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Original scientific paper**STRAIN-SPECIFIC DIFFERENCES IN AGE-RELATED CHANGES IN RAT  
SUSCEPTIBILITY TO EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS  
AND DENDRITIC CELL CYTOKINE GENE EXPRESSION**Biljana BUFAN<sup>1</sup>, Jasmina DJIKIĆ<sup>2</sup>, Mirjana NACKA-ALEKSIĆ<sup>2</sup>, Zorica STOJIĆ-VUKANIĆ<sup>1</sup>,  
Mirjana DIMITRIJEVIĆ<sup>3</sup>, Gordana LEPOSAVIĆ<sup>2</sup><sup>1</sup> Department of Microbiology and Immunology, Faculty of Pharmacy, University of Belgrade,  
Belgrade, Serbia<sup>2</sup> Department of Physiology, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia<sup>3</sup> Immunology Research Center "Branislav Janković", Institute of Virology, Vaccines and Sera  
"Torlak", Belgrade, Serbia

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Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis, a prototype of Th1/Th17-mediated organ-specific autoimmune disease. In the rat, susceptibility to development of these diseases is shown to be strain- and age-dependent. In adult rats of distinct strains, it correlates with splenic dendritic cell (DC) subset composition, which also exhibit age-related changes. The aim of this study was to examine influence of aging on: i) Albino Oxford (relatively resistant to EAE) and Dark Agouti (susceptible to EAE) rat development of EAE and ii) their splenic conventional (OX62+) DC population in respect to its subset composition and expression of mRNAs for proinflammatory and immunosuppressive cytokines. We used 3-month-old (young) and 26-month-old (aged) rats of AO and DA strain. The rats were immunized for EAE with rat spinal cord homogenate in complete Freund's adjuvant and clinical course of the disease was followed. Fresh OX62+DCs were examined for the expression of CD4 (using flow cytometry) and genes encoding cytokines influencing DC activation/maturation (TNF- $\alpha$  and IL-6) using RT-PCR. Additionally, *in vitro* lipopolysaccharide (LPS) activated/matured DCs were examined for the expression of genes encoding cytokines controlling Th1/Th17 cell polarization using RT-PCR. With aging,

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**Corresponding author:** Gordana Leposavić, Department of Physiology, Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe, 11221 Belgrade, Serbia, Tel. +381 11 3951 209, E-Mail: Gordana.Leposavic@pharmacy.bg.ac.rs

AO rats became more susceptible, whereas DA rats largely lose their susceptibility to the induction of EAE. In AO rats aging shifted CD4<sup>+</sup>:CD4<sup>-</sup> DC ratio towards CD4<sup>-</sup> cells, producing large amount of proinflammatory cytokines, whereas in DA rats CD4<sup>+</sup>:CD4<sup>-</sup> DC ratio remained stable with aging. In fresh DCs from rats of both the strains the expression of TNF- $\alpha$  mRNA increased with aging, whereas that of IL-6 mRNA decreased and increased in DCs from AO and DA rats, respectively. Following *in vitro* LPS stimulation OX62<sup>+</sup> DCs from aged AO rats up-regulated the expression of mRNA for IL-23p19 (specific subunit of IL-23; crucial for sustained IL-17 production) and IL-1 $\beta$  (positive IL-17 regulator), whereas down-regulated the expression of IL-10 (negative IL-17 regulator) when compared with young strain-matched rats. In DA rats aging increased IL-23p19 mRNA expression in LPS-stimulated DCs, whereas exerted the opposing effects on the expression of mRNAs for IL-10 and IL-1 $\beta$  compared to AO rats. Irrespective of the rat strain, aging did not influence mRNA expression for IL-12p35 (driving Th1 polarization) in DCs. Overall, results suggest role of changes in the expression of genes encoding proinflammatory and immunosuppressive cytokines in development of age-related alterations in rat susceptibility to EAE induction.

*Key words:* Aging, Cytokine gene expression, Dendritic cells, Experimental autoimmune encephalomyelitis, Rat strain differences

