Neuroimmunomodulation

Research Article

Neuroimmunomodulation 2023;30:346–373
DOI: 10.1159/000535150

Received: June 30, 2023
Accepted: November 9, 2023
Published online: November 10, 2023

Ageing Affects Thymopoiesis and Experimental Autoimmune Encephalomyelitis Development in a Strain-Dependent Manner

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Keywords
Ageing · Rat strain differences · Negative selection · Natural regulatory T cells · Experimental autoimmune encephalomyelitis susceptibility

Abstract

Introduction: Considering significance of mechanisms of central tolerance for development of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), and suppressive influence of circulating proinflammatory cytokines and alterations in brain-thymus communication, characteristic for the central nervous system (CNS) autoimmune diseases, on thymopoiesis, the study interogated putative strain-based thymus-related specificities relevant for the opposite effects of ageing on susceptibility of Dark Agouti (DA) and Albino Oxford (AO) rats to EAE. ***Methods:*** Quantitative and qualitative changes in thymopoiesis including underlying mechanisms were examined using flow cytometry and RT-qPCR quantification of mRNAs for molecules relevant for integrity of stroma and T-cell development, respectively. ***Results:*** With ageing, differently from DA rats, in AO rats the surface density of CD90, a negative regulator of selection threshold, on thymocytes undergoing lineage commitment was upregulated (consistent with TGF-β expression downregulation), whereas the generation of natural CD4+CD25+Foxp3+ regulatory T cells (nTregs) was impaired reflecting differences in thymic expression of cytokines supporting their development. Additionally, specifically in old AO rats, in whom EAE development depends on IL-17-producing CD8+ T cells, their thymic differentiation was augmented, reflecting augmented thymic IL-4 expression. In turn, differently from old DA rats developing self-limiting EAE, in age-matched AO rats developing EAE of prolonged duration, EAE development led to impaired generation of nTregs and accumulation of proinflammatory, cytotoxic CD28–CD4+ T cells in the periphery. ***Discussion:*** The study indicates that strain differences in age-related changes in the efficacy of central tolerance, in addition to enhanced thymic generation of CD8+ T cells prone to differentiate into IL-17-producing cells, could partly explain the opposite effect of ageing on DA and AO rat susceptibility to EAE induction. Additionally, it suggested that EAE development leading to a less efficient thymic output of CD4+ cells and nTregs in old AO rats than their DA counterparts could contribute to prolonged EAE duration in AO compared with DA rats. ***Conclusion:*** The study warns to caution when designing therapeutic interventions to

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enhance thymic activity in genetically diverse populations, e.g., humans, and interpreting their outcomes. Furthermore, it indicates that CNS autoimmune pathology may additionally worsen thymic involution and age-related immune changes.

Introduction

Thymus is a primary lymphoid organ providing specific microenvironment where bone marrow-derived precursors mature into cells which acquire the ability to proliferate in response to TCR stimulation, and undergo licensing for cytokine production, prior to joining the peripheral T-cell pool as recent thymic emigrants (RTEs) [1]. This maturation also encompasses establishment of central tolerance through two complementary mechanisms. First is the thymocyte negative selection, during which the majority of self (auto)-reactive developing T cells are depleted from the repertoire via apoptosis, so only those cells bearing TCR with a low affinity for self enter the circulation [2]. Accordingly, generally, deregulation of this process leads to autoimmune disease [3]. Second is the generation of CD4+CD8− single positive (SP) natural (thymic) regulatory T cells (nTregs), which in rats are best identified by the expression of CD25 and Foxp3 [4–6]. Their primary function is to suppress T cell-mediated self-reactivity by the cells which somehow escaped negative selection [7]. In other words, they should compensate for imperfections of negative selection and thereby preserve immune homeostasis in the periphery, viz., prevent development of autoimmune disorders [7].

Of note, the thymus is organ shown to undergo atrophy with age, a phenomenon termed as thymic involution, which is associated with a low output of naïve T cells leading to increased oligoclonal expansion of memory T cells [8] followed by accumulation of CD28− (null) CD4+ and CD8+ T cells, which acquire expression of several receptors commonly associated with natural killer cells, secrete large amounts of proinflammatory cytokines, and express high amounts of perforin and granzyme B (viz., acquire a high cytotoxic capability) in the elderly and old experimental animals, contributing to development of chronic inflammatory diseases in old age [9–11]. Additionally, although it is yet relatively little known, ageing impacts quantitative changes not only in thymopoiesis, but also in the efficacy of negative selection [12–15] and the generation of nTregs [15, 16]. However, despite enhanced autoimmunity, there is no increase in the incidence of T-cell-mediated autoimmune diseases, including multiple sclerosis (MS), in old age, most likely due to activation/augmentation of mechanisms of peripheral tolerance as the intricate details associating self-tolerance mechanisms to autoimmunity have been challenging to decipher [17]. Namely, the first signs of MS typically appear between the ages of 20 and 40 years, whereas they rarely occur after the age of 60 years [18]. Consistently, the susceptibility to development of clinically manifested experimental autoimmune encephalomyelitis (EAE), the most commonly used animal models of MS, varies with age in an animal strain-specific manner [19].

Our previous study showed that with ageing susceptibility to EAE development decreases in Dark Agouti (DA) rats, whereas Albino Oxford (AO) rats, who do not exhibit neurological signs of the disease upon immunisation for EAE in young adult age, develop mild disease of prolonged duration in old age [20]. This phenomenon has been ascribed to strain differences in multiple mechanisms controlling activation of neuroantigen-specific CD4+ T cells in draining lymph nodes (LNs), including the frequency of interleukin (IL)-17-producing CD8+ T cells shown to be important in the pathogenesis of clinically mild models of EAE [20–22], and the frequency of subsets of regulatory T cells [20, 23].

It is also noteworthy that development of chronic inflammatory diseases leads to thymic atrophy and increased generation of proinflammatory CD28− T cells. This reflects action of proinflammatory cytokines not only on the thymus [24, 25], but also their direct action on CD28 expression in mature T cells, and thereby contributes to maintenance/progression of these diseases [26, 27]. The disease-induced changes could be particularly important for chronic inflammatory diseases affecting the central nervous system (CNS), such as MS/EAE as significant alterations in the activity of sympathetic nervous system identified even in preclinical/prodromal phase of MS/EAE [rev. in 28] could contribute to thymic premature involution [29]. This seems to be quite conceivable as noradrenaline, the end-point mediator of the sympathetic nervous system is shown to have an important role in shaping the efficacy of thymopoiesis and particularly the efficacy of negative selection and generation of nTreg during gerontogenesis [30–32].

Finally, it should be pointed out that individual/strain-specific differences in susceptibility to autoimmune diseases, including MS/EAE, have been observed [18–20]. However, notwithstanding the thymic size/weight variations between different strains of rats [25, 33] and mice [34], studies encompassing systematic analysis of potential strain-specific differences in thymic function, particularly in mechanisms underlying strain differences in the central tolerance, are rather limited. There are some

findings indicating impairment in the induction of the negative selection response gene set in non-obese diabetic (NOD) mouse strain [35]. Additionally, strain-specific differences have been reported in the number and functional properties of nTregs from mouse strains (C57BL/6 [B6], BALB/c, and NOD) most frequently used in immunological research, being particularly prominent in NOD mice, a strain characterised by high predisposition to develop spontaneous and induced autoimmune diseases [36, 37]. We also revealed differences in the generation of nTregs in thymi of young adult AO rats compared with age-matched DA rats, which corresponded to their susceptibility for EAE induction [25]. In the same line are data indicating significance of genetic background for the number of thymic stromal cells and their thymopoietic activity in adult mice [38]. Furthermore, at the present even less is known about individual/the strain differences in the impact of ageing on qualitative changes in T-cell maturation and selection. There is only indirect evidence indicating strain differences in thymic involution rate and thymic output [38, 39].

From all aforementioned, it seems clear that, as suggested [36], the genetic background of an inbred animal strain may have a profound impact on the immune response in the animal. Considering that genetic background may influence thymopoietic efficiency and central tolerance in rats, hereby reported study was undertaken to compare (i) influence of ageing on thymopoietic efficiency, changes in developmental route from CD4+CD8+ double positive (DP) thymocyte stage to mature CD4+CD8– or CD4–CD8+ SP stage encompassing the negative selection, the generation of nTregs and CD8+ T cells endorsed with capacity to differentiate into IL-17-producing ones in DA and AO rats, and (ii) the effects of EAE development on thymopoietic efficacy and generation of nTregs in these animals. It is noteworthy that without such studies it is difficult to interpret the outcome of therapeutic interventions aimed at improving thymic function in situations of immunodeficiency/immunoderegulation and prevention/attenuation of thymic involution.

Materials and Methods

Experimental Animals

In this study, two strains of young and aged female rats were used, DA and AO. The rats were kept in the animal facility of the Immunology Research Centre "Branislav Janković" at the Institute of Virology, Vaccines and Sera "Torlak" (Belgrade, Serbia), which was validated by the Veterinary Department of the Ministry of Agriculture and Environmental Protection of the Republic of Serbia. At the beginning of the experiments, the animals were either 2–3 months old (young) or 22–24 months old (old). Three experimental animals were housed per cage, with bedding of sterilised wood shavings, under standard laboratory conditions encompassing controlled humidity, temperature, and lighting, and free access to regular food pellets and fresh water.

