Cellular Immunology 336 (2019) 48–57

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

Noradrenaline through β-adrenoceptor contributes to sexual dimorphism in primary CD4+ T-cell response in DA rat EAE model?

Ivana Vujnović[a], Ivan Pilipović[a], Nebojša Jasnić[b], Raisa Petrović[a], Veljko Blagojević[a], Nevena Arsenović-Ranin[c], Zorica Stojić-Vukanić[c], Jelena Djordjević[b], Gordana Leposavić[d,*]

[a] Immunology Research Centre "Branislav Janković", Institute of Virology, Vaccines and Sera "Torlak", 458 Vojvode Stepe, 11221 Belgrade, Serbia
[b] Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, 16 Studentski trg, 11000 Belgrade, Serbia
[c] Department of Microbiology and Immunology, Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe, 11221 Belgrade, Serbia
[d] Department of Physiology, Faculty of Pharmacy, University of Belgrade, 450 Vojvode Stepe, 11221 Belgrade, Serbia

ARTICLE INFO

Keywords:
EAE
Noradrenaline
β-Adrenoceptor
Draining lymph nodes
CD4+ cell proliferation
Th17 differentiation

ABSTRACT

Males exhibit stronger sympathetic nervous system (SNS) activity, but weaker primary CD4+ T-cell (auto) immune responses. To test the role of catecholamines, major end-point SNS mediators, in this dimorphism, influence of propranolol (β-adrenoceptor blocker) on mitogen/neuroantigen-stimulated CD4+ T cells from female and male EAE rat draining lymph node (dLN) cell cultures was examined. Male rat dLNs exhibited higher noradrenaline concentration and frequency of β_2-adrenoceptor–expressing CD4+ T lymphocytes and antigen presenting cells. Propranolol, irrespective of exogenous noradrenaline presence, more prominently augmented IL-2 production and proliferation of CD4+ lymphocytes in male than female rat dLN cell cultures. In neuroantigen-stimulated dLN cells of both sexes propranolol increased IL-1β and IL-23/p19 expression and IL-17+ CD4+ cell frequency, but enhanced IL-17 production only in male rat CD4+ lymphocytes, thereby abrogating sexual dimorphism in IL-17 concentration observed in propranolol-free cultures. Thus, β-adrenoceptor–mediated signalling may contribute to sex bias in rat IL-17–producing cell secretory capacity.

1. Introduction

Generally, as many other organ-specific autoimmune diseases, multiple sclerosis (MS) is more prevalent in women than in men [1]. The sex bias in the incidence of MS is linked with greater T- and B-cell–mediated immune responses in women compared with men [1,2]. However, the factors determining the magnitude of (auto)immune responses and mechanisms of their action, have not been fully elucidated, yet [1,2]. Animal studies could provide opportunity to identify them, and thereby to bridge the gap in our knowledge.

Experimental autoimmune encephalomyelitis (EAE) is widely used animal model for studying MS pathogenesis, and for developing and testing drugs [3]. Typically, EAE is a CD4+ T lymphocyte-mediated autoimmune disease [3]. The development of EAE starts in the preclinical phase with activation/proliferation of neuroantigen-specific T lymphocytes in the draining lymph nodes (dLNs) and their migration into the central nervous system (CNS) [3]. In the CNS, cytokine-secreting T lymphocytes further recruit immune cells (monocytes) from the blood into the CNS and activate microglia [3]. This leads to the tissue damage and inflammation, and consequently transition from the preclinical to clinical phase of EAE [3].

Sex-based differences in the incidence and clinical course of EAE have also been recognized [4–7]. In Dark Agouti (DA) rat EAE model, the lower incidence and postponed onset of the clinical phase of the disease were observed in male compared with female rats [5]. This was linked with a less efficient generation of IL-17–producing T helper

Abbreviations: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; CD, cluster of differentiation; dLNs, draining lymph nodes; CNS, central nervous system; DA, Dark Agouti; IL, interleukin; Th, T helper; AR, adrenoceptor; MBP, myelin basic protein; PBS, phosphate buffered saline; d.p.i., day post immunization; Abs, antibodies; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll-protein; HPLC, high performance liquid chromatography; RPMI, Roswell Park Memorial Institute; ConA, concanavalin A; AMPT, alpha-methyl-DL-tyrosine; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; mRNA, messenger ribonucleic acid; DNase, deoxyribonuclease; cDNA, complementary deoxyribonucleic acid; UDG, Uracil-DNA glycosylase; RORγt, retinoic acid-related orphan receptor gamma t; Ct, threshold cycle; NGF, nerve growth factor; MFI, mean fluorescence intensity; 7-AAD, 7-aminoactinomycin D; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; IFN, interferon

* Corresponding author.
E-mail address: gordana.leposavic@pharmacy.bg.ac.rs (G. Leposavić).

https://doi.org/10.1016/j.cellimm.2018.12.009
Received 3 August 2018; Received in revised form 20 December 2018; Accepted 24 December 2018
Available online 26 December 2018

(Th17) cells, the key driver in EAE pathogenesis [5,8], in dLNs in the preclinical phase of EAE in male compared with female rats [5]. However, the factors contributing to this sexual dimorphism have not yet been identified.

It is widely accepted that noradrenaline, predominantly acting through β_2-adrenoceptor (AR), modulates innate and adaptive immune cell proliferation, differentiation, migration/homing and effector functions [9–12]. The major sources of noradrenaline in secondary lymphoid organs are noradrenergic nerve fibers [11,13], and noradrenaline synthesizing, storing and releasing "noradrenergic" immune cells [14,15]. Several lines of evidence suggest sex differences in noradrenaline immunoregulatory action. Firstly, sympathetic nervous system activity is greater in men compared with women [16]. Secondly, in rat secondary lymphoid organs age-dependent sex differences in the density of noradrenergic nervous fibers and noradrenaline content in immune cells have been observed [17,18]. Thirdly, the density of β_2-AR on immune cells and many β_2-AR–mediated catecholamine effects on these cells are sexually dimorphic in healthy rodents and humans [17,19,20].

Furthermore, not less important, alterations in noradrenaline immunomodulatory action are suggested to contribute to MS pathogenesis [21,22]. Namely, MS patients exhibit: i) sympathetic nervous system dysfunction [23], ii) changes in the circulating noradrenaline levels [14] and iii) alterations in peripheral blood lymphocyte noradrenaline content and β-AR expression/responsiveness [14,22]. Additionally, pharmacological manipulations with β-AR–mediated noradrenaline action affect EAE development in rodents [24–26]. Furthermore, the decline in splenic noradrenaline level associated with up-regulation of β-AR expression in splenocytes in the preclinical phase of EAE [27,28] suggests a role for noradrenaline in the modulation of early steps in EAE pathogenesis.

Having in mind all the aforementioned, we hypothesized that sexual diergism in β-AR–mediated noradrenergic immunomodulation could contribute to the previously observed sex differences in the primary CD4+ T-lymphocyte response to immunization for EAE in DA rats (higher in females), and consequently in the incidence and onset of clinical disease [5]. To test this hypothesis, β-AR–mediated effects of noradrenaline on mitogen- and myelin basic protein (MBP)-stimulated proliferation of CD4+ lymphocytes and the frequency of Th17 cells in cultures of dLN cells retrieved in the preclinical phase of EAE from DA rats of both sexes were examined.

2. Material and methods

2.1. Experimental animals and experimental design

Three-month-old male and female DA rats from the Immunology Research Centre "Branislav Janković" breeding colony were used in the study. The animal facilities were endorsed by the Ministry of Agriculture and Environmental Protection of the Republic of Serbia (Veterinary Department). The animals were housed under the standard laboratory conditions. All experimental procedures and animal care were performed in accordance with the directive 2010/63/EU of the European Parliament and the Council on the protection of animals used for scientific purposes, and the governmental regulations (Law on Animal Welfare, "Official Gazette of RS", no. 41/2009). The study protocol was approved by the Animal Care and Use Committee of the Faculty of Pharmacy (Etički komitet Farmaceutskog fakulteta, permit number 6/12). The experiments complied with the ARRIVE guidelines for reporting animal research. Animal health monitoring was performed on a daily basis by animal care staff and a veterinarian.

2.2. Induction and clinical evaluation of EAE

For induction of EAE, the rats were administered with 100 µl emulsion of equal volumes of rat spinal cord homogenate in phosphate-buffered saline (PBS) and complete Freund's adjuvant containing 1 mg/ml of heat-killed and dried *M. tuberculosis* H37Ra (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) followed by an injection of 5×10^8 *B. pertussis* (Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia), as described in detail [5]. All rats were sacrificed on the 7th day post immunization (d.p.i.) through transcardial perfusion in deep anesthesia by an intraperitoneal injection of ketamine/xylazine anesthetizing cocktail (80 mg/kg body weight/8 mg/kg body weight). Two independent experiments (6 rats/sex, total 12 rats/sex) were performed.

2.3. Antibodies and immunoconjugates

For immunolabeling, the following monoclonal antibodies (Abs): fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-conjugated anti-CD4 (clone OX-38), FITC-conjugated anti-CD8 (clone OX-8), biotin-conjugated anti-CD11b (clone WT.5), PE-conjugated anti-IL17A (clone TC11-18H10), as well as FITC-conjugated goat anti-rabbit IgG, peridinin chlorophyll-protein (PerCP)-conjugated streptavidin and isotype controls were obtained from BD Biosciences Pharmingen (Mountain View, CA, USA). Unconjugated rabbit polyclonal anti-β_2-AR Ab (H-73) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 647-conjugated monoclonal anti-TCRαβ (clone R73) Ab was provided by BioLegend (San Diego, CA, USA). Unconjugated rabbit polyclonal anti-tyrosine hydroxylase Ab was obtained from EMD Millipore (Billerica, MA, USA).

