Experimental Gerontology 171 (2023) 112009

Contents lists available at ScienceDirect

Experimental Gerontology

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

Thymic changes as a contributing factor in the increased susceptibility of old Albino Oxford rats to EAE development

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ARTICLE INFO

Section Editor: Daniela Frasca

Keywords:
Ageing
EAE susceptibility
Thymic atrophy
Natural Treg
Tc17 cell thymic generation
CD28- T cells

ABSTRACT

The study was aimed to examine putative contribution of thymic involution to ageing-associated increase in susceptibility of Albino Oxford (AO) rats to the development of clinical EAE, and vice versa influence of the disease on the progression of thymic involution. To this end we examined (i) the parameters of thymocyte negative selection efficacy, the thymic generation of CD4+CD25+Foxp3+ T regulatory cells (Tregs) and thymic capacity to instruct/predetermine IL-17-producing T-cell differentiation, and thymopietic efficacy-associated accumulation of "inflammescent" cytotoxic CD28- T cells in the periphery, and (ii) the key underlying mechanisms in young and old non-immunised AO rats and their counterparts immunised for EAE (on the 16th day post-immunisation when the disease in old rats reached the plateau) using flow cytometry analysis and/or RT-qPCR. It was found that thymic involution impairs: (i) the efficacy of negative selection (by affecting thymocyte expression of CD90, negative regulator of selection threshold and the expression of thymic stromal cell integrity factors) and (ii) Treg generation (by diminishing expression of cytokines supporting their differentiation/maturation). Additionally, the results suggest that thymic involution facilitates CD8+ T-cell differentiation into IL-17-producing cells (previously linked to the development of clinical EAE in old AO rats). Furthermore, they confirmed that ageing-related decrease in thymic T-cell output (as indicated by diminished frequency of recent thymic emigrants in peripheral blood) resulted in the accumulation of CD28- T cells in peripheral blood and, upon immunisation, in the target organ. On the other hand, the development of EAE (most likely by increasing circulatory levels of proinflammatory cytokines) contributed to the decline in thymic output of T cells, including Tregs, and thereby to the progression/maintenance of clinical EAE. Thus, in AO rats thymic involution via multi-layered mechanisms may favour the development of clinically manifested autoimmunity, which, in turn, precipitates the thymus atrophy.

Abbreviations: AO, Albino Oxford; MS, multiple sclerosis; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; IFN, interferon; IL, interleukin; Tregs, regulatory T cells; nTregs, natural Tregs; tTregs, thymic Tregs; TCRs, T-cell receptors; Teff, T effector cell; NK, natural killer; TNF, tumour necrosis factor; TEC, thymic epithelial cell; T-PBL, peripheral blood T lymphocyte; RTEs, recent thymic emigrants; PBS, phosphate-buffered saline; CFA, Complete Freund's Adjuvant; mAbs, monoclonal antibodies; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; PerCP, peridinin–chlorophyll–protein; PMA, phorbol 12-myristate 13-acetate; MC540, merocyanine 540; FCA, flow cytometry analysis; MFI, mean fluorescence intensity; SD, standard deviation; Foxp3, forkhead box P3 protein; Foxn1, forkhead box N1 protein; DN, double negative; DP, double positive; SP, single positive; TCF-1, T-cell factor 1; CXCL12, C-X-C motif chemokine ligand 12; MAF, MAF BZIP transcription factor; RORγt, retinoic acid-related orphan receptor gamma t; γc, gamma chain; TGF-β, transforming growth factor beta; AIRE, autoimmune regulator; MHC, major histocompatibility complex; NKG2D, natural-killer group 2 member D; FCS, foetal calf serum; FACS, fluorescence-activated cell sorting; RPMI, Roswell Park Memorial Institute; RT-qPCR, reverse transcription-quantitative real-time polymerase chain reaction; CD, cluster of differentiation; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

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https://doi.org/10.1016/j.exger.2022.112009
Received 29 July 2022; Received in revised form 26 October 2022; Accepted 27 October 2022
Available online 2 November 2022

1. Introduction

Multiple sclerosis (MS) is the most common inflammatory autoimmune disease of the central nervous system (CNS) (Tullman, 2013). This disease typically begins between the ages of 20 and 40 years, whereas initial symptoms rarely occur after the age of 60 years (termed late-onset MS) (Tullman, 2013). The influence of ageing on MS development is still uderinvestigated. The most commonly used animal models of MS are referred to as experimental autoimmune encephalomyelitis (EAE) (Lassmann and Bradl, 2017). Like MS, EAE is elicited in genetically susceptible hosts that exhibit histopathological lesions and a spectrum of clinical disease subtypes that are genetically controlled and recapitulate those observed in MS (Butterfield et al., 1999). Data on the influence of ageing on the incidence and clinical presentation of EAE are limited, and largely inconsistent reflecting, at least partly, strain differences (Ditamo et al., 2005; Endoh et al., 1990; Källén and Nilsson, 1989; Ludowyk et al., 1993; Matejuk et al., 2005; Stojić-Vukanić et al., 2018; Wang et al., 2022). We showed that the susceptibility of Albino Oxford (AO) rats to the development of clinically manifested EAE increases with ageing, so differently from young rats, which do not develop clinically manifested disease, old rats upon autoimmune challenge exhibit neurological signs of the disease, thereby „modelling“ the late onset of human disease in some individuals (Stojić-Vukanić et al., 2015). This phenomenon was associated with an age-related increase in the frequency of interferon (IFN)-γ- and interleukin (IL)-17-producing CD8+ T cells in draining lymph nodes and spinal cord followed by a decrease in the frequency of regulatory CD4+CD25+Foxp3+ T cells (Tregs) (Stojić-Vukanić et al., 2018). To corroborate these findings are data indicating that IFN-γ- and IL-17-producing CD8+ T cells have an important role in the pathogenesis of clinically mild models of EAE (Camara et al., 2013; Huber et al., 2013; Huber and Lohoff, 2015). Specifically, it has been shown that these cells, in addition to direct encephalitogenic action, enhance weak neuroantigen-specific response of CD4+ T cells in draining lymph nodes by helping them to fully differentiate into CNS-infiltrating effector cells, the phenomenon termed “reverse help” (Camara et al., 2013; Huber et al., 2013; Huber and Lohoff, 2015). Taken as a whole, these findings are consistent with the notion that clinically overt autoimmune disease ultimately results from an imbalance between effector damaging and regulatory immune responses (that keep the former under control) (Bano et al., 2019). More specifically, it is believed that autoimmune disease occurs only if the damaging response is boosted and/or the regulatory response for some reason is impaired (Bano et al., 2019).

The thymus plays a central role in establishing and maintaining self-tolerance and preventing the development of autoimmune diseases by eliminating developing T lymphocytes that react with “self” antigens through selection mechanisms that operate in the cortex and medulla, and the generation of Tregs termed natural (nTregs) or thymic Tregs (tTregs) (von Boehmer, 2009; Xing and Hogquist, 2012). Namely, thymocytes bearing T-cell receptors (TCRs) that are autoreactive to self-peptides presented in the context of self-MHC molecules are deleted or may be diverted to become Tregs (von Boehmer, 2009). Tregs in the periphery compensate for imperfections in negative selection by keeping under control self-reactive T cells which slip through the negative selection process (von Boehmer, 2009). Progressive thymic atrophy with ageing termed thymic involution affects both the negative selection and the generation of Tregs, possibly leading to an imbalance in T effector cell (Teff)/Treg ratio in the periphery (Coder et al., 2015; Leposavić et al., 2006). The mechanisms underlying these changes have not fully been elucidated, yet. Additionally, thymic involution ultimately results in significantly decreased thymic output of naïve T cells leading to amplified oligoclonal expansion of memory T cells (Hale et al., 2006; Palmer, 2013; Rezzani et al., 2014). The latter is recognized in both the major T-cell compartments, but particularly within the CD8+ T-cell compartment, with gradual accumulation of oligoclonal cells characterized by critically shortened telomeres (often classified as senescent), the loss of CD28 (CD28-, $CD28^{null}$) and enhanced cytotoxicity (Effros et al., 1994; Strioga et al., 2011; Tedeschi et al., 2022; Weng et al., 2009). Noteworthy, CD4+CD28- cells acquire a large cytoplasmic store of granzymes and perforin, similarly to CD8+ T cells and natural killer (NK) cells (Thewissen et al., 2007a). CD28- T cells have a memory effector phenotype and hence do not require a co-stimulatory input to be re-activated, so they exhibit a more rapid response (Thewissen et al., 2007b). Finally, CD28- T cells express NK cell markers, such as NKG2D, which possibly further contribute to the amplification of the pro-inflammatory signals (Warrington et al., 2001). These extra receptors also lower their threshold for the activation by specific and non-specific stimuli (Lindstrom and Robinson, 2010). In addition, they produce large amounts of pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, which potentially contribute to host tissue damage (Bano et al., 2019). The loss of CD28 expression in T cells with ageing has also been attributed to repeated antigenic stimulation (Vallejo, 2005). Of note, this ageing-associated loss of CD28 is suggested to contribute to the state of low-grade chronic inflammation termed inflammaging in old animals and the elderly (Franceschi and Campisi, 2014), and the development of various inflammatory diseases including autoimmune diseases (Elyahu and Monsonego, 2021; Strioga et al., 2011; Tedeschi et al., 2022; Weng et al., 2009).

On the other hand, regardless of the chronological age, chronic inflammatory autoimmune diseases, including MS and EAE (Bolton and Flower, 1989; Duszczyszyn et al., 2010; Eschborn et al., 2021; Levine et al., 1980; Nacka-Aleksić et al., 2018) are shown to accelerate the expansion of peripheral “inflammescent” CD28- T-cell compartment (Fulop et al., 2018) by inducing thymic atrophy, which then, in turn, contributes to their perpetuation/progression (Bolton and Smith, 2018; Fulop et al., 2018). Of note, this phenomenon is linked not only with an accelerated decline in thymopoietic efficiency and consequent loss of CD28 expression on T cells (Duszczyszyn et al., 2010; Haegert et al., 2011), but also to a direct inhibitory action of proinflammatory cytokines on their CD28 expression (Duszczyszyn et al., 2010; Haegert et al., 2011; Lee and Lee, 2016; Maly and Schirmer, 2015).

Multiple cellular and molecular pathways are suggested to be involved in thymic involution and atrophy in autoimmune diseases, some of which are suggested to be common (Dooley and Liston, 2012). However, although it could be therapeutically important, they have not fully been elucidated, yet. Additionally, the role of thymic changes in the development of autoimmune diseases is still underinvestigated. Thus, the present study was undertaken to examine putative contribution of ageing-related changes in thymopoiesis to the increased susceptibility of AO rats to EAE development and vice versa the influence of EAE development on thymopoiesis in old AO rats and the disease progression. To this end, distribution of thymocyte subsets at distinct stages of development, their expression of CD90 (Thy1) molecule controlling thymocyte selection threshold (Hueber et al., 1997), and the development of nTregs (Mabarrack et al., 2008) was examined. To complement the picture of thymic changes, thymic expression of distinct molecules controlling the integrity of thymic epithelial cell (TEC) compartment, thymocyte survival/apoptosis, proliferation, selection, Treg development, and possibly predetermining some mature CD8+ cell fate (differentiation into IL-17-producing cells) was also investigated. In addition, the major peripheral blood T lymphocyte (T-PBL) subpopulations were examined for the frequency of recent thymic emigrants (RTEs), suggested to be a surrogate marker of thymic output, and activated/memory cells and CD28- cells.

2. Material and methods

2.1. Experimental animals

Female AO rats used in the study were bred in the Immunology Research Centre “Branislav Janković“ animal facilities (Belgrade, Serbia), validated by the Ministry of Agriculture and Environmental

Protection of the Republic of Serbia (Veterinary Department). The animals were 2-3-month-old (young) and 22-24-month-old (old) at the beginning of the experiments. They were housed under standard laboratory conditions: 3 rats per cage containing sterilized wood shavings, with controlled humidity, temperature and lighting conditions, and free access to standard food and fresh water. The study was conducted in compliance with the directive 2010/63/EU of the European Parliament and the Council on the protection of animals used for scientific purposes (revising Directive 86/609/EEC) and the governmental regulations (Law on Animal Welfare, "Official Gazette of RS", no. 41/2009). Experimental protocol was approved by the institutional Ethical Committee and Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Economy of the Republic of Serbia (permit no. 323-07-01577/2016-05/14). Animal health status was monitored by animal care staff and a veterinarian on a daily basis. The study adhered to ARRIVE recommendations for reporting in vivo animal research.