Induction and Clinical Evaluation of EAE

Randomly chosen rats of both ages and strains (6 rats per group) were either immunised for EAE (immunised rat group) or left intact (non-immunised [NIM] rat group). Before the immunisation for EAE, rats were anaesthetised using 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) anaesthetising cocktail (50 mg/kg body weight [BW] of ketamine/5 mg/kg BW xylazine), in order to ease the stress and pain. As in our previous studies [10, 20, 23, 40, 41], rats of both ages and strains were immunised by an intradermal injection of 100 μL of an emulsion composed of equal volumes of syngeneic rat spinal cord homogenate in phosphate-buffered saline (PBS), mixed in 1:2.5 (weight:volume) ratio, and complete Freund's adjuvant containing 1 mg/mL of killed *Mycobacterium tuberculosis* (strain H37Ra) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) into the left hind foot pad, and a 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. This emulsion was always freshly prepared before immunisation using a traditional manual syringe-based method (which is the gold standard for most laboratories) and a drop-test to confirm that a water-in-oil emulsion had been formed. Starting from the 1st day post-immunisation (d.p.i.), on a daily basis, the rats were monitored for BW and clinical signs of EAE by two independent experienced evaluators. EAE severity was scored using numerical scale as follows: 0, no clinical signs; 0.5, distal tail atony; 1, complete tail atony; 2, paraparesis; 3, paraplegia; 4, tetraplegia or moribund state. None of the rats exhibiting clinical signs of EAE reached either a moribund state or death during the experiments.

The rats were sacrificed when, according to the previously published preliminary experiment, the neurological signs of the disease reached the peak and plateau value, i.e., on the 14th and 16th d.p.i. in DA and AO rats, respectively [23]. Animals were deeply anaesthetised with an intraperitoneal injection of ketamine/xylazine anaesthetising mixture (80 mg/kg BW ketamine/8 mg/kg BW xylazine), blood samples were taken by cardiac puncture, and the animals were then sacrificed by transcardial perfusion.

Antibodies

In order to conduct flow cytometry analysis (FCA), we used the following monoclonal antibodies (mAbs) supplied by BD Biosciences Pharmingen, Mountain View, CA, USA: fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/allophycocyanin-labelled anti-CD8 (clone OX-8), FITC/PE-labelled anti-CD4 (clone OX-38), peridinin-chlorophyll-protein (PerCP)-labelled anti-TCRαβ (clone R73), PerCP-labelled anti-CD90 (Thy-1.1, clone OX-7), PE-labelled anti-CD45RC (clone OX-22), FITC-labelled anti-Ki-67 (clone B56), PE-labelled anti-CD28 (clone JJ319), PE-labelled anti-IL-17A (clone TC11-18H10), FITC-labelled anti-CD161a (clone 10/78). Additionally, FITC-labelled anti-Foxp3 (clone FJK-16s), PerCP eFluor 710-labelled anti-CD25 (clone OX-39), and allophycocyanin-labelled anti-CD4 (clone OX-35)

mAbs were obtained from eBioscience (San Diego, CA, USA), while Alexa Fluor 647-labelled anti-TCRαβ (clone R73) mAb was acquired from BioLegend (San Diego, CA, USA).

Preparation of Mononuclear Cell Suspension from the Lymphoid Tissues and Peripheral Blood for (Immune) staining and FCA

To prepare single-cell suspensions, thymi and popliteal and inguinal LNs were removed with caution, weighed, and afterwards finely minced and pressed through 70 μm nylon cell strainers (BD Biosciences, Erembodegem, Belgium). Cell suspensions were then washed three times in ice-cold fluorescence-activated cell sorting buffer, i.e., PBS containing 2% foetal calf serum (FCS, Gibco, Grand Island, NY, USA) and 0.1% sodium azide (Sigma-Aldrich Chemie GmbH).

For FCA of peripheral blood T lymphocyte (T-PBL) subsets, peripheral blood samples were treated with isotonic solution of ammonium chloride for erythrocyte lysis (5:1 lysis buffer-to-blood volume ratio). The samples were incubated for 10 min and then centrifuged at 350 *g* for 5 min at 4°C. Cells were washed three times in ice-cold PBS containing 2% FCS. In all cell suspensions, cells were enumerated using 0.2% trypan blue solution (for identification of viable cells) and an improved Neubauer haemocytometer.

LN Mononuclear Cell Stimulation for the Analysis of IL-17 Production

Freshly isolated mononuclear LN cells of NIM DA and AO rats of both ages were grown in 24-well plates (Sarstedt AG & Co., Nümbrecht, Germany) using RPMI 1640 cell culture medium (Sigma-Aldrich Chemie GmbH). Basal RPMI 1640 medium was completed by supplementing with 2 mM L-glutamine (Serva, Heidelberg, Germany), 1 mM sodium pyruvate (Serva), 100 units/mL penicillin (ICN, Costa Mesa, CA, USA), 100 μg/mL streptomycin (ICN), and 10% FCS. Plated cells were stimulated by adding 200 ng/mL of phorbol 12-myristate 13-acetate (Sigma-Aldrich Chemie GmbH) and 400 ng/mL of ionomycin (Sigma-Aldrich Chemie GmbH), along with 3 μg/mL of brefeldin A (eBioscience). Following 4-hour-long incubation in a humidified atmosphere of 5% v/v CO_2 at 37°C, the cells were collected for intracellular IL-17 immunostaining.

Thymocyte Apoptosis Analysis

Given that assessment of thymocyte apoptosis ex vivo may be hampered by (i) prompt phagocytosis of apoptotic thymocytes by macrophages occurring in vivo [42] and (ii) mechanical stress during cell isolation and downstream experimental manipulations which could initiate apoptosis [43], freshly isolated thymocytes were plated in 96-well flat-bottom plates (Nunc A/S, Roskilde, Denmark) and incubated in complete RPMI 1640 medium for 18 h, as it has been previously recommended [44]. Samples were then collected and stained with 5 μL of merocyanine 540 dye (MC 540; Sigma-Aldrich Chemie GmbH) for FCA of apoptotic (MC 540+) cells.

Surface Antigen Immunostaining for FCA

For immunostaining of cell surface antigens, fluorochrome-labelled mAbs were added to thymocyte, T-PBL, and LN mononuclear cell samples, incubated for 30 min at 4°C and then washed with fluorescence-activated cell sorting buffer prior to FCA.

Intracellular Antigen Immunostaining for FCA

For immunostaining of intracellular antigens, cell samples were fixed and permeabilised using the fixation/permeabilisation solution from the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Fixed and permeabilised thymocytes were incubated with anti-Foxp3 or anti-Ki-67 mAbs, while LN cell samples were immunostained using anti-IL-17 mAb. After 30 min of incubation time, at 4°C for Foxp3, or at room temperature for Ki-67 and IL-17 immunolabelling, samples were washed using permeabilisation buffer prior to FCA.

Flow Cytometry Analysis

FACSCalibur (Becton Dickinson, Mountain View, CA, USA) or FACSVerse flow cytometer was used to acquire 50,000 events per sample for FCA. All antibodies used in the experiments specifically react with rat antigens and have been tested for use in flow cytometry by the manufacturers. Target cell antigen specificity for all the antibodies used was also confirmed in our previous [10, 20] and the present study, in single- or multi-colour analyses, using antigen-positive and antigen-negative cell types. Unstained cells and isotype-matched controls were also used. For antigens exhibiting low expression, or those showing continuous distributions from negative to positive cells, fluorescence minus one controls were used to determine gating boundaries. Specifics of the fluorescence minus one- and isotype control-based flow cytometry gating strategies are available from the authors on reasonable request.

Flow cytometry data were analysed for the frequencies of cells displaying distinct phenotype or mean fluorescence intensity (MFI) of a cell population expressing distinct antigen, indicative of a marker expression density, using FlowJo software version 7.8 (TreeStar Inc, Ashland, OR, USA). MFI data are expressed as MFI ratios calculated as MFI of mAb-labelled cells/MFI of unstained cells (negative control) [45].

RT-qPCR

For the isolation of total thymic tissue RNA, TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used, as instructed by the manufacturer. Absorbance spectrometry was utilised to determine RNA concentrations (absorbance at 260 nm) and purity (absorbance at 280 nm) using Orion AquaMate 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesised with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's manual. Each RT-qPCR reaction consisted of 12.5 μL of TaqMan Gene Expression Master Mix (Applied Biosystems), 1.25 μL of commercially available TaqMan Gene Expression Assay (Applied Biosystems), 6.25 μL of PCR-certified water, and 5 μL of cDNA template. Samples were set up in triplicate, and amplification was carried out using 7500 Real-Time PCR Instrument (Applied Biosystems), running under the default, pre-optimised conditions. 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), Tgfb1 (Rn00572010_m1), Foxn1 (Rn01460454_m1), Il21 (Rn01755623_m1), and Actb (Rn00667869_m1). Target mRNA levels were quantified using β-actin as a reference gene. For reference and target genes, threshold cycle (Ct) values were calculated using SDS v1.4.0. software (Applied Biosystems). Relative expression level of each target mRNA was reported as 2^{-dCt} value, with dCt = Ct target − Ct β-actin.

ELISA

IL-6 and TNF-α concentrations were measured in the plasma samples using ELISA kits sourced from BioLegend. Minimum detectable concentrations of IL-6 and TNF-α were 5.3 pg/mL and 2 pg/mL, respectively. Blood samples were collected in heparin-coated tubes and centrifuged for 15 min at 1,500 *g* to separate the plasma. Plasma samples collected into polypropylene tubes were immediately stored at −70°C until analysis. Analyses were performed following the manufacturer's instructions.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). In order to calculate the influence of strain and ageing or strain and immunisation for EAE on the thymic, peripheral blood and LN immunological parameters, two-way ANOVA followed by the Bonferroni post hoc test was used. To estimate the influence of ageing and immunisation for EAE on the values of examined parameters, regression analysis was used, and the slope values (quantifying the steepness) of the lines depicting age- and EAE-related alterations in values of examined parameters were compared. Values of $p \leq 0.05$ were regarded as statistically significant. All data are given as mean ± standard deviation.

Animal group sizes were determined using "resource equation" method. This method suggests that, when ANOVA is used to analyse the data, number of required animals is estimated according to the formula: E = total number of animals – total number of animal groups [46, 47]. Sample sizes with E values between 10 and 20 are considered adequate, given that for E values less than 10 the chance of false-negative results increases substantially, while for values over 20, the cost and use of animals increase for only a modest gain [44, 45]. The method is considered reliable in whole animal studies [46] and is used when multiple endpoints are measured or more complex statistical analysis is involved [48]. For the experimental design used in this study, calculated E value was 20 which corresponds to 6 rats per group.