2.4. Isolation of mononuclear cells

Freshly isolated popliteal dLNs were weighed and divided into pieces that were used for isolation of mononuclear cells and high performance liquid chromatography (HPLC). To obtain single-cell mononuclear cell suspensions, dLN tissue was passed through a 70 µm nylon cell strainer (BD Biosciences, Erembodegem, Belgium) in ice-cold PBS supplemented with 2% fetal calf serum (Gibco, Grand Island, NY, USA). Thereby obtained dLN cells were counted in 0.2% trypan blue solution using an improved Neubauer hemacytometer.

2.5. Cultivation of dLN cells for analyses of cell proliferation and cytokine production

Mononuclear cells from dLNs were cultured for 72 h in complete RPMI 1640 medium supplemented with 100 µM ascorbic acid in the presence or in the absence of 2.5 µg/ml of Concanavalin A (ConA, Sigma-Aldrich Chemie GmbH) or 20 µg/ml MBP (Sigma-Aldrich Chemie GmbH) in a 5% CO_2 humidified air atmosphere at 37 °C. To ConA- and MBP-stimulated cultures 10^{-6} M of arterenol [(±)-noradrenaline (+)-bitartrate salt, Sigma-Aldrich Chemie GmbH] and/or 10^{-5} M of non-selective β-AR antagonist propranolol [(±)-propranolol hydrochloride, Sigma-Aldrich Chemie GmbH] were added. Cells were preincubated at 37 °C in a 5% CO_2 humidified air atmosphere with: i) arterenol (1 h) or propranolol (15 min) alone and ii) arterenol and propranolol (arterenol was added 15 min after propranolol). This dose of arterenol was chosen considering that noradrenaline concentration in direct vicinity of lymphocytes in secondary lymphoid organs is on the order of 0.3–3 mM [29], and that its supraphysiological (i.e. ≥ 10^{-6} M) concentrations are required for functional changes in immune cells *in vitro* [11]. Additionally, dLN cells were pretreated with 10^{-5} M of alpha-methyl-DL-tyrosine (AMPT, Sigma-Aldrich Chemie GmbH) for 1 h (to inhibit catecholamine synthesis), and then stimulated with ConA in the absence or in the presence of propranolol. After cultivation, the cells were processed for proliferation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses, or restimulated with 200 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Chemie GmbH) and 400 ng/ml ionomycin (Sigma-Aldrich Chemie GmbH) in the complete culture medium. The cells were restimulated in the presence of 3 µg/ml of brefeldin A (eBioscience, San

Diego, CA, USA) in a 5% CO_2 humidified atmosphere for 4 h at 37 °C and examined for IL-17 synthesis using flow cytometry analysis. Cell-free culture supernatants were assayed for IL-17 and IL-2 concentrations by ELISA.

2.6. Noradrenaline measurement

For HPLC analysis, dLN cells (1×10^7) were harvested and dLN tissue (10 mg) was homogenized, in DEPROT solution containing 2% ethylene glycol tetraacetic acid, 0.1 N $HClO_4$ and 0.2% $MgCl_2$, sonicated and centrifuged (30 min, 18.000 rpm, +4 °C). The supernatants were injected using the autosampler of a Dionex UltiMate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) equipped with a Hibar 125–4 LiCrospher100 RP-18 (5 μm) HPLC column (Merck Millipore, Darmstadt, Germany). Instrument control and data acquisition were carried out by the Chromeleon7 Chromatography Data System (Thermo Scientific). The flow rate of mobile phase consisting of 98% ammonium formate buffer (Fisher Scientific, Cambridge, UK, pH 3.6) and 2% methanol (J.T.Baker, Griesheim, Germany) was set at 500 μl/min. The potential for electrochemical measurements and the separation temperature were set at +850 mV and 25 °C, respectively. Standard solutions of noradrenaline (DL-noradrenaline hydrochloride) in 0.5–25 μg/ml concentration range were prepared from the stock standard solution (1 mg/ml noradrenaline in methanol) in DEPROT. All chemicals, if not stated otherwise, were obtained from Sigma-Aldrich Chemie GmbH.

2.7. RT-qPCR

Total RNA was extracted by ABI Prism 6100 Nucleic Acid PrepStation system (Applied Biosystems, Foster City, CA, USA) using Total RNA Chemistry Starter Kit (Applied Biosystems) and DNase wash solution (Absolute RNA Wash Solution, Applied Biosystems). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), under the following thermal cycler conditions: 10 min at 25 °C, 120 min at 37 °C and 5 s at 85 °C. Reaction mixtures for RT-qPCR consisted of 5 μl of cDNA template, 1x TaqMan Gene Expression Master Mix with Uracil-DNA glycosylase (UDG) (Applied Biosystems) and 1x mix of premade primer and hydrolysis probe sets (TaqMan Gene Expression Assays, Applied Biosystems), in a final volume of 25 μl. RT-qPCR reactions were carried out in triplicate using Applied Biosystems 7500 Real-Time PCR System, as previously described [5]. The following TaqMan Gene Expression Assays were used: IL-1β (Il1b, Rn99999009_m1), IL-2 (Il2, Rn00587673_m1), IL-23/p19 (Il23a, Rn00590334_g1), RORγt (Rorc, Rn01261022_m1), nerve growth factor (NGF; Ngf, Rn01533872_m1), and β-actin (Actb, Rn00667869_m1). Target mRNA expression was determined using the comparative threshold cycle (dCt) method with β-actin as a reference and SDS v1.4.0. software (Applied Biosystems). Relative amounts of target mRNAs were shown as 2^{-dCt} values, representing the ratio of target to reference gene, where dCt = Ct target – Ct reference.

2.8. Flow cytometry analysis

Aliquots of freshly isolated cells (for surface antigen immunostaining) or fixed/permeabilized according to eBioscience protocol (http://www.ebioscience.com/resources/best-protocols/flow-cytometry-protocols.htm) cells (for intracellular antigen immunostaining) were incubated with saturating concentrations of either fluorochrome-labeled Abs or biotin-conjugated/unconjugated Abs. When biotin-conjugated/unconjugated Abs were used, after the incubation the cells were washed and subjected to an additional incubation with the appropriate second-step reagents.

The proliferating lymphocytes among cultured dLN cells were identified using 7-aminoactinomycin D (7-AAD) staining of DNA, and their frequency was determined using Dean-Jett-Fox model of the FlowJo software version 7.8. (TreeStar Inc, Ashland, OR, USA). For this purpose, upon surface antigen staining, cells were fixed/permeabilized overnight using 70% ethanol at 4 °C, and then incubated with 7-AAD (BD Biosciences Pharmingen), at 4 °C for 30 min.

For analyses, 50.000 events per sample were acquired on FACSVerse flow cytometer (Becton Dickinson, Mountain View, CA, USA) and used to determine the frequency and/or absolute number of marker positive cells and/or mean fluorescence intensity (MFI), using FlowJo software version 7.8.

2.9. ELISA

For measuring the concentration of cytokines, commercial kits for IL-17A (BioLegend) and IL-2 (R&D Systems, Minneapolis, MN, USA) were used. The assays were performed following the manufacturers' instructions. A standard curve was generated for each assay, with the limits of detection for IL-17 = 8 pg/ml and IL-2 < 15 pg/ml.

2.10. Statistical analysis

To assess sex differences unpaired Student's *t*-test was used. To examine influence of sex and culturing conditions on the examined parameters, two-way ANOVA followed by Bonferroni test for *post-hoc* comparisons was used. Data were analyzed using GraphPad Prism Software (version 5.00; GraphPad Software, San Diego, CA, USA). The results were shown as mean ± SEM. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Sex-based differences in noradrenaline concentration in dLN tissue and β_2-AR expression on dLN cells from EAE rats

3.1.1. Higher noradrenaline concentration in male rat dLN tissue

On the 7th d.p.i. for EAE (the preclinical phase of EAE) dLNs were isolated from female DA rats, which exhibit higher incidence and shorter duration of the preclinical phase of EAE than age- and strain-matched male rats [5], and from their male counterparts. The concentration of noradrenaline was higher in male than in female rat dLNs (Fig. 1A). Considering that noradrenergic nerve fibers are the main source of noradrenaline in lymphoid tissues and that noradrenergic nerve fiber density in peripheral tissues correlates with NGF expression [30–32], the amount of NGF mRNA in dLNs was examined. NGF expression was upregulated in dLNs from male rats compared with their female counterparts (Fig. 1A). Given that, apart from noradrenergic nerve fibers, various types of immune cells also synthesize noradrenaline [33–35], their contribution to the sexual dimorphism in noradrenaline concentration in dLNs of EAE rats was also investigated. The overall noradrenaline content was greater in male than in female rat dLN cells (Fig. 1B). Consistently, the frequency of cells expressing tyrosine hydroxylase, the key rate-limiting enzyme in catecholamine biosynthesis [36], was higher among dLN cells from male compared with female rats (Fig. 1C; Suppl. Fig. 1A). The greater number of tyrosine hydroxylase+ cells isolated from male rat dLNs suggested that the augmented "intrinsic" noradrenaline supply also contributed to the higher concentration of this neurotransmitter/hormone in dLNs of male compared with female DA rats. It is noteworthy that we failed to detect measurable levels of dopamine in dLN cells.