2.2. Induction and clinical evaluation of EAE

Rats of both ages (6 rats per group) were randomly selected and either subjected to immunisation for EAE (immunised rat group) or left intact (non-immunised rat group). To relieve stress and pain, rats were anaesthetised prior to immunisation for EAE by an intraperitoneal injection of ketamine (Ketamidor, Richter Pharma AG, Wels, Austria; 100 mg/ml) / xylazine (Xylased, Bioveta, Ivanovice na Hané, Czech Republic; 20 mg/ml) anaesthetizing cocktail [50 mg/kg body weight (BW) of ketamine/5 mg/kg BW xylazine]. Immunisation for EAE was performed by application of 100 μl of an emulsion made of equal volumes of syngeneic rat spinal cord homogenate in phosphate-buffered saline (PBS) and complete Freund's adjuvant (CFA) with 1 mg/ml of heat-killed and dried *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) into the left hind foot pad, followed by subcutaneous administration of 0.25 ml of 5×10^8 *Bordetella pertussis* saline suspension (Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia) into the dorsum of the same paw. From the 1st day post-immunisation, two independent experienced observers recorded animal weight and EAE scores (0, no clinical signs; 0.5, distal tail atony; 1, complete tail atony; 2, paraparesis; 3, paraplegia; 4, tetraplegia or moribund state) on a daily basis. None of the EAE-inflicted rats reached moribund state or death during the experiments.

All animals were sacrificed by transcardial perfusion. Rats immunised for EAE were sacrificed on the 16th day post immunisation, when the disease in aged rats reached the plateau (Supplementary Fig. 1). Before the transcardial perfusion, the animals were deeply anaesthetized with an intraperitoneal injection of ketamine/xylazine anaesthetizing cocktail (80 mg/kg BW ketamine/8 mg/kg BW xylazine) and blood samples were taken by cardiac puncture.

2.3. Antibodies and secondary reagents

For flow cytometry analysis (FCA), the following monoclonal antibodies (mAbs) and secondary reagents were supplied by BD Biosciences Pharmingen (Mountain View, CA, USA): fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/allophycocyanin (APC)/biotin-labelled anti-CD8 (clone OX-8), PE-labelled anti-CD4 (clone OX-38), peridinin–-chlorophyll–protein (PerCP)-labelled anti-CD90 (Thy-1.1, clone OX-7), PerCP-labelled anti-TCRαβ (clone R73), PE-labelled anti-CD45RC (clone OX-22), Alexa Fluor® 488-labelled anti-Ki-67 (clone B56), PE-labelled anti-CD28 (clone JJ319), PE-labelled anti-IL-17A (clone TC11-18H10), PerCP-labelled streptavidin and FITC-labelled goat anti-mouse Ig Ab. In addition, FITC-labelled anti-Foxp3 (clone FJK-16s), PerCP eFluor® 710-labelled anti-CD25 (clone OX-39), APC-labelled anti-CD4 (clone OX-35) were obtained from eBioscience (San Diego, CA, USA), while FITC-labelled anti-granzyme B (clone GB11) and Alexa Fluor® 647-labelled anti-TCRαβ (clone R73) were acquired from BioLegend (San Diego, CA, USA). FITC-conjugated rabbit anti-goat IgG Ab was purchased from Sigma-Aldrich Chemie GmbH.

2.4. Preparation of lymphoid tissue, spinal cord and peripheral blood cell suspensions for (immune)staining and FCA

The cell counts across all cell suspensions were determined using 0.2 % Trypan blue solution (for identification of viable cells) and an improved Neubauer hemacytometer.

2.4.1. Isolation of thymus and lymph nodes and preparation of thymocyte and lymph node mononuclear cell suspensions

Thymi and popliteal and inguinal lymph nodes were carefully removed, weighed and then finely minced and pressed through 70 μm nylon cell strainers (BD Biosciences, Erembodegem, Belgium) to obtain single cell suspensions. Thymic and lymph node suspensions were washed three times in ice-cold fluorescence-activated cell sorting (FACS) buffer, i.e. PBS supplemented with 2 % foetal calf serum (FCS, Gibco, Grand Island, NY, USA) and 0.1 % sodium azide (Sigma-Aldrich Chemie GmbH).

2.4.2. Isolation of spinal cords and preparation of spinal cord mononuclear cell suspensions

After thorough perfusion with ice-cold PBS pH 7.4 to ensure that no blood remains in the blood vessels irrigating the CNS, the careful lamynectomy, starting from the cervical to the sacral region, was performed, and entire spinal cords (SCs) were detached from the spinal meninges and placed in Petri dishes containing ice-cold PBS (Mothe and Tator, 2015). To acquire single-cell mononuclear cell suspensions, SCs were ground on 70 μm nylon cell strainer (BD Biosciences, Erembodegem, Belgium) and collected in RPMI 1640 medium (Sigma-Aldrich Chemie GmbH) supplemented with 5 % FCS (SC cells). Afterwards, the SC cells were fractioned on a discontinuous 40/70 % Percoll (Sigma-Aldrich Chemie GmbH) gradient at 1000 ×g for 50 min, and mononuclear cells were collected from the interface.

2.4.3. Preparation of peripheral blood cell suspensions

For FCA of T-PBL subsets, peripheral blood samples were subjected to erythrocyte lysis using isotonic solution of ammonium chloride (5:1 lysis buffer to blood volume ratio). During 10-min incubation, the tubes were inverted several times and then centrifuged at 350 ×*g* for 5 min at 4 °C. Cells were washed three times in ice-cold PBS containing 2 % FCS.

2.5. Stimulation of lymph node mononuclear cells for analysis of IL-17

Cells isolated from lymph nodes of non-immunised rats were seeded in 24-well plates (Sarstedt AG & Co., Nümbrecht, Germany) and cultured in RPMI 1640 complete medium containing 2 mM L-glutamine (Serva, Heidelberg, Germany), 1 mM sodium pyruvate (Serva), 100 units/ml penicillin (ICN, Costa Mesa, CA, USA), 100 μg/ml streptomycin (ICN) and 10 % FCS. The medium was supplemented with 200 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Chemie GmbH) and 400 ng/ml ionomycin (Sigma-Aldrich Chemie GmbH), and 3 μg/ml of brefeldin A (eBioscience). Plates were incubated in a humidified atmosphere of 5 % *v*/v CO_2 for 4 h at 37 °C and the cells were subsequently harvested for intracellular IL-17 immunostaining.

2.6. Analysis of thymocyte apoptosis

To secure reliable estimation of apoptotic cells, i.e. to overcome limitations related to their swift elimination by macrophages in vivo (Savill et al., 1993) and to secure exclusion of mechanically stressed cells during isolation and subsequent experimental manipulations, which could trigger apoptosis (Hsieh and Nguyen, 2005), freshly isolated thymocytes were plated in 96-well flat-bottom plates and incubated with RPMI 1640 complete culture medium (Nunc A/S, Roskilde, Denmark) for 18 h, as it has been previously suggested (Kamath et al.,

1997). After the incubation, thymocyte samples were harvested and stained with 5 μl of merocyanine 540 dye (MC 540; Sigma-Aldrich Chemie GmbH) stock solution (1 mg/ml) for FCA.

2.7. Cell staining and FCA

2.7.1. Immunostaining of surface antigens

For cell surface staining, thymocyte, T-PBL, lymph node and spinal cord mononuclear cell samples were immunostained with either fluorochrome-labelled or unconjugated/biotin-conjugated mAbs for 30 min and then washed using FACS buffer. Following immunolabelling with unconjugated/biotin-conjugated mAbs, the cells were incubated with fluorochrome-conjugated second step reagents for another 30 min, then washed and used for FCA. All incubations were performed at 4 °C.

2.7.2. Intracellular antigen immunostaining

For immunolabelling of intracellular antigens, cell samples were fixed and permeabilised using the reagents from the Foxp3/transcription factor staining buffer set (eBioscience). Thymocytes were subsequently stained with anti-Foxp3 or anti-Ki-67 mAbs, while lymph node and spinal cord cell samples were immunostained using anti-IL17 and anti-granzyme B mAbs, respectively. All mAbs were used according to manufacturers' instructions.

2.7.3. FCA

For FCA, 50.000 events per sample were acquired using a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) or a FACSVerse flow cytometer. Primary antibodies used in the study are specific for rat antigens and have been validated for flow cytometry application by the manufacturers. Target cell antigen specificity for all antibodies was further confirmed in our previous (Stojić-Vukanić et al., 2018) and the present study using either single- or multi-colour analyses and involving positive and negative cell types. Additionally, other controls were also used including unstained cells, isotype staining and secondary antibody only controls. To settle gating boundaries, fluorescence minus one controls were applied.

Flow cytometry data were assessed for frequencies of cells exhibiting particular phenotype or mean fluorescence intensity (MFI), which corresponds to the density of a marker expression, using FlowJo software version 7.8. (TreeStarInc, Ashland, OR, USA). All MFI data are presented as MFI ratio, calculated by dividing MFI of mAb-labelled cells by MFI of unstained cells (negative control), as suggested by Reguzzoni et al. (2002).

2.8. RT-qPCR

TRIzol™ Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for thymic tissue total RNA isolation. Sample RNA concentrations were measured by absorbance spectrometry at 260 nm, while the RNA purity was assessed by measuring absorbance at 280 nm. RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and resulting cDNA served as a template in triplicate RT-qPCR reactions. Each reaction contained 12.5 μl of TaqMan Gene Expression Master Mix (Applied Biosystems), 1.25 μl of predesigned TaqMan Gene Expression assay (Applied Biosystems), 6.25 μl of PCR-grade water and 5 μl of cDNA. Samples were ran under the default, pre-optimised, conditions of 7500 Real-Time PCR instrument (Applied Biosystems). All the reagents were used as instructed by vendors. TaqMan Gene Expression Assays were used in the study as follows: *Il2* (Rn00587673_m1), *Il4* (Rn99999010_m1), *Il6* (Rn99999011_m1), *Il7* (Rn00681900-m1), *Il15* (Rn00689964_m1), *Il17a* (Rn01757168_m1), *Il21* (Rn01755623_m1), *Tgfb1* (Rn00572010_m1), *Cxcl12* (Rn00573260_m1), *Foxn1* (Rn01460454_m1) and *Actb* (Rn00667869_m1). Relative quantification of target mRNA levels, normalised against β-actin, was performed by the comparative threshold cycle (Ct) method using SDS v1.4.0. software (Applied Biosystems). Relative amounts of target mRNAs were given as 2^{-dCt} values, with dCt = Ct target − Ct β-actin.

2.9. ELISAs

For estimation of plasma IL-6 and TNF-α concentrations, blood samples were collected in heparinised tubes and then centrifuged for 15 min at 1500 ×*g* to separate plasma. Separated plasma was stored in polypropylene tubes and held at −70 °C until analysis. ELISA kits used for quantification of plasma IL-6 and TNF-α concentrations were provided by BioLegend. Declared detection sensitivity values were 5.3 pg/ml and 2 pg/ml for IL-6 and TNF-α, respectively. Test procedures were performed according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical testing was performed using GraphPad Prism 5 software (GraphPadSoftware, Inc., La Jolla, CA, USA). Two-way ANOVA followed by the Bonferroni post hoc test was used to evaluate the influence of ageing and immunisation on the thymic, peripheral blood and spinal cord immunological parameters. The influence of ageing on the thymic IL-17 mRNA expression and the frequencies of IL-17-producing cells among lymph node CD8+TCRαβ+ lymphocytes from non-immunised rats, as well as granzyme B+ cells within spinal cord CD4+ and CD8+ T lymphocytes from immunised rats was assessed with Student's *t*-test. Values of $p \leq 0.05$ were regarded as statistically significant. All data are given as mean ± standard deviation (SD).

3. Results

3.1. Ageing increased AO rat susceptibility to EAE development

As previously shown (Stojić-Vukanić et al., 2015), differently from young AO rats, who were relatively resistant to the induction of clinical EAE, old AO animals developed mild signs of the disease reaching the plateau value between the 15th and the 16th d.p.i. (Supplementary Fig. 1).

3.2. Ageing and immunisation for EAE diminished thymic weight and thymocyte yield

As expected (Leposavić et al., 2006; Perišić et al., 2009; Quaglino et al., 1998), the absolute thymic weight ($p < 0.001$) and absolute thymocyte yield ($p < 0.001$) decreased with ageing (Fig. 1a). Immunisation for EAE (in further text immunisation) in young and old rats also reduced ($p < 0.05$) the thymic weight and thymocyte yield (Fig. 1a). Consequently, both the parameters were found to be lower ($p < 0.001$) in old rats immunised for EAE than in young rats subjected to the same treatment (Fig. 1a). More important, the thymic weight and thymocyte yield normalised to 100 g body weight exhibited the same pattern of ageing- and immunisation-associated changes (Fig. 1a), suggesting that the observed changes were thymocyte yield- and thymic weight-specific, respectively.

