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ORIGINAL ARTICLE

Noradrenaline modulates CD4+ T cell priming in rat experimental autoimmune encephalomyelitis: a role for the α_1-adrenoceptor

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Abstract

Pharmacological blockade of α_1-adrenoceptor is shown to influence development of experimental autoimmune encephalomyelitis (EAE), an IL-17-producing CD4+TCR+ (Th17) cell-mediated disease mimicking multiple sclerosis. Considering significance of CD4+ cell priming for the clinical outcome of EAE, the study examined α_1-adrenoceptor-mediated influence of catecholamines, particularly those derived from draining lymph node (dLN) cells (as catecholamine supply from nerve fibers decreases with the initiation of autoimmune diseases) for CD4+ cell priming. The results confirmed diminishing effect of immunization on nerve fiber-derived noradrenaline supply and showed that antigen presenting and CD4+ cells synthesize catecholamines, while antigen presenting cells and only CD4+CD25+Foxp3+ regulatory T cells (Tregs) express α_1-adrenoceptor. The analysis of influence of α_1-adrenoceptor antagonist prazosin on the myelin basic protein (MBP)-stimulated CD4+ lymphocytes in dLN cell culture showed their diminished proliferation in the presence of prazosin. This was consistent with prazosin enhancing effect on Treg frequency and their Foxp3 expression in these cultures. The latter was associated with upregulation of TGF-β expression. Additionally, prazosin decreased antigen presenting cell activation and affected their cytokine profile by diminishing the frequency of cells that produce Th17 polarizing cytokines (IL-1β and IL-23) and increasing that of IL-10-producing cells. Consistently, the frequency of all IL-17A+ cells and those co-expressing GM-CSF within CD4+ lymphocytes was decreased in prazosin-supplemented MBP-stimulated dLN cell cultures. Collectively, the results indicated that dLN cell-derived catecholamines may influence EAE development by modulating interactions between distinct subtypes of CD4+ T cells and antigen presenting cells through α_1-adrenoceptor and consequently CD4+ T cell priming.

Keywords Noradrenaline · α_1-Adrenoceptor · EAE · Tregs · Th17 · CD4+ lymphocyte proliferation

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a widely used model of multiple sclerosis. In most EAE models, autoreactive CD4+ T lymphocytes are actively induced by immunization with spinal cord tissue or myelin antigens in draining lymph nodes (dLNs) [1, 2]. These cells migrate to the central nervous system, where they recognize their cognate antigens, are reactivated, and start an inflammatory cascade leading to tissue injury [1, 2]. Myelin antigen-specific CD4+ T helper (Th)17 cells are involved in triggering the inflammatory cascade [2]. Their pathogenicity has been ascribed to the production of granulocyte–macrophage colony-stimulating factor (GM-CSF) [3, 4]. Thus, the development of EAE can be modulated by affecting the development of autoreactive CD4+ T cell response at either the dLN or the target organ [5–7].

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It has been suggested that dysregulation of β-adrenoceptor-mediated immunomodulatory action of noradrenaline, the main end point mediator of sympathetic nervous system, is involved in the development of not only multiple sclerosis but also rheumatoid arthritis, a CD4+ T cell–mediated autoimmune disease [8, 9]. Additionally, it has been shown that the density of noradrenaline-synthesizing ("noradrenergic") cells in the lymphoid target organ tissues increases with the development of collagen-induced arthritis, an experimental model of rheumatoid arthritis, most likely compensating for the loss of sympathetic nerve fibers [10–12]. However, to the best of our knowledge, there is no data on the influence of EAE development on "noradrenergic" immune cells in either the dLN or the target organ. However, pharmacological manipulations with β-adrenoceptor-mediated noradrenaline action have been shown to influence EAE development [13–15]. Our study on the effects of β-adrenoceptor blockade on CD4+ T cell proliferation and frequency of IL-17A+CD4+ cells in cultures of dLN cells isolated from Dark Agouti (DA) rats in the inductive phase of EAE suggested that sympathetic nerve/dLN cell-derived noradrenaline affects both CD4+ T cell proliferation and the frequency of IL-17A+CD4+ cells acting through α-adrenoceptor [16]. This is consistent with previous studies indicating that prazosin, α_1-adrenoceptor antagonist, suppresses the clinical and histological signs of EAE in Lewis rats [13, 17–19].

Bearing all aforementioned in mind, this study was undertaken to examine the following: (i) α_{1B}-adrenoceptor expression on dLN cells involved in the primary CD4+ T cell response and (ii) influence of α_1-adrenoceptor blockade on CD4+ lymphocyte proliferation and their IL-17A and GM-CSF synthesis in cultures of dLN cells recovered from DA rats in the inductive phase of EAE. Considering sexual dimorphism in CD4+ T cell response in dLNs and EAE development in DA rats, both female and male rats were included in this study [20].

Materials and methods

Experimental animals

Female (weighting between 150 and 160 g) and male (weighting between 200 and 225 g) 3-month-old DA rats from the breeding colony of the Immunology Research Centre "Branislav Janković" were used in the study. Rats were housed under a 12-h light/dark cycle, with controlled humidity and temperature conditions, free access to standard laboratory food and tap water. All experimental procedures and animal care were performed 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) and approved by the Animal Care and Use Committee of the Faculty of Pharmacy (permit number 6/12). The experiments complied with the ARRIVE guidelines for reporting animal research.

Induction of EAE

To induce EAE, the rats were inoculated with an intradermal application of an emulsion (100 μl) made from equal volumes of DA rat spinal cord homogenate in phosphate buffered saline (PBS) and complete Freund's adjuvant (CFA), containing 1 mg/ml of heat-killed and dried *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in the left hind paw, followed by a subcutaneous injection of 5×10^8 *Bordetella pertussis* (obtained from Institute of Virology, Vaccines, and Sera "Torlak," Belgrade, Serbia) saline suspension (250 μl) on the dorsum of the same paw. This immunization protocol leads to development of an acute monophasic disease in DA rats, followed by full recovery of all of the animals [20]. In order to minimize stress, pain, and injury, all animals were anesthetized before immunization with an intraperitoneal injection of 50 mg/kg body weight (BW) of ketamine (Ketamidor, Richter Pharma AG, Wels, Austria; 100 mg/ml) and 5 mg/kg BW of xylazine (Xylased, Bioveta, Ivanovice na Hané, Czech Republic; 20 mg/ml). Immunized rats were sacrificed in the preclinical phase of EAE, on the 7th day post immunization (d.p.i.), through transcardial perfusion preceded by deep anesthesia with an intraperitoneal injection of ketamine/xylazine anesthetizing cocktail (80 mg/kg BW/8 mg/kg BW). Experimental groups consisted of six rats, and experiments were repeated three times. Intact (non-immunized) animals were used as controls.

Antibodies and immunoconjugates

The following monoclonal antibodies (mAbs) were used for immunolabeling: fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-conjugated anti-CD4 (clone OX-38), FITC-conjugated anti-CD8 (clone OX-8), biotin-conjugated anti-CD134 (clone OX-40), biotin-conjugated anti-CD11b (clone WT.5), PE-conjugated anti-IL17A (clone TC11-18H10), PE-conjugated anti-IL-10 (clone A5–4), biotin-conjugated anti-CD86 (clone 24F), biotin-conjugated anti-CD80 (clone 3H5), and FITC-conjugated anti Ki-67 (clone B56). All of the mAbs were obtained from BD Biosciences Pharmingen (Mountain View, CA, USA). The second-step reagents, FITC-conjugated goat anti-rabbit IgG, PE-conjugated F (ab′)2 donkey anti-rabbit IgG, peridinin chlorophyll–protein (PerCP)-conjugated streptavidin, and isotype controls, were also purchased from BD Biosciences Pharmingen. Unconjugated goat polyclonal anti-α_{1B}-adrenoceptor (N-20) and PE-conjugated donkey anti-goat IgG Abs were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 647-conjugated anti-TCRαβ (clone R73) Ab was purchased from

BioLegend (San Diego, CA, USA). Alexa Fluor 647-conjugated anti-GM-CSF (clone 83308) and unconjugated rabbit polyclonal anti-IL-1β Abs were provided by Novus Biologicals (Littleton, CO, USA). Unconjugated rabbit polyclonal anti-tyrosine 3-monooxygenase (EC 1.14.16.2; Trivial name: tyrosine hydroxylase) Ab was obtained from EMD Millipore (Billerica, MA, USA) and FITC-conjugated rabbit anti-goat IgG Ab from Sigma-Aldrich Chemie GmbH. FITC-conjugated anti-CD11b (clone ED8) and PE-conjugated anti-OX-62 (clone OX-62) mAbs were supplied by Serotec (Oxford, UK). Unconjugated rabbit polyclonal anti-IL-23 and unconjugated rabbit polyclonal anti-PD-1 Abs were purchased from Bioss (Woburn, MA, USA). Foxp3 Staining Set, PerCP-eFluor710-conjugated anti-CD25 (clone OX39), and allophycocyanin (APC)-conjugated anti-CD4 (clone OX35) mAbs were purchased from eBioscience (San Diego, CA, USA). Biotin-conjugated anti-CD25 (clone OX-39) mAb was provided by Thermo Fisher (Rockford, IL, USA).

Isolation of mononuclear cells

Freshly isolated popliteal dLNs from immunized rats, and popliteal and inguinal LNs from non-immunized rats were weighed, and tissue pieces were sampled for high performance liquid chromatography (HPLC) analysis or passed through a 70-μm nylon cell strainer (BD Biosciences, Erembodegem, Belgium) in ice-cold PBS supplemented with 2% fetal calf serum (FCS, Gibco, Grand Island, NY, USA) in order to obtain single-cell mononuclear cell suspensions. Cells were counted in 0.2% trypan blue solution using an improved Neubauer hemacytometer.

Magnetic activated cell sorting (MACS) of CD4+CD25- dLN cells

A two-step procedure was used to purify conventional CD4+ CD25− lymphocytes. In the first step, dLN mononuclear cells were incubated with biotin-conjugated anti-CD11b mAb (BD Biosciences Pharmingen) for 30 min at 4 °C, washed in MACS buffer (degassed PBS with 0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid), and magnetically labeled with a cocktail of rat CD8a (clone G28, Miltenyi Biotec), rat CD45RA (clone OX-33), and anti-biotin microbeads (Miltenyi Biotec) for 15 min at 4 °C. Following another wash in MACS buffer, dLN cells were loaded onto LS column (Miltenyi Biotec) placed in the magnetic field of the Quadro MACS separator (Miltenyi Biotec) for negative selection. Flow cytometry analysis showed that the unlabeled cell fraction contained 90–95% CD4+ cells. In the second step, these cells were indirectly labeled using biotin-conjugated anti-CD25 mAb (Thermo Fisher) and anti-biotin microbeads (Miltenyi Biotec) and subjected to further negative selection. On average, CD4+CD25− fraction displayed 85% purity.