Considering that CD4+ T lymphocytes, the key cell driver in EAE pathogenesis [5,8], synthesize noradrenaline [37], their expression of tyrosine hydroxylase was also explored. The results showed higher frequency of tyrosine hydroxylase+ cells within dLN CD4+ T lymphocytes from male compared with female rats (Fig. 1D; Suppl. Fig. 1B). Given that more tyrosine hydroxylase+ CD4+ T lymphocytes were retrieved from male rat dLNs (Fig. 1D), it was assumed that CD4+ T lymphocytes contributed to the sexual dimorphism in dLN "intrinsic" noradrenaline supply.

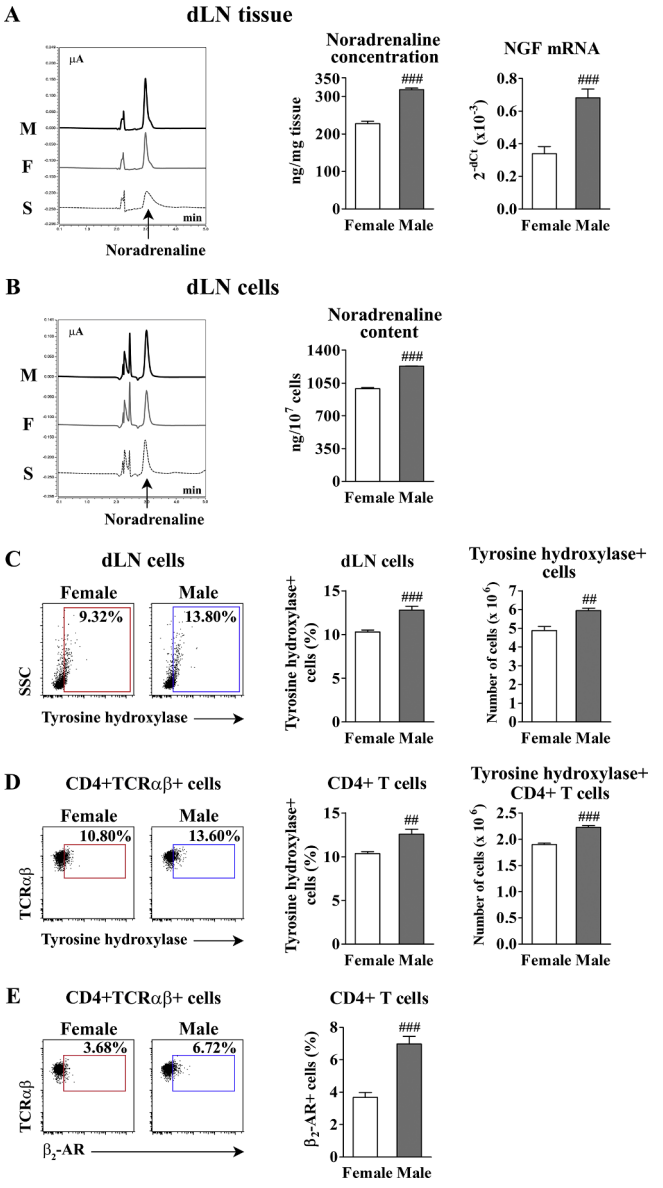
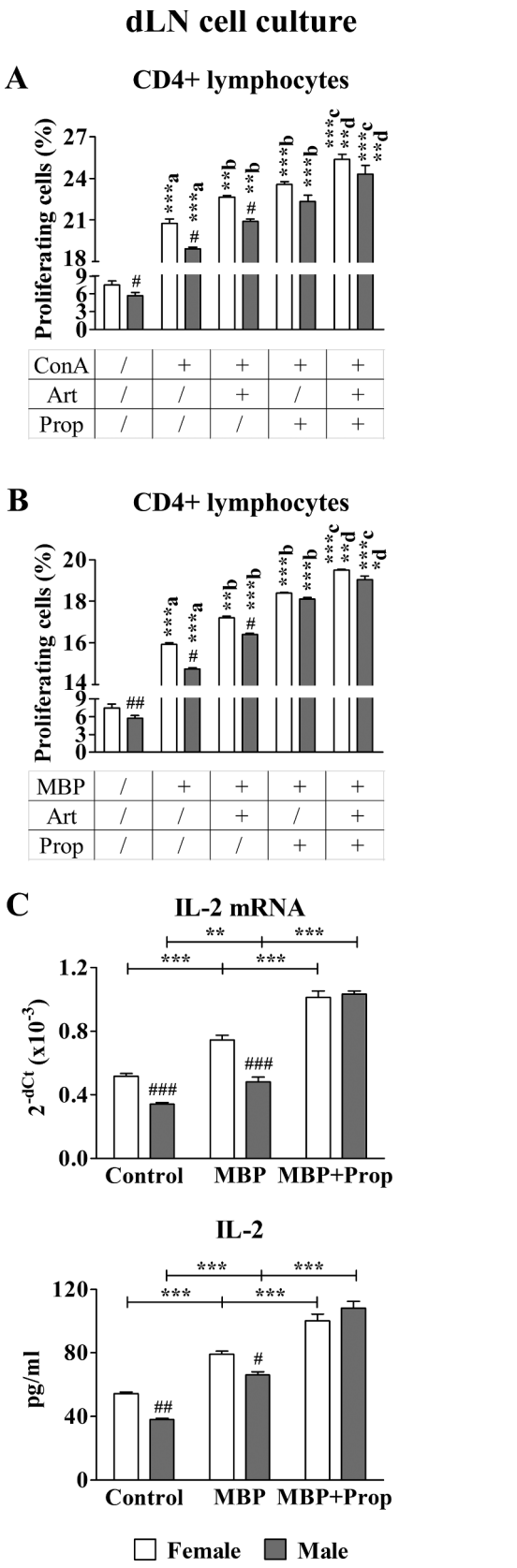


Fig. 1. Sex differences in noradrenaline concentration and cell content in dLNs from rats immunized for EAE on the 7th d.p.i., and tyrosine hydroxylase and β_2-AR expression in/on their dLN cells. (A, B) Chromatograms of (A) tissue and (B) cell samples retrieved from dLNs of male (bold black line, M) and female (gray line, F) rats. Dashed lines (S) correspond to standard samples containing (A) 8 µg/ml and (B) 4 µg/ml of noradrenaline. Bar graphs indicate (A) noradrenaline concentration and the relative expression of NGF mRNA (determined by RT-qPCR and shown as 2^{-dCt} relative to β-actin) in dLN tissue and (B) noradrenaline content in dLN cells. (C, D) Representative flow cytometry dot plots indicate the frequency of tyrosine hydroxylase+ cells among (C) all dLN cells and (D) dLN CD4+ TCRαβ+ (T) lymphocytes from female and male rats. FMO controls for these analyses are shown in Suppl. Fig. 1A, B. Bar graphs indicate (left) the frequency and (right) the number of tyrosine hydroxylase+ cells among (C) all dLN cells and (D) dLN CD4+ T lymphocytes from female and male rats. (E) Representative flow cytometry dot plots indicate the frequency of β_2-AR+ cells among dLN CD4+ T lymphocytes from female and male rats. FMO controls for β_2-AR expression analysis are shown in Suppl. Fig. 1C. Bar graph indicates the frequency of β_2-AR expressing cells in CD4+ T cells from female and male rats. The data are represented as mean ± SEM (6 pools/group, total 12 rats). $^{\#\#}p \le 0.01$; $^{\#\#\#}p \le 0.001$.



(caption on next page)

3.1.2. Higher frequency of β_2-AR+ cells among dLN CD4+ T lymphocytes from male rats

Given that noradrenaline exerts immunomodulatory effects on T lymphocytes mainly through β_2-AR [9,11], its expression on dLN CD4+ T lymphocytes was also examined. In the preclinical phase of EAE, the frequency of β_2-AR+ cells was higher among CD4+ T lymphocytes from male compared with female rat dLNs (Fig. 1E; Suppl. Fig. 1C).

Fig. 2. β-AR–mediated influence of noradrenaline on proliferation of CD4+ lymphocytes in cultures of dLN cells retrieved from female and male rats immunized for EAE on the 7th d.p.i. (A, B) Bar graphs indicate the frequency of proliferating cells (cells in S+ G2/M phases of the cell cycle) among CD4+ lymphocytes from dLN cell cultures of female and male rats in the absence of cognate stimuli (Control) or following stimulation with (A) ConA or (B) MBP in the absence or in the presence of 10^{-6} M of arterenol (Art) and/or 10^{-5} M of propranolol (Prop), as determined by 7-AAD staining. (C) Bar graphs indicate (upper) the relative expression of IL-2 mRNA (determined by RT-qPCR and shown as 2^{-dCt} relative to β-actin) in cells and (lower) the concentration of IL-2 in supernatants from female and male rat dLN cell cultures stimulated with MBP in the presence of Prop or in its absence and corresponding Control cultures, as determined by ELISA. Two-way ANOVA showed significant interactions between the effects of sex and culturing conditions for the frequency of proliferating CD4+ cells in ConA- ($F_{(4,50)} = 2.60$; $p \leq 0.05$) and MBP-stimulated ($F_{(4,50)} = 2.65$; $p \leq 0.05$) cultures, and for IL-2 mRNA expression ($F_{(2,30)} = 14.19$; $p \leq 0.001$) and IL-2 concentration ($F_{(2,30)} = 10.15$; $p \leq 0.001$) in MBP-stimulated cultures. The data are represented as mean ± SEM (6 pools/group, total 12 rats). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$; $^{\#\#\#}p \leq 0.001$. # vs Female. a, vs unstimulated; b, vs ConA/MBP; c, vs ConA/MBP + Art; d, vs ConA/MBP + Prop.