#### INTRODUCTION

Dendritic cells (DCs) have been recognized as highly potent antigen presenting cells (APCs). These cells have important role within immune system, being not only initiators, but also regulators of immune response (HART, 1997; BANCHEREAU and STEINMAN, 1998). In unperturbed tissues, there are primarily immature DCs, which are specialized for up taking environmental antigens by different routes and processing them (CELLA *et al.*, 1997; BANCHEREAU *et al.*, 2000). It is supposed that they are involved in maintenance of tolerance (CELLA *et al.*, 1997; BANCHEREAU *et al.*, 2000). Upon encountering antigen in the presence of inflammation and danger signals (dying cells, microorganisms, proinflammatory cytokines), DCs undergo complex maturation/activation process (BANCHEREAU and STEINMAN, 1998; REISE SOUSA, 2006). This enables them to prime naive T cells, and initiate primary immune response (BANCHEREAU *et al.*, 2000). In addition, they produce specific constellation of cytokines, which is decisive for T cells polarization into different subsets of Th (Th1, Th2, Th17) effector cells (KAPSENBERG, 2003; PULENDRAN, 2005; FEILI-HARIRIR *et al.*, 2005; REISE SOUSA, 2006). Considering origin, morphology, phenotype, maturational and functional capacity, DCs represent extremely heterogeneous population (NAIK, 2008; SATHE and SHORTMAN, 2008). In rat secondary lymphoid organs, at least three different subsets of DCs: CD4<sup>+</sup> and CD4<sup>-</sup> conventional DCs (cDCs) expressing CD103 ( $\alpha_{OX62}$  integrin subunit; recognised by MRC OX62 monoclonal antibody) and plasmacytoid DCs (pDCs) can be distinguished (VOISINE *et al.*, 2002; HUBERT *et al.*, 2004). It has been demonstrated that CD4<sup>-</sup> DCs (OX62<sup>+</sup>CD11b<sup>+</sup>CD4<sup>-</sup>) have myeloid morphology, produce large amounts of the proinflammatory cytokines and induce a Th1 response in co-culture with allogeneic CD4<sup>+</sup> T cells (VOISINE *et al.*, 2002). On the other hand, the CD4<sup>+</sup> DCs (OX62<sup>+</sup>CD11b<sup>+</sup>CD4<sup>+</sup>) have more heterogeneous morphology than CD4<sup>-</sup> DCs,

induce CD4+ and CD8+ cell proliferation, produce low levels of proinflammatory cytokines and are capable of induction of both Th1 and Th2 responses (VOISINE *et al.*, 2002).

It is noteworthy that adult inbred rodent strains differs greatly according to susceptibility to the induction of experimental autoimmune encephalomyelitis (EAE), ranging from almost complete resistant to extremely high susceptible (MILJKOVIC and MOSTARICA STOJKOVIC, 2006). Furthermore, as sensitivity to autoimmune diseases, including EAE development, is shown to be at least partly determined by DCs functional capacity (WALDNER *et al.*, 2004; REIS E SOUSSA, 2006), a predisposition of experimental animals for development of EAE could be associated with strain-dependent specificities of these cells (YANG *and* HOU, 2006). Moreover, in the rat, the proportion of distinct splenic DC subsets is strain-dependent (FOURNIE *et al.*, 2001; HUBERT *et al.*, 2006), and highly predictive for strain-dependent susceptibility to organ specific autoimmune diseases (HUBERT *et al.*, 2006).

Aging is associated with progressive immune system changes, which involve all types of immune cells (NIKOLICH-ZUGICH, 2005; GRUVER *et al.*, 2007; SHAW *et al.*, 2010; SOLANA *et al.*, 2012). The data on age-associated DC alterations, and, in particular, those affecting Th-polarizing cytokine gene expression, origin mainly from studies in mice and humans, and are largely inconsistent (AGRAWAL *et al.*, 2008; SHAW *et al.*, 2010; AGRAWAL *et al.*, 2012; GUPTA, 2014). At clinical level, age-related immune changes manifest as: i) increased susceptibility to infections and reduced response to vaccination, ii) altered incidence of autoimmune diseases (VOLLMER and WAXMAN, 1991; CASTLE *et al.*, 2000; LESLIE and DELLI CASTELLI, 2004) and iii) increased incidence of cancer (CASTLE *et al.*, 2000). Although incidence of many diseases increases with aging, it is not the case with the incidence of multiple sclerosis, a prototype of Th1/Th17-mediated organ-specific autoimmune disease (FLETCHER *et al.*, 2010), and diabetes mellitus type I (LESLIE and DELLI CASTELLI, 2004). A prominent feature of multiple sclerosis is its high (50%) incidence of onset between the ages of 20 and 40 years. Onset in persons younger than 10 and older than 50 accounts for less than 5% of cases (VOLLMER and WAXMAN, 1991; FAROOQI *et al.*, 2010). This is suggestive of age-related restriction of multiple sclerosis clinical expression (VOLLMER and WAXMAN, 1991; FAROOQI *et al.*, 2010). Emerging clues suggest that DC dysregulation might be involved in the development of various autoimmune disorders (LUDEWIG *et al.*, 2001; MEHLING and BEISSERT, 2003; HARDIN, 2005). It has been reported that age-related genetic factors not only influence the risk for development of autoimmune diseases, but also their clinical presentation and the rate of progression (LESLIE and DELLI CASTELLI, 2004). Therefore it may be hypothesized that age-related changes in DC gene expression contribute to alterations in susceptibility to autoimmune disease induction in aged rodent (KALLEN and NILSSON, 1989; LUDOWYK *et al.*, 1993; DITAMO *et al.*, 2005; TARJANYJ *et al.*, 2009).