Results

Total Thymocyte Number, Thymic Weight, and Thymic Function Exhibited Different Age-Related Changes in DA and AO Rats

Ageing Exerted the Opposite Effects on DA and AO Rat Susceptibility to EAE

As previously shown [41], injection of SC homogenate in complete Freund's adjuvant induced neurologically manifested EAE in all young adult (young) DA rats, while none of the AO rats developed clinically manifested signs of neurological deficit during the observation period (online suppl. Fig. 1a; for all online suppl. material, see https://doi.org/10.1159/000535150). Also, in accordance with previous study [23], with ageing the incidence of EAE decreased in DA rats, so in old animals it was markedly lower (100% in young vs. 50% in old) compared with their young counterparts (online suppl. Fig. 1a). In accordance with previous study showing that old DA rats developed monophasic self-limiting disease with mild neurological deficit at the maximum on the 14th d.p.i. [23], EAE-inflicted old DA rats showed mild neurological signs of the disease (online suppl. Fig. 1b). Also, consistent with our previous findings indicating that majority (66.67%) of old AO rats develop EAE of prolonged duration reaching plateau of neurological deficit on the 16th d.p.i. [23], old AO rats inflicted with EAE developed mild neurological signs of EAE (online suppl. Fig. 1b).

Kinetics of the Thymic Involution Depends on Rat Strain

Given that, classically, involution can be defined in terms of decline in the whole thymus weight and/or the total number of thymocytes [49], these parameters were examined in young and old rats. As expected [10, 50], with ageing, judging by the steepness of the slopes of regression lines, the thymic weight decreased in DA ($p < 0.001$) and AO ($p < 0.001$) rats (Fig. 1a). Thymic weight did not differ between young AO and DA rats (Fig. 1a). Given that the age-related decrease in thymic weight was steeper ($p < 0.05$) in DA rats than in AO rats, it was greater ($p < 0.01$) in old AO rats than in age-matched DA ones (Fig. 1a).

Additionally, with ageing, judging by the regression line slope analyses, thymocyte yield also decreased in DA ($p < 0.001$) and AO ($p < 0.001$) rats, but this decrease was markedly more prominent ($p < 0.001$) in DA rats (Fig. 1a). Consequently, contrary to young rats, in whom thymocyte yield was less ($p < 0.001$) in AO rats than in DA ones, in old ones it was comparable between these two strains (Fig. 1a).

To get mechanistic insight into these strain specificities, circulating levels of IL-6, a highly potent thymosuppressive agent associated with acute thymic atrophy and thymic involution [24, 51], were examined. In accordance with other studies [52, 53], strain differences in circulating levels of IL-6 in young rats were found. Namely, its level was lower ($p < 0.01$) in young AO rats when compared with age-matched DA rats (Fig. 1b). With ageing, the circulating levels of IL-6 raised ($p < 0.001$) in DA and AO rats, but to a greater extent in AO ones (Fig. 1b). Thus, differently from young rats, in old rats IL-6 level in circulation of AO rats exceeded ($p < 0.001$) that in age-matched DA rats (Fig. 1b). Besides, circulating levels of TNF-α, the cytokine also associated with thymic atrophy [54], were investigated. In accordance with data indicating that TNF-α does not exhibit age-related increase in mouse serum [55], TNF-α plasma concentration did not significantly differ across examined groups of rats (Fig. 1b).

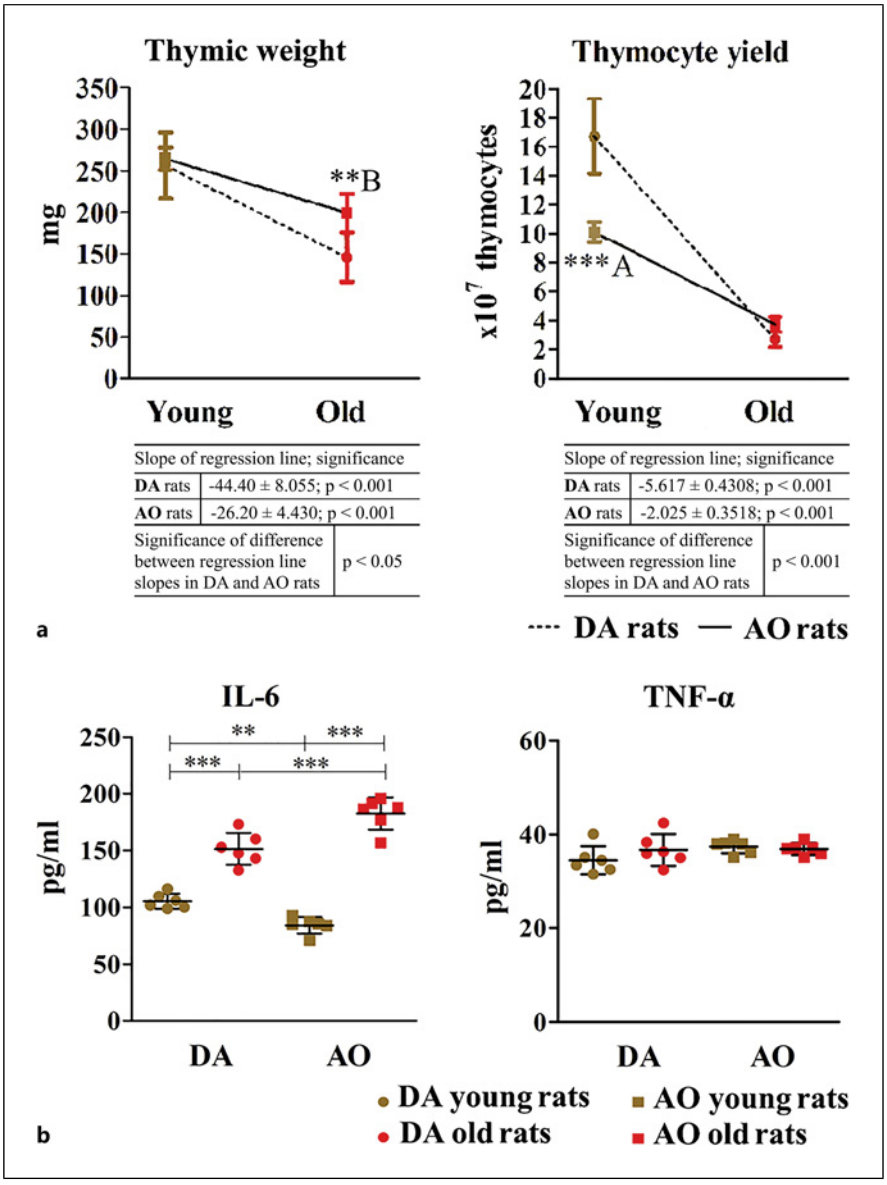


Fig. 1. Strain-specific differences in the age-related changes in the thymic involution, and circulating levels of thymosuppressive IL-6. **a** Linear graphs depict linear regression analyses of age-related changes in (left) thymic weight and (right) thymocyte yield in rats of DA and AO strains. Significances of differences in the thymic weight and thymocyte yield between DA and age-matched AO rats are shown in the linear graphs (A, young; B, old). In the tables below the linear graphs are displayed the values for slopes of linear regression lines (indicating the steepness of the lines and thereby indirectly the kinetics of the age-related changes in the analysed parameters) and their significances in DA and AO rats, and significance of difference in the regression line slopes between the examined strains. **b** Scatter plots indicate concentrations of (left) IL-6 and (right) TNF-α in plasma of young and old DA and AO rats. Two-way ANOVA showed significant interactions between the effects of age and strain for thymocyte yield [$F_{(1,20)}$ = 41.70, p <0.001], and circulating IL-6 level [$F_{(1,20)}$ = 32.99, p <0.001]. Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. **p <0.01; ***p <0.001.

Strain Differences in Age-Related Changes in the Thymic Expression of Genes Relevant for Thymic Involution

As circulating IL-6 level does not reflect solely its thymic production [56], the transcriptional levels of IL-6 mRNA were investigated in thymi from young and old rats of DA and AO strain. As expected [10, 50, 51, 57], with ageing the expression of IL-6 mRNA in thymi from DA (p <0.001) and AO (p <0.05) rats increased (Fig. 2a). However, at odds with its circulating concentration, thymic IL-6 expression was lower not only in young (p <0.05) but also in old (p <0.001) AO rats compared with age-matched DA rats (Fig. 2a). This suggested that thymic IL-6 production does not exclusively depend on circulating IL-6 levels [58], but also on some other factors, such as the proportion of IL-6-producing cells in the thymus [24], and thymic expression of some other cytokines influencing thymic IL-6 expression [51, 57].