3.2. β-AR blockade was more efficient in enhancing proliferation of CD4+ lymphocytes in male compared with female rat dLN cell cultures

Next, β-AR–mediated effects of noradrenaline on proliferation of dLN CD4+ lymphocytes retrieved from female and male rats in the preclinical phase of EAE were investigated. Their proliferation was analyzed in dLN cell cultures following stimulation with either mitogen (ConA) or neuroantigen (MBP), in the presence of exogenous noradrenaline (arterenol) and/or propranolol (β-AR blocker) and in their absence. In the presence of either ConA or MBP alone the frequency of cells in S+G2/M phases of cell cycle (proliferating cells) was lower among CD4+ lymphocytes from male compared with female rat dLN cell cultures (Fig. 2A, B). Arterenol increased their frequency in ConA- and MBP-stimulated dLN cell cultures from rats of both sexes (Fig. 2A, B). However, propranolol also augmented CD4+ lymphocyte proliferation in ConA- and MBP-stimulated dLN cell cultures from rats of both sexes (Fig. 2A, B). These effects of propranolol were more pronounced in dLN cell cultures from male rats (Fig. 2A, B). This resulted in the loss of sex differences in the frequency of proliferating cells among CD4+ lymphocytes observed in the presence of either ConA or MBP alone (Fig. 2A, B). The effects of the β-AR blocker in dLN cell cultures in the absence of arterenol could be ascribed to noradrenaline synthesis in dLN cells. To corroborate this assumption was the lack of propranolol effects on CD4+ lymphocyte proliferation in dLN cell cultures pretreated with AMPT, an inhibitor tyrosine hydroxylase [38], as the key and rate-limiting enzyme in catecholamine synthesis (Suppl. Fig. 2).

Considering that noradrenaline influences mitogen-induced CD4+ cell proliferation by diminishing their ÌL-2 synthesis [39,40], IL-2 mRNA expression in cells from dLN cell cultures stimulated with MBP in the absence and the presence of propranolol and IL-2 protein concentration in cell free supernatants from the same cultures were examined. The stimulation with MBP increased IL-2 expression at both mRNA and protein level in dLN cell cultures from both female and male rats, without affecting sex bias in its expression (Fig. 2C). IL-2 concentration was lower in male rat compared with female rat dLN cell cultures (Fig. 2C). As expected [39,40], propranolol up-regulated IL-2 mRNA expression in cells from MBP-stimulated dLN cell cultures of male and female rats, and increased the protein levels in their supernatants (Fig. 2C). These effects of propranolol were more prominent in dLN cell cultures from male rats, so none of these two parameters significantly differed between sexes in propranolol-supplemented dLN cell cultures (Fig. 2C).

Furthermore, in both ConA- and MBP-stimulated dLN cell cultures the frequency of proliferating cells among CD4+ lymphocytes was higher in the co-presence of arterenol and propranolol than in the presence of either arterenol or propranolol alone (Fig. 2A, B). This suggested that noradrenaline (arterenol) affects CD4+ lymphocyte proliferation in dLN cell cultures acting not only through β-AR, but also through α-AR, as previously suggested [41]. Additionally, in the co-presence of arterenol and propranolol there was no sex difference in the frequency of proliferating cells among CD4+ lymphocytes (Fig. 2A, B).

3.3. Effects of β-AR blockade on the frequency of IL-17+ cells among CD4+ lymphocytes in male and female rat dLN cell cultures

Considering data indicating (i) dominant pathogenetic role of Th17 lymphocytes in EAE model used in this study [8] and (ii) a strong correlation between tyrosine hydroxylase expression level and Th17 cell frequency in spleen of mice with collagen-induced arthritis [12], influence of arterenol and/or propranolol on the frequency of IL-17+ cells among CD4+ lymphocytes from dLN cell cultures was also examined. In the presence of either ConA or MBP their frequency increased in dLN cell cultures from rats of both sexes when compared with control cultures without any cognate proliferative stimulus (Fig. 3A, B; Suppl. Fig. 3A). However, it remained lower in male rat cultures (Fig. 3A, B; Suppl. Fig. 3A). Arterenol decreased the frequency of IL-17+ cells among CD4+ lymphocytes in both ConA- and MBP-stimulated male and female rat dLN cell cultures without affecting the sex bias in their frequency (Fig. 3A, B). On the contrary, propranolol, irrespective of the proliferative stimulus, increased the frequency of IL-17+ cells among CD4+ lymphocytes in both female and male rat dLN cell cultures (Fig. 3A, B). To exclude non-AR–mediated action of propranolol, we found that it did not significantly influence the frequency of IL-17+ cells among CD4+ lymphocytes in dLN cell cultures pretreated with AMPT, a blocker of catecholamine synthesis (Suppl. Fig. 3B). Additionally, propranolol elevated the production of IL-17, as it was shown by determining its concentrations in supernatants of MBP-stimulated dLN cells cultures from female and male rats (Fig. 3B). However, this effect of propranolol was more prominent in male rat dLN cell cultures, so IL-17 concentration was comparable in male and female rat MBP-stimulated dLN cell cultures supplemented with propranolol (Fig. 3B). It is noteworthy that MBP alone increased the concentration of IL-17 in both female and male rat dLN cell cultures, so that its concentration was lower in male rat compared with female rat dLN cell cultures (Fig. 3B). To explain obvious discrepancy in the frequency of IL-17+ cells among CD4+ lymphocytes from female and male dLN cell cultures and IL-17 concentration in these cultures, IL-17 MFI, as a measure of the protein expression, in IL-17+ CD4+ cells was examined. The results showed that IL-17 MFI in IL-17+ CD4+ cells was comparable between female and male control and MBP-stimulated dLN cell cultures (Fig. 3B). Propranolol increased IL-17 MFI only in MBP-stimulated dLN cell cultures from male rats (Fig. 3B). Consequently, IL-17 MFI was higher in IL-17+ CD4+ cells from male compared with female propranolol-supplemented MBP-stimulated dLN cell cultures (Fig. 3B). Thus, the lack of sexual dimorphism in IL-17 concentration in propranolol-supplemented MBP-stimulated dLN cell cultures exhibiting sexual dimorphism in IL-17+ cell frequency (lower frequency of IL-17+ cells in male compared with female cultures) could be associated with greater IL-17 production in male compared with female IL-17+ CD4+ cells. In the co-presence of arterenol and propranolol the frequency of IL-17+ cells among CD4+ lymphocytes was higher than in the presence of either propranolol or arterenol alone (Fig. 3A, B). This suggests that arterenol (noradrenaline) may also affect IL-17+ cell frequency in dLN cell cultures through α-AR, but that its β-AR–mediated inhibitory action is dominant.

Next, the putative mechanisms underlying propranolol influence on IL-17+ cell frequency in MBP-stimulated dLN cell cultures were investigated. Considering data suggesting a direct catecholamine action on Th17 cell differentiation [12], dLN cells were examined for the expression of mRNA for RORγt, the key transcription factor in Th17 cell