Cultivation of dLN cells for cell proliferation and cytokine production analyses

Mononuclear cells from dLNs were cultured in complete RPMI 1640 culture medium (Sigma-Aldrich Chemie GmbH) without or with 2.5 μg/ml of concanavalin A (ConA, Sigma-Aldrich Chemie GmbH) or 20 μg/ml myelin basic protein (MBP, Sigma-Aldrich Chemie GmbH) in a 5% CO_2 humidified air atmosphere at 37 °C for 72 h. The culture medium contained 10% heat-inactivated FCS, 200 mM L-glutamine (Serva, Heidelberg, Germany), 100 IU/ml penicillin (ICN, Costa Mesa, CA, USA), 100 μg/ml streptomycin (ICN), 50 μM β-mercaptoethanol, and 100 μM ascorbic acid. Prior to stimulation with ConA or MBP, the cells were preincubated at 37 °C in a 5% CO_2 humidified air atmosphere with α_1-adrenoceptor antagonist prazosin (prazosin hydrochloride, Sigma-Aldrich Chemie GmbH; 10^{-5} M) for 15 min and/or 10^{-6} M of arterenol ((±)-noradrenaline (+)-bitartrate salt; Sigma-Aldrich Chemie GmbH) for 1 h. When both antagonist and agonist were used, prazosin was added 15 min prior to arterenol. The dose of prazosin was chosen based on previous studies, indicating that prazosin at this concentration is effective in modulating immune cell function [21]. Arterenol dosage was based on the facts that (i) noradrenaline concentration in direct vicinity of lymphocytes in secondary lymphoid organs is on the order of 0.3–3 mM, and (ii) its supraphysiological concentrations (i.e., ≥ 10^{-6} M) are required for functional changes in immune cells in vitro [22]. Cells from dLN cell cultures were processed for cell cycle and/or reverse transcription–quantitative real-time PCR (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 presence of 3 μg/ml of brefeldin A (eBioscience) or in its absence, in a 5% CO_2 humidified atmosphere for 4 h at 37 °C. Cells from brefeldin-supplemented cultures were analyzed for intracellular cytokine immunostaining. Cell-free culture supernatants were used for measuring the concentration of cytokines by ELISA.

MACS-sorted CD4+CD25− cells were cultured in complete RPMI 1640 culture medium in a 5% CO_2 humidified air atmosphere at 37 °C for 72 h, as described above. The cells were grown in the medium alone or stimulated with plate bound anti-CD3 mAb (NA/LE, clone G4.18, BD Biosciences Pharmingen; 1 μg/ml) and soluble anti-CD28 mAb (NA/LE, clone JJ319, BD Biosciences Pharmingen; 1 μg/ml), in the absence or in the presence of 10^{-5} M of prazosin. Prior to stimulation with anti-CD28 mAb, the cells were preincubated with prazosin for 15 min, as described above. Following the cultivation, the cells were harvested for intracellular Ki-67 immunostaining to assess cell proliferation.

Noradrenaline measurement

For HPLC analysis, aliquots of 1×10^7 (d)LN cells were pelleted and resuspended in 300 μl of DEPROT solution, containing 2% ethylene glycol tetraacetic acid (Sigma-Aldrich), 0.1 N $HClO_4$ (Sigma-Aldrich) and 0.2% $MgCl_2$ (Sigma-Aldrich), while (d)LNs were homogenized in DEPROT (1 mg of tissue: 40 μl of DEPROT) using an ultra-turrax homogenizer. After sonication (3 × 10 s) and centrifugation (30 min, 18.000 rpm, + 4 °C), the collected supernatants (20 μl for the tissue and 50 μl for the cells) were placed in the autosampler of a Dionex UltiMate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA). The HPLC system consisted of degasser unit, binary pump, autosampler, column compartment, and RS electrochemical detector equipped with a glassy carbon working electrode. A Hibar 125-4 LiCrospher100 RP-18 (5 μm) HPLC column (Merck Millipore, Darmstadt, Germany) was used. Instrument control and data acquisition were performed with the Chromeleon7 Chromatography Data System (Thermo Scientific).

The mobile phase consisted of 98% ammonium formate buffer (Fisher Scientific, Cambridge, UK, 100 mM, pH 3.6) and 2% methanol (J.T.Baker, Griesheim, Germany). To obtain the ammonium formate buffer, ammonium formate was dissolved in purified water and the pH was adjusted to a value of 3.6 with formic acid (49–51%, Fluka, Buchs, Switzerland). The mobile phase was pumped at a flow rate of 500 μl/min, the applied potential for electrochemical measurements was + 850 mV, and the separation temperature was set at 25 °C. Stock standard solution (1 mg/ml) of noradrenaline (DL-noradrenaline hydrochloride; Sigma-Aldrich) was prepared in methanol and kept at − 20 °C. Standard solutions in concentration range of 0.5–25 μg/ml were made by diluting the stock standard solution in DEPROT.

RT-qPCR

Cell samples were collected using Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA, USA) and immediately stored at − 70 °C until RNA purification. For extraction of total RNA, ABI Prism 6100 Nucleic Acid PrepStation system (Applied Biosystems) and Total RNA Chemistry Starter Kit (Applied Biosystems) were used. DNAse (Absolute RNA Wash Solution, Applied Biosystems) treatment was included to prevent genomic DNA contamination, as confirmed using no-reverse transcription controls. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), with a 20-μl reaction volume under the following thermal cycler conditions: 10 min at 25 °C, 120 min at 37 °C and 5 s at 85 °C. RT-qPCR reactions (25 μl, run in triplicate) contained 1× TaqMan Gene Expression Master Mix with Uracil-DNA glycosylase (UDG) (Applied Biosystems), 1× mix of premade primer and hydrolysis probe sets (TaqMan Gene Expression Assays, Applied Biosystems), and 5 μl of cDNA template. They were carried out using Applied Biosystems 7500 Real-Time PCR System under preoptimized conditions: 2 min at 50 °C (UDG incubation), 10 min at 95 °C (AmpliTaq Gold DNA Polymerase activation), and 40 cycles involving 15 s at 95 °C (template denaturation) and 1 min at 60 °C (primer annealing/extension). The TaqMan Gene Expression Assays (Applied Biosystems) used in this study are indicated in Table 1. Target mRNA levels were quantified with SDS v1.4.0. software (Applied Biosystems), using the comparative threshold cycle (dCt) method and β-actin as a reference gene. Relative amounts of target mRNA were expressed as 2^{-dCt} values, showing the target to reference gene ratio, where dCt = Ct target − Ct reference.

Immunostaining and flow cytometry analysis

For surface antigen immunostaining, cell aliquots were incubated with saturating concentrations of either fluorochrome-labeled mAbs or biotin-conjugated/unconjugated Abs. Appropriate 20% normal serum in PBS containing 0.1% NaN_3 was added to the cells (15 min, room temperature) prior to anti-α_{1B}-adrenoceptor Ab labeling. After the incubation with biotin-conjugated/unconjugated Abs, the cells were washed in PBS supplemented with 2% FCS and 0.1% NaN_3 and the appropriate second-step reagents were used. For intracellular antigen immunostaining, cells were fixed/permeabilized overnight at 4 °C prior to their incubation with Abs, using the fixation/permeabilization buffer kit, following the manufacturer's instructions (eBioscience; http://www.ebioscience.com/resources/best-protocols/flow-cytometry-protocols.htm). All of the Ab incubations were performed for 30 min in the dark at 4 °C, except for the intracellular cytokine content assessment and Ki-67 immunostaining (room temperature).

7-Aminoactinomycin D (7-AAD; BD Biosciences Pharmingen) staining of DNA was used to identify the proliferating lymphocytes among cultured dLN cells. After surface antigen staining and overnight fixation/permeabilization using 70% ethanol at 4 °C, the cells were incubated with 7-AAD at 4 °C for 30 min.

Samples (50,000–100,000 events) were acquired on a FACSCalibur or FACSVerse flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed for the frequency of marker positive cells or used for cell cycle analysis (according to the Dean–Jet–Fox model) with FlowJo software version 7.8. (TreeStar Inc., Ashland, OR, USA). To settle gating boundaries, fluorescence minus one gating controls were used, except for markers exhibiting clear bimodal staining [23].

Table 1 Summary of mRNA targets and reference gene for RT-qPCR analysis

	Symbol	Gene name	Accession No.[a]	Assay ID	Size (bp)
1	Foxp3	Forkhead box P3	NM_001108250.1	Rn01525092_m1	72
2	Tgfb1	Transforming growth factor, beta 1	NM_021578.2	Rn00572010_m1	65
3	Rorc	RAR-related orphan receptor C	XM_006232926.2	Rn01261022_m1	54
4	Il1b	Interleukin 1 beta	NM_031512.2	Rn99999009_m1	100
5	Il23a	Interleukin 23, alpha subunit p19	NM_130410.2	Rn00590334_g1	72
6	Il10	Interleukin 10	NM_012854.2	Rn00563409_m1	70
7	Actb	Actin, beta	NM_031144.3	Rn00667869_m1	91

[a] RefSeq: NCBI Reference Sequence Database

ELISA

Commercial kits for IL-17A (BioLegend; 8 pg/ml detection limit), GM-CSF (Elabscience Biotechnology Co., Ltd., Wuhan, China; 9.375 pg/ml detection limit), and TGF-β (Invitrogen, Waltham, MA, USA; 31.25 pg/ml detection limit) were used to assess the cytokine concentrations in dLN cell culture supernatatnts, according to the manufacturers' instructions.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism Version software (San Diego, CA, USA) by analysis of variance (ANOVA). Analyses were performed using two-way (sex × treatment) ANOVA, if not stated otherwise. A post hoc analysis was made by the Bonferroni multiple comparisons test. The data are presented as mean ± SEM; p values < 0.05 were considered statistically significant.

Results

The influence of immunization on noradrenaline content in dLN cells

On the 7th d.p.i. for EAE, dLN cells from female and male DA rats, which have previously been shown to exhibit different magnitudes of the primary (auto)reactive CD4+ T cell response after immunization with spinal cord in CFA [20], were retrieved to examine α_1-adrenoceptor-mediated noradrenaline immunomodulatory autocrine/paracrine action.

The concentration of noradrenaline in dLN tissue diminished in female ($p < 0.05$) and male ($p < 0.001$) rats following immunization (Fig. 1a). However, noradrenaline levels were higher ($p < 0.001$) in male compared with female rats in (d)LN tissue from both immunized (EAE rats) and non-immunized rats (controls) (Fig. 1a).

Irrespective of sex, the overall noradrenaline content in dLN cells increased ($p < 0.001$) in the response to immunization (Fig. 1b). Additionally, it remained greater ($p < 0.001$) in male compared with female rats (Fig. 1b). The analysis of noradrenaline content in cells recovered from mg (d)LN tissue showed that it was markedly lower in both control (approx. 20-fold and 14-fold in female and male rats, respectively) and EAE rats (approx. 5-fold and 4-fold in female and male rats, respectively) than noradrenaline content per mg whole (d)LN tissue. Thus, it seems clear that, in accordance with the previous studies [24], the main source of noradrenaline in (d)LNs was not immune cells, but nerve fibers. Additionally, the obtained findings indicated that, irrespective of sex, immunization for EAE had the opposite effect on noradrenaline level in whole dLN tissue and cells, but that the latter could not fully compensate for the immunization-induced decrease in nerve noradrenaline supply.