The influence of aging on rodent susceptibility to development of EAE, the most commonly used experimental model of multiple sclerosis, is shown to be strain-dependent (KALLEN *and* NILSSON, 1989; LUDOWYK *et al.*, 1993; DITAMO *et al.*, 2005; TARJANYJ *et al.*, 2009).

Having in mind all aforementioned, we designed the present study to investigate influence of aging on: i) EAE development in two rat strains characterized by differential susceptibility to the induction of this disease in adult ages and ii) splenic DCs in respect of the subset composition and proinflammatory and immunosuppressive cytokine gene expression. For this purpose we chose 3-month-old (young) and 26-month-old (aged) Albino Oxford (AO) and

Dark Agouti (DA) inbred rats, which are relatively resistant and susceptible to induction of several Th1/Th17-mediated autoimmune diseases, including EAE, respectively (DIMITRIJEVIĆ *et al.*, 2001; MILJKOVIC and MOSTARICA STOJKOVIC, 2006; MILJKOVIC *et al.*, 2006).

## MATERIALS AND METHODS

### *Animals*

In this study we used 3- and 26-month-old female AO and DA rats, bred in the Immunology Research Centre „Branislav Janković“ animal facility. We performed two sets of experiments. In the first set of experiments we immunized rats for induction of EAE and monitored them for clinical signs of disease. In the second set of experiments, we analyzed phenotypic characteristics and/or cytokine mRNA expression in freshly isolated and cultivated DCs. Rats showing evident signs of illness, tumors or splenomegaly at autopsy were excluded from the study. All animals were handled in accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (revising Directive 86/609/EEC). The experimental protocol was approved by the Institutional Experimental Animal Committee.

### *Reagents and antibodies*

The culture medium consisted of RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 2 mM L-glutamine (Serva, Heidelberg, Germany), 1 mM sodium pyruvate (Serva), 100 units/ml penicillin (ICN, Costa Mesa, CA, USA), 100 µg/ml streptomycin (ICN) and 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA) was used. Collagenase D was obtained from Roche Diagnostics (Meylan, France). The complete Freund's adjuvant (CFA), density gradient medium Optiprep™, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), lipopolysaccharide from *Escherichia coli* (LPS) and sodium azide were purchased from Sigma Aldrich Chemie GmbH. Ethylenediamine-tetraacetic acid (EDTA) and bovine serum albumin (BSA) were obtained from Fluka AG (Chemie) GmbH (Buchs SG, Switzerland). Suspension of *Bordetella pertussis* cells was obtained from Institute of Virology, Vaccines and Sera “Torlak” (Belgrade, Serbia).

In addition, we used the following mouse anti-rat monoclonal antibodies (mAbs): fluorescein-isothiocyanate (FITC)-conjugated anti-CD4 (clone OX-38) and FITC/biotin-conjugated anti-CD11b (clone WT.5) were purchased from BD Biosciences Pharmingen (San Diego, CA, USA) and phycoerythrin (PE)-conjugated anti- $\alpha_{OX-62}$  integrin subunit (anti-OX62; clone OX-62), purchased from AbD Serotec (Oxford, UK). Streptavidin-PerCP from BD Biosciences Pharmingen was used as secondary step reagent. The appropriate isotype controls were also purchased from BD Biosciences Pharmingen.

### *Induction and clinical assessment of EAE*

EAE was induced by an intracutaneous injection (into the left hind foot) of 0.1 ml of an emulsion containing rat spinal cord homogenate (20 mg/rat) in CFA (0.3 mg *Mycobacterium tuberculosis*/rat). In addition, rats received an injection of  $9 \times 10^9$  *Bordetella pertussis* cells subcutaneously.

The monitoring of rats for clinical signs of the disease was conducted daily. Clinical signs were graded as follows: 0- no clinical signs, 0.5 - partial loss of tail tonicity, 1- paralysis of

tail, 2- paraparesis of hind limbs, 3- paraplegia and 4- tetraplegia or moribund state. The clinical signs are presented as a maximum clinical score reflecting the overall severity of disease.

#### *Isolation and preparation of DCs*

Spleens, dissected from rats euthanized by exposure to increasing doses of CO<sub>2</sub>, were minced and digested in 2 mg/ml collagenase D in RPMI/2% FCS for 30 min at 37°C. After washing in PBS/2% FCS, cells were resuspended in RPMI/0.04% EDTA and mixed with Optiprep™ (3:1 v/v) to give a 15% solution ( $\rho=1.085$  g/ml). This cell suspension (4 ml) was overlaid with 5 ml of 11.5% Optiprep™ ( $\rho=1.068$  g/ml) and 2 ml RPMI/0.02% EDTA and centrifuged at 600 g for 15 min at 4°C without braking. The low density cell fraction (LDF) at the interface of the top two layers was collected and washed. These cells were used for phenotypic analysis or were subjected to immunomagnetic separation in order to isolate OX62+ cells.