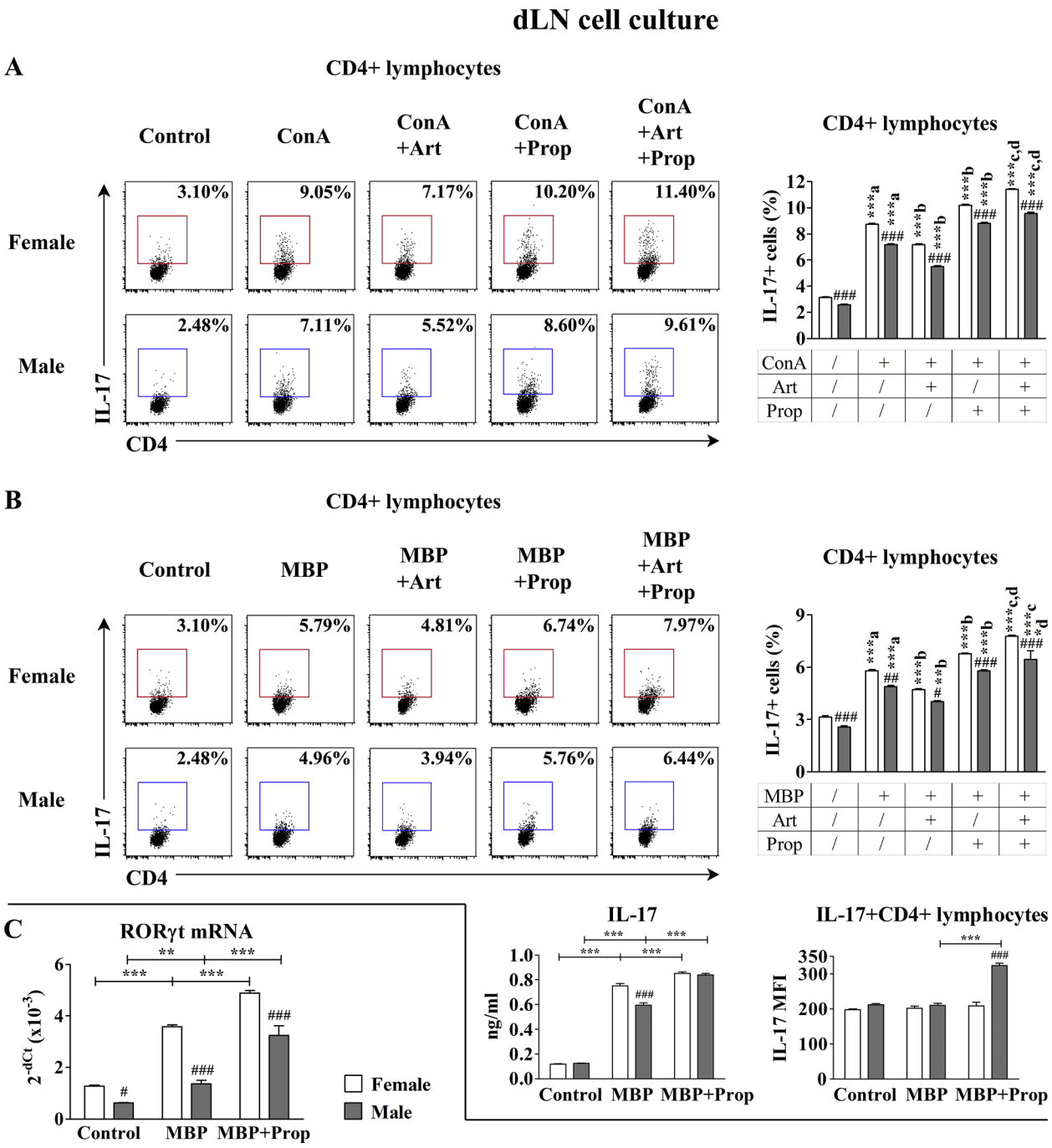


Fig. 3. β-AR–mediated influence of noradrenaline on the frequency of IL-17+ cells within CD4+ lymphocytes in cultures of dLN cells retrieved from female and male rats immunized for EAE on the 7th d.p.i. (A, B) Representative flow cytometry dot plots indicate the frequency of IL-17+ cells among CD4+ lymphocytes from dLN cell cultures of female and male rats in the absence of cognate stimuli (Control) or following stimulation with (A) ConA or (B) MBP in the absence or in the presence of 10^{-6} M of arterenol (Art) and/or 10^{-5} M of propranolol (Prop). Isotype/FMO control for these analyses is shown in Suppl. Fig. 3A. Bar graphs indicate the frequency of IL-17+ cells within CD4+ lymphocytes from female and male rat dLN cell cultures stimulated with (A) ConA or (B) MBP in the absence or in the presence of Art and/or Prop and corresponding Control cultures. Bar graphs (B, inserts) indicate (right) IL-17 mean intensity of fluorescence (MFI) in IL-17+ CD4+ cells and (left) IL-17 concentration in supernatants from female and male rat dLN cell cultures stimulated with MBP in the presence of Prop and in its absence and corresponding Control cultures. (C) Bar graph indicates the relative expression of RORγt mRNA in cells from female and male rat dLN cell cultures stimulated with MBP in the presence of Prop and in its absence and corresponding Control cultures (determined by RT-qPCR and represented as 2^{-dCt} relative to β-actin). Two-way ANOVA showed significant interactions between the effects of sex and culturing conditions for the frequency of IL-17+ cells within CD4+ lymphocytes in ConA- ($F_{(4,50)} = 24.94$; $p \leq 0.001$) and MBP-stimulated ($F_{(4,50)} = 2.59$; $p \leq 0.05$) cultures, and for RORγt mRNA expression ($F_{(2,30)} = 11.24$; $p \leq 0.001$), IL-17 concentration ($F_{(2,30)} = 23.29$; $p \leq 0.001$) and IL-17 MFI in IL-17+ CD4+ cells ($F_{(2,30)} = 46.34$; $p \leq 0.001$) in MBP-stimulated cultures. The data are represented as mean ± SEM (6 pools/group, total 12 rats). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$; $^{\#\#\#}p \leq 0.001$. # vs Female. a, vs unstimulated; b, vs ConA/MBP; c, vs ConA/MBP + Art; d, vs ConA/MBP + Prop.

development [42]. In control cultures the less amount of RORγt mRNA was found in dLN cells from male rats compared with female rats (Fig. 3C). MBP upregulated RORγt mRNA expression in cells from female and male rat dLN cell cultures (Fig. 3C). In the presences of propranolol these effects of MBP were further augmented in both female (by approx. 37%) and male (by approx. 160%) rats (Fig. 3C). However, irrespective of propranolol presence, the expression of RORγt mRNA remained less in MBP-stimulated dLN cells from male compared with female rats (Fig. 3C).

Next, considering the central role of cytokines secreted by antigen presenting cells in CD4+ T lymphocyte differentiation, and AR expression on their surface [43–45], the expression of mRNAs for IL-1β and IL-23/p19, the key cytokines driving/maintaining Th17 cell polarization [46], was examined in dLN cell cultures stimulated with MBP in the presence of propranolol and in its absence. The expression of both cytokines was lower in dLN cells from female and male rat control cultures compared with corresponding cultures stimulated with MBP (Fig. 4A). However, irrespective of MBP presence their expression was lower in male rat dLN cells (Fig. 4A). In the presence of propranolol the expression of mRNAs for IL-1β and IL-23/p19 was enhanced in dLN cells from rats of both sexes (Fig. 4A). In the presence of propranolol the expression of IL-1β transcript was lower in dLN cells from male compared with female rats (Fig. 4A). However, in the presence of propranolol the expression of IL-23/p19 mRNA was comparable in dLN cells from female and male rats (Fig. 4A).

To corroborate the previous findings, the expression of tyrosine hydroxylase and β_2-AR in/on CD11b+ cells from dLNs isolated on the 7th d.p.i. were examined. Indeed, irrespective of sex, tyrosine hydroxylase+ and β_2-AR+ cells were found among dLN CD11b+ cells. The frequency of tyrosine hydroxylase+ cells was comparable among CD11b+ cells from male and female rat dLNs (Fig. 4B; Suppl. Fig. 4A). On the other hand, the frequency of β_2-AR+ cells was slightly higher among CD11b+ cells from male compared with female rat dLNs (Fig. 4C; Suppl. Fig. 4B).

4. Discussion

The study indicated that dLN CD4+ T lymphocytes and CD11b+ cells expressed not only β_2-AR, but also synthesized noradrenaline ("noradrenergic cells"), so that propranolol, irrespective of arterenol presence, augmented CD4+ lymphocyte proliferation and increased the frequency of Th17 cells in MBP-stimulated cultures of dLN cells isolated from EAE rats in the preclinical phase of EAE. Thus, our results indicated that noradrenaline released not only from noradrenergic nerve fiber endings, but also from "noradrenergic" dLN cells could have an important role in modulating the primary CD4+ T-lymphocyte