Given that the elevation of serum IL-6 and/or thymic transcriptional IL-6 level (through autocrine/paracrine signalling) can damage the thymic microenvironment enhancing thymic involution/atrophy [24, 51, 57], thymic expression of mRNAs for Foxn1, a master regulator in the thymic epithelial cell (TEC) lineage development and TEC maintenance,

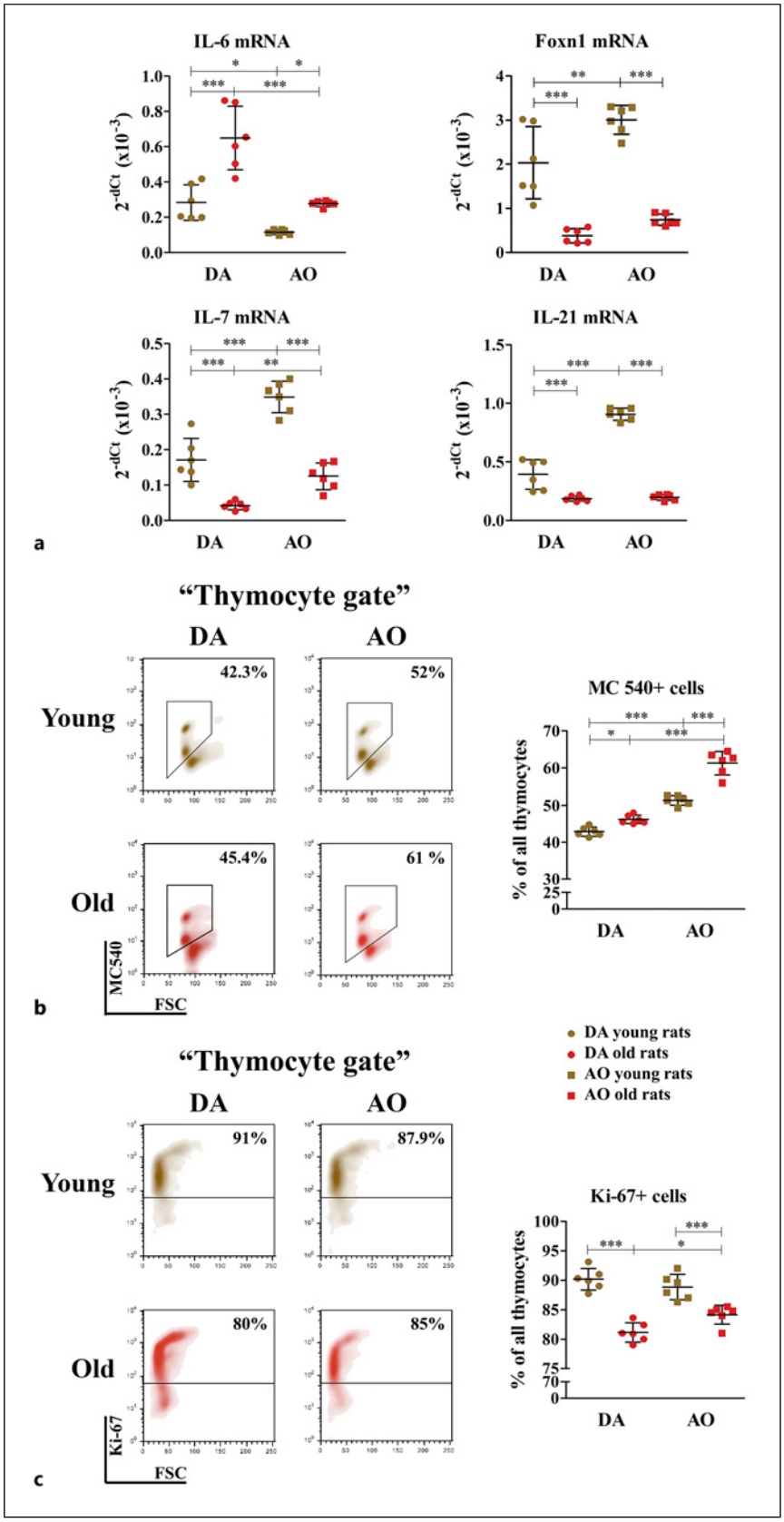


Fig. 2. Strain-specific differences in the age-related changes in the thymic expression of major factors controlling thymic stroma development/maintenance and thymocyte survival/expansion, and thymocyte apoptosis and proliferation. **a** Scatter plots display the expression of mRNAs encoding (upper left) IL-6, (upper right) Foxn1, (lower left) IL-7, and (lower right) IL-21 in the thymi from young and old rats of DA and AO strains. **b**, **c** Representative flow cytometry density plots indicate merocyanine 540 (MC 540) (**b**) and Ki-67 staining (**c**) of thymocytes retrieved from young and old DA and AO rats (see Materials and Methods section). Corresponding scatter plots display the frequencies of apoptotic MC 540+ (**b**) and proliferating Ki-67+ cells (**c**) among all thymocytes from young and old DA and AO rats. Two-way ANOVA showed significant interactions between the effects of age and strain for thymic IL-6 [$F_{(1,20)}$ = 5.805, p <0.05], IL-7 [$F_{(1,20)}$ = 7.350, p <0.05], and IL-21 [$F_{(1,20)}$ = 77.04, p <0.001] mRNA expression, frequencies of MC 540+ [$F_{(1,20)}$ = 17,75, p <0.001] and Ki-67+ [$F_{(1,20)}$ = 8.422, p <0.01] cells. Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *p <0.05; **p <0.01; ***p <0.001.

and thereby RTE level [59, 60], was examined. The expression level of mRNA for Foxn1 was less (p <0.001) in thymi of old DA and particularly AO rats compared with strain-matched young rats suggesting greater age-related decline in AO rats (Fig. 2a). Consistently, although greater (p <0.01) amount of Foxn1 mRNA was found in thymi of young AO rats compared with age-matched DA ones, its expression in old rats did not significantly differ between these two strains (Fig. 2a).

Additionally, thymic expression of IL-7, the cytokine (mainly) secreted by TECs and exhibiting a critical role in survival and proliferation of the most immature thymocytes [61], was investigated. With ageing, its expression decreased (p <0.001) in DA and AO rats without affecting strain difference (Fig. 2a). Namely, thymic IL-7 mRNA expression was greater in young (p <0.001) and old (p <0.01) AO rats compared with age-matched DA rats (Fig. 2a).

Moreover, given that administration of rIL-21, the cytokine expressed by thymocytes, enhances thymopoiesis in old mice through proliferative expansion of thymocyte compartments, and consequently the stromal ones [62], thymic expression of IL-21 mRNA was also quantified. With ageing, IL-21 transcript level also decreased (p <0.001) in thymi from DA and AO rats, but this decrease was more prominent in AO rats (Fig. 2a). Consequently, the strain difference observed in young rats, which was mirrored in greater (p <0.001) IL-21 mRNA expression in AO rats, disappeared in old animals (Fig. 2a).

Considering the previous findings related to the influence of ageing and strain on the expression of thymic factors regulating survival and expansion of rat thymocytes, their apoptosis and proliferation were also examined. The frequency of apoptotic MC 540+ cells in thymocyte cultures from old DA (p <0.05) and AO (p <0.001) rats was higher than in thymocyte cultures from strain-matched young rats (Fig. 2b). Additionally, their frequency was higher (p <0.001) in thymocyte cultures from young and old AO rats when compared with thymocyte cultures from age-matched DA rats (Fig. 2b).

To the contrary, with ageing the frequency of proliferating Ki-67+ cells (which did not differ between thymocytes from young AO and DA rats) decreased (p <0.001) among thymocytes from DA rats and AO rats (Fig. 2c). This decline was less prominent in AO rats (Fig. 2c). Consequently, their frequency was higher (p <0.05) among thymocytes from old AO rats compared with age-matched DA rats (Fig. 2c).

Strain-Specific Differences in the Influence of Ageing on the Thymocyte Differentiation/Maturation

Given that thymus provides the unique cellular and molecular platform not only for thymocyte survival and proliferation, but also for thymocyte differentiation/maturation [63], strain-specific differences in influence of ageing on thymocyte phenotypic profile were also investigated.

Ageing Influenced Strain Specificities in the Composition of the Major Thymocyte Subsets Delineated by CD4/CD8 Expression. With ageing in DA (p <0.001) and AO (p <0.01) rats, the increase in the frequency of the most immature CD4–CD8– double negative (DN) cells among thymocytes was found (Fig. 3). This increase was strikingly less prominent in AO rats (Fig. 3). Thus, differently from young animals in whom their frequency did not significantly differ between DA rats and AO rats, it was markedly lower (p <0.001) in old AO rats compared with age-matched DA rats (Fig. 3).

On the other hand, with ageing the frequency of CD4+CD8+ DP cells among thymocytes decreased in DA (p <0.05) and AO (p <0.001) rats, but to a greater extent in AO rats (Fig. 3). Consequently, although in young rats their frequency was higher (p <0.001) in AO rats compared with DA rats, in old rats it did not significantly differ between them (Fig. 3).

The frequencies of both CD4+CD8– and CD4–CD8+ SP cells among thymocytes from DA (p <0.05) and AO (p <0.05 and p <0.001 for CD4+CD8– and CD4–CD8+ cells, respectively) rats increased with ageing (Fig. 3). Additionally, ageing influenced strain specificities in the frequency of CD4+CD8– and CD4–CD8+ SP cells among thymocytes (Fig. 3). Namely, the frequency of CD4+CD8– SP cells which was lower (p <0.01) in young AO rats compared with age-matched DA rats was comparable between old rats of these two strains (Fig. 3). Differently, the frequency of CD4–CD8+ SP cells did not differ between young DA and AO rats, but was higher (p <0.001) in old AO rats when compared with age-matched DA ones (Fig. 3).