Next, CD4+ T cells, a key driver of EAE pathogenesis [2, 25], were analyzed for the expression of tyrosine hydroxylase, the key

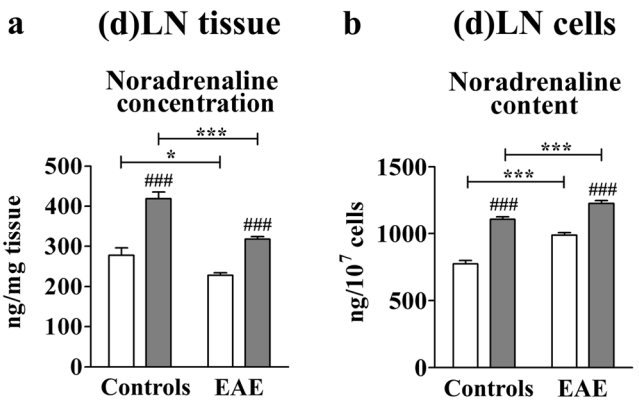


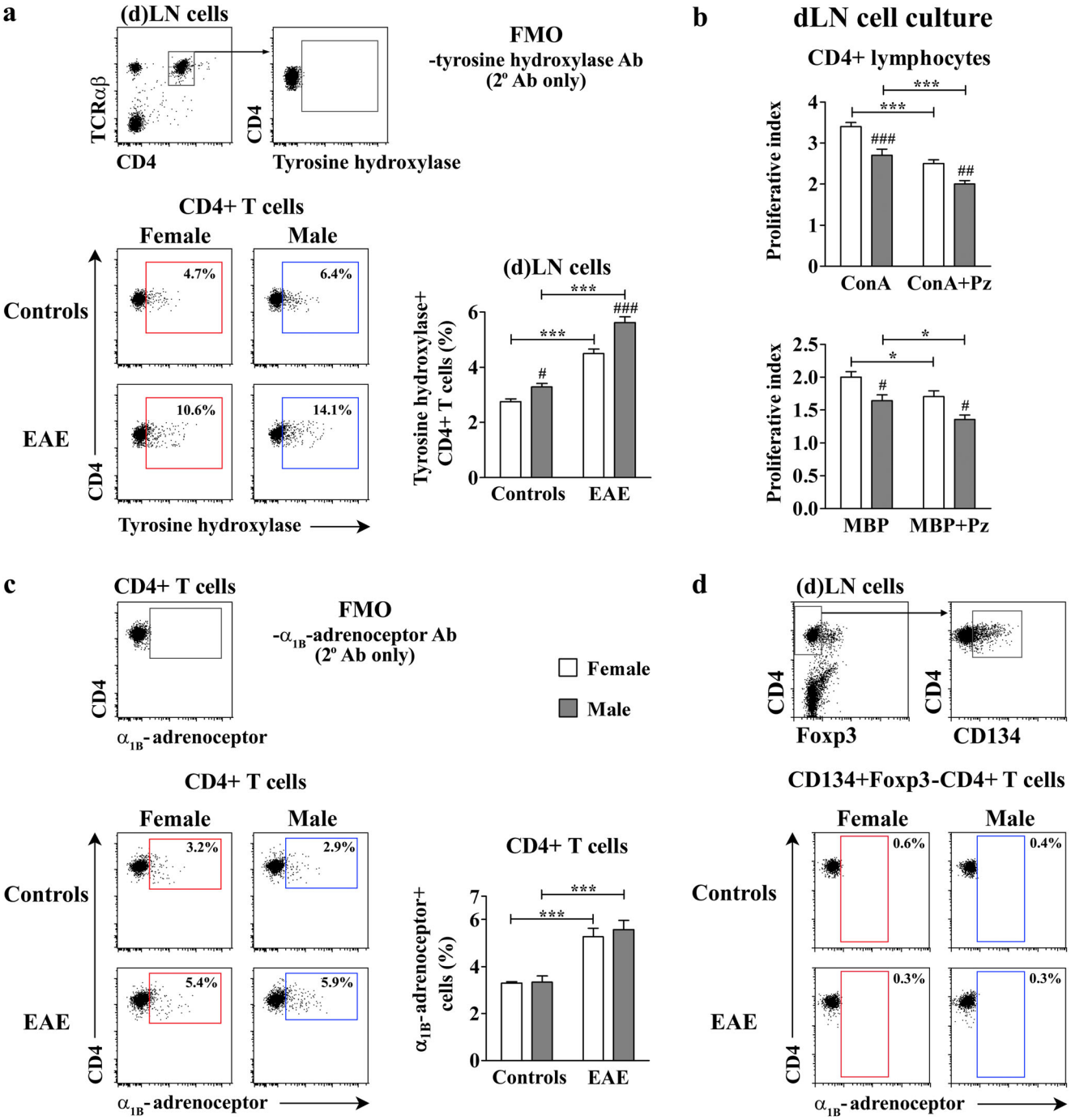
Fig. 1 Opposing effects of immunization for EAE on noradrenaline concentration in (d)LN tissue and noradrenaline content in (d)LN cells. Bar graphs indicate **a** noradrenaline concentrations in (d)LN tissue and **b** noradrenaline content in (d)LN cells retrieved on the 7th d.p.i. from male and female rats immunized for EAE (EAE rats) and non-immunized sex-matched control rats (controls). Two-way ANOVA showed significant interactions between the effects of sex and immunization for the (d)LN cell NA content ($F_{(1,20)} = 7.62$; $p < 0.05$). The data are shown as mean ± SEM ($n = 6$). $^{*}p < 0.05$; $^{***}p < 0.001$; $^{\#\#\#}p < 0.001$. Number sign, vs female

rate-limiting enzyme in catecholamine biosynthesis [26] using flow cytometry. The results indicated that, irrespective of sex, the increase in (d)LN cell-derived noradrenaline with immunization at least partly could be ascribed to the rise ($p < 0.001$) in the frequency of CD4+ T cells immunoreactive for tyrosine hydroxylase (tyrosine hydroxylase+) (Fig. 2a). Similarly, the greater noradrenaline content in male rat (d)LN cells from both EAE rats and controls reflected the higher ($p < 0.05$) frequency of tyrosine hydroxylase+ CD4+ T cells compared with female rats (Fig. 2a).

α_1-Adrenoceptor blockade diminished CD4+ lymphocyte proliferation in dLN cell cultures

Prazosin affected cells influencing CD4+ lymphocyte proliferation

Next, considering that (i) lymphocytes in culture retain the capacity to produce noradrenaline [27], (ii) noradrenaline can influence lymphocyte proliferation in an autocrine/



paracrine manner [28, 29], and (iii) α_1-adrenoceptor stimulation affects proliferation of many cell types [30–32], the influence of prazosin (an α_1-adrenoceptor blocker) on ConA- and MBP-stimulated CD4+ lymphocyte proliferation in cultures of dLN cells retrieved on the 7th d.p.i. was examined using flow cytometry (Fig. 2b; Online Resource 1a). The analysis of proliferative index indicating alterations in proliferation of CD4+ cells in dLN cell cultures stimulated with mitogen/MBP in the presence of prazosin or in its absence in respect to their proliferation in control cultures without cognate stimulus showed that in dLN cell cultures from rats of both sexes, ConA and MBP augmented CD4+ cell proliferation (Fig. 2b). Additionally, this analysis indicated that prazosin in dLN cells cultures stimulated with either ConA ($p < 0.001$) or MBP ($p < 0.05$) diminished the proliferation of CD4+ cells (Fig. 2b). Moreover, it is noteworthy that, irrespective of prazosin presence, the proliferation was statistically significantly less in dLN cell cultures from male rats (Fig. 2b).

To additionally confirm the specificity of prazosin action on CD4+ lymphocytes, the proliferation of CD4+ cells from dLN cell cultures stimulated with MBP in the presence of arterenol and/or prazosin was also investigated (Online Resource 1b). The results showed that arterenol in dLN cell cultures from rats of both sexes augmented CD4+ cell proliferation in respect to that in dLN cell cultures from sex-matched rats supplemented with MBP alone (Online Resource 1b). On the other hand, the addition of prazosin and arterenol or prazosin alone to dLN cell cultures from rats of both sexes diminished their proliferation to the levels below those measured in the presence of MBP alone (Online Resource 1b). This could be explained taking conjointly herein presented data indicating noradrenaline production in dLN cells with our previous data indicating that arterenol decreases CD4+ cell proliferation in dLN cell cultures through β-adrenoceptor [16].

To assess whether noradrenaline affected CD4+ lymphocyte proliferation by acting directly on CD4+ T lymphocytes, α_1-adrenoceptor expression on CD4+ T cells was examined. We focused on α_{1B}-adrenoceptor expression, given that the α_{1B}-adrenoceptor subtype is suggested to be involved in the stimulation of cell proliferation, which is different from other subtypes of this adrenoceptor type [33]. The analysis of α_{1B}-adrenoceptor expression on LN CD4+ T lymphocyte subpopulation showed the presence of the α_{1B}-adrenoceptor on these cells in controls of both sexes (Fig. 2c). Their frequency was comparable between female and male controls (Fig. 2c). Irrespective of sex, α_{1B}-adrenoceptor+ cell frequency increased ($p < 0.001$) with immunization among dLN CD4+ T lymphocytes, and this increase was comparable between sexes (Fig. 2c). Given that α_1-adrenoceptor expression is shown to change with activation [34], we explored the frequency of α_{1B}-adrenoceptor positive cells among conventional/effector CD134+Foxp3−CD4+ T lymphocytes [35]. We failed to detect α_{1B}-adrenoceptor expression on the surface of CD134+Foxp3−CD4+ T lymphocytes from either controls or EAE rats (Fig. 2d). Accordingly, we hypothesized that prazosin indirectly influenced CD4+ cell proliferation. To confirm this hypothesis, the influence of prazosin on in vitro proliferation of conventional CD4+ cells (separated using MACS) stimulated with anti-CD3/CD28 mAbs was examined. The results showed that prazosin did not influence the proliferation of conventional dLN CD4+ cells from either male or female (data not shown) rats upon stimulation with anti-CD3/CD28 mAbs (Online Resource 2). Thus, the findings supported our hypothesis and opened the question on the mechanism/s involved in prazosin modulation of CD4+ T cell response. Considering that CD25+Foxp3+CD4+ T regulatory cells (Tregs) and antigen presenting cells could affect the proliferation of CD4+ conventional/effector T cells in culture [36–39], they were considered to be the possible targets of prazosin's indirect effect on CD4+ cell proliferation.

