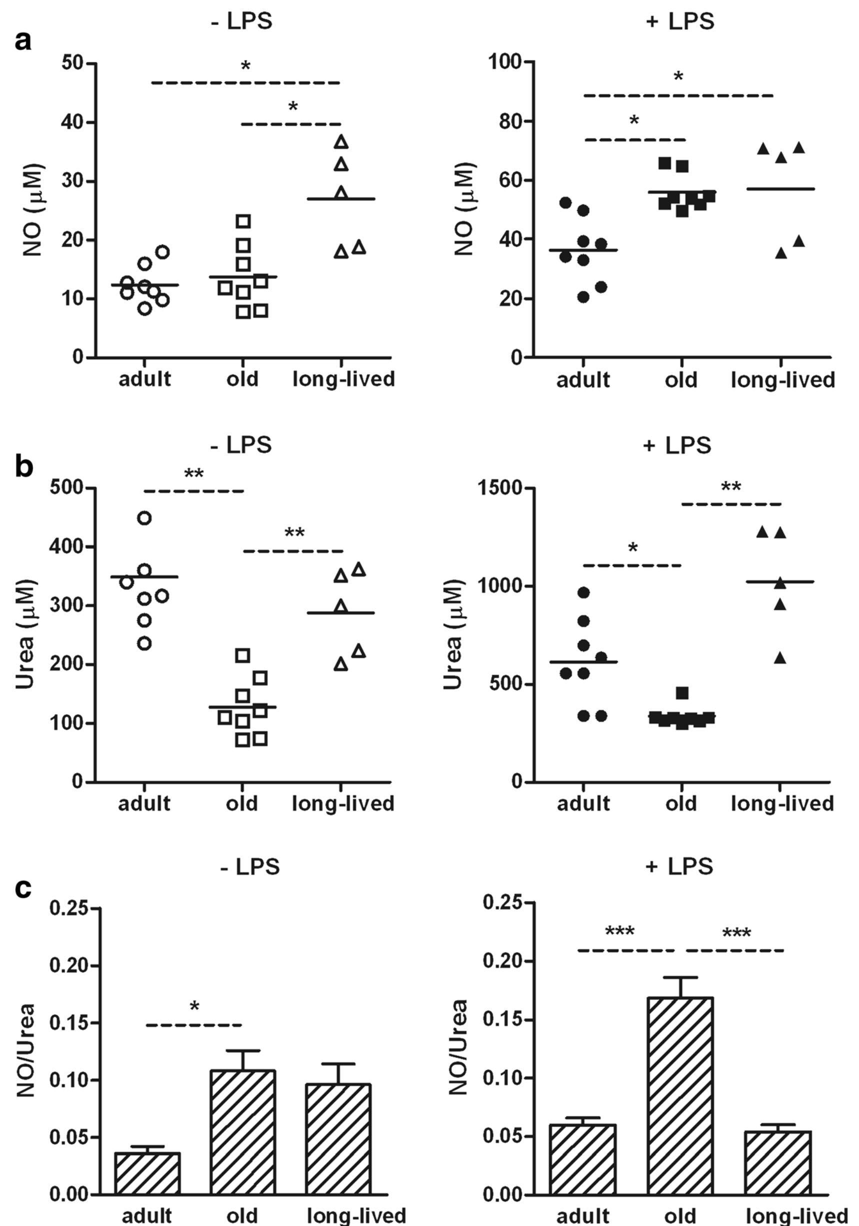



**Fig. 7** Aging alters iNOS and arginase activity in macrophages. Peritoneal macrophages of adult, old, and long-lived rats were cultured in the absence (−LPS) or in the presence of LPS (+LPS). **a** NO production (μM) measured by the Griess reaction and **b** arginase measured by a colorimetric assay; enzyme activity is the output of urea (μM) secreted from lysed macrophages. Results are presented as individual values. *Horizontal full line* indicates mean value for each age group. **c** Relationship between iNOS and arginase activity in macrophages showed as NO/Urea index (mean+SEM). Statistically significant differences: *$p<0.05$, **$p<0.01$, and ***$p<0.001$

less common proinflammatory cytokines, and exhibited lower NO/urea ratio than those from old rats.

Compared to adult rats, fresh peritoneal cells from long-lived rats were characterized by distinctively (sixfold) higher IL-10 expression, but similar IL-1β mRNA expression. Although high production of anti-inflammatory cytokines, particularly IL-10, is thought to be a distinctive characteristic of centenarians that are considered the best example of successful aging and longevity (Carrieri et al. 2004; Lio et al. 2002), it seems that not only an increase in IL-10 mRNA expression, but high IL-10/IL-1β mRNA expression ratio in fresh peritoneal cells is a distinctive characteristic of rat longevity. In addition, peritoneal cells from long-lived rats expressed smaller amount of TNF-α and IL-6 messengers than the corresponding cells from both adult and old rats, as previously shown in rat spleen and splenic dendritic cells (Pachowka et al. 2011; Stojić-Vukanić et al. 2013). Given that endogenous proinflammatory cytokines such as TNF-α, IL-1α, and IL-1β decrease

the expression of CD163 on human monocytes (Sulahian et al. 2000), whereas the expression of CD163 is strongly upregulated by anti-inflammatory mediators, in particular by IL-10 in these cells (Sulahian et al. 2000; Williams et al. 2002), the age-related changes in the expression of this marker on rat peritoneal exudate cells are fully consistent with alterations in their cytokine mRNA expression.