Ageing Influenced Strain Specificities in the Phenotypic Profile of CD4+CD8+ DP Thymocytes. Next, DP thymocytes were examined for phenotypic profile. DP thymocytes encompass TCRαβ– cells that passed β selection and progressed to DP stage [64] and TCRαβ+ cells that experience TCRαβ signals in response to self-peptides presented by major histocompatibility complex molecules [65]. Depending on the strength of this signal, they undergo death by neglect and negative selection or positive selection, resulting in maturation and

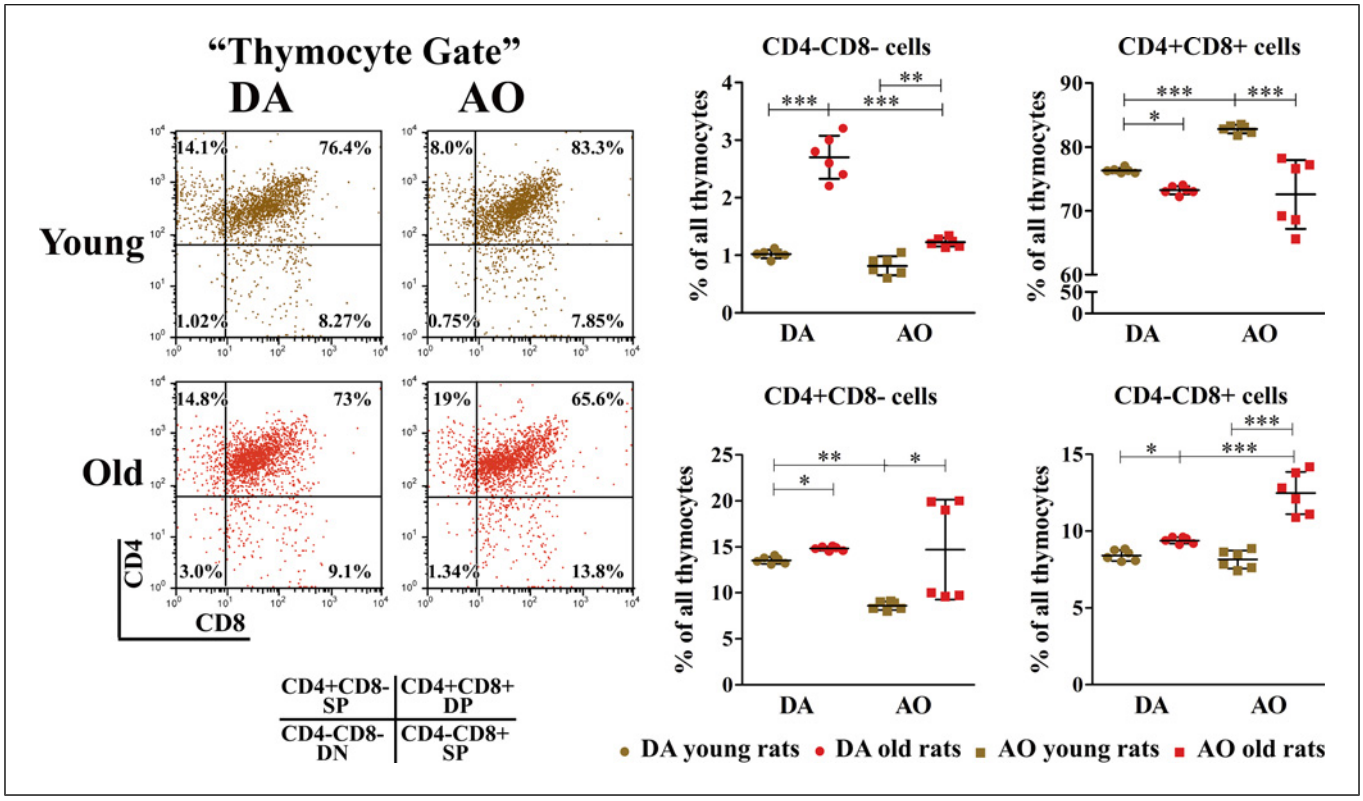


Fig. 3. Age-dependent strain specificities in the composition of the major thymocyte subsets delineated by CD4/CD8 expression. Representative flow cytometry dot plots indicate CD4/CD8 staining of thymocytes retrieved from young and old rats of DA and AO strains. According to the surface CD4/CD8 expression, thymocytes were delineated into CD4–CD8– double negative (DN), CD4+CD8+ double positive (DP), and CD4+CD8– and CD4–CD8+ single positive (SP) subsets, as shown in the schematic diagram below the flow cytometry dot plots. Corresponding scatter plots display the frequencies of (upper left) CD4–CD8– DN, (upper right) CD4+CD8+ DP, (lower left) CD4+CD8–, and (lower right) CD4–CD8+ SP cells among all thymocytes from young and old DA and AO rats. Two-way ANOVA showed significant interactions between the effects of age and strain for the frequencies of DN [$F_{(1,20)}$ = 54.34, p <0.001], DP [$F_{(1,20)}$ = 10.25, p <0.01], CD4+CD8– [$F_{(1,20)}$ = 4.693, p <0.05], and CD4–CD8+ [$F_{(1,20)}$ = 29.90, p <0.001] SP thymocytes. Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *p <0.05; **p <0.01; ***p <0.001.

commitment to either the CD4 or CD8 lineage [65]. With ageing, the frequency of TCRαβ– cells among DP thymocytes decreased (p <0.05) in DA rats, whereas it increased (p <0.001) in AO rats (Fig. 4a). Consequently, differently from young rats exhibiting similar frequency of TCRαβ– cells among DP thymocytes from DA and AO rats, in old rats their frequency was higher (p <0.001) in AO rats than in DA ones (Fig. 4a). Complementary, age-related increase and decrease in the frequency of TCRαβ+ cells among DP thymocytes from DA (p <0.05) and AO (p <0.001) rats were found, respectively (Fig. 4a). This resulted in lower (p <0.001) frequency of TCRαβ+ cells among DP cells from old AO rats compared with age-matched DA rats (Fig. 4a).

Considering our previous finding suggesting that ageing impairs thymocyte-negative selection by upregulating surface expression of CD90 (Thy-1), a negative regulator of TCR-mediated signalling and selection threshold [64] on DP TCRαβ+ thymocytes [16], CD90 surface expression on DP TCRαβ+ cells was also assessed. Indeed, with ageing, judging by CD90 MFI ratio, CD90 density increased on CD90+ DP TCRαβ+ thymocytes from DA (p <0.05) and AO (p <0.001) rats, but to a greater extent in AO rats (Fig. 4b). Thus, although there was no strain difference in the density of CD90 on CD90+ DP TCRαβ+ thymocytes in young rats, in old rats its density was markedly greater (p <0.001) in AO rats than in DA rats (Fig. 4b).

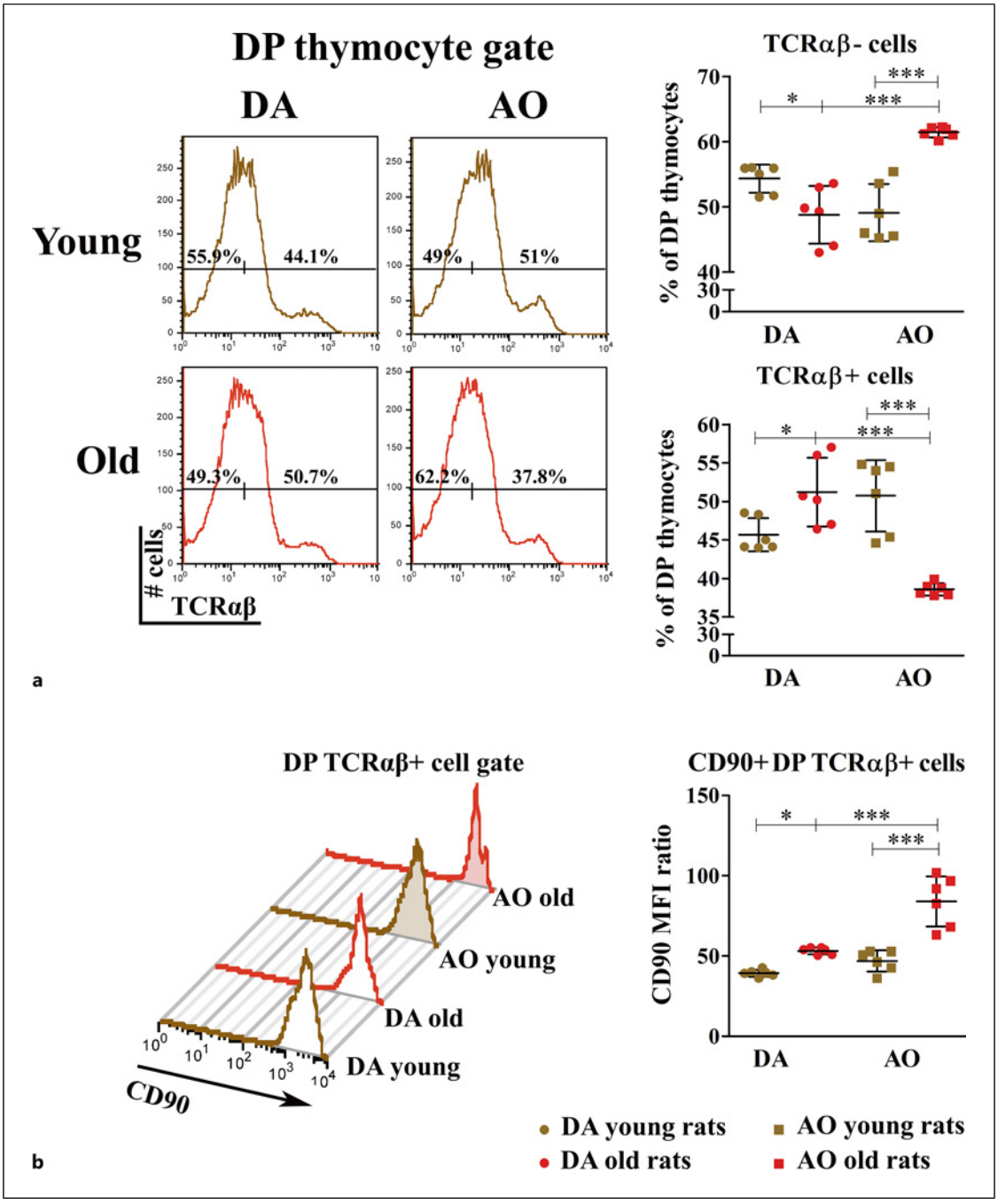
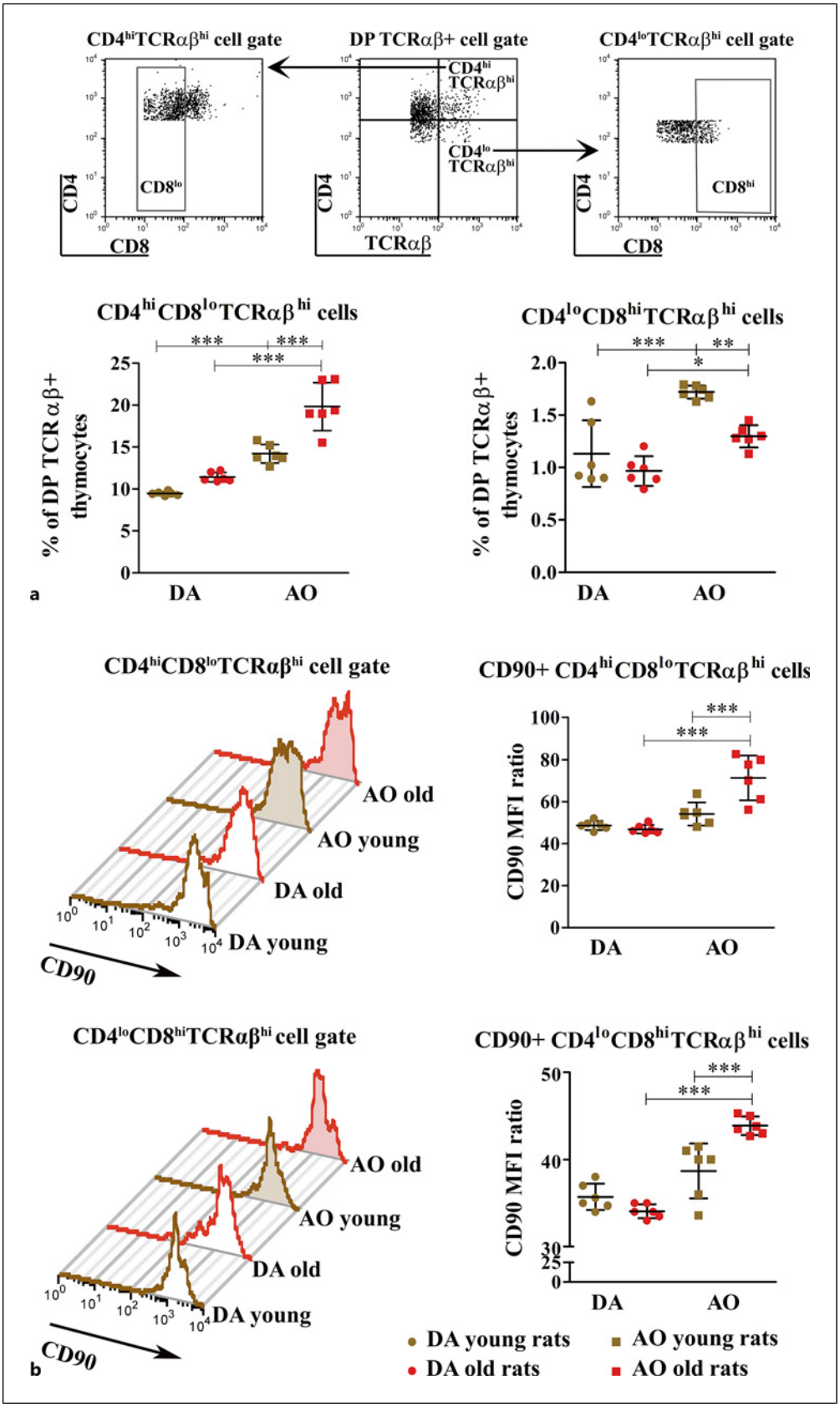