◀ **Fig. 2** α_1-Adrenoceptor blockade diminished overall CD4+ lymphocyte proliferation in dLN cell cultures from EAE rats. **a** Representative flow cytometry dot plot panel indicates the expression of tyrosine hydroxylase in (d)LN CD4+TCRαβ+ (T) cells retrieved on the 7th d.p.i. from (left) female and (right) male rats immunized for EAE (EAE rats) and non-immunized sex-matched control rats (controls). Fluorescence minus one (FMO) control without primary anti-tyrosine hydroxylase antibody (-tyrosine hydroxylase Ab; secondary antibody only) was used (right flow cytometry dot plot) to set the cutoff boundary for tyrosine hydroxylase expression analysis in CD4+ T cells, gated as shown in left flow cytometry dot plot. Bar graph indicates the frequency of tyrosine hydroxylase+ CD4+ T cells among all (d)LN cells from EAE rats and controls of both sexes. The frequency of tyrosine hydroxylase+CD4+ T cells among all (d)LN cells was determined using FlowJo software version 7.8. (TreeStar Inc., Ashland, OR, USA). **b** Bar graphs indicate the fold increase in the percentage of proliferating cells (cells in S+G2/M phases of the cell cycle) among CD4+ lymphocytes from 72-h dLN cell cultures of female and male EAE rats following stimulation with (upper) ConA or (lower) MBP in the absence or in the presence of 10^{-5} M of prazosin (Pz) over the corresponding values in cultures with medium alone. The fold increase is expressed as proliferative index (ratio between the frequency of proliferating cells from stimulated cultures and the frequency of proliferating cells in cultures with medium alone, as determined by 7-AAD staining, see Online Resource 1a). **c**, **d** Representative flow cytometry dot plot panels indicate the expression of α_{1B}-adrenoceptor on **c** CD4+ T cells and **d** CD134+Foxp3−CD4+ T cells (gated as shown in the corresponding upper flow cytometry dot plots) retrieved on the 7th d.p.i. from (left) female and (right) male EAE rats and controls of both sexes. **c** Fluorescence minus one (FMO) control without primary anti-α_{1B}-adrenoceptor antibody (-α_{1B}-adrenoceptor Ab; secondary antibody only) was used to set the cutoff boundary for α_{1B}-adrenoceptor expression analysis in CD4+ T cells, gated as shown in **a**. Bar graph indicates the frequency of α_{1B}-adrenoceptor+ cells within (d)LN CD4+ T cells of EAE rats and controls of both sexes. Two-way ANOVA showed significant interactions between the effects of sex and immunization for the frequency of tyrosine hydroxylase+ CD4+ T cells ($F_{(1,20)} = 4.47$; $p < 0.05$) in all (d)LN cells. The data are shown as mean ± SEM ($n = 6$). $^{*}p < 0.05$; $^{***}p < 0.001$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. Number sign, vs female. Ab antibody, 2° secondary

Prazosin increased Treg frequency in dLN cell cultures by elevating TGF-β concentration

Contrary to activated conventional/effector CD134+Foxp3− CD4+ T lymphocytes, Tregs were found to express α_{1B}-adrenoceptor at a detectable level (Fig. 3a). Immunization increased ($p < 0.001$) the frequency of α_{1B}-adrenoceptor+ cells among Tregs, which were shown to be equally represented in dLNs from female and male EAE rats [20] (Fig. 3a). In both controls ($p < 0.01$) and EAE rats ($p < 0.001$), the frequency of α_{1B}-adrenoceptor+ cells among Tregs was lower in male than female rats (Fig. 3a). Additionally, given that human and mouse Tregs constitutively express tyrosine hydroxylase [40, 41], Tregs fom controls and EAE rats were also examined for tyrosine hydroxylase expression. Immunoreactive tyrosine hydroxylase was found in Tregs from controls of both sexes (Fig. 3b). Immunization increased ($p < 0.001$) the frequency of tyrosine hydroxylase+ Tregs irrespective of sex (Fig. 3b), suggesting that noradrenaline may influence Tregs by acting in an autocrine manner. This increase was particularly prominent in male rats (Fig. 3b). Consequently, the frequency of tyrosine hydroxylase+ cells was higher ($p < 0.001$) in EAE rats among Tregs from male rats when compared with their female counterparts (Fig. 3b). Considering these previous findings, the influence of prazosin on Treg frequency was examined. The frequency of Tregs among CD4+ lymphocytes was higher ($p < 0.001$) in MBP-stimulated cultures than in sex-matched control cultures (Fig. 4a), and prazosin increased ($p < 0.001$) the frequency of Tregs in MBP-stimulated dLN cell cultures from both female and male rats (Fig. 4a). Irrespective of the presence of prazosin, the frequency of Tregs was comparable in MBP-stimulated dLN cell cultures from female and male rats (Fig. 4a). To support the previous findings, the expression of mRNA for Foxp3 in cells from

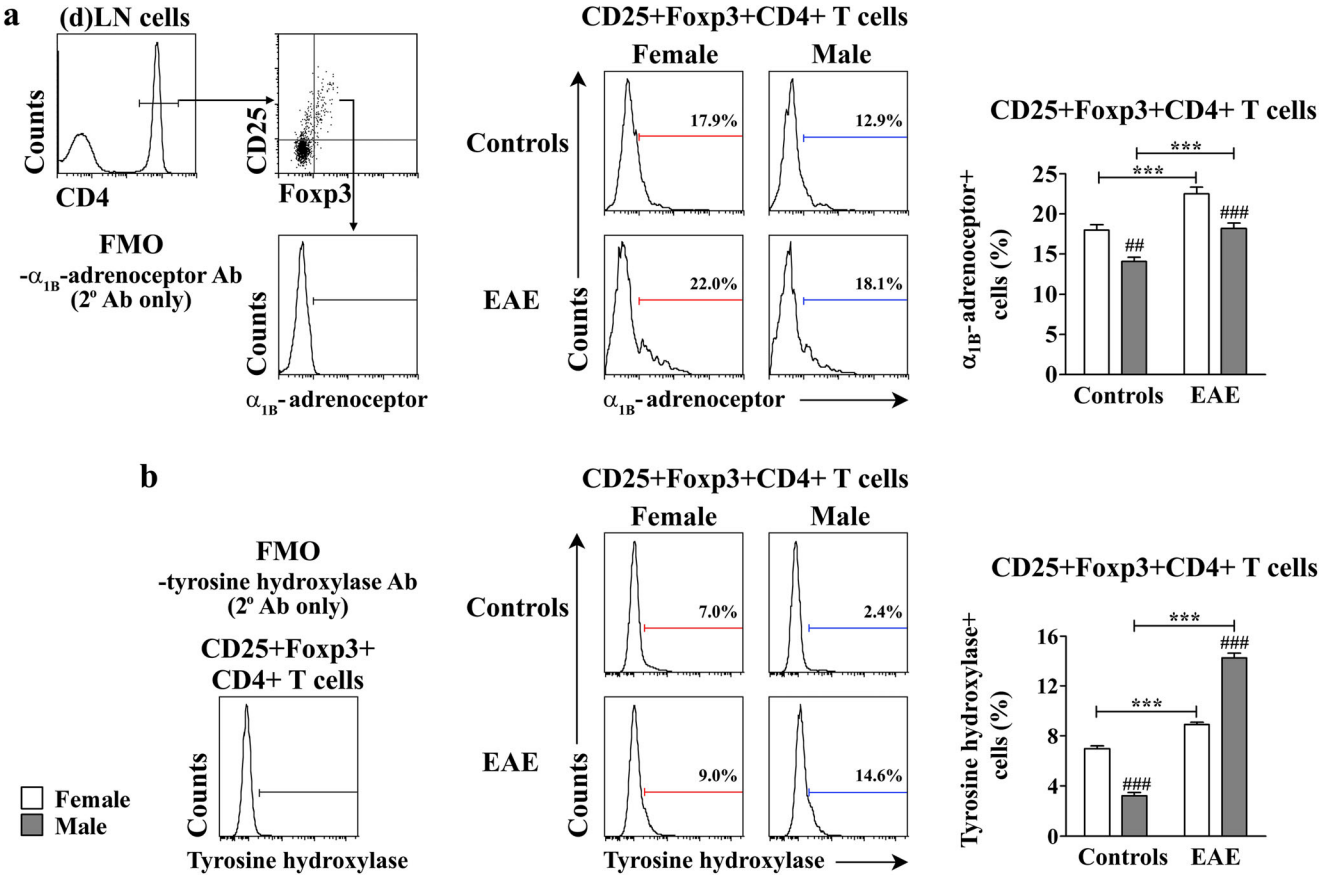


Fig. 3 Influence of immunization for EAE on α_{1B}-adrenoceptor and tyrosine hydroxylase expression on/in (d)LN CD25+Foxp3+CD4+ cells. Representative flow cytometry histogram panels indicate the expression of **a** α_{1B}-adrenoceptor and **b** tyrosine hydroxylase on/in (d)LN CD25+Foxp3+CD4+ cells (Tregs) retrieved on the 7th d.p.i from (left) female and (right) male rats immunized for EAE (EAE rats) and non-immunized sex-matched control animals (controls). Fluorescence minus one (FMO) controls without primary **a** anti-α_{1B}-adrenoceptor and **b** anti-tyrosine hydroxylase antibodies (secondary antibodies only) were used to set the cutoff boundaries for analyses of **a** α_{1B}-adrenoceptor and **b** tyrosine hydroxylase expression on/in CD25+Foxp3+CD4+ T cells, gated as shown in (**a**, left panel, upper flow cytometry profiles). Bar graphs indicate the frequency of **a** α_{1B}-adrenoceptor+ and **b** tyrosine hydroxylase+ cells within (d)LN CD25+Foxp3+CD4+ T cells of EAE rats and controls of both sexes. Two-way ANOVA showed significant interactions between the effects of sex and immunization for the frequency of tyrosine hydroxylase+ cells within CD25+Foxp3+CD4+ T cells ($F_{(1,20)} = 402.80$; $p < 0.001$). The data are shown as mean ± SEM ($n = 6$). $^{***}p < 0.001$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. Number sign, vs female. Ab antibody, 2° secondary

dLN cell cultures was also examined. The expression of Foxp3 mRNA was higher ($p < 0.05$) in dLN cells from MBP-stimulated cultures than in sex-matched control cultures (Fig. 4b). However, Foxp3 expression was comparable between female and male rat dLN cells in both control and MBP-stimulated cultures (Fig. 4b). Prazosin enhanced ($p < 0.001$) Foxp3 mRNA expression in MBP-stimulated dLN cells (Fig. 4b), and the expression of Foxp3 mRNA was similar in MBP-stimulated cells from male and female rat dLN cell cultures irrespective of antagonist presence (Fig. 4b).

Considering that noradrenaline can influence TGF-β production and subsequent Treg generation [42], the expression of TGF-β at the protein and mRNA level in dLN cell cultures was examined. Irrespective of sex, the presence of MBP increased ($p < 0.001$) TGF-β concentrations in dLN cell culture supernatants (Fig. 4c). As in control cultures, TGF-β concentration was similar in male and female MBP-stimulated cultures (Fig. 4c). In dLN cell cultures from rats of both sexes, prazosin increased ($p < 0.001$ in female and $p < 0.01$ in male) concentrations of TGF-β (Fig. 4c). Thus, the concentration of this cytokine was comparable in female and male rat dLN cell cultures supplemented with MBP and prazosin (Fig. 4c). Furthermore, the pattern of sex- and stimulation-related differences in TGF-β mRNA expression was similar to that observed in the concentration of TGF-β protein found in cells from dLN cell cultures (Fig. 4c).

In addition, to get an insight into the functional capacity of Tregs, the expression of programmed death (PD)-1 molecule, an important immune-inhibitory receptor for Treg regulatory function in an autoimmune setting [43], was investigated. The frequency of PD-1+ cells among Tregs was comparable between control dLN cell cultures from male and female rats (Fig. 4d). In rats of both sexes, stimulation with MBP enhanced ($p < 0.001$) the frequency of PD-1+ cells among Tregs (Fig. 4d). However, prazosin did not affect the frequency of PD-1+ cells within Tregs (Fig. 4d). Consequently, irrespective of the presence of prazosin, the frequency of PD-1+ cells was comparable among Tregs in MBP-stimulated dLN cell cultures from male and female rats (Fig. 4d). Thus, it is assumed that prazosin affects CD4+ lymphocyte proliferation by affecting the frequency of Tregs in dLN cell cultures.