In accordance with the previous study, indicating that proinflammatory and anti-inflammatory cytokines are both produced and utilized by macrophages in processes known as autocrine regulatory pathways (Shnyra et al. 1998), peritoneal macrophages from old and long-lived rats exhibited substantial differences in secretory response to LPS stimulation. Upon LPS stimulation, the production of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) was intensified in macrophages from old rats in respect to adult ones. High serum TNF-α level is considered to be a strong predictor of mortality in 80-year-old people (Bruunsgaard et al. 2003b) and centenarians (Bruunsgaard et al. 2003a). Furthermore, high plasma levels of IL-6 correlate with greater disability, morbidity, and mortality in the elderly (Cesari et al. 2004; Ferrucci et al. 1999), whereas high levels of IL-1β are associated with poor muscle strength and physical performance (Cesari et al. 2004), congestive heart failure, and angina pectoris in older people (Di Iorio et al. 2003). Augmented production of a proinflammatory cytokine itself is not sufficient to trigger age-related life-threatening diseases and to reduce survival, but in conjunction with a genetic predisposition (such as ApoE 4 polymorphism) adds to the onset of specific age-related diseases (Franceschi and Bonafe 2003; Franceschi et al. 2000). The increase in proinflammatory cytokine release from LPS-activated macrophages from old rats in respect to those from adult rats correlated with an increase in the average surface density of both components (i.e., TLR4 and CD14 co-receptor) of LPS receptor complex. In humans, diminished LPS responsiveness due to single-nucleotide polymorphism (Asp299Gly, A+896G) has been associated with increased risk for gram-negative infections and septic shock (Agnese et al. 2002; Lorenz et al. 2002), and a lower risk for diabetes (Kiechl et al. 2002) and atherogenesis (Kiechl et al. 2002; Kolek et al. 2004). Considering the previous findings in humans, the opposing effect of aging on macrophage LPS complex expression in mice (Renshaw et al. 2002; Vega et al. 2004) and rats could be related to differences in their genetic makeup.

In LPS-stimulated macrophages from long-lived rats, production of all the examined proinflammatory cytokines, except of IL-1β, was decreased compared to the corresponding cells from old rats. The latter finding is consistent with data indicating that in humans, quite paradoxically, some proinflammatory characteristics have also been documented in healthy centenarians (Baggio et al. 1998). It is noteworthy that the macrophage TNF-α, and IL-6 production in long-lived rats declined to the levels lower than in the corresponding cells from adult rats. Given that PGE2 production is shown to positively correlate with IL-6 production (Bouffi et al. 2012; Hinson et al. 1996), the decrease in PGE2 level in LPS-stimulated macrophage cultures from long-lived rats further support the latter finding. In LPS-activated macrophages from both old and long-lived rats, IL-10 production was increased compared to that in the corresponding cells from adult rats, but to a strikingly greater extent in those from long-lived ones. Taking into account (i) the lack of correlation between the levels of IL-6 and PGE2 in macrophage cultures from adult and old rats and (ii) data showing inhibitory effect of endogenous IL-10 on PGE2 synthesis by LPS-stimulated monocytes (Niiro et al. 1994), it seems likely that reduced PGE2 amount in macrophage cultures from long-lived rats is a consequence of exceptional increase in IL-10 level in these cell cultures. Previous findings are consistent with data indicating that IL-10 downregulates TNF-α, IL-6, and IL-1β secretion (Fiorentino et al. 1991). In addition, they suggest that LPS-stimulated macrophages from long-lived rats, unlike those from old rats, retained capacity to limit the production of proinflammatory cytokines. Unaltered and elevated NO/urea ratio in these cells from long-lived and old rats, respectively, further corroborated the previous assumption. Several parameters, specifically the level of TNF-α and IL-6 and NO/urea ratio in LPS-stimulated macrophage cultures, indicated similarities between long-lived and adult rats. It is noteworthy that similarities in many other macrophage parameters, e.g., chemotaxis, phagocytosis, NFkB activation, and activity of catalase (an antioxidant enzyme), between long-lived and adult mice have been reported (Arranz et al. 2010a, b). Since NFκB has a crucial role in the expression of genes encoding TNF-α, IL-6, and NO, it may be assumed that in long-lived rats, as in mice (Arranz et al. 2010a), the preserved control of NFκB activation stands behind relatively restrained secretion of the inflammatory mediators.

In conclusion, the present study clearly showed substantial differences in the secretory profile of macrophages from long-lived and old rats. More specifically, as opposed to macrophages from old rats, those cells from long-lived rats exhibited improved capacity to produce anti-inflammatory cytokine IL-10 and restrained that of common proinflammatory cytokines. This phenomenon could be related to their longevity, as it has been shown that deregulated production of proinflammatory/anti-inflammatory mediators is one of the main deleterious effects of aging leading to various life-threatening pathologies in advanced age (Mahbub et al. 2012; reviewed in Salvioli et al. 2006). Furthermore, the study suggests that the ratio of proinflammatory/anti-inflammatory mediator(s) is a better indicator of rat longevity than any of inflammatory mediators solely, and point to putative indicators (IL-10/IL1β messenger ratio in fresh peritoneal macrophages and NO/urea ratio in LPS-stimulated peritoneal macrophage cultures) of rat successful aging and longevity. In as much as data obtained in animal model could be extrapolated to humans, the relevance of this finding for human longevity is worth further testing.

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