Fig. 4. Age-dependent strain specificities in the phenotypic profile of CD4+CD8+ double positive (DP) thymocytes. **a** Representative flow cytometry histograms and corresponding scatter plots indicate the frequencies of TCRαβ− and TCRαβ+ cells within the CD4+CD8+ DP thymocytes (gated as shown in Fig. 3) from young and old rats of DA and AO strains. **b** Representative overlaid flow cytometry histograms indicate CD90 (Thy-1) surface expression on DP TCRαβ+ thymocytes from young and old DA and AO rats. Corresponding scatter plot displays CD90 mean fluorescence intensity (MFI) ratio, calculated for CD90+ DP TCRαβ+ cells as specified in the section Materials and Methods, and indicative of CD90 surface density. Data in the overlaid flow cytometry histograms are displayed as % of max (the cell count in each bin divided by the cell count in the bin that contained the largest number of cells; http://www.flowjo.com), to allow visual comparison of samples with different event numbers collected. Two-way ANOVA showed significant interactions between the effects of age and strain for the frequency of DP TCRαβ+ cells [$F_{(1,20)} = 40.38$, $p < 0.001$)] and CD90 MFI ratio on CD90+ DP TCRαβ+ thymocytes [$F_{(1,20)} = 10.89$, $p < 0.01$]. Data are presented as mean ± standard deviation (SD) ($n = 6$ per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *$p < 0.05$; ***$p < 0.001$.



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Next, given that at the late stage of positive selection DP thymocytes undergo lineage commitment leading to development of $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ and $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ progenitors of CD4+CD8– and CD4–CD8+ SP thymocytes, respectively [66], their frequencies among DP TCRαβ+ thymocytes were examined. In DA rats, the frequency of either $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ cells or $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ cells among DP TCRαβ+ thymocytes did not change with ageing (Fig. 5a). Differently, in AO rats the frequency of $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ cells among DP TCRαβ+ thymocytes increased (p <0.001), whereas that of $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ cells decreased (p <0.01) with ageing (Fig. 5a). Additionally, in young (p <0.001) and old (p <0.001 and p <0.05 for $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ and $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ cells, respectively) AO rats, the frequencies of these cells were higher than in age-matched DA rats (Fig. 5a).

Taking in conjunction data indicating that (i) DP thymocytes on transition into mature SP cells undergo negative selection [35] and (ii) CD90 surface density changes with thymocyte differentiation/maturation [67], so its average density on DP population may not reflect changes in its small subsets, the expression of CD90 on $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ and $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ thymocytes was also investigated. With ageing, the surface density of CD90 on these cells changed only in AO rats (Fig. 5b). It was greater (p <0.001) on both CD90+ $CD4^{hi}CD8^{lo}$ $TCR\alpha\beta^{hi}$ and CD90+ $CD4^{lo}CD8^{hi}$ $TCR\alpha\beta^{hi}$ thymocytes from old AO rats compared with strain-matched young ones (Fig. 5b). Differently from young rats, in whom CD90 density on either CD90+ $CD4^{hi}CD8^{lo}$ $TCR\alpha\beta^{hi}$ thymocytes or CD90+ $CD4^{lo}CD8^{hi}$ $TCR\alpha\beta^{hi}$ thymocytes did not significantly differ between DA and AO rats, in old rats its density on these thymocyte subsets was greater (p <0.001) in AO rats compared with DA rats (Fig. 5b).

Age-Dependent Strain Specificities in the Level of the Most Mature SP TCRαβ+ Thymocytes and the Generation of CD8+ Cells Prone to Differentiate into IL-17-Producing Cells and nTregs

SP TCRαβ+ Thymocyte Level. In the next step, the frequency of the most mature SP TCRαβ+ thymocytes was examined. In assessing these parameters, findings indicating that mature (mainly) activated/memory CD4+ and CD8+ T lymphocytes exhibiting CD90– phenotype can recirculate and persist in the thymus of humans and laboratory animals, including the rat [68, 69], were considered. Of note, these findings were confirmed in hereby presented study (online suppl. Fig. 2). Thus, to get insight into the relative proportion of the most mature thymocyte subsets, thymocytes from young and old DA and AO rats were examined for the frequency of SP CD90+TCRαβ+ cells; viz., recirculating SP CD90–TCRαβ+ cells were excluded from the analysis (online suppl. Fig. 2). With ageing, the frequency of CD4+CD8–CD90+TCRαβ+ cells increased among (p <0.001) thymocytes from both DA and AO rats, but this increase was more prominent in AO rats (Fig. 6a, online suppl. Fig. 2). Accordingly, in old rats the frequency of CD4+CD8–CD90+TCRαβ+ thymocytes was higher (p <0.001) in AO rats compared with DA rats (Fig. 6a, online suppl. Fig. 2). On the other hand, with ageing the frequency of CD4–CD8+CD90+TCRαβ+ cells increased (p <0.001) only in AO rats, whereas it was unaffected in DA rats (Fig. 6a, online suppl. Fig. 2). Consequently, as their frequency was comparable in young rats of these two strains, in old rats it was higher (p <0.001) in AO rats than in DA rats (Fig. 6a, online suppl. Fig. 2).

As expected from the changes in thymocyte yield, with ageing the absolute numbers of both the most mature CD4+CD8–CD90+TCRαβ+ and CD4–CD8+CD90+TCRαβ+ thymocytes decreased (p <0.001) in DA and AO rats (Fig. 6a). However, judging by the regression analysis, the slopes

Fig. 5. Age-dependent strain specificities in the phenotypic profile of $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ and $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ thymocytes. **a** Representative flow cytometry dot plots indicate the gating strategy for (left) $CD8^{lo}$ and (right) $CD8^{hi}$ cells within (middle) $CD4^{hi}TCR\alpha\beta^{hi}$ and $CD4^{lo}TCR\alpha\beta^{hi}$ thymocytes, respectively. $CD4^{hi}TCR\alpha\beta^{hi}$ and $CD4^{lo}TCR\alpha\beta^{hi}$ cell subsets were delineated within CD4+CD8+ double positive (DP) TCRαβ+ thymocytes, gated as shown in Figures 3 and 4. Scatter plots display the frequencies of $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ and $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ cells within DP TCRαβ+ thymocytes from young and old DA and AO rats. **b** Representative overlaid flow cytometry histograms indicate CD90 surface expression on (upper) $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ and (lower) $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ thymocytes from young and old DA and AO rats, whereas corresponding scatter plots display CD90 MFI ratio for (upper) CD90+ $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ and (lower) CD90+ $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ thymocytes. Data in the overlaid flow cytometry histograms are displayed as % of max (the cell count in each bin divided by the cell count in the bin that contained the largest number of cells; http://www.flowjo.com), to allow visual comparison of samples with different event numbers collected. Two-way ANOVA showed significant interactions between the effects of age and strain for CD90 MFI ratio on CD90+ $CD4^{hi}CD8^{lo}TCR\alpha\beta^{hi}$ [$F_{(1,20)}$ = 13.88, p <0.01] and CD90+ $CD4^{lo}CD8^{hi}TCR\alpha\beta^{hi}$ [$F_{(1,20)}$ = 20.06, p <0.001] thymocytes. Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *p <0.05; **p <0.01; ***p <0.001.