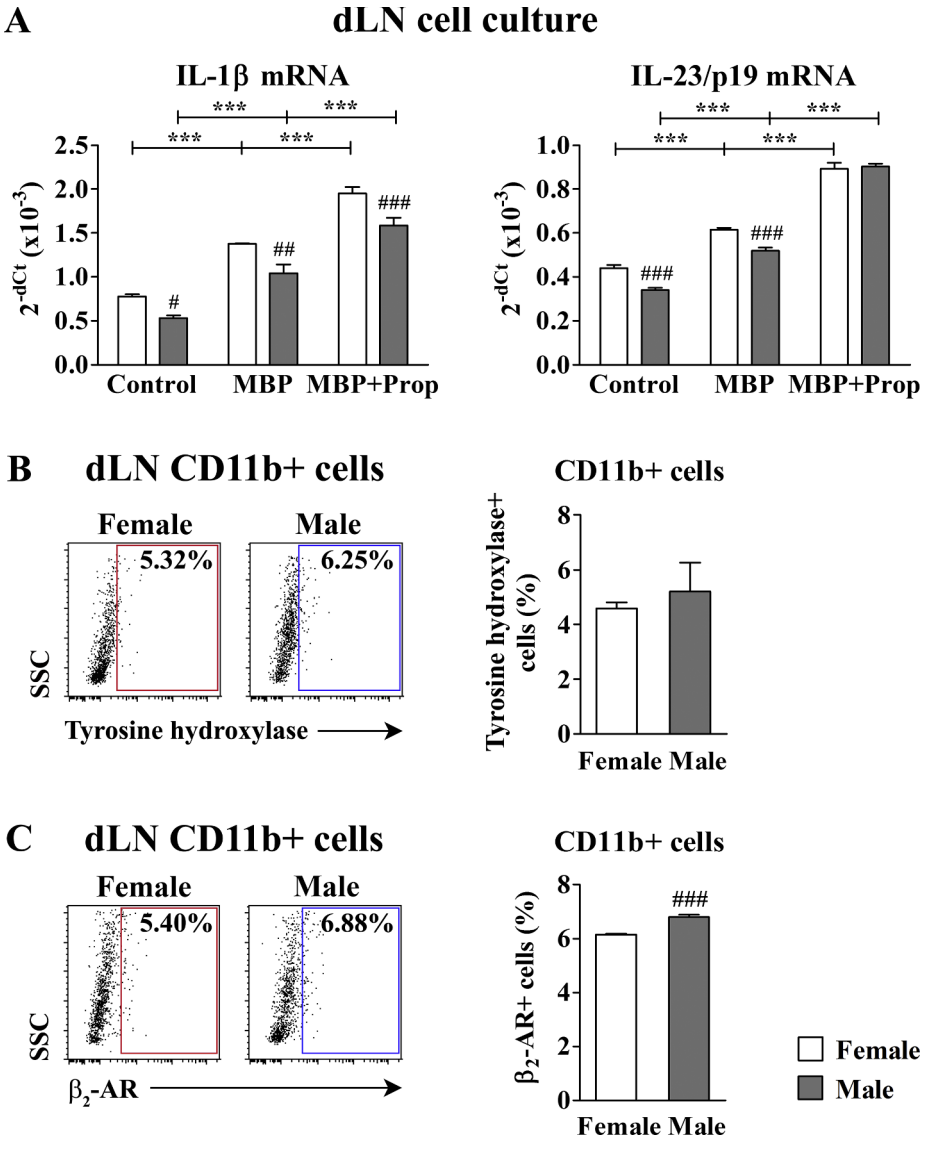


Fig. 4. Tyrosine hydroxylase and β_2-AR expression in/on CD11b+ dLN cells retrieved from female and male rats immunized for EAE on the 7th d.p.i. and β-AR-mediated influence of noradrenaline on the expression of Th17-polarizing cytokines in cells from dLN cell cultures of these animals. (A) Bar graphs indicate the relative expression of (left) IL-1β and (right) IL-23/p19 mRNAs in the cells from dLN cell cultures of female and male rats in the absence of cognate stimuli (Control) or following stimulation with MBP in the presence of 10^{-5} M of propranolol (Prop) and in its absence, as determined by RT-qPCR. Results are shown as 2^{-dCt} relative to β-actin. (B, C) Representative flow cytometry dot plots indicate the frequency of (B) tyrosine hydroxylase+ and (C) β_2-AR+ cells among CD11b+ antigen presenting cells retrieved from dLNs of female and male rats. FMO controls for these flow cytometry analyses are shown in Suppl. Fig. 4. Bar graphs indicate the frequency of (B) tyrosine hydroxylase+ and (C) β_2-AR + cells among dLN CD11b+ cells from female and male rats. Two-way ANOVA showed significant interactions between the effects of sex and culturing conditions for IL-23/p19 mRNA expression ($F_{(2,30)} = 7.67$; $p \leq 0.01$). The data are represented as mean ± SEM (6 pools/group, total 12 rats). $^{***}p \leq 0.001$; $^{\#}p \leq 0.05$; $^{\#\#}p \leq 0.01$; $^{\#\#\#}p \leq 0.001$. # vs Female.

response. To give an extra weight to this notion, are data indicating that noradrenaline from "noradrenergic" immune cells has a particularly important role in dLNs and some other tissues following sympathectomy, during autoimmune pathologies and under stress, when its release from nerve fibers decreases [47–50]. Indeed, we found that noradrenaline concentration (reflecting mainly its "extrinsic" supply) declines in dLN tissue from rats of both sexes after immunization for EAE (data not shown). In favor of the stimulatory influence of propranolol on CD4+ *T*-lymphocyte proliferation are data indicating that β-AR agonists inhibit proliferation of CD4+ T lymphocytes from both healthy rodents and mice with collagen-induced arthritis [13,24,51]. Given that, despite the lower frequency of activated antigen presenting CD11b+ cells in male compared with female DA rat dLN cells on the 7th d.p.i. [5], propranolol exerted more prominent effects on CD4+ lymphocyte proliferation in both ConA-stimulated (where CD4+ cell proliferation was not dependent on the antigen presenting cells) and MBP-stimulated (where CD4+ cell proliferation was dependent on the antigen presenting cells) dLN cell cultures, a direct effect of propranolol on CD4+ T lymphocytes could be assumed. To corroborate this assumption are data showing that β_2-AR-mediated noradrenaline influence on T-cell proliferation is not dependent on the presence of antigen presenting cells [52]. Given that CD4+ lymphocytes are the main producers of IL-2 [53], the stimulatory effect of propranolol on CD4+ lymphocyte proliferation in dLN cell cultures was linked with their increased IL-2 production. In favor of this assumption are data indicating that noradrenaline diminishes IL-2 production in mitogen-activated splenocytes acting through β_2-AR [54]. The greater proliferative response of CD4+ lymphocytes to propranolol stimulation in male compared with female rat dLN cell cultures (leading to the loss of sexual dimorphism in this parameter observed in cultures stimulated with ConA or MBP alone) could be associated with the higher frequency of tyrosine hydroxylase+ and β_2-AR+ cells among dLN CD4+ T lymphocytes from male compared with female rats. In favor of sexual diergism in CD4+ T lymphocyte proliferation are data indicating that CD4+ T lymphocytes from female mice exhibit greater proliferative response to stimulation with anti-CD3 and anti-CD28 Abs compared with those from male mice [55]. Considering that the concentration of noradrenaline was higher in male than in female dLNs (reflecting greater noradrenaline supply from both noradrenergic nerve fibers, as shown by analysis of NGF expression, and "noradrenergic" immune cells, as indicated by the frequency of tyrosine hydroxylase+ cells), the previous finding could explain lower proliferation of dLN CD4+ T lymphocytes and consequently neuroantigen-specific cell generation in male compared with female rats immunized for EAE [5]. To corroborate sex bias in noradrenergic nerve supply are sex differences in: i) the structural organization and number of tyrosine hydroxylase-expressing neurons in the locus coeruleus having a pivotal role in the regulation of sympathetic nervous system activity [56] and ii) regulation of tyrosine hydroxylase expression and consequently noradrenaline synthesis in the locus coeruleus neurons [57]. To the best of our knowledge there is no data on sex differences in the frequency of "noradrenergic" cells in dLNs. The higher frequency of β_2-AR+ cells among CD4+ T lymphocytes from male rats is also consistent with their less efficient generation of neuroantigen-specific cells in male compared with female DA rats [5].

Additionally, propranolol increased the frequency of IL-17+ cells among CD4+ lymphocytes from MBP-stimulated dLN cell cultures, and RORγt expression in the cells from these cultures. Without excluding direct stimulatory effect of propranolol on Th17 differentiating cells in MBP-stimulated dLN cell cultures [12,52], our findings suggested that the increased frequency of Th17 cells in dLN cell cultures in the presence of propranolol could also reflect its enhancing effect on the expression of Th17 polarizing cytokines in antigen presenting cells. In favor of direct action of propranolol on CD4+ T cell differentiation towards Th17 cells are findings showing that β_2-AR antagonst ICI118551 inhibits noradrenaline-induced decrease in the percentage of IL-17+ cells and the expression of ROR-γt in anti-CD3/CD28 antibody-stimulated CD4+ T-cell cultures from mice with collagen-induced arthritis [51]. Additionally, there are data indicating that tyrosine hydroxylase gene overexpression in CD4+ T lymphocytes diminishes RORγt expression, and consequently Th17 cell differentiation in mice with collagen-induced arthritis [12]. On the other hand, in favor of propranolol indirect action on CD4+ T cell differentiation into Th17 cells are data indicating that noradrenaline may reduce Th17 cell differentiation acting on antigen presenting cell polarizing capacity [58–60]. Specifically, noradrenaline is shown to inhibit lipopolysaccharide- or IFN-γ-stimulated IL-1β production in macrophages through β_2-AR [58,59]. Additionally, it has been shown that: i) noradrenaline inhibits lipopolysaccharide-stimulated IL-23 production in a mixed population of human dendritic cells [61] and ii) adrenaline pretreatment diminishes IL-23–producing capacity of bone marrow dendritic cells maturing in the presence of lipopolysaccharide [62]. To additionally support indirect, antigen presenting cell-mediated, action of propranolol on CD4+ lymphocytes we found β_2-AR expression on dLN CD11b+ cells. This was fully consisted with data obtained in some previous studies examining β_2-AR expression on dendritic cells and macrophages [62–64]. The greater effects of propranolol on the expression of cytokines driving/maintaining Th17 cell differentiation, which are particularly obvious if one takes into account the lower frequency of activated dLN antigen presenting cells in male compared with female rats [5], could be related to the higher frequency of β_2-AR + cells among CD11b+ cells from male rat dLNs. Finally, given that despite lower frequency of IL-17+ cells in male compared with female rat MBP-stimulated dLN cell cultures supplemented with propranolol, IL-17 concentration was comparable in these cultures, it seems obvious that propranolol influenced not only the frequency, but also secretory capacity of IL-17-producing cells. Given that the concentration of IL-17 was lower in male than in female dLN cell cultures stimulated with MBP alone, it seems obvious that this effect of propranolol, differently from that on IL-17+ CD4+ cell frequency, was sexually dimorphic. To support this assumption are our findings obtained by examining influence of propranolol on IL-17 MFI. To additionally corroborate differential effect of propranolol in female and male rat dLN cell cultures are data indicating that: i) while RORγt is considered as a IL-17-specific transcription factor, an accumulating body of evidence clearly shows that IL-17 transcription is regulated by multiple transcription factors exerting not only positive, but also negative regulatory effects [65], and ii) among them some selectively inhibit IL-17 expression in male CD4+ T lymphocytes [66].