3.3. Ageing and immunisation for EAE increased plasma and thymic levels of IL-6

Considering that the increase in circulating level of IL-6 during ageing and inflammatory diseases has been associated with the reduction in thymic weight and thymocyte yield (Billard et al., 2011; Dooley and Liston, 2012; Sempowski et al., 2000), the plasma levels of this cytokine were quantified. The plasma levels of IL-6 rose ($p < 0.001$) with ageing (Fig. 1b). Additionally, immunisation increased its level in young rats ($p < 0.05$) (Fig. 1b). This effect of immunisation was more prominent ($p < 0.001$) in old rats (Fig. 1b). Accordingly, in immunised rats IL-6 level was also higher ($p < 0.001$) in old rats compared with young ones

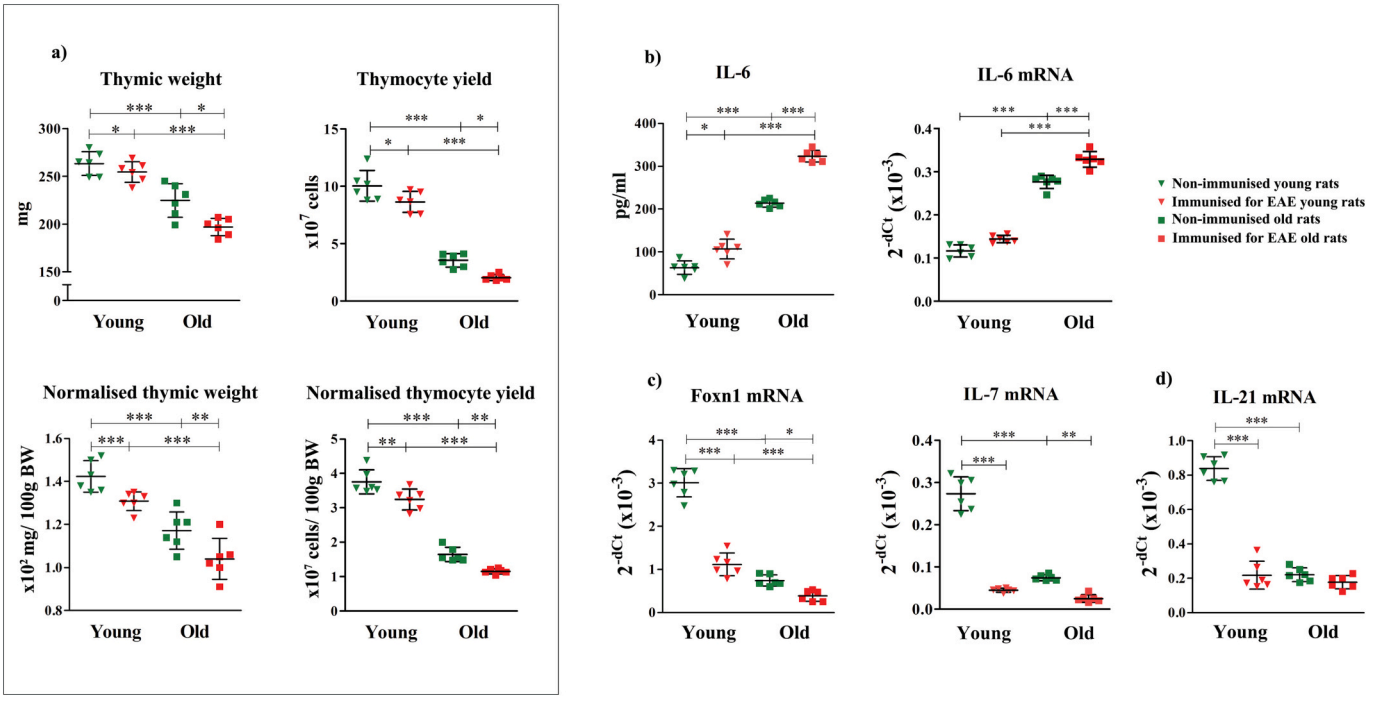

Fig. 1. Ageing and immunisation for EAE in AO rats diminished thymic weight and thymocyte yield by affecting the thymic expression of factors involved in thymic epithelial cell maintenance and thymocyte survival and proliferation.
Scatter plots indicate: a) thymic weight and thymocyte yield (upper), and normalised thymic weight (thymic weight per 100 g body weight; BW) and normalised thymocyte yield (thymocyte yield per 100 g BW) (lower); b) circulating levels of IL-6 and thymic IL-6 mRNA expression; c) Foxn1, IL-7 and d) IL-21 mRNA expression in thymic tissue of non-immunised and immunised for EAE young and old rats. Two-way ANOVA showed significant interactions between the effects of ageing and immunisation for normalised thymic weight [$F_{(1,20)} = 316.7$, $p < 0.001$], circulating levels of IL-6 [$F_{(1,20)} = 26.65$, $p < 0.001$], thymic expression of Foxn1 [$F_{(1,20)} = 146.3$, $p < 0.001$], IL-7 [$F_{(1,20)} = 162.8$, $p < 0.001$] and IL-21 [$F_{(1,20)} = 122.8$, $p < 0.001$] mRNAs. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(Fig. 1b).

Considering that systemic increase in proinflammatory cytokines is associated with upregulation of thymic IL-6 mRNA expression (Cohen-Kaminsky et al., 1991; Sempowski et al., 2000), the amount of IL-6 transcript in thymic tissue was also investigated. Indeed, with ageing thymic IL-6 mRNA expression increased ($p < 0.001$) in non-immunised rats (Fig. 1b). Immunisation amplified the amount of IL-6 mRNA in thymic tissue of young and old rats, but this increase reached statistical significance ($p < 0.001$) only in old rats (Fig. 1b). Thus, the greater ($p < 0.001$) amount of IL-6 mRNA was found in thymi of old immunised rats when compared with their young counterparts (Fig. 1b).

3.4. Ageing and immunisation for EAE affected thymic expression of factors influencing TEC maintenance and thymocyte yield

Given that the elevation in circulating IL-6 levels and/or thymic IL-6 expression leads to direct TEC damage and thymic atrophy (Cohen-Kaminsky et al., 1991; Gruver and Sempowski, 2008; Sempowski et al., 2000), the expression of mRNA for Foxn1, the key regulator of TEC development and maintenance (Rode et al., 2015; Vaidya et al., 2016), was also examined. As expected (Rode et al., 2015; Vaidya et al., 2016), in non-immunised rats the expression of Foxn1 transcript dramatically decreased ($p < 0.001$) with ageing (Fig. 1c). Immunisation additionally decreased ($p < 0.05$) its expression in thymi of old rats (Fig. 1c). The thymic expression of Foxn1 mRNA also decreased ($p < 0.001$) upon immunisation in young rats, but it remained greater ($p < 0.001$) in young immunised rats compared with their old counterparts (Fig. 1c).

Given that IL-7, a critical immature thymocyte pro-survival/anti-apoptotic factor is mainly produced by TECs (Hong et al., 2012), its expression in the thymus was also examined. Ageing ($p < 0.001$) and immunisation ($p < 0.01$) in young and old rats diminished thymic IL-7 mRNA expression (Fig. 1c).

Considering finding indicating that administration of rIL-21 enhances thymopoiesis in old mice through proliferative expansion of thymocyte compartments primarily, and consequently the stromal compartment (Al-Chami et al., 2016), the amount of IL-21 transcript in thymus was also measured. Indeed, the expression of IL-21 mRNA in thymus markedly ($p < 0.001$) decreased with ageing (Fig. 1d). Immunisation diminished ($p < 0.001$) the amount of IL-21 mRNA in thymus of young rats, while in old rats it was not efficient in this respect (Fig. 1d). Thus, the expression of this cytokine was comparable in thymi from young and old immunised rats (Fig. 1d).

3.5. Ageing and immunisation for EAE affected thymocyte apoptosis and proliferation

To get a mechanistic view of the previous findings, thymocyte apoptosis and proliferation were also examined. In accordance with the changes in the thymocyte yield, the frequency of apoptotic cells was markedly higher ($p < 0.01$) among thymocytes from old non-immunised rats compared with young non-immunised rats (Fig. 2a). Additionally, the frequency of apoptotic cells was higher ($p < 0.05$) among thymocytes from young and old immunised rats compared with age-matched non-immunised rats (Fig. 2a). Of note, their frequency was markedly higher ($p < 0.01$) in old immunised rats compared with their young counterparts (Fig. 2a). On the other hand, in non-immunised rats the frequency of Ki-67+ proliferating cells among thymocytes decreased ($p < 0.01$) with ageing (Fig. 2b). Immunisation did not significantly affect their frequency in either young or old rats, so it was lower in old compared with young immunised rats, but this difference did not reach statistical significance (Fig. 2b).

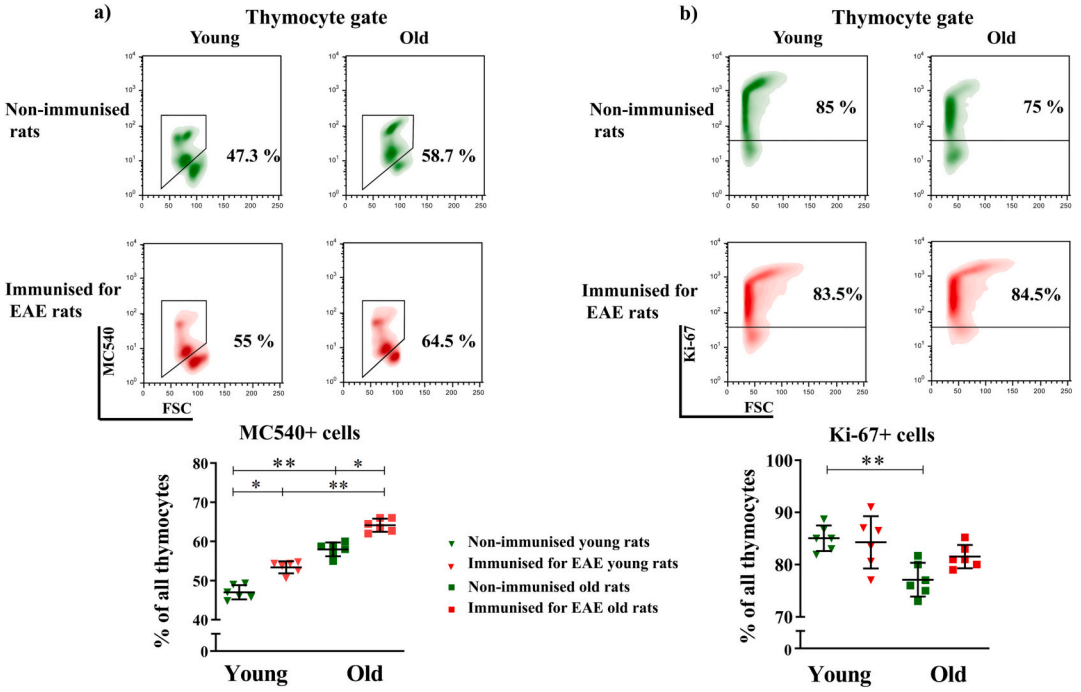

Fig. 2. Ageing and immunisation for EAE affected thymocyte apoptosis and/or proliferation in AO rats.
Representative flow cytometry density plots (upper) and scatter plots (lower) indicate: a) MC540 staining of thymocytes (cultured for 18 h to evaluate apoptosis; see Material and methods section) of non-immunised and immunised for EAE young and old rats, and the frequency of apoptotic MC540+ cells among thymocytes; b) Ki-67 staining of thymocytes retrieved from non-immunised and immunised young and old rats (gating strategy is displayed in Supplementary Fig. 2) and the frequency of proliferating Ki-67+ cells among thymocytes. Data points and means ± SD are from one of two experiments with similar results (n = 6). * $p < 0.05$; ** $p < 0.01$.