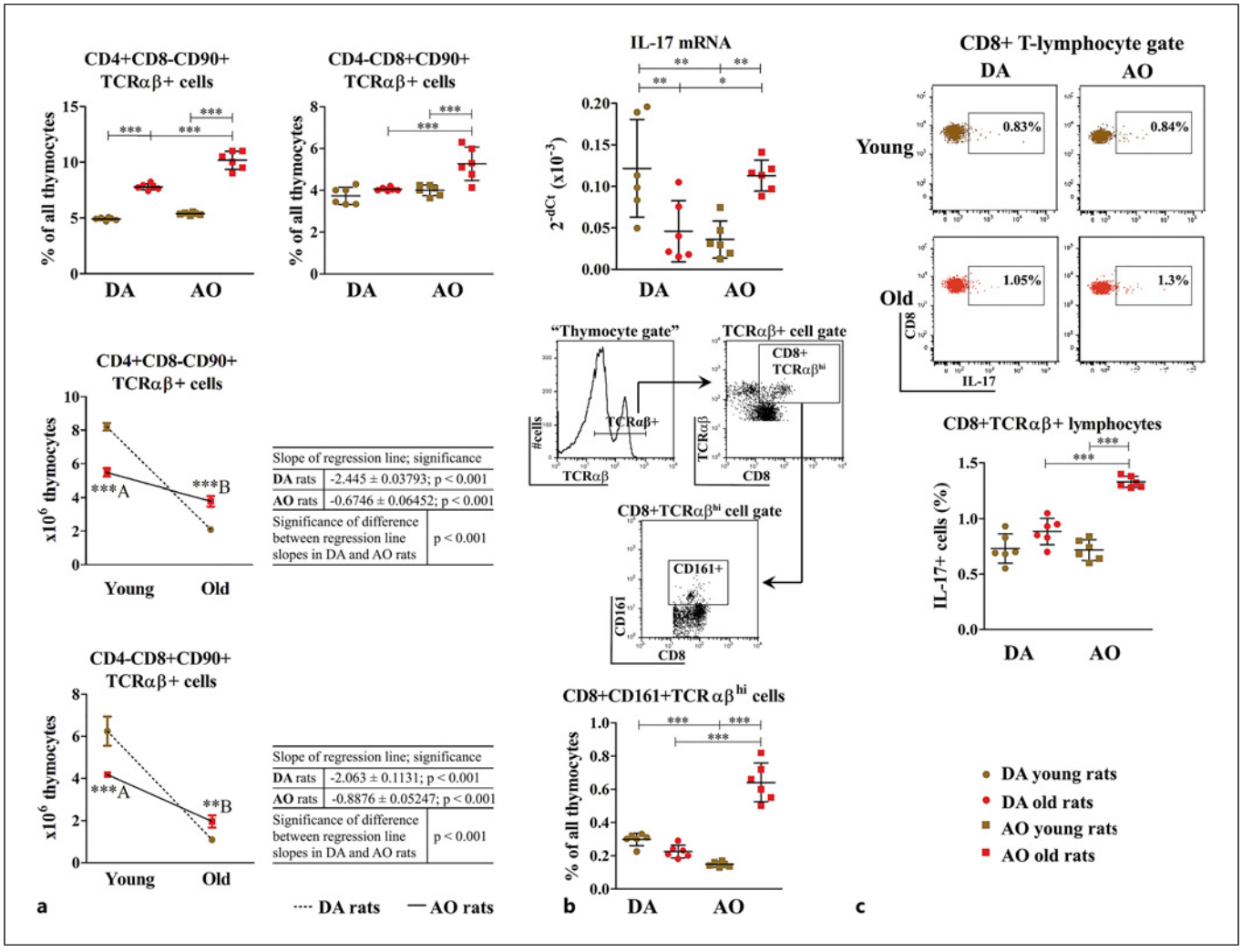


Fig. 6. Ageing modulated the proportion of the most mature thymocyte subsets and thymic generation of IL-17-producing CD8+ T cells in a strain-specific manner. **a** Scatter plots indicate the frequencies of the most mature (left) CD4+CD8– and (right) CD4–CD8+ single positive (SP) CD90+TCRαβ+ cells among all thymocytes in rats of DA and AO strains. Linear graphs depict linear regression analyses of age-related changes in the absolute numbers of the most mature (upper) CD4+CD8– and (lower) CD4–CD8+ SP CD90+TCRαβ+ cells in rats of DA and AO strains. Significances of differences in the absolute numbers of the most mature (upper) CD4+CD8– and (lower) CD4–CD8+ SP CD90+TCRαβ+ cells between DA and age-matched AO rats are shown in the linear graphs (A, young; B, old). In the tables next to the linear graphs are displayed the values for slopes of linear regression lines (indicating the steepness of the lines and thereby indirectly the kinetics of the age-related changes in the analysed parameters) and their significances in DA and AO rats, and significance of difference in the regression line slopes between the examined strains. **b** Scatter plots indicate (upper) IL-17 mRNA expression in the thymus and (lower) the frequency of CD8+CD161+TCRαβ^{hi} cells among all thymocytes from young and old DA and AO rats. Representative flow cytometry dot plots indicate the gating strategy for (lower) CD161+ cells within (upper) CD8+TCRαβ^{hi} thymocytes. CD8+TCRαβ^{hi} cell subset was delineated within TCRαβ+ thymocytes, gated as shown on flow cytometry histogram. **c** Representative flow cytometry dot plots and scatter plot indicate the frequency of IL-17+ cells among CD8+TCRαβ+ lymphocytes retrieved from the lymph nodes (LNs) of young and old DA and AO rats. Two-way ANOVA showed a significant interaction between the effects of age and strain for the frequencies of CD4+CD8–CD90+TCRαβ+ [$F_{(1,20)}$ = 28.44, p <0.001] and CD4–CD8+CD90+TCRαβ+ [$F_{(1,20)}$ = 6.164, p <0.05] cells, absolute numbers of CD4+CD8–CD90+TCRαβ+ [$F_{(1,20)}$ = 558.9, p <0.001] and CD4–CD8+CD90+TCRαβ+ [$F_{(1,20)}$ = 126.5, p <0.001] cells, thymic IL-17 mRNA expression [$F_{(1,20)}$ = 24.78, p <0.001], and the frequencies of CD8+CD161+TCRαβ+ thymocytes [$F_{(1,20)}$ = 114.7, p <0.001] and IL-17+ CD8+TCRαβ+ LN cells [$F_{(1,20)}$ = 19.27, p <0.001]. Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *p <0.05; **p <0.01; ***p <0.001.

(steepness of these declines) were markedly milder (p <0.001) in AO rats than in DA rats (Fig. 6a). Thus, although the absolute numbers of these cells were lower (p <0.001) in young AO compared with age-matched DA rats, in old rats they were greater (p <0.001 and p <0.01 for CD4+CD8−CD90+TCRαβ+ and CD4−CD8+CD90+TCRαβ+ cells, respectively) in old AO rats compared with age-matched DA rats (Fig. 6a).

Generation of CD8+ Cells Prone to Differentiate into IL-17-Producing Cells. Next, considering our previous studies suggesting an important role of IL-17-producing CD8+ T cells in pathogenesis of EAE in old AO rats [20], the expression of IL-17 mRNA in thymi of young and old DA and AO rats was quantified. Interestingly, it was found that ageing exerted the opposite effect on its expression in thymi of DA rats and AO rats (Fig. 6b). Namely, compared with young strain-matched rats, its expression was downregulated (p <0.01) and upregulated (p <0.01) in old DA and old AO rats, respectively (Fig. 6b). Thus, thymic IL-17 mRNA expression, which was markedly downregulated (p <0.01) in young AO rats compared with age-matched DA rats, in old rats was greater (p <0.05) in AO rats than in DA rats (Fig. 6b).

Age-related increase in thymic expression of IL-17 mRNA in AO rats was already associated with a dramatic increase in thymic expression of mRNA for IL-4 [10]. Namely, this cytokine is shown to inhibit transcription factor T-cell factor 1 [70], which regulates CD8+ T-cell fate decisions (in terms of IL-17-producing capacity) in DP thymocyte stage, through the sequential suppression of MAF and RORγt via transcription factor T-cell factor 1-driven modulation of chromatin state [71]. This finding was confirmed in herein reported study (online suppl. Fig. 3). On the other hand, consistent with data obtained in mice [72], we observed strain differences in the expression of IL-4 mRNA in old rats. Namely, the expression of IL-4 mRNA was not reliably quantifiable in thymi of young DA rats and AO rats, as well as in old DA rats (online suppl. Fig. 3).

To additionally specify the changes in thymic expression of IL-17 mRNA, it was considered that T-cell subsets with a capacity to produce IL-17 and their precursors exhibit surface expression of CD161 [73]. This was ascribed to dual control of genes (for IL-17 and CD161) by transcription factor RORγt [74], and CD161 is agreed to serve as a marker of IL-17-producing cells [73]. Thus, the frequency of CD8+CD161+TCRαβ^{hi} cells among thymocytes was also examined. With ageing, their frequency among thymocytes increased (p <0.001) in AO rats, whereas in DA rats it did not significantly change (Fig. 6b). Consequently, although in young rats the frequency of CD8+CD161+TCRαβ^{hi} cells among thymocytes was lower (p <0.001) in AO rats, their frequency in old AO rats was higher (p <0.001) than in age-matched DA rats (Fig. 6b). To envisage the significance of these findings, the frequency of IL-17−producing (IL-17+) cells among CD8+TCRαβ+ cells from LN was also attested. As expected [10], age-related rise (p <0.001) in their frequency was found in AO rats, whereas it did not significantly differ between young and old DA rats (Fig. 6c). Given that in young rats the frequency of IL-17+ cells among CD8+TCRαβ+ LN cells was comparable between the strains, in old rats their frequency was higher (p <0.001) in AO rats than in DA rats (Fig. 6c).

nTreg Thymic Level. In the next step, given that the thymus generates a lineage-committed subset of nTregs with the important role in the maintenance of self-tolerance and prevention of autoimmune disorders [5, 6], the thymic expression of mRNAs for IL-2, IL-15, and TGF-β, the cytokines supporting nTreg differentiation/maturation [75–77], was quantified. The amount of IL-2 transcript in thymi of DA and AO rats exhibited age-related decrease (p <0.001), which was more prominent in AO rats (Fig. 7a). Consequently, strain-specific difference mirrored in its greater (p <0.001) amount in young AO rats compared with age-matched DA rats disappeared in old animals (Fig. 7a).