Prazosin diminished the frequency of activated/matured CD11b+ antigen presenting cells in dLN cell cultures

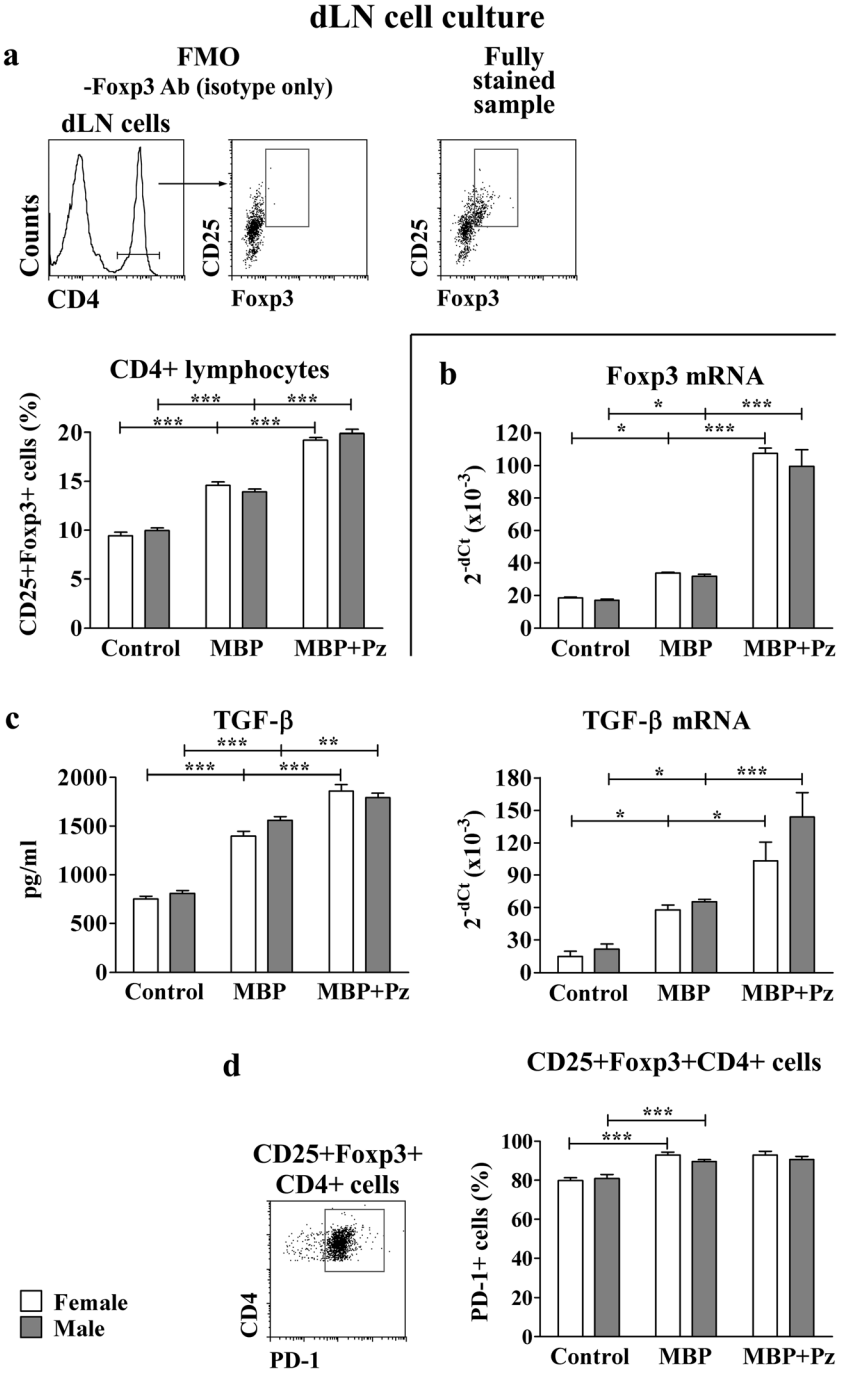
Following immunization, the frequency of α_{1B}-adrenoceptor+ cells among dLN CD11b+ cells, whose frequency was shown to be lower in dLNs from male rats [20], increased ($p < 0.001$) in rats of both sexes when compared with sex-matched controls (Fig. 5a). Additionally, their frequency among CD11b+ cells was comparable between dLNs from male and female EAE rats as in LNs from controls (Fig. 5a). Moreover, the same pattern of immunization-induced differences showed the frequency of α_{1B}-adrenoceptor+ cells among conventional myeloid dendritic cells (OX62+), which are shown to be highly efficient in priming and polarizing encephalitogenic CD4+ T lymphocytes in EAE models [44] (Fig. 5b). Considering that innate immune cells are also shown to synthesize noradrenaline [45], the expression of tyrosine hydroxylase in innate immune antigen presenting CD11b+ cells from controls and EAE rats in the preclinical phase of the disease was examined. Irrespective of sex, CD11b+ cells from LNs of both controls and EAE rats expressed tyrosine hydroxylase (Fig. 5c). Their frequency among dLN CD11b+ cells from EAE rats of both sexes was lower ($p < 0.001$) when compared with sex-matched controls (Fig. 5c). However, the frequency of tyrosine hydroxylase+ cells among CD11b+ cells was comparable in dLNs from male and female EAE rats, as in LNs from controls (Fig. 5c).

Based on our previous findings, it was assumed that noradrenaline might also affect CD4+ cell proliferation by acting on antigen presenting cells. To test this assumption, the expression of activational/maturational markers on dLN CD11b+ cells from dLN cell cultures was examined. When stimulated with MBP, the frequencies of CD80+ and CD86+ cells among CD11b+ cells in dLN cell cultures from rats of both sexes were increased ($p < 0.001$) when compared with control cultures from sex-matched rats (Fig. 6). Their frequency was comparable in both control and MBP-stimulated dLN cell cultures from males when compared with the corresponding cultures from female rats (Fig. 6). Prazosin diminished ($p < 0.001$) the frequency of CD80+ and CD86+ cells among dLN CD11b+ cells in MBP-stimulated cultures from rats of both sexes (Fig. 6). However, irrespective of prazosin presence, the frequency of activated/matured cells (judging by expression of co-stimulatory molecules) was comparable between MBP-stimulated cultures from male and female rats (Fig. 6). Moreover, prazosin did not change the frequency of MHC II+ cells among dLN CD11b+ cells from MBP-stimulated dLN cell cultures in rats of both sexes (data not shown).

α_1-Adrenoceptor blockade diminished the frequency of IL-17A+ and GM-CSF+ cells among CD4+ cells from dLN cell cultures

Prazosin diminished the frequency of all IL-17A+ and GM-CSF+ CD4+ T cells and that of highly pathogenic IL-17A+GM-CSF+ CD4+ T cells

Given that IL-17A-producing cells have a main role in the EAE model used in this study [25], α_1-adrenoceptor-mediated influence of dLN cell-derived noradrenaline on the frequency of IL-17A+ cells among CD4+ lymphocytes in cultures of dLN cells isolated in the preclinical phase of EAE was



examined. Their frequency increased ($p < 0.001$) in the presence of MBP in dLN cell cultures from rats of both sexes when compared to control cultures from sex-matched rats (Fig. 7a). However, their frequency was lower ($p < 0.001$) in MBP-stimulated cultures from male rats as in the control cultures (Fig. 7a). The frequency of IL-17A+ cells among CD4+ lymphocytes diminished ($p < 0.001$) in MBP-stimulated dLN cell cultures in the presence of prazosin when compared with sex-matched MBP-stimulated cultures without prazosin (Fig. 7a). Additionally, their frequency was lower in the co-presence of prazosin and MBP ($p < 0.001$) in the cultures from male compared to female rats (Fig. 7a). Additionally, the concentration of IL-17A in MBP-stimulated dLN cell culture supernatants exhibited a similar pattern of changes to that observed in the frequency of IL-17A+ cells (Fig. 7b).

To corroborate the previous findings, the expression of mRNA for RORγt (the Th17-related transcription factor) in cells from dLN cell cultures was examined. In the presence of MBP, RORγt mRNA expression increased ($p < 0.001$) in dLN cell cultures from rats of both sexes compared with control

◀ **Fig. 4** Influence of α_1-adrenoceptor blockade on Treg frequency in dLN cell cultures from EAE rats on the 7th d.p.i. **a** Bar graph indicates the frequency of CD25+Foxp3+ cells among CD4+ lymphocytes from 72-h dLN cell cultures of female and male rats immunized for EAE (EAE rats), in the absence of cognate stimuli (control) or following stimulation with MBP in the absence or in the presence of 10^{-5} M of prazosin (Pz) ("Materials and methods"). Right flow cytometry dot plot (fully stained sample) indicates the gating strategy for CD25+Foxp3+ cells within CD4+ lymphocytes, gated as shown in flow cytometry histogram. Fluorescence minus one (FMO) control incubated with isotype-matched control instead of anti-Foxp3 antibody (-Foxp3 Ab) was used to set the cutoff boundary for Foxp3 expression analysis. **b** Bar graph indicates the relative expression of Foxp3 mRNA in 72-h dLN cell cultures of female and male EAE rats, following stimulation with MBP in the absence or in the presence of Pz, as determined by RT-qPCR. **c** Bar graphs indicate (left) supernatant concentration of TGF-β and (right) the relative expression of TGF-β mRNA in 72-h dLN cell cultures of female and male EAE rats, following stimulation with MBP in the absence or in the presence of Pz, as determined by ELISA and RT-qPCR, respectively. Results of RT-qPCR are represented as 2^{-dCt} relative to β-actin. **d** Bar graph indicates the frequency of PD-1+ cells among CD25+Foxp3+CD4+ lymphocytes from 72-h dLN cell cultures of female and male EAE rats, following stimulation with MBP in the absence or in the presence of Pz. Flow cytometry dot plot indicates the gating strategy for PD-1+ cells within CD25+Foxp3+CD4+ cells, gated as shown in **a**. Two-way ANOVA showed significant interactions between the effects of sex and culturing conditions for the frequency of CD25+Foxp3+cells within CD4+ lymphocytes ($F_{(2,30)} = 6.27$; $p < 0.01$). The data are shown as mean ± SEM ($n = 6$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Ab antibody

cultures from sex-matched rats (Fig. 7c). RORγt expression was lower ($p < 0.001$) in MBP-stimulated cultures from males compared with those from female rats, similar to the control cultures (Fig. 7c). Prazosin diminished RORγt mRNA expression in MBP-stimulated female ($p < 0.001$) and male ($p < 0.01$) dLN cells (Fig. 7c). However, prazosin did not influence the sex difference in RORγt mRNA expression observed in dLN cells stimulated with MBP only (Fig. 7c). Thus, irrespective of prazosin presence, RORγt mRNA was downregulated ($p < 0.001$) in MBP-stimulated dLN cells from male compared with female rats (Fig. 7c).

Given that the pathogenic potential of autoreactive Th17 cells in mouse and rat EAE models is ascribed to their production of GM-CSF [3, 4, 46], we investigated the α_1-adrenoceptor-mediated influence of dLN cell-derived noradrenaline on the frequency of GM-CSF+ cells within Th17 cells. The frequency of GM-CSF+ cells among IL-17A+ CD4+ T cells and GM-CSF+IL-17A+ cells among CD4+ T cells was higher ($p < 0.001$) in MBP-stimulated dLN cell cultures when compared with sex-matched control cultures (Fig. 7d). However, in both control ($p < 0.05$) and MBP-stimulated ($p < 0.001$) cultures from male rats, the frequencies of these cells were lower than in the corresponding cultures from female rats (Fig. 7d). It the co-presence of MBP and prazosin, the frequency of GM-CSF+ cells among IL-17A+ CD4+ cells and that of GM-CSF+IL-17A+ cells among CD4+ cells diminished ($p < 0.001$) when compared with sex-matched cultures stimulated with MBP only (Fig. 7d). Prazosin did not affect sex bias in their frequency observed in the cultures stimulated with MBP only (Fig. 7d).