3.6. Ageing and immunisation for EAE affected thymocyte differentiation/maturation

3.6.1. Influence of ageing and immunisation for EAE on the distribution of the major thymocyte subsets delineated by CD4/CD8 expression

Considering that thymic stroma provides a unique platform of growth factors, chemokines, and receptor ligands that not only sustain thymocyte survival, proliferation, but also their differentiation/maturation (Alves et al., 2009), the composition of thymocyte subsets delineated by characteristic constellation of surface CD4/CD8/TCRαβ expression (Tsuchida et al., 1994) was examined. Ageing increased ($p < 0.001$) the frequency of the most immature CD4-CD8- double negative (DN) cells among thymocytes (Fig. 3a). Immunisation increased ($p < 0.001$) their frequency in young rats, whereas in old rats it was inefficient in this respect (Fig. 3a). Thus, their frequency was lower ($p < 0.05$) among thymocytes from old immunised rats compared with their young counterparts (Fig. 3a). On the other hand, the frequency of more mature CD4+CD8+ double positive (DP) cells among thymocytes decreased ($p < 0.001$) with ageing (Fig. 3a). Immunisation diminished ($p < 0.05$) the frequency of DP cells in young rats, whereas in old rats it had the opposite effect ($p < 0.05$), so their frequency was comparable between young and old immunised rats (Fig. 3a). The frequencies of both CD4+CD8- and CD4-CD8+ single positive (SP) cells among thymocytes increased ($p < 0.001$) with ageing (Fig. 3a). In young rats immunisation affected only the frequency of CD4-CD8+ cells, so it was lower ($p < 0.05$) in immunised young rats compared with age-matched non-immunised rats (Fig. 3a). However, in old rats immunisation decreased the frequency of both CD4+CD8- ($p < 0.001$) and CD4-CD8+ ($p < 0.01$) SP cells among thymocytes (Fig. 3a). Consequently, the frequency of CD4+CD8- SP cells was comparable between young and old immunised rats, whereas that of CD4-CD8+ cells was higher ($p < 0.01$) among thymocytes from old immunised rats compared with their young counterparts (Fig. 3a).

Given that the overall thymocyte number dramatically decreased with ageing, the number of thymocytes within all analysed thymocyte subsets was lower ($p < 0.001$) in old compared with young non-immunised rats (Supplementary Fig. 4). The cellularity of all thymocyte subsets exept for DN subset diminished ($p < 0.001$) upon immunisation in young rats (Supplementary Fig. 4). The number of cells within DN subset was greater ($p < 0.001$) in immunised compared with non-immunised young rats (Supplementary Fig. 4), suggesting prominent accumulation of cells at this stage of development. Differently, in old immunised rats, the number of cells of all thymocyte subsets was lower ($p < 0.001$) compared with non-immunised old rats (Supplementary Fig. 4).

Next, considering that CXCL12 is an indispensable factor for proper localization of early lymphoid progenitors in the cortex and consequently their successful steady state differentiation (Plotkin et al., 2003), the amount of CXCL12 transcript in thymus was quantified. The expression of CXCL12 mRNA in thymus increased ($p < 0.001$) with ageing (Fig. 3b). Immunisation for EAE did not significantly affect its thymic expression in either young or old rats (Fig. 3b). Thus, in immunised rats thymic CXCL12 mRNA expression was higher ($p < 0.01$) in old than in young ones (Fig. 3b).

3.6.2. Influence of ageing and immunisation for EAE on thymocyte selection

To elucidate putative influence of ageing and immunisation for EAE on thymocyte selection, the frequency of DP thymocytes expressing distinct surface TCRαβ levels: i) undetectable (TCRαβ-) - thymocytes which successfully arranged TCR β chain and passed β-selection (Zamoyska and Lovatt, 2004), ii) low ($TCR\alpha\beta^{lo}$) - thymocytes entering positive selection processes (Jameson et al., 1995; Zamoyska and Lovatt, 2004) and iii) high ($TCR\alpha\beta^{hi}$) - thymocytes in an intermediate post-selection stage between DP $TCR\alpha\beta^{lo}$ and SP $TCR\alpha\beta^{hi}$ cells (Jameson et al., 1995; Zamoyska and Lovatt, 2004), was examined. The frequency of DP TCRαβ- cells was markedly higher ($p < 0.001$) among thymocytes from old non-immunised rats compared with young non-immunised rats

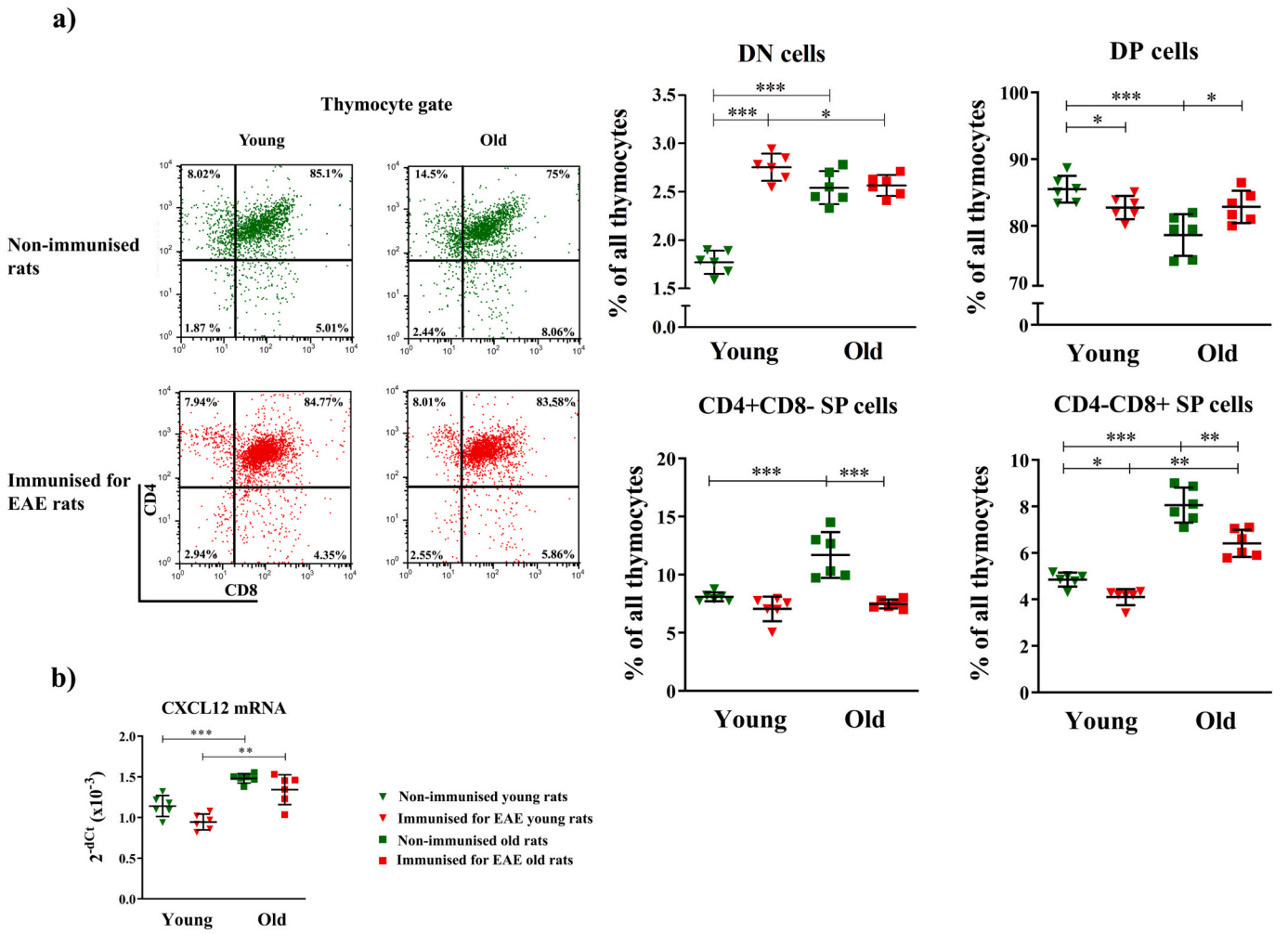

Fig. 3. Ageing and immunisation for EAE affected the composition of the major thymocyte subsets delineated by CD4/CD8 expression in AO rats.
(a) Representative flow cytometry dot plots show CD4/CD8 staining of thymocytes retrieved from non-immunised and immunised for EAE young and old rats (gating strategy is displayed in Supplementary Fig. 3). Scatter plots show the frequency of CD4-CD8- double negative (DN), CD4+CD8+ double positive (DP), and CD4+CD8- and CD4-CD8+ single positive (SP) thymocytes from non-immunised and immunised young and old rats. (b) Scatter plot indicates the expression of CXCL12 mRNA in thymic tissue from non-immunised and immunised young and old rats. Two-way ANOVA showed a significant interaction between the effects of ageing and immunisation on the frequency of CD4+CD8- SP thymocytes [$F_{(1,20)} = 9.376$, $p < 0.001$]. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(Fig. 4a). Immunisation for EAE increased ($p < 0.001$) their frequency in young and old rats, so it was higher ($p < 0.001$) among old immunised rats compared with their young counterparts (Fig. 4a). The frequency of DP TCRαβ^{lo} cells among thymocytes markedly decreased ($p < 0.001$) with ageing in non-immunised rats (Fig. 4a). Given that immunisation influenced the frequency of DP TCRαβ^{lo} cells in neither young nor old rats, their frequency was higher ($p < 0.001$) in young compared with old immunised rats (Fig. 4a). The frequency of DP TCRαβ^{hi} cells among thymocytes decreased ($p < 0.001$) with ageing (Fig. 4a). Their frequency also decreased upon immunisation ($p < 0.001$ and $p < 0.05$ in young and old rats, respectively); the effect particularly prominent in young rats (Fig. 4a). Consequently, the frequency of DP TCRαβ^{hi} cells was lower ($p < 0.05$) among thymocytes from young immunised rats compared with their old counterparts (Fig. 4a).

To get better insight into the influence of ageing and immunisation for EAE on thymocyte selection, the surface density of Thy-1 (CD90), which is highly expressed on the surface of DP thymocytes to negatively regulate TCR-mediated signaling and selection threshold during thymocyte differentiation (Hueber et al., 1997), on CD90+ DP thymocytes was examined. The average surface density of CD90 expression (judging by MFI) on CD90+ DP TCRαβ^{lo} thymocytes ($p < 0.01$) and CD90+TCRαβ^{hi} DP thymocytes increased ($p < 0.01$) with ageing (Fig. 4b). Upon immunisation in young and old rats CD90 density on both of the examined thymocyte subsets markedly decreased ($p < 0.001$), but to a greater extent in old rats, so CD90 surface density was similar on CD90+ DP TCRαβ+ thymocytes from young and old immunised rats (Fig. 4b).

3.6.3. *Ageing and immunisation for EAE decreased the level of the most mature SP TCRαβ^{hi} thymocytes in thymus and affected generation of CD8+ T cells prone to differentiate into Tc17*

Next, considering that within both SP thymocyte subsets, apart from the most mature TCRαβ^{hi} cells, immature SP TCRαβ$^{-/lo}$ cells are also identified (Hugo and Petrie, 1992; Lucas and Germain, 1996), SP thymocytes were examined for the frequency of TCRαβ^{hi} cells. The frequency of the most mature CD4+CD8- TCRαβ^{hi} ($p < 0.05$) and CD4-CD8+ TCRαβ^{hi} ($p < 0.001$) cells among thymocytes increased with ageing (Fig. 5). In young rats their frequencies were comparable in non-immunised rats and immunised rats (Fig. 5). However, in old rats, the frequencies of both CD4+CD8- TCRαβ^{hi} and CD4-CD8+ TCRαβ^{hi} cells decreased ($p < 0.001$) upon immunisation (Fig. 5). In immunised rats, the frequency of CD4+CD8- TCRαβ^{hi} cells was lower ($p < 0.01$) in old