Next, OX62+ cells were separated from splenic LDF using immunomagnetic cell separation technique and anti-rat OX62 microbeads (Miltenyi Biotec, Teterow, Germany). The separation procedure was conducted according to manufacturer's instructions (Miltenyi Biotec). Briefly, after incubation of LDF with anti-rat OX62 microbeads (4°C, 15 min), positive selection was performed on magnetic cell separation column by using QuadroMACS magnet (Miltenyi Biotec). The purity of DCs obtained in this manner was up to 85% (as shown by staining with FITC--conjugated anti-CD11b).

Next, magnetically sorted OX62+ cells were analysed for cytokines expression or cultured in 24-well plates ( $1 \times 10^6$  cells/ml per well) in the RPMI/10% FCS with LPS (1  $\mu$ g/ml) or without LPS. After 16-18 h cells were collected and used for analyses of cytokines expression.

#### *Flow cytometry analysis (FCA) of surface markers*

For phenotypic analysis, LDF cells ( $1 \times 10^5$  cells per sample) were incubated with fluorochrome-labeled mAbs (direct labeling) and/or with unlabeled Abs (indirect labeling) for 30 min. Cells subjected to indirect labeling were incubated with second step reagent for further 30 min. All incubations were performed in the dark on ice. After incubations, cells were washed three times with PBS/2% FCS/0.1% sodium azide. Non-specific 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 anterior and right-angle scatter. The percentage of positive cells was determined by using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and post-acquisition data analysis was performed with FlowJo software, v.7.8. (Three Star, Inc., Ashland, OR, USA).

#### *Real time PCR analysis of cytokine mRNA expression*

Total RNA was isolated from  $1 \times 10^5$  fresh and cultured OX62+ DCs by using the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA) and Total RNA Chemistry (Applied Biosystems). Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and 5  $\mu$ l of cDNA was used for real-time PCR. Triplicate 25  $\mu$ l reactions were ran under Applied Biosystems 7500 universal cycling conditions. Gene Expression Master Mix and commercial TaqMan Gene Expression Assays for rat TNF- $\alpha$  (Rn99999017\_m1), IL-10 (Rn00563409\_m1), IL-12a (Rn00584538\_m1), IL-23a (Rn00590334\_g1), IL-6 (Rn99999011\_m1), IL-1 $\beta$  (Rn99999009\_m1) and hypoxanthine phosphoribosyltransferase 1 (HPRT 1) (Rn01527840\_m1) were obtained from Applied

Biosystems. All procedures were performed according to manufacturer's instructions. Input cDNA was normalized to housekeeping gene (HPRT 1) as it displayed an optimal stability among various samples tested. Quantitative differences in gene expression levels were assessed using Applied Biosystems SDS software (v 1.4.0.) and  $2^{-\Delta\Delta Ct}$  method.

#### Statistical analysis

To assess significances between groups, we used: Student's unpaired t-test for testing the influence of aging on fresh OX62+ DCs subset distribution and Mann-Whitney U test for testing the influence of aging on OX62+ DCs cytokine mRNA expression. All analyses were performed using SPSS version 18.0 software. Differences were considered to be significant when  $p < 0.05$ .

## RESULTS

### *Aging alters resistance/susceptibility to EAE induction in AO and DA rats*

In the first set of experiments, we examined the influence of aging on EAE development. Our results showed that, with aging, relatively resistant AO rats became more susceptible to EAE induction (Table 1). Namely, differently from young AO rats, which did not express observable symptoms of the disease, the incidence of EAE raised to 60% in aged rats of this strain (Table 1). The diseased rats exhibited mild clinical signs judging by maximum clinical score (Table 1). Contrary to AO rats, DA rats, which were highly susceptible to the induction of EAE in young age, became more resistant to the disease induction with aging (Table 1). Specifically, there was no significant difference in the disease onset, but aged DA exhibited a lower incidence of the disease and lower maximum clinical score, when compared with strain-matched young animals (Table 1).

*Table 1. The effect of aging on experimental autoimmune encephalomyelitis (EAE) in Albino Oxford (AO) and Dark Agouti (DA) rats*

		Incidence of EAE	Day of onset (mean±SEM)	Maximal clinical score
AO	Young	0/14 (0%)	-	0
	Aged	6/10 (60%)	10.3±1.36	2
DA	Young	9/9 (100%)	11.67±0.41	4
	Aged	3/11 (27.3%)	11.33±0.41	2

### *Influence of strain and aging on splenic OX62+CD11b+ DC subset composition and expression of mRNAs for cytokines influencing Th1/Th17 polarization*

Next, we examined the influence of aging on the relative proportion of CD4+ and CD4- cells within OX62+ splenic DC population from AO and DA rats, as well as on their expression of cytokine mRNAs.

### *Aging exhibits strain-specific influence on rat splenic OX62+CD11b+ DC subset composition*

In fresh OX62+CD11b+ DC population from young AO rats, CD4+ cells represented the predominant cell type (Figure 1). However, comparing with OX62+CD11b+ DCs from young AO rats, in this cell population from aged rats of the same strain, the relative proportion of

CD4+ cells was reduced ( $p < 0.001$ ), whereas that of CD4- cells was increased ( $p < 0.001$ ), so that the latter cells become predominant (Figure 1).