Next, the frequency of all GM-CSF+ cells among CD4+ lymphocytes in dLN cell cultures was examined. Stimulation with MBP increased ($p < 0.001$) the frequencies of GM-CSF+ cells among CD4+ cells in dLN cell cultures from rats of both sexes when compared with those from control cultures from sex-matched rats (Fig. 8a). The presence of prazosin diminished ($p < 0.01$ in female and $p < 0.001$ in male rats) the frequency of GM-CSF+ cells among CD4+ lymphocytes in MBP-stimulated dLN cell cultures from rats of both sexes (Fig. 8a). Irrespective of the presence of prazosin, their frequency was lower ($p < 0.05$) in MBP-stimulated dLN cell cultures from male compared with female rats (Fig. 8a). The diminishing action of prazosin on the frequency of GM-CSF-producing cells was confirmed by measuring the concentration of GM-CSF in the culture supernatants. Namely, the pattern of differences in the concentrations of GM-CSF in the supernatants from dLN cell cultures was similar to that observed in the frequency of GM-CSF+ cells among CD4+ T lymphocytes from the corresponding cultures (Fig. 8b).

Prazosin affected the expression of cytokines influencing the polarization of Th17 and GM-CSF-producing CD4+ T cells

Next, we explored the frequencies of cells expressing the key cytokines (IL-1β and IL-23) driving/stabilizing Th17 phenotype and promoting Th17 cell expansion [47] among CD11b+ cells from dLN cell cultures and the expression levels of mRNA for IL-1β and IL-23/p19 in cells from these cultures. In MBP-stimulated cultures from rats of both sexes, the frequencies of IL-1β+ and IL-23+ cells within CD11b+ cells were higher ($p < 0.001$) than in corresponding control cultures from sex-matched rats (Fig. 9a, b). In both control and MBP-stimulated cultures, the frequencies of IL-1β+ and IL-23+ cells were lower ($p < 0.001$) within CD11b+ dLN cells from male compared with female rats (Fig. 9a, b). Prazosin diminished their frequencies ($p < 0.001$ for IL-1β+ and $p < 0.01$ for IL-23+ cells) among dLN CD11b+ cells in MBP-stimulated cultures, so that they remained lower ($p < 0.001$) in cells from male rat dLN cell cultures (Fig. 9a, b). The expression of IL-1β and IL-23/p19 mRNA in cells from dLN cell cultures exhibited a similar pattern of differences to that observed in the frequencies of the corresponding cytokine-producing cells (Fig. 9a, b).

Stimulation with MBP increased the frequency of IL-10+ cells within dLN CD11b+ cells ($p < 0.001$) and IL-10 mRNA expression ($p < 0.05$) in cells from dLN cell cultures from rats of both sexes (Fig. 9c). Moreover, as in the control cultures, the frequency of IL-10+ cells among CD11b+ cells ($p < 0.05$) and IL-10 mRNA expression ($p < 0.001$) in cells was higher in MBP-stimulated cultures from male when compared to female

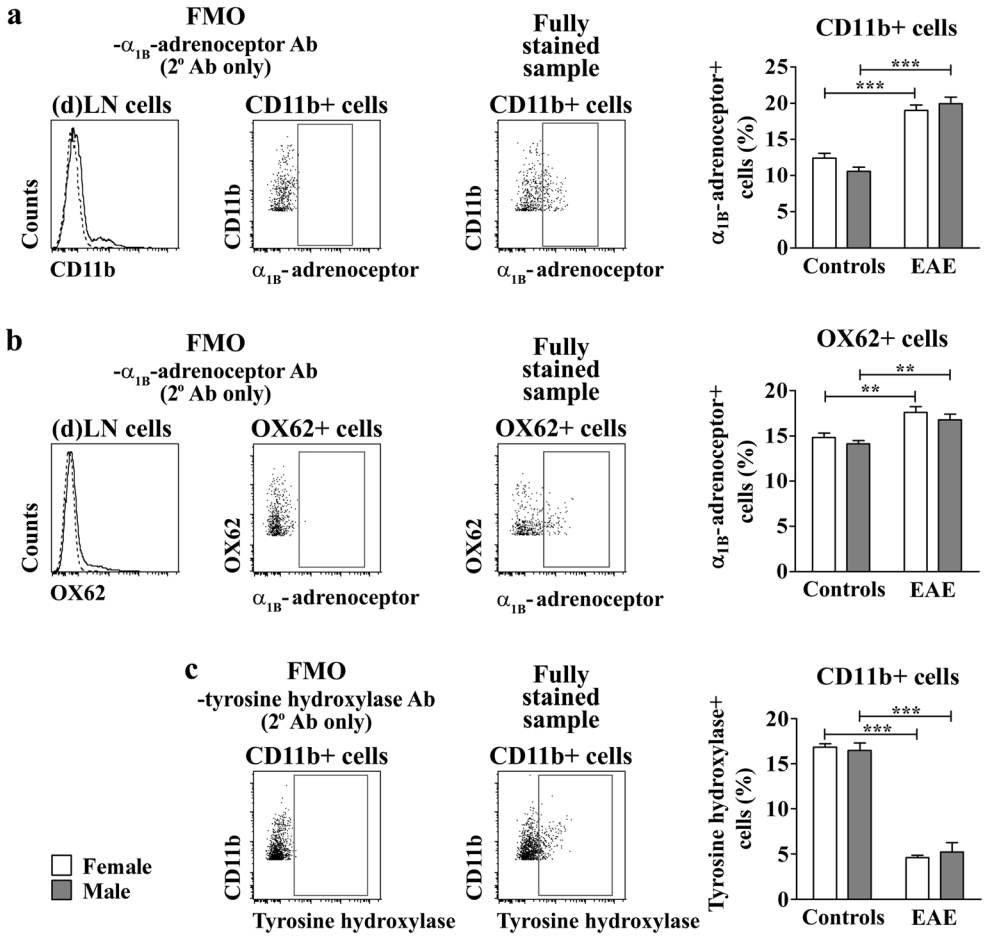


Fig. 5 Influence of immunization for EAE on α_{1B}-adrenoceptor and tyrosine hydroxylase expression on/in rat (d)LN antigen presenting cells. Bar graphs indicate the frequency of α_{1B}-adrenoceptor+ cells among **a** CD11b+ antigen presenting cells and **b** OX62+ conventional myeloid dendritic cells and **c** the frequency of tyrosine hydroxylase+ cells among CD11b+ cells retrieved on the 7th d.p.i. from female and male rats immunized for EAE (EAE rats) and sex-matched non-immunized control rats (controls). Fluorescence minus one (FMO) controls without primary **a**, **b** anti-α_{1B}-adrenoceptor or **c** anti-tyrosine hydroxylase antibodies (secondary antibodies only) were used to set the cutoff boundaries for analyses of **a**, **b** α_{1B}-adrenoceptor or **c** tyrosine hydroxylase expression on/in **a**, **c** CD11b+ and/or **b** OX62+ cells in fully stained samples. **a** CD11b+ and **b** OX62+ cells were gated as shown in overlaid flow cytometry histograms. Two-way ANOVA showed significant interactions between the effects of sex and immunization for the frequency of α_{1B}-adrenoceptor+ cells within CD11b+ (d)LN cells ($F_{(1,20)} = 5.19$; $p < 0.05$). The data are shown as mean ± SEM ($n = 6$). $^{**}p < 0.01$; $^{***}p < 0.001$. Ab antibody, 2° secondary

rats (Fig. 9c). Prazosin, in MBP-stimulated dLN cell cultures, contrary to its influence on the expression of polarizing cytokines, increased ($p < 0.001$) the frequency of IL-10+ cells among CD11b+ cells and augmented ($p < 0.001$) IL-10 mRNA expression in cultivated cells (Fig. 9c). However, irrespective of the presence of an α_1-adrenoceptor antagonist, the frequency of IL-10+ cells among CD11b+ cells ($p < 0.05$) and IL-10 mRNA expression ($p < 0.001$) in cells was higher in dLN cell cultures from male compared with female rats (Fig. 9c).

Discussion

The study showed that in EAE, as in experimental models of some other autoimmune diseases [10–12], noradrenaline synthesis increases in dLN cells most likely allowing for local compensation of the decrease in its release from nerve fibers. Moreover, it showed that this reflected increase in the frequency of tyrosine hydroxylase+ "noradrenergic" cells among CD4+ T cells, including Tregs. In favor of these findings is tyrosine hydroxylase expression in human and mouse Tregs [40, 41]. On the other hand, the frequency of "noradrenergic" cells among CD11b+ cells (encompassing macrophages) diminished with immunization. This disparity is consistent with specific regulation of tyrosine hydroxylase expression in macrophages with respect to other types of immune cells [48].

The results from our study confirmed that noradrenaline derived from "noradrenergic" cells augmented CD4+ lymphocyte proliferation and Th17 cell frequency in MBP-stimulated dLN cell cultures from EAE rats of both sexes [16].

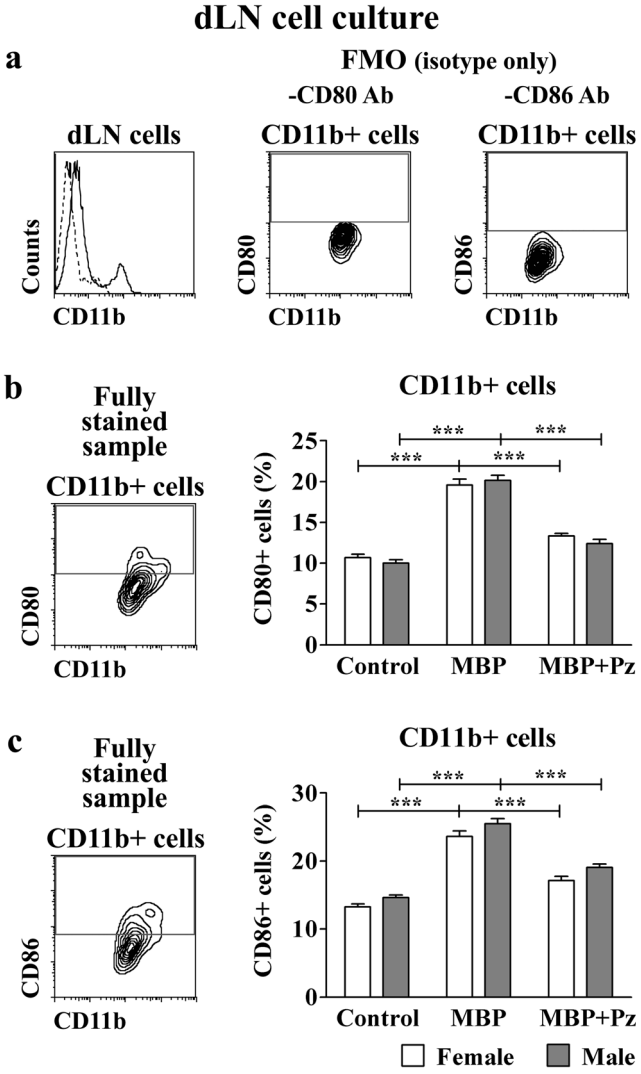


Fig. 6 α_1-Adrenoceptor blockade diminished the expression of co-stimulatory molecules on CD11b+ cells in dLN cell cultures from EAE rats on the 7th d.p.i. Bar graphs indicate the frequency of **b** CD80+ cells and **c** CD86+ cells among CD11b+ cells from 72-h dLN cell cultures of female and male rats immunized for EAE (EAE rats), in the absence of cognate stimuli (control) or following stimulation with MBP in the absence or in the presence of 10^{-5} M of prazosin (Pz) ("Materials and methods"). **a** Fluorescence minus one (FMO) controls incubated with isotype-matched controls instead of (left) CD80 (-CD80 Ab) or (right) CD86 (-CD86 Ab) antibodies were used to set the cutoff boundaries for analyses of CD80 or CD86 expression on CD11b+ cells in fully stained samples. CD11b+ cells were gated as shown in overlaid flow cytometry histogram. The data are shown as mean ± SEM ($n = 6$). ***$p < 0.001$. Ab antibody