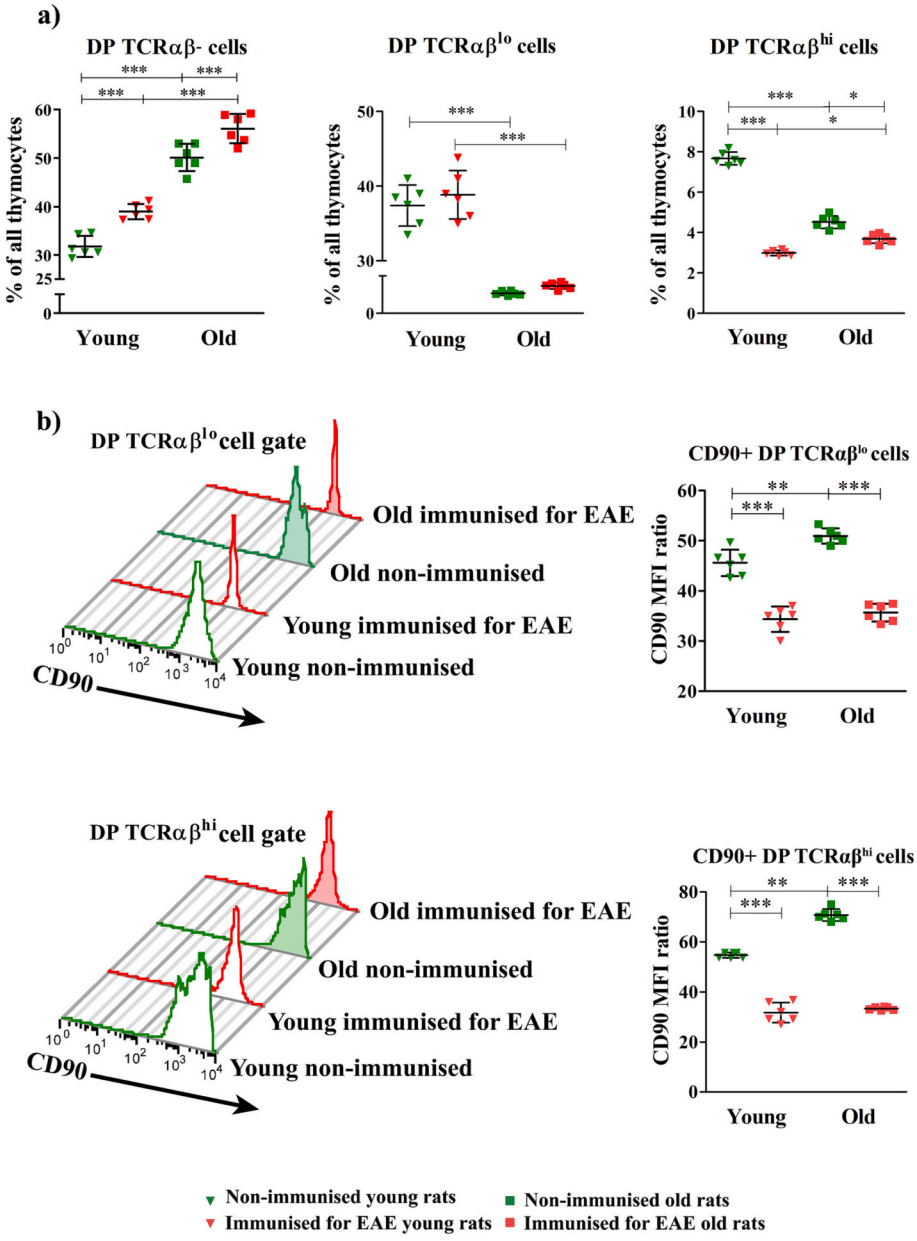

Fig. 4. Ageing and immunisation for EAE affected thymocyte selection in AO rats.
(a) Scatter plots indicate the frequency of CD4+CD8+ double positive (DP) TCRαβ^{-}, TCRαβ^{lo} and TCRαβ^{hi} cells among all thymocytes retrieved from non-immunised and immunised for EAE young and old rats. Gating strategy for distinct thymocyte subsets is displayed in Supplementary Fig. 3. (b) Representative overlaid flow cytometry histograms display CD90 (Thy-1) surface expression on DP TCRαβ^{lo} and TCRαβ^{hi} thymocytes of (green histograms) non-immunised and (red histograms) immunised young and old rats. Scatter plots show the CD90 mean fluorescence intensity (MFI) ratio (see Material and methods section) on CD90+ DP TCRαβ^{lo} and TCRαβ^{hi} thymocytes from non-immunised and immunised young and old rats. Two-way ANOVA showed significant interaction between the effects of ageing and immunisation for the frequency of DP TCRαβ^{hi} cells [$F_{(1,20)} = 55.28$, $p < 0.001$)], CD90 MFI on CD90+ DP TCRαβ^{lo} [$F_{(1,20)} = 8.297$, $p < 0.01$] and CD90+ DP TCRαβ^{hi} [$F_{(1,20)} = 5.451$, $p < 0.05$] thymocytes. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

rats than in young rats, whereas that of CD4-CD8+ TCRαβ^{hi} was comparable between these two groups of rats.

Additionally, herein presented findings showed that the absolute numbers of both CD4+CD8- TCRαβ^{hi} ($p < 0.001$) and CD4-CD8+ TCRαβ^{hi} ($p < 0.05$) cells decreased with ageing (Fig. 5). Immunisation in young and old rats diminished ($p < 0.001$) the absolute numbers of these cells (Fig. 5). However, their numbers were markedly lower ($p < 0.001$) in old immunised rats compared with their young counterparts (Fig. 5).

Next, taking into account putative role of Tc17, viz. IL-17-producing CD8+ T cells, in pathogenesis of EAE in old AO rats (Stojić-Vukanić et al., 2018) and data indicating that an increase in thymic expression of IL-4, such as after systemic inflammatory diseases or infectious Th1 diseases (Baez et al., 2019), may inhibit transcription factor T-cell factor 1 (TCF-1) (Maier et al., 2011), which regulates CD8+ T-cell fate decisions in DP thymocytes through the sequential suppression of MAF and RORγt, in parallel with TCF-1–driven modulation of chromatin state (viz. TCF-1 as instrumental in suppressing the MAF–RORγt axis in developing T cells to limit Tc17 formation), so its ablation leads to enhanced Tc17 cell development (Mielke et al., 2019), IL-4 mRNA in thymus was examined (Fig. 6a). Indeed, differently from young non-immunised rats, IL-4 mRNA expression was detectable in thymi from old non-immunised rats (Fig. 6a). The expression of IL-4 mRNA was additionally augmented ($p < 0.001$) upon immunisation for EAE (Fig. 6a). Consistently, the thymic expression of IL-17 mRNA markedly increased ($p < 0.001$) in thymi of old non-immunised rats compared with non-immunised young rats (Fig. 6b). Consistently, the frequency of Tc17 cells in lymph nodes of non-immunised rats was higher ($p < 0.001$) in old compared with young rats (Fig. 6c).

3.6.4. Ageing and immunisation for EAE diminished the level of regulatory CD4+CD25+Foxp3+ cell in thymus by affecting expression of cytokines driving their differentiation

Considering that the thymus generates a lineage-committed subset of nTregs, best identified by the expression of the transcription factor Foxp3 and CD25 in the rat (Mabarrack et al., 2008; Stephens et al., 2004), their frequency and number in thymus were also investigated. The frequency of these cells which are involved in the maintenance of self-tolerance and prevention of autoimmune disorders (Mabarrack

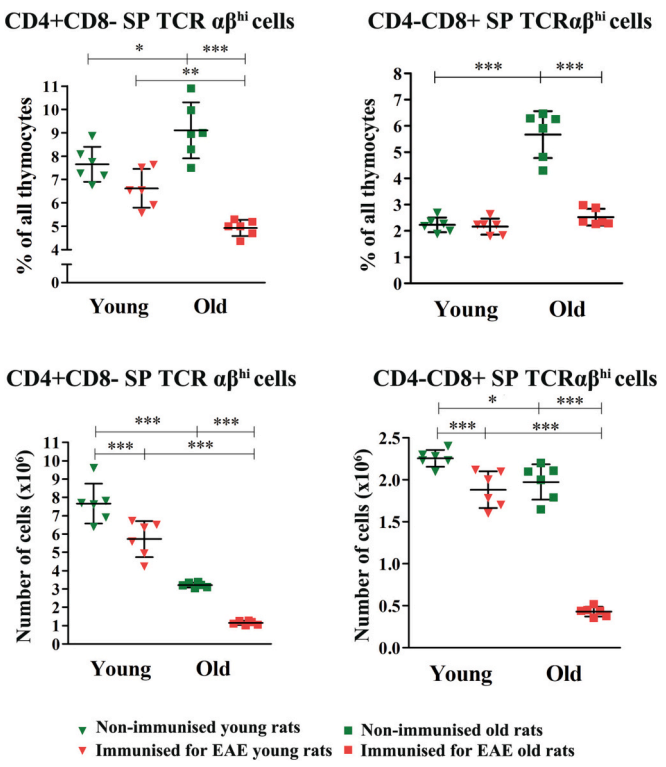

Fig. 5. Ageing increased the frequency of the most mature CD4+CD8- and CD4-CD8+ SP TCRαβ^{hi} thymocytes in AO rats, whereas immunisation for EAE decreased their frequency in old animals.
Scatter plots indicate (upper) the frequency and (lower) the absolute number of CD4+CD8- and CD4-CD8+ single positive (SP) TCRαβ^{hi} thymocytes of non-immunised and immunised for EAE young and old rats. Two-way ANOVA showed significant interaction between the effects of ageing and immunisation for the frequency of CD4+CD8- [$F_{(1,20)} = 55.12$, $p < 0.001$] and CD4-CD8+ [$F_{(1,20)} = 20.97$, $p < 0.001$] SP TCRαβ^{hi} thymocytes, and the number of CD4+CD8- [$F_{(1,20)} = 229.8$, $p < 0.001$] and CD4-CD8+ [$F_{(1,20)} = 64.32$, $p < 0.001$] SP TCRαβ^{hi} cells. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

et al., 2008; Stephens et al., 2004) was found to decrease ($p < 0.01$) among thymocytes with ageing (Fig. 7a). Additionally, their frequency decreased upon immunisation in both young ($p < 0.01$) and old ($p < 0.05$) rats, so in old immunised rats it was lower than in their young counterparts, but this decrease did not reach statistical significance (Fig. 7a). Similarly, the number of CD4+CD25+Foxp3+ thymocytes diminished ($p < 0.001$) with ageing and upon immunisation for EAE in both young and old rats (Fig. 7a). In immunised rats their number was markedly lower ($p < 0.001$) in old rats than in their young counterparts (Fig. 7a).

Next, the expression of mRNAs for IL-2 and IL-15 gamma chain (γc) cytokines (Apert et al., 2018; Hu et al., 2017), which in addition to FoxN1 and IL-7 (Coder et al., 2015; Vang et al., 2008), influence Treg differentiation/maturation, was examined. In accordance with age-related decrease in the frequency of Tregs, the expression of both IL-2 and IL-15 mRNAs was diminished ($p < 0.001$) in thymi of old compared with young non-immunised rats (Fig. 7b). It was also down-regulated in thymi of young ($p < 0.001$) and old ($p < 0.01$ and $p < 0.001$ for IL-2 mRNA and IL-15 mRNA, respectively) immunised rats compared with age-matched non-immunised rats (Fig. 7b). Of note, the expression of both IL-2 mRNA and IL-15 mRNA was lowered ($p < 0.05$) in thymi of old immunised rats compared with their young counterparts (Fig. 7b).

The quantification of TGF-β mRNA expression showed prominent age-related decrease ($p < 0.001$) (Fig. 7b). Immunisation down-regulated its expression in young ($p < 0.001$) and old ($p < 0.05$) rats (Fig. 7b). Thus, less ($p < 0.001$) amount of TGF-β mRNA was found in thymi from old immunised rats compared with their young counterparts (Fig. 7b).

3.7. Ageing and immunisation for EAE affected the phenotypic profile of T-PBLs

3.7.1. Ageing and immunisation for EAE influenced the frequency of CD90+CD45RC- RTEs and cells expressing CD90-CD45RC- memory phenotype among T-PBLs

To evaluate the impact of ageing- and immunisation-related changes in thymopoiesis on the peripheral T-cell compartment, the frequency of CD90+CD45RC- RTEs, suggested to be thymic function surrogates (Hosseinzadeh and Goldschneider, 1993), was evaluated. Indeed, with ageing the frequency of RTEs among both CD4+ T-PBLs and CD8+ T-PBLs markedly decreased ($p < 0.001$) (Fig. 8a). Additionally, immunisation for EAE in young ($p < 0.001$) and old ($p < 0.01$) rats diminished their frequency among the major subpopulations of T-PBLs (Fig. 8a). It should be pointed out that the frequency of RTEs was lower among both CD4+ T-PBLs ($p < 0.01$) and CD8+ T-PBLs ($p < 0.001$) from old immunised rats compared with their young counterparts (Fig. 8a). Collectively, these findings supported the hypothesis based on our

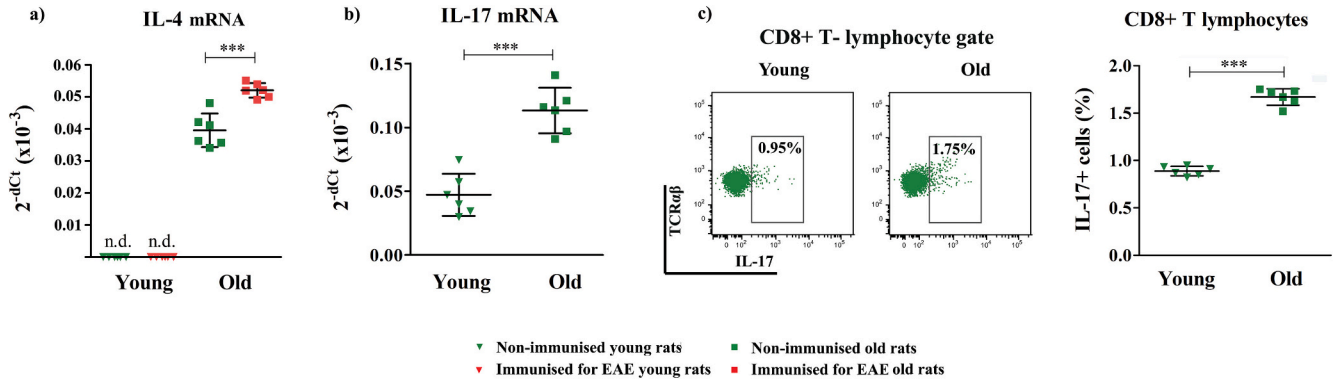

Fig. 6. Ageing increased thymic expression of IL-4 and IL-17 mRNAs and the frequency of IL-17+ cells among lymph node CD8+ T lymphocytes in AO rats.
Scatter plots indicate the expression levels of (a) IL-4 mRNA in the thymi of non-immunised and immunised for EAE young and old rats, and (b) IL-17 mRNA in the thymic tissue of non-immunised young and old rats. (c) Representative flow cytometry dot plots and scatter plot show the frequency of IL-17+ cells among CD8+TCRαβ+ (T) cells retrieved from the lymph nodes of non-immunised young and old rats (gating strategy is displayed in Supplementary Fig. 5). Significance of differences between groups in thymic IL-17 mRNA expression and the frequency of IL-17+ cells among lymph node CD8+ T cells was assessed using Student's *t*-test. Two-way ANOVA showed a significant interaction between the effects of ageing and immunisation for thymic IL-4 mRNA expression [$F_{(1,20)} = 28.57$, $p < 0.001$]. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). *** $p < 0.001$.