On the other hand, in DA rats, CD4- subset was the predominant subset in fresh splenic OX62+CD11b+ DC population of young animals, and aging did not significantly influence the relative representation of these cells within OX62+CD11b+ DCs (Figure 1).

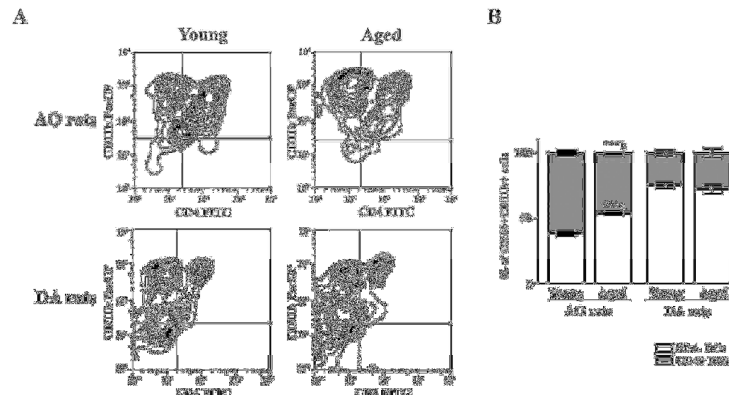


Figure 1. Strain-specific influence of aging on rat splenic OX62+ DC subsets composition

The figure shows percentages of CD4+ and CD4- cells within OX62+CD11b+ DCs from young and aged rats of Albino Oxford (AO) and Dark Agouti (DA) strains. (A) The flow cytometric contour histograms represent the expression of CD4 and CD11b on gated OX62+ DCs from splenic low density fraction from (left) young and (right) aged rats from (upper) AO and (lower) DA strains. (B) The stacked bar graph indicates the percentage of CD4+ and CD4- OX62+CD11b+ DCs. Results are expressed as mean  $\pm$  SEM ( $n=6$ ); <sup>a</sup> CD4- DCs from aged vs. CD4- DCs from young strain-matched rats; <sup>b</sup> CD4+ DCs from aged vs. CD4+ DCs from young strain-matched rats; \*\*\* $p < 0.001$ . The figure indicates results from a single experiment. Similar data were obtained in three independent experiments.

#### *Aging exhibits strain-specific influence on the expression of mRNAs for TNF- $\alpha$ and IL-6 in fresh OX62+ splenic DCs*

Considering putative importance of the steady-state immature DCs in lymphoid organs for the maintenance of peripheral tolerance (WILSON *et al.*, 2003), we examined the expression of mRNAs for cytokines that might influence their maturational status.

We found that fresh OX62+ DCs from aged AO rats expressed increased amount ( $p < 0.05$ ) of mRNA for TNF- $\alpha$  and decreased ( $p < 0.05$ ) that for IL-6 when compared with strain-matched young rats (Figure 2). On the other hand, in the same cells of aged DA rats, the expression of mRNAs for both TNF- $\alpha$  and IL-6 mRNAs was augmented ( $p < 0.05$  and  $p < 0.01$ ,

respectively) relative to that of corresponding mRNA in OX62+ DCs from strain-matched young animals (Figure 2).

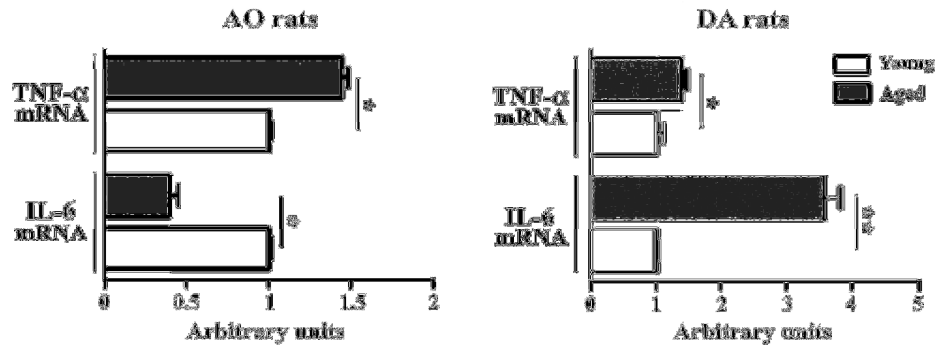


Figure 2. Strain-specific influence of aging on the expression of mRNAs for TNF- $\alpha$  and IL-6 in fresh OX62+ splenic DCs

The bar graphs represent fold change in mRNA expression for TNF- $\alpha$  and IL-6 in fresh splenic OX62+ DCs from (left) AO and (right) DA aged rats relative to those from strain-matched young rats. HPRT was selected as the housekeeping gene to normalize for input cDNA variation as it displayed an optimal stability in our experimental system. The data are presented as mean  $\pm$  SEM (n=6); \*p<0.05; \*\*p<0.01. The figure indicates results from a single experiment. Similar data were obtained in three independent experiments.