Given that we failed to detect the expression of the α_{1B}-adrenoceptor on activated conventional CD4+ T lymphocytes, it may be assumed that noradrenaline does not affect their proliferation directly. The lack of prazosin effects on anti-CD3/CD28 mAb-stimulated proliferation of conventional CD4+ cells (separated using MACS) supported the previous assumption. However, consistent with previous findings showing α_1-adrenoceptor expression on unfractionated rat LN CD4+ T lymphocytes [49], α_{1B}-adrenoceptor was detected on Tregs. In accordance with data indicating the upregulation of α_{1B}-adrenoceptor expression when lymphocytes are actively involved in immune response [34], the frequency of α_{1B}-adrenoceptor+ cells among Tregs was higher in dLN from EAE rats compared with sex-matched controls. Thus, it may be hypothesized that prazosin diminished MBP-stimulated CD4+ lymphocyte proliferation indirectly, acting on Tregs. Indeed, prazosin increased the frequency of Tregs and the expression of mRNA for Foxp3 in MPB-stimulated dLN cell cultures from rats of both sexes. These changes correlated with increase in TGF-β levels. To corroborate these findings are data indicating that chemical sympathectomy in mice inhibited the development of EAE by increasing the TGF-β level and consequently the frequency and number of Tregs in the spleen and dLNs [42]. Additionally, it was shown that noradrenaline affects the production of TGF-β, the cytokine which helps the conversion of naïve T cells into functional Tregs and induces expansion of Tregs [50, 51], in some non-immune cells [52].

In accordance with previous studies, α_{1B}-adrenoceptor expression was found not only on Tregs but also on antigen presenting cells. To support this finding are data showing the expression of the α_{1B}-adrenoceptor on the monocytic cell line THP-1 [34], human and rodent macrophages [53, 54], and mouse bone marrow-derived dendritic cells [55]. The expression of the α_{1B}-adrenoceptor is upregulated on LPS-stimulated monocytes and on peripheral blood mononuclear cells during the development of inflammatory disease [34, 56]. Thus, it may be supposed that prazosin augmented induction/expansion of Tregs in dLN cell cultures by enhancing autocrine/paracrine TGF-β action. The effects of TGF-β could be potentiated by the increased concentration of IL-10 in prazosin-supplemented dLN cell cultures [57]. The lack of sex specificity in prazosin effect on Treg frequency may be associated with contrasting differences in the frequency of α_{1B}-adrenoceptor- and tyrosine hydroxylase-synthesizing cells among dLN Tregs from female and male EAE rats and the lack of sex-based differences in the frequency of either α_{1B}-adrenoceptor+ cells or tyrosine hydroxylase+ cells among CD11b+ dLN cells from EAE rats. Additionally, consistent with previous studies indicating that chemical sympathectomy affects the frequency of Tregs in lymphoid tissues, but not their regulatory capacity at single-cell level [42], we failed to observe any significant changes in PD-1 expression on Tregs from prazosin-supplemented MBP-stimulated dLN cell cultures when compared with the corresponding cultures supplemented with MBP only.

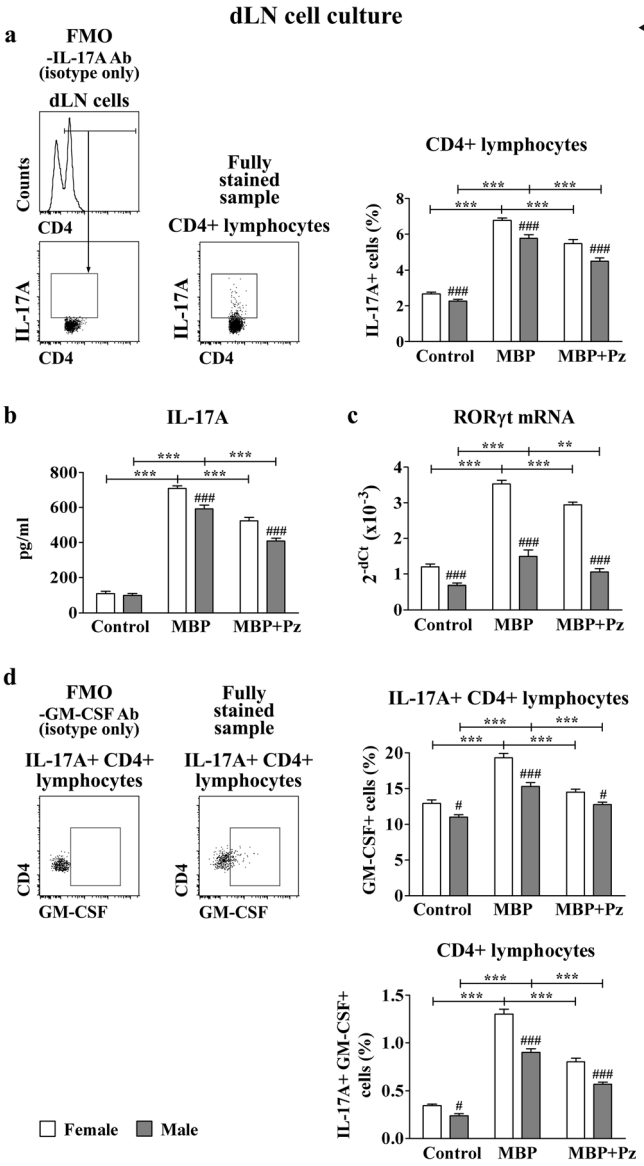
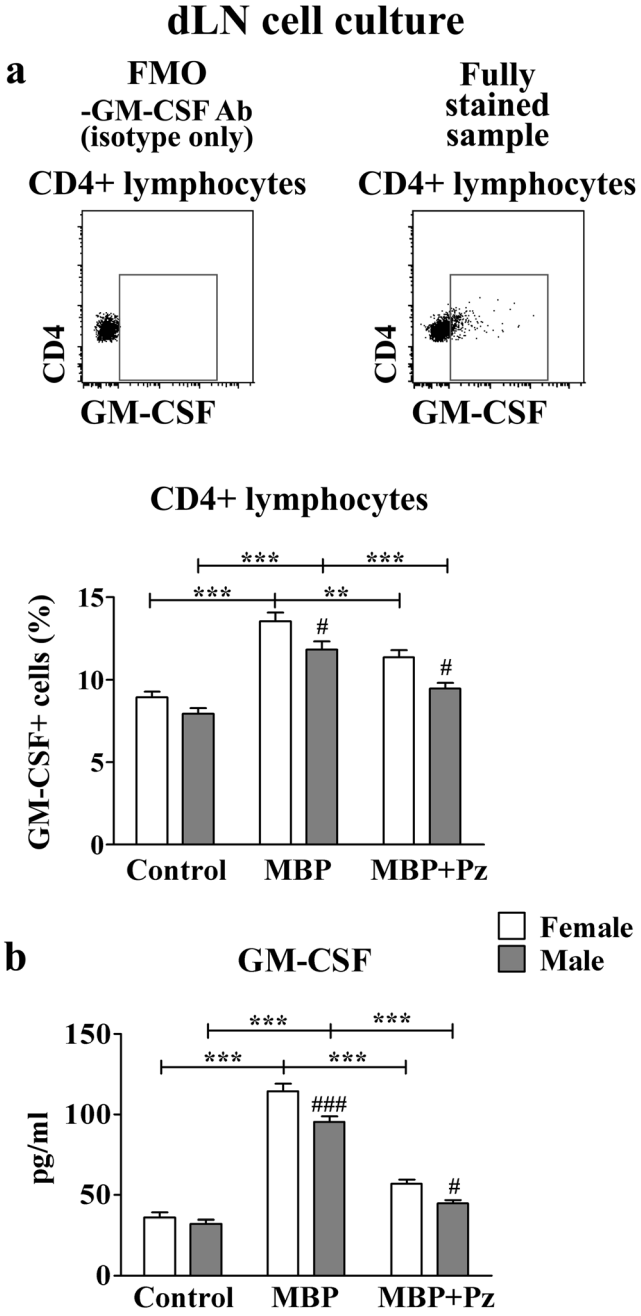


Fig. 7 α_1-Adrenoceptor blockade diminished the frequency of all IL-17A+ cells and IL-17A+GM-CSF+ cells among CD4+ lymphocytes in dLN cell cultures from EAE rats on the 7th d.p.i. **a** Bar graph indicates the frequency of IL-17A+ cells within CD4+ lymphocytes from 72-h dLN cell cultures of female and male rats immunized for EAE (EAE rats), in the absence of cognate stimuli (control) or following stimulation with MBP in the absence or in the presence of 10^{-5} M of prazosin (Pz) ("Materials and methods"). Fluorescence minus one (FMO) control incubated with isotype-matched control instead of anti-IL-17A antibody (-IL-17A Ab) was used to set the cutoff boundary for analysis of IL-17A expression in CD4+ lymphocytes in fully stained samples. CD4+ lymphocytes were gated as shown in flow cytometry histogram. **b**, **c** Bar graphs indicate **b** supernatant concentrations of IL-17A and **c** the relative expression of RORγt mRNA in 72-h dLN cell cultures of female and male EAE rats, in the absence of cognate stimuli (control) or following stimulation with MBP in the absence or in the presence of Pz, as determined by ELISA and RT-qPCR, respectively. Results of RT-qPCR are represented as 2^{-dCt} relative to β-actin. **d** Bar graphs indicate the frequency of (upper) GM-CSF+ cells among IL-17A+CD4+ lymphocytes and (lower) IL-17A+GM-CSF+ cells among CD4+ lymphocytes from 72-h dLN cell cultures of female and male EAE rats, following stimulation with MBP in the absence or in the presence of Pz. Fluorescence minus one (FMO) control incubated with isotype-matched control instead of anti-GM-CSF antibody (−GM-CSF Ab) was used to set the cutoff boundary for analysis of GM-CSF expression in IL-17A+CD4+ lymphocytes in fully stained samples. IL-17A+CD4+ lymphocytes were gated as shown in **a**. Two-way ANOVA showed significant interactions between the effects of sex and culturing conditions for the concentrations of IL-17A ($F_{(2,30)} = 9.50$; $p < 0.001$), the expression of RORγt mRNA ($F_{(2,30)} = 43.76$; $p < 0.001$), the frequency of GM-CSF+ cells within IL-17A+ CD4+ lymphocytes ($F_{(2,30)} = 4.07$; $p < 0.05$), and IL-17A+GM-CSF+ cells within CD4+lymphocytes ($F_{(2,30)} = 10.05$; $p < 0.001$). The data are shown as mean ± SEM ($n = 6$). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$. Number sign, vs female. Ab antibody

Next, given that prazosin diminished the frequency of CD80/CD86-expressing cells among CD11b+ cells in dLN cell cultures from rats of both sexes and that a decline in CD80/CD86 expression was associated with a decreased capacity of antigen presenting cells to stimulate T cell expansion [37, 39], it may be assumed that this prazosin effect also contributed to the diminished proliferation of CD4+ lymphocytes in MBP-stimulated dLN cell cultures. Considering that noradrenaline does not affect the expression of CD80/CD86 on antigen presenting cells [58], downregulation of their expression in prazosin-supplemented MBP-stimulated dLN cell cultures could be linked with findings indicating that Tregs affect co-stimulatory molecule expression through direct contact with dendritic cells [59, 60]. Moreover, given that IL-10 inhibits T cell proliferation in response to mitogen [61], lower CD4+ lymphocyte proliferation in prazosin-supplemented MBP-stimulated dLN cell cultures could also be ascribed to the higher concentration of IL-10 when compared to cultures without prazosin. The extra rise in IL-10 concentration in MBP-stimulated cultures when supplemented with prazosin is consistent with previous findings showing that prazosin additionally increases LPS-induced immune cell secretion of IL-10 [62].