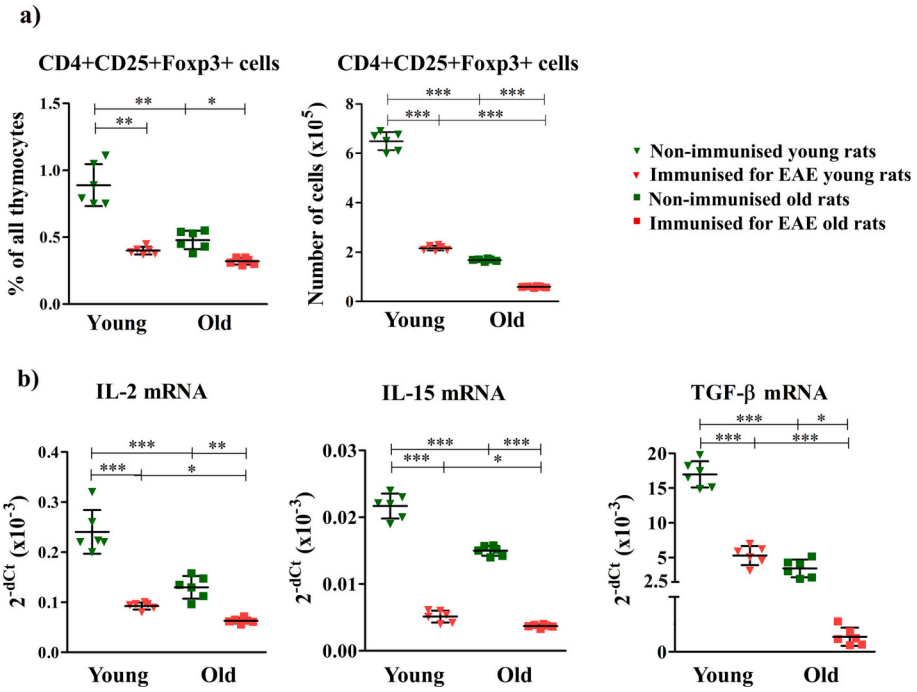

Fig. 7. Ageing and immunisation for EAE decreased the frequency and number of thymocytes with regulatory CD4+CD25+Foxp3+ phenotype in AO rats by affecting the thymic expression of mRNAs for IL-2, IL-15 and TGF-β.

Scatter plots show (a) the frequency and the number of CD4+ thymocytes exhibiting regulatory CD25+Foxp3+ phenotype in thymi of non-immunised and immunised for EAE young and old rats (gating strategy for CD25+Foxp3+ cells among CD4+ thymocytes is displayed in Supplementary Fig. 6), and (b) the expression of mRNAs encoding IL-2, IL-15 and TGF-β in thymic tissue of non-immunised and immunised young and old rats. Two-way ANOVA showed significant interaction between the effects of ageing and immunisation for the frequency [$F_{(1,20)} = 32.4$, $p < 0.001$] and number [$F_{(1,20)} = 428.4$, $p < 0.05$] of CD4+CD25+Foxp3+ thymocytes, and for thymic IL-2 [$F_{(1,20)} = 59.94$, $p < 0.001$], IL-15 [$F_{(1,20)} = 4.672$, $p < 0.05$] and TGF-β ($F_{(1,20)} = 77.6$, $p < 0.001$) mRNA expression. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

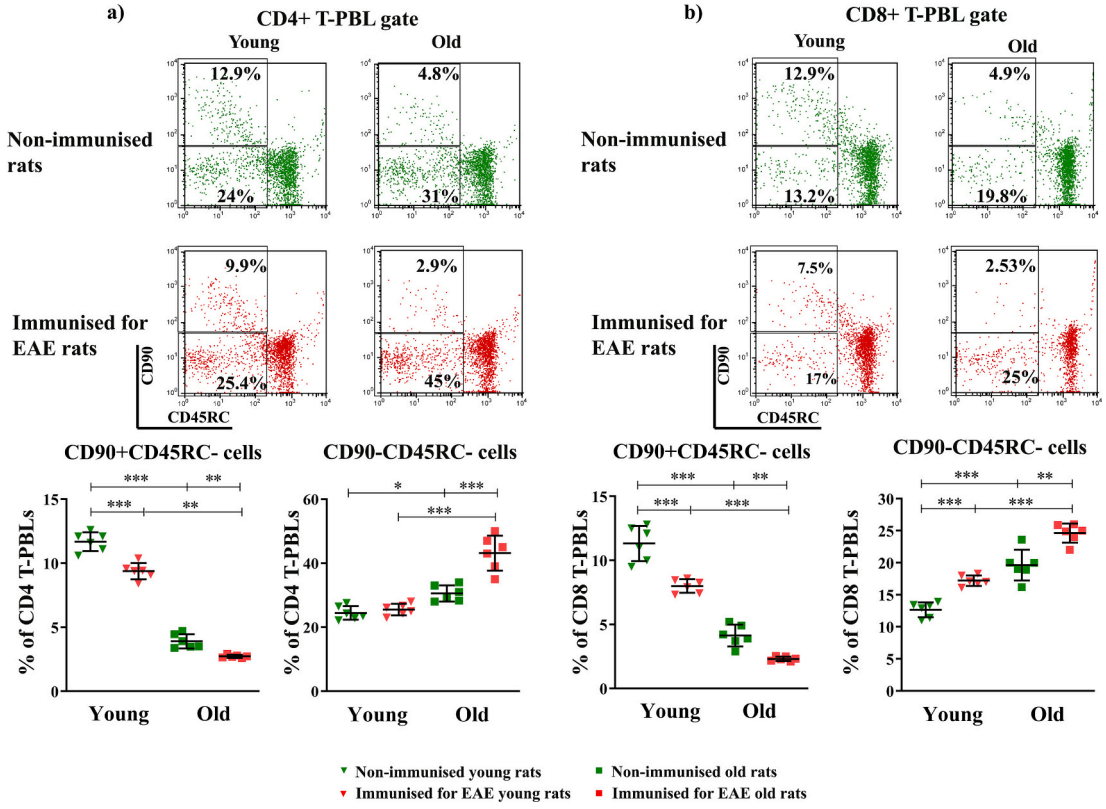

Fig. 8. Ageing and immunisation for EAE decreased the frequency of CD90+CD45RC- RTEs, but increased the frequency of activated/memory CD90-CD45RC- cells among CD4+ and CD8+ T-PBLs in AO rats.

Representative flow cytometry dot plots show CD90/CD45RC staining of (a) CD4+ and (b) CD8+ T-peripheral blood lymphocytes (T-PBLs) of non-immunised and immunised for EAE young and old rats (gating strategy for CD4+ and CD8+ T-PBLs is displayed in Supplementary Fig. 7). Scatter plots indicate the frequency of CD90+CD45RC- recent thymic emigrants (RTEs) and memory/activated CD90-CD45RC- cells within (a) CD4+ and (b) CD8+ T-PBLs. Two-way ANOVA showed a significant interaction between the effects of ageing and immunisation for the frequency of CD90-CD45RC- cells within CD4+ T-PBLs [$F_{(1,20)} = 28.52$, $p < 0.001$]. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

previous findings which suggest that ageing and immunisation decrease the thymic T-cell output.

Next, given that diminished thymopoiesis is associated with expansion of certain T-cell clones in the periphery, and consequently a relative enlargement of the pool of memory/activated T cells (Naylor et al., 2005; Theofilopoulos et al., 2001), the frequency of cells exhibiting memory/activated CD90-CD45RC- phenotype (Hosseinzadeh and Goldschneider, 1993) among both CD4+ and CD8+ T-PBLs was examined. With ageing the frequency of memory/activated CD90-CD45RC- cells raised among both CD4+ T-PBLs ($p < 0.05$) and CD8+ T-PBLs ($p < 0.001$) (Fig. 8b). Upon immunisation for EAE in young rats their frequency increased ($p < 0.001$) only among CD8+ T-PBLs (Fig. 8b). Differently, in old rats upon immunisation the frequency of CD90-CD45RC- cells raised among both CD4+ T-PBLs ($p < 0.001$) and CD8+ T-PBLs ($p < 0.01$) (Fig. 8b). Consequently, the frequency of CD90-CD45RC- cells was higher ($p < 0.001$) among both CD4+ T-PBLs and CD8+ T-PBLs from old immunised rats when compared with their young counterparts (Fig. 8b).

3.7.2. Ageing and immunisation for EAE increased the frequency of CD28- cells among CD8+ T-PBLs and/or CD4+ T-PBLs

Taking herein reported findings in conjunction with data showing that the decrease in the thymic output of T cells results in their homeostatic oligoclonal proliferation (Ge et al., 2001; Jameson, 2002) and loss of CD28 surface expression (Vallejo et al., 1999), the frequency of CD28-(null) cells among CD4+ and CD8+ T-PBLs was also examined. With ageing, the frequency of CD28- cells increased within both CD4+ T-PBLs ($p < 0.001$) and CD8+ T-PBLs ($p < 0.01$) (Fig. 9a). In old rats immunisation additionally increased ($p < 0.001$) their frequency within both CD4+ T-PBLs and CD8+ T-PBLs (Fig. 9a). On the other hand, in young rats upon immunisation the frequency of CD28- cells increased ($p < 0.05$) only within CD8+ T-PBLs (Fig. 9a). Consequently, their frequency was markedly higher ($p < 0.001$) among CD4+ T-PBLs and CD8+ T-PBLs from old immunised rats compared with their young counterparts (Fig. 9a).

Next, given that the development of inflammatory autoimmune diseases is associated with the rise in circulating levels of TNF-α, the cytokine which directly downregulates CD28 expression on T cells (Bryl et al., 2001; Lee and Lee, 2016; Maly and Schirmer, 2015), its plasma level was also determined. Differently from young rats, in old rats the plasma level of TNF-α was higher ($p < 0.001$) in immunised rats compared with age-matched non-immunised animals (Fig. 9b). Its concentration was also higher ($p < 0.001$) in old immunised rats than in their young counterparts (Fig. 9b).