*Strain-specific influence of aging on the expression of mRNA for immunostimulatory and immunosuppressive cytokines in LPS-stimulated DCs*

Since mature/activated DCs are shown not only to initiate immune response, but also to influence Th polarization (KAPSENBERG, 2003), we investigated the influence of aging on OX62+ DCs expression of mRNAs for cytokines driving/influencing Th17 and/or Th1 polarization.

Our results showed that in the presence of LPS, a Toll like receptor (TLR4) agonist that acts as maturation factor, OX62+ DCs from aged AO rats expressed greater (p<0.001) amount of mRNA for IL-23p19, specific subunit of the heterodimeric IL-23 cytokine, than the corresponding cells from young strain-matched rats (Figure 3). However, these cells exhibited lower (p<0.001) expression of mRNA for immunosuppressive IL-10 cytokine, which was accompanied with a greater (p<0.001) expression of IL-1 $\beta$  mRNA compared with young strain-matched rats (Figure 3). Unlike LPS-stimulated OX62+ DCs from aged AO rats, the corresponding cells from DA rats exhibited increased expression of mRNAs for IL-23p19 (p<0.001) and IL-10 (p<0.001), whereas the expression of IL-1 $\beta$  mRNA was diminished (p<0.05) when compared with young strain-matched rats (Figure 3). Finally, irrespective of rat



strain there was no significant difference in the expression of mRNA for IL-12p35, a subunit of heterodimeric Th1 polarizing IL-12 (MACATONIA *et al.*, 1995; BECKER *et al.*, 2005), between LPS-stimulated OX62+ DCs from aged and young rats (data not shown).

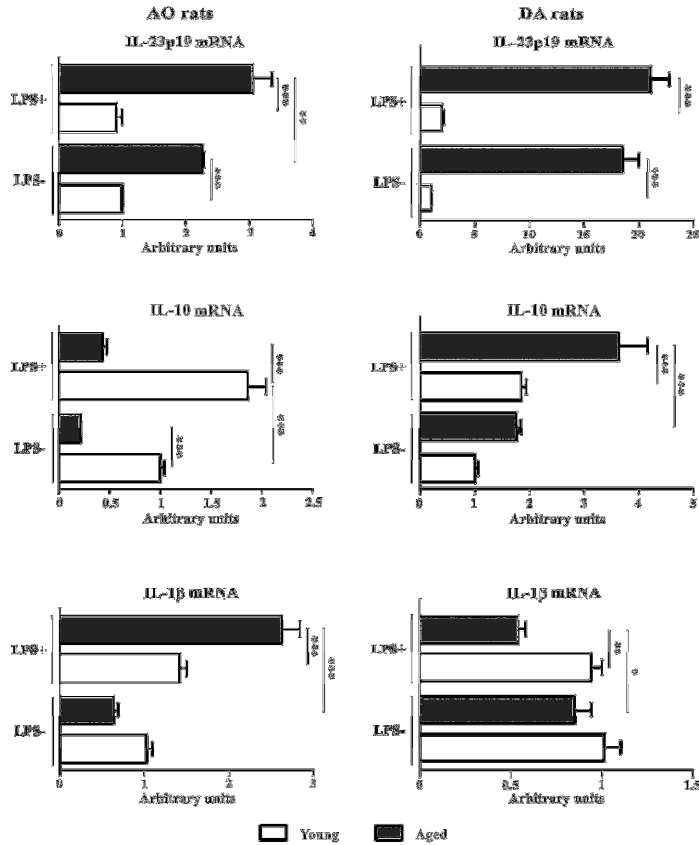


Figure 3. Strain-specific influence of aging on mRNA expression for IL-23p19, IL-10 and IL-1 $\beta$  in LPS-stimulated OX62+ DCs

The bar graphs represent fold change in mRNA expression for IL-23p19, IL-10 and IL-1 $\beta$  in control (LPS-) OX62+ DCs from aged rats and LPS-stimulated (LPS+) OX62+ DCs from young and aged rats from (left) AO and (right) DA rats relative to those from young strain-matched control rats. HPRT was selected as the housekeeping gene to normalize for input cDNA variation as it displayed an optimal stability in our experimental system. The data are presented as mean  $\pm$  SEM (n=6); \*p<0.05; \*\*p<0.01; \*\*\*p<0.001. The figure indicates results from a single experiment. Similar data were obtained in three independent experiments.

It should be mentioned, that quite expectably, as rat DCs put in culture medium alone exhibit significant degree of “spontaneous activation/maturation” (VREMEC *et al.*, 2011), the expression of cytokine mRNA was not always greater in DCs from LPS-stimulated cultures when compared to that in control (LPS- cultures) (Figure 3).