In conclusion, the study showed that: i) noradrenaline from rat dLN noradrenergic nerve fibers and "noradrenergic" cells alike, acting through β-AR, exerts an inhibitory influence on the primary CD4+ T-cell response to rat inoculation with (auto)antigens, in particular on Th17 cell differentiation and IL-17 production by Th17 cells and ii) noradrenaline, differently from Th17 cell differentiation, influences IL-17 production by Th17 cells, in a sex-specific manner (more efficiently in male rats). However, considering that pathogenic effects of autoreactive CD4+ T cells induced in dLNs following immunization for EAE largely depend on the target tissue capacity to control their migration, autoimmune attack and cytoprotection, and thereby tissue damage [67], it is obvious that further *in vivo* studies to evaluate not only influence of propranolol on the shaping of Th17 cell pathogenic profile, but also their target tissue damaging capacity (and thereby its relevance for the outbreaking of EAE and possibly MS) are required. Such investigations have already been launched in our laboratory.

Funding

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia [grant number 175050].

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.12.009.

References

[1] E. Ortona, M. Pierdominici, A. Maselli, C. Veroni, F. Aloisi, Y. Shoenfeld, Sex-based differences in autoimmune diseases, Ann. Ist. Super. Sanita 52 (2016) 205–212.
[2] A. Nicot, Gender and sex hormones in multiple sclerosis pathology and therapy, Front. Biosci. (Landmark Ed.) 14 (2009) 4477–4515.
[3] A.P. Robinson, C.T. Harp, A. Noronha, S.D. Miller, The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment, Handb. Clin. Neurol. 122 (2014) 173–189.
[4] I. Harpaz, S. Abutbul, A. Nemirovsky, R. Gal, H. Cohen, A. Monsonego, Chronic exposure to stress predisposes to higher autoimmune susceptibility in C57BL/6 mice: glucocorticoids as a double-edged sword, Eur. J. Immunol. 43 (2013) 758–769.
[5] M. Nacka-Aleksić, J. Djikić, I. Pilipović, Z. Stojić-Vukanić, D. Kosec, B. Bufan, N. Arsenović-Ranin, M. Dimitrijević, G. Leposavić, Male rats develop more severe experimental autoimmune encephalomyelitis than female rats: sexual dimorphism and diergism at the spinal cord level, Brain Behav. Immun. 49 (2015) 101–118.
[6] T.L. Papenfuss, C.J. Rogers, I. Gienapp, M. Yurrita, M. McClain, N. Damico, J. Valo, F. Song, C.C. Whitacre, Sex differences in experimental autoimmune encephalomyelitis in multiple murine strains, J. Neuroimmunol. 150 (2004) 59–69.
[7] J. Reddy, H. Waldner, X. Zhang, Z. Illes, K.W. Wucherpfennig, R.A. Sobel, V.K. Kuchroo, Cutting edge: CD4+CD25+ regulatory T cells contribute to gender differences in susceptibility to experimental autoimmune encephalomyelitis, J. Immunol. 175 (2005) 5591–5595.
[8] J.M. Damsker, A.M. Hansen, R.R. Caspi, Th1 and Th17 cells: adversaries and collaborators, Ann. N. Y. Acad. Sci. 1183 (2010) 211–221.
[9] A.P. Kohm, V.M. Sanders, Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo, Pharmacol. Rev. 53 (2001) 487–525.
[10] F. Marino, M. Cosentino, Adrenergic modulation of immune cells: an update, Amino Acids 45 (2013) 55–71.
[11] C.J. Padro, V.M. Sanders, Neuroendocrine regulation of inflammation, Semin. Immunol. 26 (2014) 357–368.
[12] X.Q. Wang, Y. Liu, H.H. Cai, Y.P. Peng, Y.H. Qiu, Expression of tyrosine hydroxylase in CD4+ T cells contributes to alleviation of Th17/Treg imbalance in collagen-induced arthritis, Exp. Biol. Med. 241 (2016) 2094–2103.
[13] I.J. Elenkov, R.L. Wilder, G.P. Chrousos, E.S. Vizi, The sympathetic nerve—an integrative interface between two supersystems: the brain and the immune system, Pharmacol. Rev. 52 (2000) 595–638.
[14] M. Cosentino, F. Marino, Adrenergic and dopaminergic modulation of immunity in multiple sclerosis: teaching old drugs new tricks? J. Neuroimmune Pharmacol. 8 (2013) 163–179.
[15] M.A. Flierl, D. Rittirsch, M. Huber-Lang, J.V. Sarma, P.A. Ward, Catecholamines-crafty weapons in the inflammatory arsenal of immune/inflammatory cells or opening pandora's box? Mol. Med. 14 (2008) 195–204.
[16] C. Hinojosa-Laborde, I. Chapa, D. Lange, J.R. Haywood, Gender differences in sympathetic nervous system regulation, Clin. Exp. Pharmacol. Physiol. 26 (1999) 122–126.
[17] G. Leposavić, I. Pilipović, M. Perišić, Cellular and nerve fibre catecholaminergic thymic network: steroid hormone dependent activity, Physiol. Res. 60 (2011) 71–82.
[18] S. ThyagaRajan, K.S. Madden, B. Teruya, S.Y. Stevens, D.L. Felten, D.L. Bellinger, Age-associated alterations in sympathetic noradrenergic innervation of primary and secondary lymphoid organs in female Fischer 344 rats, J. Neuroimmunol. 233 (2011) 54–64.
[19] C. de Coupade, R.W. Gear, P.F. Dazin, H.Y. Sroussi, P.G. Green, J.D. Levine, Beta 2-adrenergic receptor regulation of human neutrophil function is sexually dimorphic, Br. J. Pharmacol. 143 (2004) 1033–1041.
[20] C. de Coupade, A.S. Brown, P.F. Dazin, J.D. Levine, P.G. Green, Beta(2)-Adrenergic receptor-dependent sexual dimorphism for murine leukocyte migration, J. Neuroimmunol. 186 (2007) 54–62.
[21] P.J. Fitzgerald, Noradrenaline transmission reducing drugs may protect against a broad range of diseases, Auton. Autacoid Pharmacol. 34 (2015) 15–26.
[22] S.M. Gold, D.C. Mohr, I. Huitinga, P. Flachenecker, E.M. Sternberg, C. Heesen, The role of stress-response systems for the pathogenesis and progression of MS, Trends Immunol. 26 (2005) 644–652.
[23] J.M. Racosta, M. Kremenchutzky, The role of autonomic dysregulation from pathophysiology to therapeutics of multiple sclerosis: a putative novel treatment target? J. Neurol. Neurophysiol. 5 (2014) 212.
[24] E. Chelmicka-Schorr, M.N. Kwasniewski, B.E. Thomas, B.G.W. Arnason, The β-adrenergic agonist isoproterenol suppresses experimental allergic encephalomyelitis in Lewis rats, J. Neuroimmunol. 25 (1989) 203–207.
[25] E. Chelmicka-Schorr, M.N. Kwasniewski, R.L. Wollmann, Sympathectomy augments adoptively transferred experimental allergic encephalomyelitis, J. Neuroimmunol. 37 (1992) 99–103.
[26] M. Dimitrijević, A. Rauški, K. Radojević, D. Kosec, S. Stanojević, I. Pilipović, G. Leposavić, Beta-adrenoceptor blockade ameliorates the clinical course of experimental allergic encephalomyelitis and diminishes its aggravation in adrenalectomized rats, Eur. J. Pharmacol. 577 (2007) 170–182.
[27] J.P. Leonard, F.J. MacKenzie, H.A. Patel, M.L. Cuzner, Splenic noradrenergic and adrenocortical responses during the preclinical and clinical stages of adoptively transferred experimental autoimmune encephalomyelitis (EAE), J. Neuroimmunol. 26 (1990) 183–186.
[28] F.J. Mackenzie, J.P. Leonard, M.L. Cuzner, Changes in lymphocyte beta-adrenergic receptor density and noradrenaline content of the spleen are early indicators of immune reactivity in acute experimental allergic encephalomyelitis in the Lewis rat, J. Neuroimmunol. 23 (1989) 93–100.
[29] S.Y. Felten, J. Housel, D.L. Felten, Use of in vivo dialysis for evaluation of splenic norepinephrine and serotonin, Soc. Neurosci. Abstr. 12 (1986) 1065.
[30] S.L. Carlson, K.M. Albers, D.J. Beiting, M. Parish, J.M. Conner, B.M. Davis, NGF modulates sympathetic innervation of lymphoid tissues, J. Neurosci. 15 (1995) 5892–5899.
[31] G. Minnone, F. De Benedetti, L. Bracci-Laudiero, NGF and its receptors in the regulation of inflammatory response, Int. J. Mol. Sci. 18 (2017) 1028.
[32] D.L. Shelton, L.F. Reichardt, Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 7951–7955.
[33] E. Josefsson, J. Bergquist, R. Ekman, A. Tarkowski, Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis, Immunology 88 (1996) 140–146.
[34] N.R. Musso, S. Brenci, F. Indiveri, G. Lotti, L-tyrosine and nicotine induce synthesis of L-Dopa and norepinephrine in human lymphocytes, J. Neuroimmunol. 74 (1997) 117–120.
[35] I. Shaked, R.N. Hanna, H. Shaked, G. Chodaczek, H.N. Nowyhed, G. Tweet, R. Tacke, A.B. Basat, Z. Mikulski, S. Togher, J. Miller, A. Blatchley, S. Salek-Ardakani, M. Darvas, M.U. Kaikkonen, G. Thomas, S. Lai-Wing-Sun, A. Rezk, A. Bar-Or, C.K. Glass, H. Bandukwala, C.C. Hedrick, The orphan nuclear receptor Nr4a1 couples sympathetic and inflammatory cues in CNS-recruited macrophages to limit neuroinflammation, Nat. Immunol. 16 (2015) 1228–1234.
[36] T. Flatmark, R.C. Stevens, Structural insight into the aromatic amino acid hydroxylases and their disease-related mutant forms, Chem. Rev. 99 (1999) 2137–2160.
[37] J. Chen, Y.P. Peng, S.W. Cui, J.Y. Bao, Y.H. Qiu, Changes in expression of tyrosine hydroxylase by CD4+ T lymphocytes in lymphoid tissues of DBA/1 mice with collagen-induced arthritis, Zhongguo Ying Yong Sheng Li Xue Za Zhi 29 (2013) 214–218.
[38] R. Ankenman, M.F. Salvatore, Low dose alpha-methyl-para-tyrosine (AMPT) in the treatment of dystonia and dyskinesia, J. Neuropsychiatry Clin. Neurosci. 19 (2007) 65–69.
[39] V.V. Ganusov, D. Milutinović, R.J. De Boer, IL-2 regulates expansion of CD4+ T cell populations by affecting cell death: insights from modeling CFSE data, J. Immunol. 179 (2007) 950–957.
[40] Y.P. Peng, Y.H. Qiu, J.L. Jiang, J.J. Wang, Effect of catecholamines on IL-2 production and NK cytotoxicity of rats in vitro, Acta Pharmacol. Sin. 25 (2004) 1354–1360.
[41] J.W. Hadden, E.M. Hadden, E. Middleton, Lymphocyte blast transformation: I. Demonstration of adrenergic receptors in human peripheral lymphocytes, Cell. Immunol. 1 (1970) 583–595.
[42] M. Ciofani, M.A. Madar, C. Galan, M. Sellars, K. Mace, F. Pauli, A. Agarwal, W. Huang, C.N. Parkhurst, M. Muratet, K.M. Newberry, S. Meadows, A. Greenfield, Y. Yang, P. Jain, F.K. Kirigin, C. Birchmeier, E.F. Wagner, K.M. Murphy, R.M. Myers, R. Bonneau, D.R. Littman, A validated regulatory network for Th17 cell specification, Cell 151 (2012) 289–303.
[43] M. Dimitrijević, I. Pilipović, S. Stanojević, K. Mitić, K. Radojević, V. Pešić, G. Leposavić, Chronic propranolol treatment affects expression of adrenoceptors on peritoneal macrophages and their ability to produce hydrogen peroxide and nitric oxide, J. Neuroimmunol. 211 (2009) 56–65.
[44] D. Lorton, D.L. Bellinger, Molecular mechanisms underlying β-adrenergic receptor-mediated cross-talk between sympathetic neurons and immune cells, Int. J. Mol. Sci. 16 (2015) 5635–5665.
[45] A. Scanzano, M. Cosentino, Adrenergic regulation of innate immunity: a review, Front. Pharmacol. 6 (2015) 171.
[46] M.J. McGeachy, D.J. Cua, Th17 cell differentiation: the long and winding road, Immunity 28 (2008) 445–453.
[47] S. Capellino, K. Weber, M. Gelder, P. Härle, R.H. Straub, First appearance and location of catecholaminergic cells during experimental arthritis and elimination by chemical sympathectomy, Arthritis Rheum. 64 (2012) 1110–1118.
[48] M.H. Huang, D.S. Friend, M.E. Sunday, K. Singh, K. Haley, K.F. Austen, R.A. Kelly, T.W. Smith, An intrinsic adrenergic system in mammalian heart, J. Clin. Invest. 98 (1996) 1298–1303.
[49] M.H. Huang, J.J. Bahl, Y. Wu, F. Hu, D.F. Larson, W.R. Roeske, G.A. Ewy, Neuroendocrine properties of intrinsic cardiac adrenergic cells in fetal rat heart, Am. J. Physiol. Heart Circ. Physiol. 288 (2005) H497–H503.
[50] Y. Tamura, M. Sano, H. Nakamura, K. Ito, Y. Sato, K. Shinmura, M. Ieda, J. Fujita, H. Kurosawa, S. Ogawa, S. Nakano, M. Matsuzaki, K. Fukuda, Neural crest-derived resident cardiac cells contribute to the restoration of adrenergic function of transplanted heart in rodent, Cardiovasc. Res. 109 (2016) 350–357.
[51] Y. Liu, X. Rui, H. Shi, Y.H. Qiu, Y.P. Peng, Norepinephrine inhibits Th17 cells via β2-adrenergic receptor (β2-AR) signaling in a mouse model of rheumatoid arthritis, Med. Sci. Monit. 24 (2018) 1196–1204.