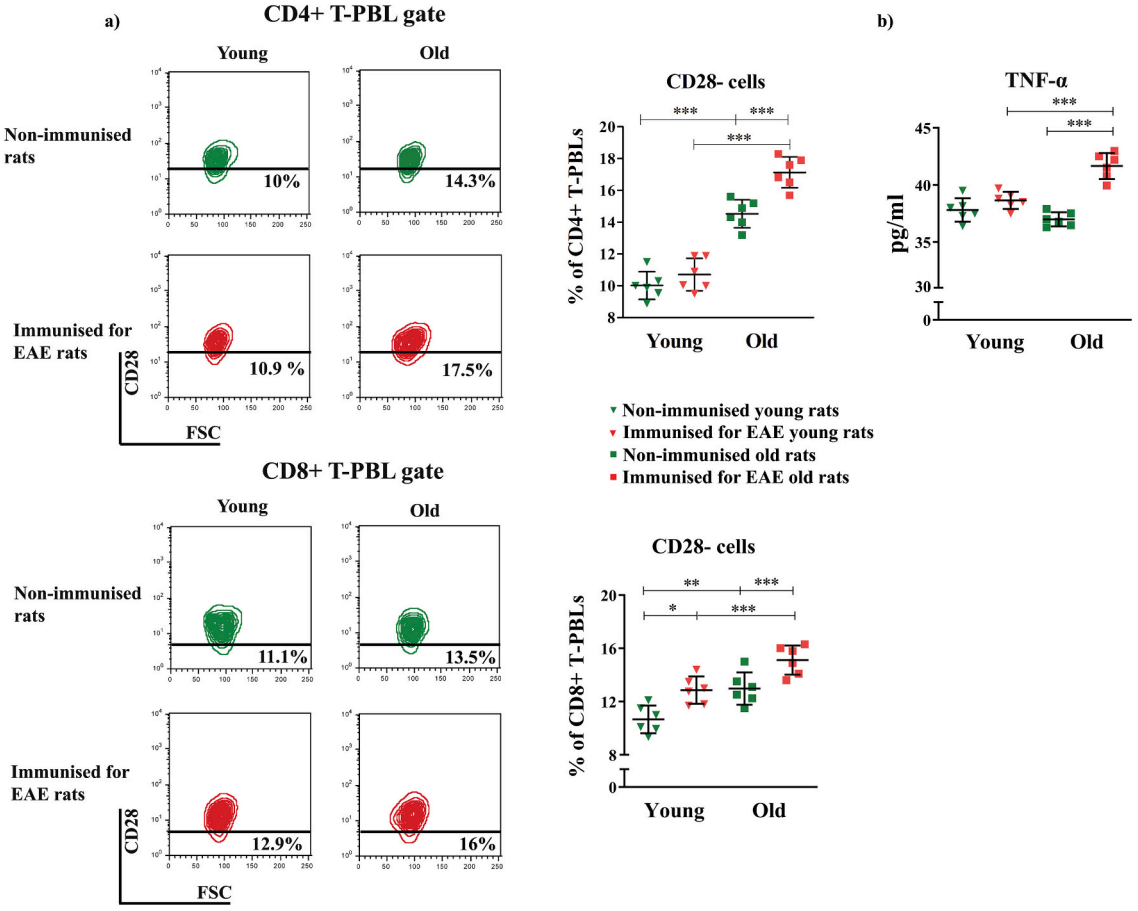

Fig. 9. Ageing and immunisation for EAE increased the frequency of CD28- cells among CD4+ and CD8+ T-PBLs from AO rats partly through elevation of circulating TNF-α level.

(a) Representative flow cytometry contour plots show CD28 staining of (upper panel) CD4+ and (lower panel) CD8+ T-peripheral blood lymphocytes (T-PBLs) from non-immunised and immunised for EAE young and old rats (gating strategy for CD28- cells among CD4+ and CD8+ T-PBLs is displayed in Supplementary Fig. 8). Scatter plots indicate the frequency of CD28- cells among (upper) CD4+ and (lower) CD8+ T-PBLs from non-immunised and immunised young and old rats. (b) Scatter plot indicates the concentration of TNF-α in plasma of non-immunised and immunised young and old rats. Two-way ANOVA showed significant interaction between the effects of ageing and immunisation for the frequency of CD28- cells among CD4+ [$F_{(1,20)} = 125.8$, $p < 0.001$] and CD8+ [$F_{(1,20)} = 44.12$, $p < 0.001$] T-PBLs, and plasma TNF-α concentration [$F_{(1,20)} = 26.65$, $p < 0.001$]. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). * $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$.

3.8. Greater frequency of granzyme B-producing CD4+ and CD8+ T lymphocytes in the spinal cord of old rats immunised for EAE

Given that cytotoxic granzyme B-producing (granzyme B+) CD28- T cells are shown to migrate to sites of inflammation in MS to contribute to tissue damage (Broux et al., 2012; Maly and Schirmer, 2015; Peeters et al., 2017; Tedeschi et al., 2022), the frequency of granzyme B+ CD4+ and CD8+ T cells infiltrating spinal cord of immunised rats was examined. The frequencies of granzyme B-producing cells within CD4+TCRαβ+ and CD8+TCRαβ+ lymphocytes were considerably higher ($p < 0.001$) in the spinal cord of old immunised rats compared with their young counterparts (Fig. 10a, b). Old immunised rats also exhibited higher ($p < 0.001$) Granzyme B expression density in CD8+TCRαβ+ spinal cord cells than young immunised animals (Fig. 10b).

4. Discussion

The study further extended our previous work on the influence of ageing on the development of EAE in AO rats (Stojić-Vukanić et al., 2015, 2018) by showing that ageing-related thymic changes may contribute to the increased susceptibility of AO rats to the development of clinically manifested EAE through multilayered mechanisms. These mechanisms include: i) deepened imperfect thymocyte negative selection, ii) impaired generation of Tregs, iii) facilitated development of CD8+ T cells licensed to differentiate into Tc17 cells and iv) diminished thymocyte output leading to augmented generation of "inflammescent" cytotoxic CD28- T cells. Besides, the study suggests that development of EAE potentiates thymic atrophy and decline in thymic T-cell output leading to the enlargement of the peripheral CD28- T-cell pool, and aggravates already diminished nTreg generation thereby leading to the disease perpetuation.

4.1. Influence of ageing on thymus of non-immunised rats

Ageing-associated decrease in thymic weight and thymocyte yield in non-immunised rats correlated with the increase in circulating levels of IL-6 and thymic expression of IL-6 mRNA. This was consistent with the findings indicating that (i) administration of IL-6 into a young, healthy mouse causes thymic atrophy (Gruver and Sempowski, 2008), and (ii) the increase in its thymic expression with ageing correlates with thymic involution and impaired thymic T-cell output (Sempowski et al., 2000). The rise in circulating levels of IL-6 with ageing has been found in humans and rodents alike, so IL-6 is termed "gerontologist's cytokine" (Ershler and Keller, 2000; Jergović et al., 2021; Nacka-Aleksić et al., 2017). The effects of IL-6 on thymus have been linked with direct damage of TECs which with other thymic stromal cells form network-like structure that regulates the development, differentiation, maturation and migration of thymocytes (Cohen-Kaminsky et al., 1991; Gruver and Sempowski, 2008; Sempowski et al., 2000). Consistently, as previously shown in mice (Ortman et al., 2002), the expression of mRNA for Foxn1, the key regulator of TEC development and maintenance (Rode et al., 2015; Vaidya et al., 2016), was dramatically diminished in thymi from old non-immunised rats compared with young non-immunised rats. Given that, differently from IL-6 which is produced by distinct subsets of thymic stromal cells including thymic adipocytes (Sempowski

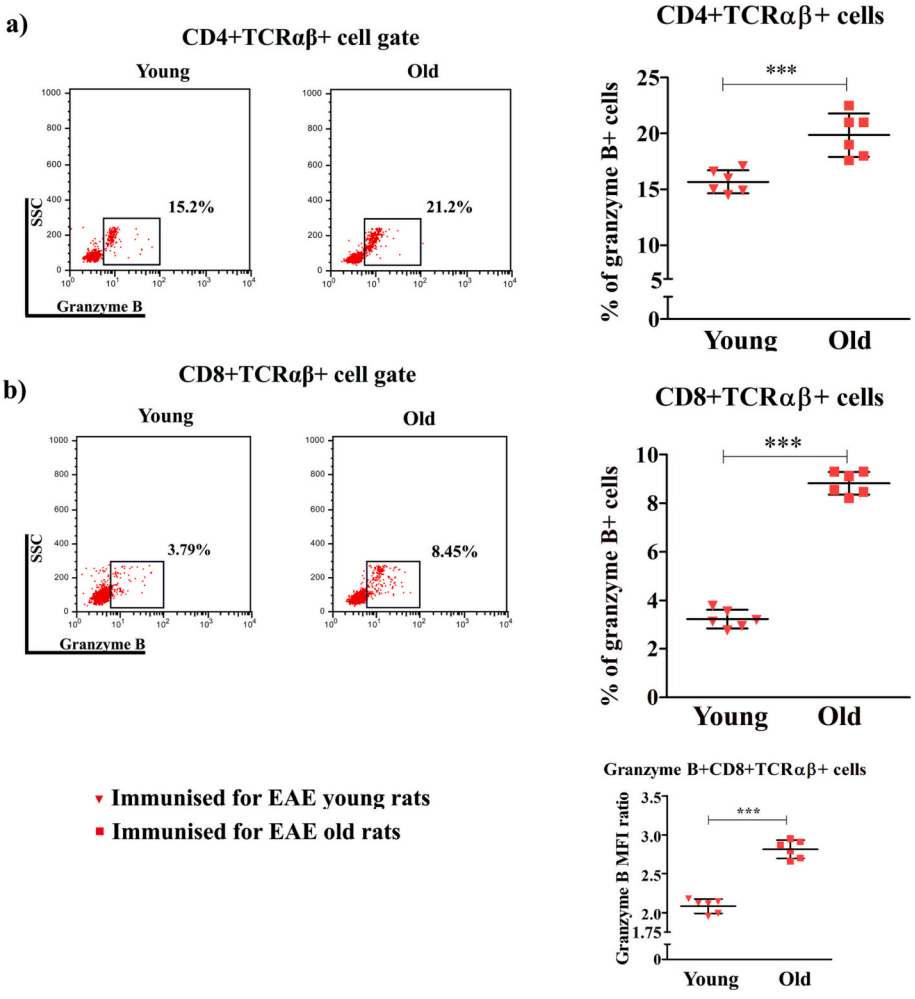

Fig. 10. Greater frequency of granzyme B+ cells within CD4+ and CD8+ T lymphocytes in spinal cords of old AO rats immunised for EAE.
Representative flow cytometry dot plots and scatter plots show the frequency of granzyme B+ cells among (a) CD4+ and (b) CD8+ TCRαβ+ (T) lymphocytes retrieved from the spinal cord of young and old rats immunised for EAE (gating strategy for Granzyme B+ cells among CD4+ and CD8+ T lymphocytes is displayed in Supplementary Fig. 9). (b, lower) Scatter plot indicates Granzyme B mean fluorescence intensity (MFI) ratio (see Material and methods section) in spinal cord Granzyme B+ CD8+ T lymphocytes of immunised young and old rats. Student's *t*-test was used to assess the statistical significance of differences between groups. Data points and means ± SD are from one of two experiments with similar results ($n = 6$). *** $p < 0.001$.

et al., 2000), IL-7 is mainly produced by TECs (Hong et al., 2012), the age-related decrease in its thymic expression was in keeping with the previous data. To indirectly corroborate this finding are data indicating that (i) total thymocyte numbers are indicative of in vivo IL-7 availability, and (ii) the complete blockade of IL-7 signaling or genetic deletion of IL-7Rα usually results in prominent decrease of thymocyte numbers (Hong et al., 2012). In the same vein was the increased frequency of apoptotic cells among thymocytes, as it was shown that during ageing up-regulated production of suppressive cytokines (such as IL-6) acting directly or indirectly overcomes anti-apoptotic signals of trophic cytokines such as IL-7, and contributes to thymic involution (Sempowski et al., 2000). In favour of this option are data indicating that thymic involution in rapid-involuting strains of mice could be ascribed to a premature downregulation of IL-7 gene expression in TECs leading to diminished thymocyte anti-apoptotic Bcl-2 gene expression, followed by upregulation of pro-apoptotic BAD gene expression (Wang et al., 2006). Furthermore, in accordance with some previous studies (Min et al., 2004), the age-related decrease in the thymocyte yield in AO rats could also be linked to the reduced frequency of proliferating cells among thymocytes. This correlated with prominent downregulation of IL-21 mRNA expression in thymus with ageing. Namely, IL-21 is shown to exert mitogenic function on peptide-mediated TCR-engaged DP thymocytes in an in vitro coculture system designed for T-cell differentiation (Rafei et al., 2013a), whereas its administration to mice with glucocorticoid-induced thymic atrophy dramatically accelerates thymic function recovery by stimulating the proliferation of DN and positively selected DP thymocytes (Rafei et al., 2013b). It is noteworthy that age-related reduction of thymocyte compartment in turn leads to the reduction of the delicate thymic stromal compartment (and thereby affects IL-7 synthesis), as it is dependent on crosstalk interactions with thymocytes for sustained survival and vice versa (Ritter and Boyd, 1993). Additionally, as the main source of IL-21 in thymus is a subset of CD4+ T cells termed natural (n)Th21 cells, which are dependent on autoimmune regulator (AIRE) expression in a highly dosage-sensitive manner (Marnik et al., 2017), it may be speculated that diminished thymic IL-21 expression in old AO rats reflected impaired AIRE expression. To corroborate this assumption are data indicating that thymic AIRE expression decreases with ageing (Thomas et al., 2020).