#### DISCUSSION

As expected from the previous studies (MOSTARICA-STOJKOVIC *et al.*, 1982; STOSIC-GRUJICIC *et al.*, 2004), differently from young AO rats, all young DA rats developed clinical signs of EAE. On the other hand, for the first time to our best knowledge, we showed that susceptibility of both AO and DA rats to induction of EAE changes with aging, so it increases in AO rats and decreases in DA rats. This is consistent with earlier reports indicating that in rodents, aging influences susceptibility to EAE development in a strain-specific manner (KALLEN and NILSSON, 1989; LUDOWYK *et al.*, 1993; DITAMO *et al.*, 2005; TARJANYI *et al.*, 2009). This phenomenon can be related to the data demonstrating that age-related genetic factors influence not only the risk for development of autoimmune diseases, but also their clinical presentation and the rate of progression (LESLIE and DELLI CASTELLI, 2004). It is also consistent with findings indicating that genetic component has a strong impact on aging process (LARBI *et al.*, 2008). However, considering that all types of immune cells suffer age-related changes (GRUVER *et al.*, 2007; SHAW *et al.*, 2010), our findings inevitably imposed question which type/s is/are responsible for the age-related changes in the development of EAE. Given that DCs are considered to be key players in immunity and tolerance (BANCHEREAU and STEINMAN, 1998; STEINMAN *et al.*, 2003), we focused on these cells.

Confirming the previous studies indicating that DC subset composition correlates with the rat susceptibility to autoimmune disease induction and Th polarization (FOURNIE *et al.*, 2001; HUBERT *et al.*, 2006), we found that, opposing to OX62+CD11b+ DC population from young AO rats, this population from DA rats exhibited the predominance of CD4- cells. These cells produce large amount of proinflammatory cytokines (VOISINE *et al.*, 2002; HUBERT *et al.*, 2006), and the increase in their proportion is associated with a greater susceptibility to Th1/Th17-mediated diseases (FOURNIE *et al.*, 2001; HUBERT *et al.*, 2006). Considering these findings, the age-related shift in CD4+:CD4- subset ratio toward CD4- subset in AO rats could be linked with a greater susceptibility of aged rats of this strain to EAE. On the other hand, despite the age-related changes in EAE development in DA rats, we failed to observe any changes in OX62+CD11b+ DC subset composition. This could suggest that, differently from AO rats, in DA rats rather age-related changes at cellular level than those at population level influence susceptibility to EAE induction. The former might reflect age-related both intrinsic (AGRAWAL *et al.*, 2007; PANDA *et al.*, 2010) and extrinsic DC changes (CHELVARAJAN *et al.*, 2005; MÜNZ *et al.*, 2005; LIAO *et al.*, 2007) affecting their capacity to produce polarizing cytokines. Alternatively, it may be also assumed that alterations in the abundance of pDCs, which is also shown to be strain dependent (HUBERT *et al.*, 2006), contribute to the age-related changes in susceptibility of DA rats to EAE induction.

Next, we analyzed the expression of mRNAs for TNF- $\alpha$  and IL-6 in fresh (presumably steady-state) OX62+ DCs, which are: i) supposed to be phenotypically and functionally immature, and ii) suggested to be involved in maintaining of tolerance (WILSON *et al.*, 2003). In agreement with the age-related increase in the basal production of proinflammatory TNF- $\alpha$  (FRANCESCHI *et al.*, 2000; GOMEZ *et al.*, 2006; VASTO *et al.*, 2007), which exerts a pro-activating

influence on DCs, acting in an autocrine manner (MORRISON *et al.*, 2003; SLOBODIN *et al.*, 2009), we found the enhanced expression of TNF- $\alpha$  in OX62+ DCs from rats from both strains comparing with the corresponding cells from young strain-matched rats. Differently from TNF- $\alpha$ , aging had differential impact on the expression of mRNA for IL-6, which is shown to stimulate DC maturation (JONULEIT *et al.*, 1997), in fresh OX62+ DCs from genetically distinct organisms. Namely, comparing with fresh OX62+ DCs from strain-matched young rats, the expression of IL-6 mRNA in the corresponding cells from aged DA was enhanced, whereas in those from AO rats was diminished. Therefore, it may be assumed that aging differentially affects maturational status of DCs from AO and DA rats. This is particularly relevant in the light of data indicating that the ability of the DC to induce tolerance is highly dependent on the stage of their maturation (REIS E SOUSSA, 2006). Furthermore, to explore capacity of activated/mature OX62+ DCs to produce cytokines driving/influencing Th1 and Th17 polarization, we examined the expression of mRNAs for relevant cytokines in these cells following LPS stimulation *in vitro*. We found that in LPS-stimulated OX62+ DCs from aged rats of both the strains the expression of IL-23p19 gene was augmented when compared with the corresponding cells from young strain-matched rats. However, differently from the expression of IL-23p19 mRNA, the expression of mRNA for IL-10, which is shown to be negative regulator of Th17 production (CORSETTI *et al.*, 2013), was differentially affected by aging in LPS-stimulated OX62+ DCs from AO and DA rats. Namely, the expression of this mRNA in LPS-stimulated OX62+ DCs from DA rats increased with aging, whereas in these cells from AO rats it showed a prominent age-related decrease. Furthermore, given that endogenous IL-10 constrains Th17 cell development through down-regulation of IL-1 $\beta$  production by DCs (WILKE *et al.*, 2011), the age-associated changes in IL-1 $\beta$  mRNA expression in LPS-stimulated OX62+ DCs from AO and DA rats were fully consistent with the changes in their expression of IL-10 mRNA. Thus, our findings strongly suggest that aging differentially affects Th-polarizing capacity of the activated DCs from AO and DA rats, and therefore, possibly their susceptibility to EAE.