[52] M.C. Takenaka, L.P. Araujo, J.T. Maricato, V.M. Nascimento, M.G. Guereschi, R.M. Rezende, F.J. Quintana, A.S. Basso, Norepinephrine controls effector T cell differentiation through β2-adrenergic receptor-mediated inhibition of NF-κB and AP-1 in dendritic cells, J. Immunol. 196 (2016) 637–644.
[53] B.H. Nelson, IL-2, regulatory T cells, and tolerance, J. Immunol. 172 (2004) 3983–3988.
[54] D.S. Ramer-Quinn, M.A. Swanson, W.T. Lee, V.M. Sanders, Cytokine production by naïve and primary effector CD4+ T cells exposed to norepinephrine, Brain Behav. Immun. 14 (2000) 239–255.
[55] M.A. De León-Nava, K. Nava, G. Soldevila, L. López-Griego, J.R. Chávez-Ríos, J.A. Vargas-Villavicencio, J. Morales-Montor, Immune sexual dimorphism: effect of gonadal steroids on the expression of cytokines, sex steroid receptors, and lymphocyte proliferation, J. Steroid Biochem. Mol. Biol. 113 (2009) 57–64.
[56] H. Pinos, P. Collado, M. Rodrıguez-Zafra, C. Rodrıguez, S. Segovia, A. Guillamon, The development of sex differences in the locus coeruleus of the rat, Brain Res. Bull. 56 (2001) 73–78.
[57] N.R. Thanky, J.H. Son, A.E. Herbison, Sex differences in the regulation of tyrosine hydroxylase gene transcription by estrogen in the locus coeruleus of TH9-LacZ transgenic mice, Brain Res. Mol. Brain Res. 104 (2002) 220–226.
[58] K.L. Engler, M.L. Rudd, J.J. Ryan, J.K. Stewart, K. Fischer-Stenger, Autocrine actions of macrophage-derived catecholamines on interleukin-1 beta, J. Neuroimmunol. 160 (2005) 87–91.
[59] E. Hetier, J. Ayala, A. Bousseau, A. Prochiantz, Modulation of interleukin-1 and tumor necrosis factor expression by beta-adrenergic agonists in mouse ameboid microglial cells, Exp. Brain Res. 86 (1991) 407–413.
[60] J. Roewe, M. Higer, D.R. Riehl, A. Gericke, M.P. Radsak, M. Bosmann, Neuroendocrine Modulation of IL-27 in Macrophages, J. Immunol. 199 (2017) 2503–2514.
[61] E. Goyarts, M. Matsui, T. Mammone, A.M. Bender, J.A. Wagner, D. Maes, R.D. Granstein, Norepinephrine modulates human dendritic cell activation by altering cytokine release, Exp. Dermatol. 17 (2008) 188–196.
[62] L.E. Nijhuis, B.J. Olivier, S. Dhawan, F.W. Hilbers, L. Boon, M.C. Wolkers, J.N. Samsom, W.J. De Jonge, Adrenergic β2 receptor activation stimulates anti-inflammatory properties of dendritic cells in vitro, PLoS One 9 (2014) e85086.
[63] C.A. Izeboud, J.A. Mocking, M. Monshouwer, A.S. Van Miert, R.F. Witkamp, Participation of beta-adrenergic receptors on macrophages in modulation of LPS-induced cytokine release, J. Recept. Signal Transduct. Res. 19 (1999) 191–202.
[64] P. Panina-Bordignon, D. Mazzeo, P.D. Lucia, D. D'Ambrosio, R. Lang, L. Fabbri, C. Self, F. Sinigaglia, Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12, J. Clin. Invest. 100 (1997) 1513–1519.
[65] D. Khan, S.A. Ahmed, Regulation of IL-17 in autoimmune diseases by transcriptional factors and microRNAs, Front. Genet. 6 (2015) 236.
[66] H.-J. Park, H.-S. Park, J.-U. Lee, A.L.M. Boyhwell, J.-M. Choi, Sex-based selectivity of PPARγ regulation in Th1, Th2, and Th17 differentiation, Int. J. Mol. Sci. 8 (2016) 1347.
[67] N.J. Hill, M. Hultcrantz, N. Sarvetnick, M. Flodström-Tullberg, The target tissue in autoimmunity – an influential niche, Eur. J. Immunol. 37 (2007) 589–597.