In addition to alterations in thymocyte yield, the thymocyte subset composition was altered with ageing suggesting changes in multistep process of thymocyte differentiation/maturation. The rise in the frequency of the most mature DN cells among thymocytes with ageing despite age-related downregulation of the expression of IL-7 and IL-21, the cytokines expanding this thymocyte subset (Rafei et al., 2013b; Wang et al., 2006), is consistent with findings indicating that systematically and locally excessive IL-6 production leads to the inhibition of the differentiation of DN thymocytes and consequently their accumulation (Carbajosa et al., 2017). Alternatively, but not mutually exclusive, the increased proportion of DN cells may be associated with the increased thymic expression of CXCL12, as this chemokine is critical for the recruitment and homing of lymphoid progenitors in the thymus (Jenkinson et al., 2007; Plotkin et al., 2003). Of note, the increased CXCL12 expression was found in the prominently involuted human thymus (Hernández-López et al., 2010). Additionally, there are also data indicating that emptying of the thymic niches accompanied by a diminished thymic T-cell export and contraction of the peripheral T-lymphocyte pool provides feedback signals promoting increased progenitor entry (Bird, 2009). The increased frequency of DN thymocyte descendant DP TCRαβ- thymocytes encompassing mostly cells that have surpassed β selection (Zamoyska and Lovatt, 2004) among thymocytes, in conjunction with the decreased frequencies of DP TCRαβ^{lo} thymocytes entering selection (Jameson et al., 1995; Zamoyska and Lovatt, 2004) and DP TCRαβ^{hi} thymocytes that surpassed positive selection (Jameson et al., 1995) in old non-immunised rats compared with their young counterparts suggested alterations in thymocyte selection processes. The age-related decrease in IL-21 expression supports ageing-dependent decrease in positively selected DP cells as rIL-21 injection to old mice significantly enhances their expansion, and consequently de novo intrathymic T-cell development (Marnik et al., 2017). On the other hand, considering that disruption of the function of AIRE, which plays a critical role in driving expression of thousands of tissue-specific antigens that are presented in complex with MHC molecules on TECs to the maturing thymocytes and thereby their negative selection (limiting the release of self-reactive thymocytes into the peripheral pool), results in the release of self-reactive thymocytes with potential to initiate self-reactivity in both humans and mice (Anderson and Su, 2011), the increased thymocyte negative selection in thymi of old rats does not seem to be plausible. As the disruption in thymic FoxN1 expression is associated with impaired AIRE function (Xia et al., 2012), age-related decline in its expression corroborates the previous notion. Last, but not least important, given that Thy-1 acts as negative regulator of thymocyte negative selection, its increased expression on DP TCRαβ^{lo} and DP TCRαβ^{hi} thymocytes also goes in favour of decreased negative selection (Hueber et al., 1997). On the other hand, the increased frequency of the most mature CD4+CD8- and CD4-CD8+ SP TCRαβ^{hi} thymocytes may suggest that an accelerated maturation of thymocytes from DP TCR- developmental stage onward also contributed to the decreased relative representation of DP TCRαβ$^{lo/hi}$ thymocyte subsets. However, at the present there is no data to support this option. Additionally, considering diminished thymic expression of IL-21, the cytokine also playing a role in the migration of SP T cells to the periphery (Moretto et al., 2019), in old non-immunised rat thymi compared with their young counterparts, the relative accrual of the mature SP thymocytes in old thymi due to their deaccelerated egress cannot be excluded.

Furthermore, considering that impairment of thymic selection alone is insufficient for the development of CNS autoimmunity (Alberti and Handel, 2021), we examined nTreg generation. Indeed, our study revealed the decline in the frequency and absolute number of Tregs among thymocytes from old non-immunised rats. This was consistent with age-related decline in thymic expression of IL-2, IL-7 and IL-15, cytokines of γc family, which regulate distinct essential steps in thymic Treg development (Apert et al., 2018; Hu et al., 2017; Vang et al., 2008). Similar findings were obtained in mice (Srinivasan et al., 2021). The age-related decrease in thymic TGF-β expression observed in non-immunised rats also goes in favour of impaired nTreg generation (Chen and Konkel, 2015). To fully appreciate the significance of these findings it should be pointed out that newly generated Tregs in thymus are critical for Treg-mediated suppression of autoimmune response in MS (Haas et al., 2007).

Of note, despite the increase in frequency of the most mature SP TCRαβ^{hi} thymocytes in old non-immunised rat thymi compared with young ones, their absolute number was markedly diminished, as suggested by other studies (Shitara et al., 2013). More important, as expected (Hale et al., 2006; Palmer, 2013; Rezzani et al., 2014), the frequency of RTEs was decreased among both CD4+ T-PBLs and CD8+ T-PBLs suggesting diminished thymic output of naïve T cells, whereas that of memory/activated cells increased. The latter correlated with the increase in the frequency of cytotoxic, highly reactive, CD28- cells with the potential to enhance tissue damage in autoimmune diseases, among CD4+ T-PBLs and CD8+ T-PBLs (Weng et al., 2009; Strioga et al., 2011). This phenomenon that could also be important for understanding age-related increase in susceptibility of old AO rats for the development of clinically manifested EAE has been observed in some other studies, as well (Vallejo, 2005).

Last, but not least important, in accordance with the increased expression of IL-4, the cytokine licensing developing CD8+ T cells to produce IL-17 (Maier et al., 2011; Mielke et al., 2019), in thymi of old non-immunised rats compared with their young counterparts, the increased frequency of Tc17 cells, which are shown to be important for EAE development in old AO rats (Stojić-Vukanić et al., 2018), was found in lymph nodes of old rats. It is noteworthy that Tc17 cells were found in secondary lymphoid organs of healthy humans (Mielke et al., 2019).

4.2. Influence of immunisation for EAE on thymus of young and old rats

As expected from previous studies indicating that inflammation, including inflammation-associated with autoimmune diseases, accelerates thymic atrophy in experimental animals (Chalan et al., 2015; Koetz et al., 2000; Nacka-Aleksić et al., 2018; O'Sullivan et al., 2018; Thewissen et al., 2007b) immunisation for EAE induced/deepened thymic atrophy. This could be linked, at least partly, with the rise in the circulating/thymic levels of IL-6 (Gruver and Sempowski, 2008; Sempowski et al., 2000). Consistent with immunisation-induced alterations in IL-6 systemic/thymic expression, thymic expression of FoxN1 and IL-7 mRNAs decreased compared with age-matched non-immunised rats (Hong et al., 2012; Rode et al., 2015; Vaidya et al., 2016). In old rats, immunisation-induced rise in TNF-α could also contribute to thymic atrophy. Namely, in addition to being a potent inducer of thymocyte apoptosis (Pérez et al., 2007), TNF-α is shown to be potent inducer of IL-6 expression in TECs (Cohen-Kaminsky et al., 1991). The increase in the frequency of apoptotic thymocytes is consistent with data indicating that the rise of thymocyte apoptosis stands behind thymic atrophy related to inflammation associated with infection and autoimmune disease (Luo et al., 2021; Nacka-Aleksić et al., 2018). Given that immunisation did not influence the overall frequency of proliferating thymocytes in either young or old rats, the decline in the total thymocyte yield most likely reflected mainly the enhanced thymocyte apoptosis. Additionally, judging by the alterations in thymocyte subset composition, immunisation affected thymocyte differentiation and maturation. Specifically, hereby presented findings suggest that immunisation affected differentiation/maturation of thymocytes from DP TCRαβ- developmental stage, i.e. further differentiation/maturation of the cells that have surpassed β selection (Zamoyska and Lovatt, 2004). Given that the frequency of DP TCRαβ- thymocytes was increased, whereas that of that of the descendent DP TCRαβ^{lo} thymocytes entering selection (Jameson et al., 1995; Zamoyska and Lovatt, 2004) remained unaltered and the frequency of DP TCRαβ^{hi} thymocytes that surpassed positive selection (Jameson et al., 1995) decreased in immunised rats compared with age-matched non-immunised rats, it may be assumed that immunisation affected the expression of αTCR chain and thereby transition into DP TCRαβ^{lo} stage and diminished thymocyte negative selection.

Furthermore, immunisation in young and old rats decreased the generation of Tregs, which are shown to be of utmost importance in curbing autoimmune response in MS (Haas et al., 2007), by impairing thymic availability of various cytokines (IL-2, IL-7, IL-15, TGF-β) involved in their development (Apert et al., 2018; Chen and Konkel, 2015; Hu et al., 2017; Liu et al., 2008; Vang et al., 2008), the phenomenon particularly prominent in old rats developing clinically manifested disease. These findings are in keeping with data indicating that genetically mediated central tolerance defects may be potentiated by thymic inflammation with important implications for the development of autoimmune diseases (O'Sullivan et al., 2018). To additionally emphasize the significance of these findings are data indicating that thymectomy inhibits spontaneous remission in rat EAE (Ben-Nun et al., 1980).

It is also noteworthy that immunisation diminished the number of the most mature CD4+CD8- and CD4-CD8+ SP TCRαβ^{hi} thymocytes, the effect particularly prominent in old rats. Consistently, immunisation diminished the frequency of RTEs among CD4+ and CD8+ T-PBLs in young and old animals and increased those of memory cells among CD8+ T-PBLs in rats of both ages, and among CD4+ T-PBLs in old rats. These changes in distribution of T-PBL subsets at distinct stages of differentiations is consistent with findings obtained in adult EAE-inflicted Dark Agouti rats (Nacka-Aleksić et al., 2018) and in some other autoimmune diseases (Chalan et al., 2015; Koetz et al., 2000; Thewissen et al., 2007b). In rats of both ages, the rise in the frequency of memory T-PBLs most likely reflected increase in the homeostatic proliferative pressure in face of diminished thymic output. In keeping with some other studies (Yanes et al., 2017), this increase was particularly prominent in CD8+ T-PBL compartment, so that the frequency of memory cells rose among CD8+ T-PBLs from young and old rats, whereas their frequency among CD4+ T-PBLs increased only in old rats (Yanes et al., 2017). Consequently, the frequency of replicatively exhausted CD28- cells increased within both the major T-PBL subpopulations of old EAE rats, and CD8+ T-PBLs from their young counterparts. The loss of CD28 expression on CD4+ T-PBLs is particularly important as these cells are shown to produce large amounts of proinflammatory cytokines such as IFN-γ and TNF-α and also have cytotoxic potential, so they may cause tissue damage and contribute to pathogenesis of many inflammatory diseases, including EAE/MS (Lee and Lee, 2016; Nacka-Aleksić et al., 2018; Peeters et al., 2017), whereas CD28-CD8+ T cells are also suggested to contain a subset of cells with regulatory properties (Strioga et al., 2011), so their contribution to EAE development is not easy to predict. Given that TNF-α is shown to promote the loss of CD28 expression not only indirectly by contributing to thymic atrophy, but also by acting directly on CD28 gene transcription (Bryl et al., 2001), the increase in the circulating level of this cytokine could also contribute to the increased frequency of CD28- T cells in old immunised rats compared with age-matched non-immunised rats. It is noteworthy that the frequency of CD28- cells among both CD4+ and CD8+ T-PBLs was greater in old immunised rats compared with their young counterparts. Thus, it may be assumed that systemic inflammation associated with the development of clinical EAE in old rats contributed to the perpetuation of the disease not only by affecting generation of nTreg, but also by increasing the development of replicatively exhausted proinflammatory cytotoxic T cells.

5. Conclusion

In conclusion, the study revealed that ageing in AO rats (relatively resistant to EAE induction in adult age) increases susceptibility to the development of clinically manifested EAE by (i) impairing central tolerance mechanisms, (ii) favouring generation of CD8+ T cells prone to differentiate into Tc17 cells, which are suggested to be important for EAE development in rats relatively resistant to the disease (Camara et al., 2013; Huber et al., 2013; Huber and Lohoff, 2015; Stojić-Vukanić et al., 2018), and (iii) diminishing T-cell generation and thereby increasing development of potentially pathogenic CD28- T cells with proinflammatory and cytotoxic properties. In addition, it confirmed that the development of a prominent inflammatory response related to autoimmune disease (as in old AO rats) deepens thymic atrophy (Nacka-Aleksić et al., 2018) and enhances the generation of proinflammatory cytotoxic CD28- T cells leading to the perpetuation of the disease. In this context, it is important to add that the loss of CD28 transcription in inflammatory pathologies is reversible (Lee and Lee, 2016), as it could be of therapeutic relevance. These findings apart from academic value, could be (inasmuch as findings obtained in experimental animals could be translated to humans) important for understanding mechanisms standing behind the late onset MS and thereby possibly for designing new therapeutic approaches.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2022.112009.

Funding

This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant numbers 451-03-68/2022-14/200161, 451-03-68/2022-14/200177).

CRediT authorship contribution statement

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Methodology, Investigation; Ivana Prijić: Methodology, Investigation; Gordana Leposavić: Conceptualization, Formal analysis, Investigation, Writing - Original draft preparation, Supervision. All authors contributed to Writing - Reviewing and Editing.

Declaration of competing interest

None.

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