Finally, although we failed to observe any significant changes in IL-12p35 mRNA expression in either AO or DA rats, the differential effect of aging on Th1 driving capacity of DCs from these strains could not be completely ruled out. Namely, it may be assumed that in aged animals some strain-specific IL-12 independent factors (e.g. increased CD40 surface density, altered expression of other members of the IL-12 family or alterations in IL-12-independent CD27/CD70 axis component) could contribute to alterations in Th1 polarizing capacity of DC. This will be matter of our further studies.

Overall, our study supports notion that alterations in gene expression contribute to age-related risk for autoimmune disease onset, as well as to the severity of their clinical manifestations (LESLIE and DELLI CASTELLI, 2004). In addition, it points that age-related alterations in expression of genes encoding proinflammatory and immunosuppressive cytokines in DCs could contribute to risk for development of autoimmune diseases.

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## SOJNE RAZLIKE U EFEKTIMA STARENJA NA RAZVOJ EKSPERIMENTALNOG AUTOIMUNSKOG ENCEFALOMIJETISA I EKSPRESIJU GENA ZA CITOKINE U DENDRITSKIM ČELIJAMA

Biljana BUFAN<sup>1</sup>, Jasmina DJIKIĆ<sup>2</sup>, Mirjana NACKA-ALEKSIĆ<sup>2</sup>, Zorica STOJIĆ-VUKANIĆ<sup>1</sup>,  
Mirjana DIMITRIJEVIĆ<sup>3</sup>, Gordana LEPOSAVIĆ<sup>2</sup>

<sup>1</sup> Katedra za mikrobiologiju i imunologiju, Univerzitet u Beogradu- Farmaceutski fakultet, Beograd, Srbija

<sup>2</sup> Katedra za fiziologiju, Univerzitet u Beogradu- Farmaceutski fakultet, Beograd, Srbija

<sup>3</sup> Centar za imunološka istraživanja "Branislav Janković", Institut za virusologiju, vakcine i serume "Torlak", Beograd, Srbija

Immunology Research Center "Branislav Janković", Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia

### Izvod

Eksperimentalni autoimunski encefalomijelitis (EAE) je animalni model multiple skleroze i prototip autoimunske bolesti posredovane Th1/Th17 limfocitima. Pokazana je sojna- i uzrastna- zavisnost razvoja ove bolesti kod pacova kao i fenotipskih i funkcijskih promena dendritskih ćelija (DC) kod miša. Cilj rada je bio da se ispita uticaj starenja na: i) osetljivost AO i DA pacova na razvoj EAE i ii) odnos CD4+:CD4- subpopulacija OX62+ DC u slezini i njihovu ekspresiju iRNK za citokine koji utiču na polarizaciju CD4+ T-limfocita u ćelije Th1 i Th17 fenotipa. Starenjem AO pacovi postaju osetljiviji, dok se kod DA smanjuje podložnost razvoju EAE. U populaciji DC starih AO pacova je nađeno povećanje zastupljenosti CD4- ćelija (sintetišu velike količine proinflamatornih citokina), dok se kod DA pacova odnos CD4-:CD4+ ćelije nije menjao. Povećana ekspresiju iRNK za TNF- $\alpha$  je nađena u svežim DC starih pacova oba soja, dok je ekspresija iRNK za IL-6 u DC bila smanjena kod AO pacova, a povećana kod DA pacova. Nakon stimulacije LPS-om, ekspresija iRNK za IL-23p19 i IL-1 $\beta$  je bila veća u DC starih nego mladih AO životinja, dok je ekspresija iRNK za IL-10 bila manja. Kod DA pacova starenje je povećalo ekspresiju iRNK za IL-23p19, dok je na ekspresiju iRNK za IL-1 $\beta$  i IL-10 imalo suprotan efekat u odnosu na AO pacove. Rezultati pokazuju da starenje, u zavisnosti od soja, različito utiče na razvoj EAE i sugeriše da se ovo može pripisati različitom uticaju na ekspresiju gena za citokine koji utiču na polarizaciju CD4+ limfocita u Th17 ćelije.

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