Prazosin also reduced RORγt expression, the frequency of IL-17A+ CD4+ T cells and the production of IL-17A in MBP-stimulated cultures from rats of both sexes. This is fully consistent with the decrease in frequency of IL-1β- and IL-23-expressing cells among CD11b+ cells and the amount of IL-1β and IL-23/p19 transcripts in the cells from prazosin-supplemented cultures, as well as increase in their TGF-β expression [63]. To corroborate our findings are data that: (i) phenylephrine, a selective α_1-adrenoceptor agonist, augmented LPS-induced IL-1β production in human monocytes and monocyte-derived macrophages [64] and (ii) prazosin reduced LPS-stimulated plasma levels of IL-1 [62]. Additionally, the



◀ **Fig. 8** α_1-Adrenoceptor blockade diminished the frequency of GM-CSF+ cells among CD4+ lymphocytes in dLN cell cultures from EAE rats on the 7th d.p.i. **a** Bar graph indicates the frequency of GM-CSF+ cells among CD4+ lymphocytes from 72-h dLN cell cultures of female and male rats immunized for EAE (EAE rats), in the absence of cognate stimuli (control) or following stimulation with MBP in the absence or in the presence of 10^{-5} M of prazosin (Pz) ("Materials and methods"). Fluorescence minus one (FMO) control incubated with isotype-matched control instead of anti-GM-CSF antibody (-GM-CSF Ab) was used to set the cutoff boundary for analysis of GM-CSF expression in CD4+ lymphocytes in fully stained samples. CD4+ lymphocytes were gated as shown in Fig. 7a. **b** Bar graph indicates the concentrations of GM-CSF in 72-h dLN cell culture supernatants of female and male EAE rats, following stimulation with MBP in the absence or in the presence of Pz, as determined by ELISA. Two-way ANOVA showed significant interactions between the effects of sex and culturing conditions for the concentration of GM-CSF ($F_{(2,30)} = 3.61$; $p < 0.05$). The data are shown as mean ± SEM ($n = 6$). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$. Number sign, vs female. Ab antibody

diminished frequency of IL-17A+ cells among CD4+ lymphocytes from prazosin-supplemented MBP-stimulated dLN cell cultures compared with MBP-stimulated cultures without prazosin could also reflect elevated concentrations of IL-10, as an essential facilitator of Treg-mediated suppression of Th17 cell response [65, 66].

Furthermore, in accordance with data indicating that IL-23 promotes GM-CSF expression in Th17 cells whereas TGF-β inhibits its expression [3, 4, 67], the frequency of cells co-producing IL-17A and GM-CSF was reduced in the co-presence of prazosin and MBP compared with MBP alone. To diminished frequency of IL-17A+GM-CSF+ CD4+ T cells in prazosin-supplemented MBP-stimulated cultures could also contribute the downregulation of IL-1β, as myeloid cell-derived IL-1β is important for the expansion/boosting of GM-CSF+ Th17 cells rather than Th17 cell differentiation in murine secondary lymphoid organs during CD4 T cell priming [68]. Finally, in accordance with data indicating that TGF-β1 diminishes GM-CSF production in CD4+ T cells [69], the frequency of all GM-CSF+ cells comprising not only GM-CSF-producing Th17 cells but also several other CD4+ T cell subsets [46] was diminished in the co-presence of prazosin and MBP in dLN cell cultures compared to MBP alone.

In conclusion, the study revealed that noradrenaline may influence the magnitude and cytokine profile of dLN CD4+ T cell response through α_1-adrenoceptor acting in autocrine/paracrine manner. Accordingly, in dLN cell cultures, the blockade of α_1-adrenoceptor diminished CD4+ cell proliferation and shifted Treg/Th17 cell balance toward Tregs, and the effects were ascribed to the rises in TGF-β and IL-10 production. Additionally, it impaired the generation of highly pathogenic IL-17A+GM-CSF+ Th17 cells in dLN cell cultures by diminishing the frequency of IL-23+ and IL-1β+ CD11b+ cells. These findings are consistent with data indicating that prazosin ameliorates in EAE rats the clinical outcome of the disease [13]. The study also suggested that sexual dimorphism in the primary CD4+ T cell response in dLNs [20] is not linked with α_1-adrenoceptor-mediated noradrenaline immunomodulation. Thus, the study as a whole suggests a detrimental immunomodulatory α_1-adrenoceptor-mediated role for noradrenaline from dLN cells in the early phase of EAE development. This could be important for defining the immunomodulatory potential of

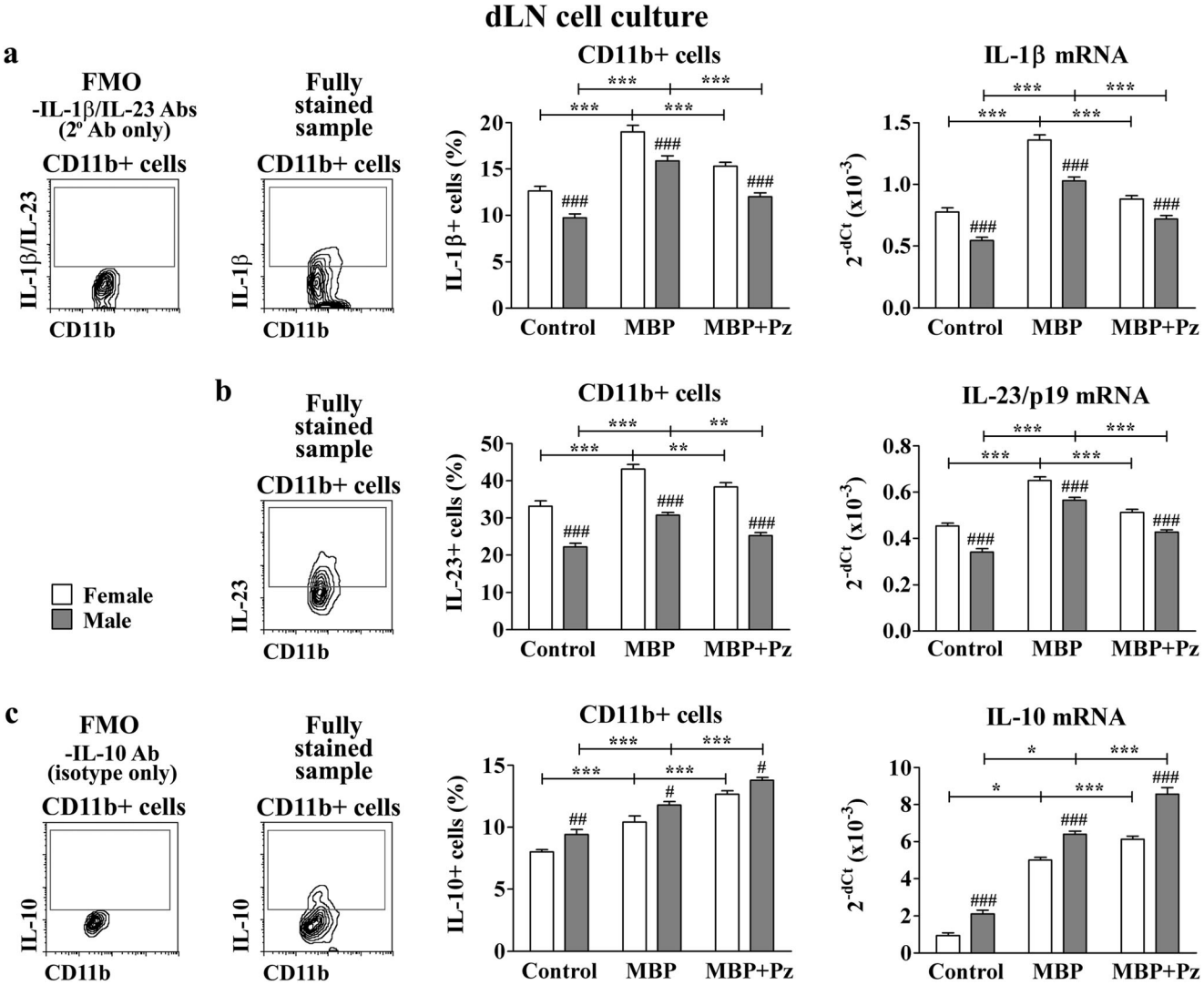


Fig. 9 Influence of α_1-adrenoceptor blockade on the expression of polarizing/immunoregulatory cytokines in dLN cell cultures from EAE rats on the 7th d.p.i. Left bar graphs indicate the frequency of **a** IL-1β+, **b** IL-23+, and **c** IL-10+ cells among CD11b+ cells from 72-h dLN cell cultures of female and male rats immunized for EAE (EAE rats), in the absence of cognate stimuli (control) or following stimulation with MBP in the absence or in the presence of 10^{-5} M of prazosin (Pz) ("Materials and methods"). Fluorescence minus one (FMO) controls **a** without primary anti-IL-1β/IL-23 antibodies (-IL-1β/IL-23 Abs; secondary antibodies only) or **c** incubated with isotype-matched control instead of anti-IL-10 antibody (-IL-10 Ab) were used to set the cutoff boundaries for analyses of **a**, **b** IL-1β/IL-23 or **c** IL-10 expression in CD11b+ cells in fully stained samples. CD11b+ cells were gated as shown in Fig. 6a. Right bar graphs indicate the relative expression of **a** IL-1β, **b** IL-23/p19, and **c** IL-10 mRNA in 72-h dLN cell cultures of female and male EAE rats, following stimulation with MBP in the absence or in the presence of Pz, as determined by RT-qPCR. Results are represented as 2^{-dCt} relative to β-actin. Two-way ANOVA showed significant interactions between the effects of sex and culturing conditions for IL-1β ($F_{(2,30)} = 5.57$; $p < 0.01$) and IL-10 ($F_{(2,30)} = 7.46$; $p < 0.01$) mRNA expression. The data are shown as mean ± SEM ($n = 6$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p \le 0.05$; $^{\#\#}p \le 0.01$; $^{\#\#\#}p \le 0.001$. Number sign, vs female. Ab antibody, 2° secondary

adrenergic drugs and possibly repurposing their use as immunomodulating agents.

Author contributions GL, ZSV, IP, and IV designed the study. GL, ZSV, and IV wrote the manuscript. GL, ZSV, IP, IV, RP, DK, MNA, and NJ participated in experiments, data analysis and/or interpretation, and critically reviewed and approved the manuscript.

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Compliance with ethical standards

All experimental procedures and animal care were performed 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) and approved by the Animal Care and Use Committee of the Faculty of Pharmacy (permit number 6/12). The experiments complied with the ARRIVE guidelines for reporting animal research.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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