With ageing, the expression of IL-15 mRNA changed in the opposite direction in DA rats and AO rats (Fig. 7a). It increased (p <0.001) in DA rats, whereas it decreased (p <0.05) in AO rats (Fig. 7a). Accordingly, contrary to young rats, in whom was found greater (p <0.05) amount of this mRNA in thymi of AO rats, its expression was less (p <0.001) in thymi from old AO rats compared with age-matched DA rats (Fig. 7a).

Additionally, a similar pattern of age- and strain-dependent changes to that observed in the thymic expression of IL-15 mRNA was found in the thymic expression of TGF-β mRNA (Fig. 7a). Namely, compared with strain-matched young rats, in old DA rats TGF-β mRNA expression was upregulated (p <0.001), whereas in old AO rats it was downregulated (p <0.001), thereby reversing strain differences in its expression observed in young animals (Fig. 7a). Specifically, thymic TGF-β mRNA expression in young AO rats exceeded (p <0.05) that in age-matched DA rats, whereas in old rats of this strain it was markedly below (p <0.001) that in age-matched DA rats (Fig. 7a).

Considering the previous findings, the frequency and absolute number of CD4+CD25+Foxp3+ nTregs among thymocytes were also examined. With ageing, the opposite changes in the frequency of CD4+CD25+Foxp3+ nTregs

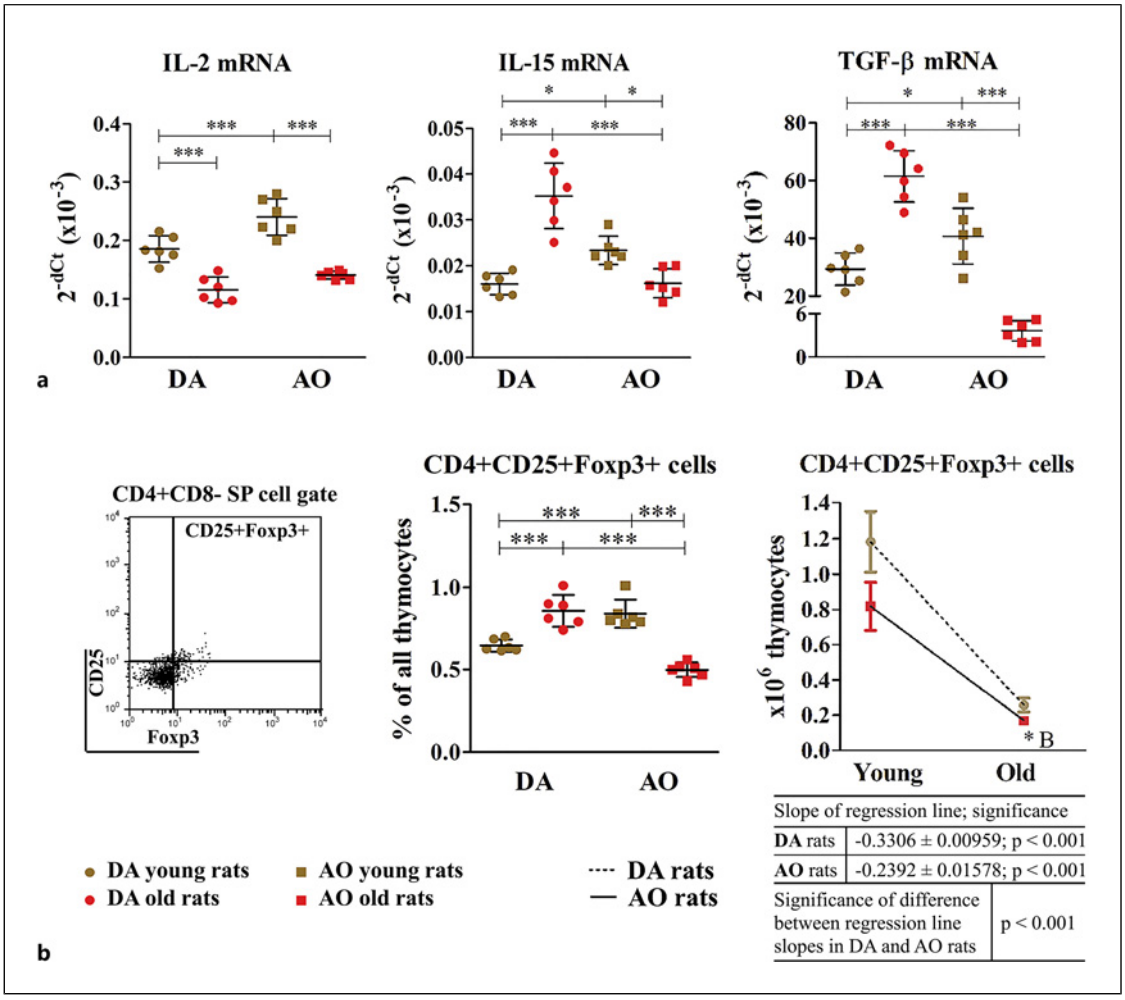
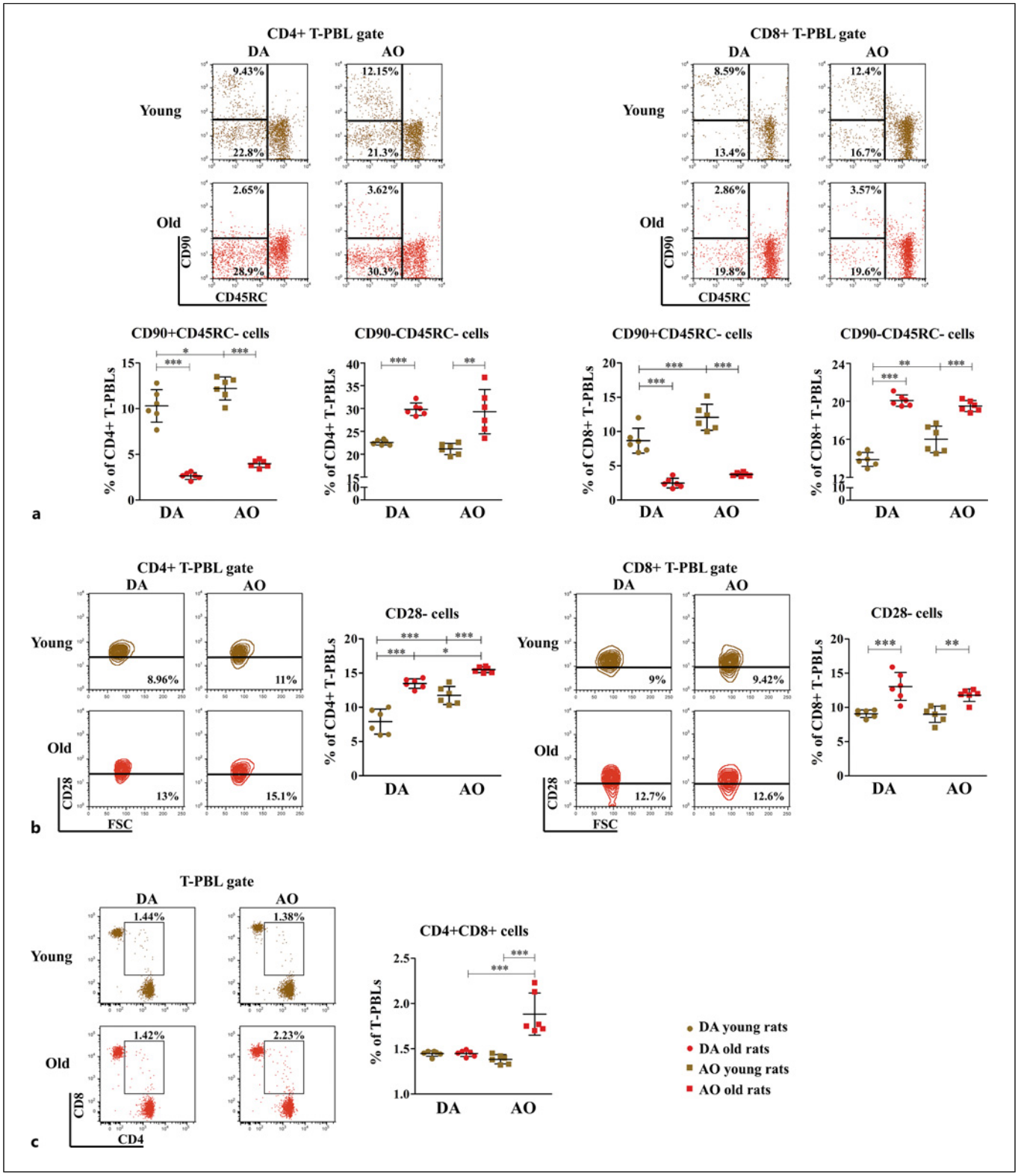


Fig. 7. Strain-specific age-related changes in the generation of CD4+CD25+Foxp3+ natural regulatory T cells (nTregs) and thymic expression of cytokines supporting their differentiation/maturation. **a** Scatter plots display the expression of mRNAs encoding (left) IL-2, (middle) IL-15, and (right) TGF-β in thymi from young and old DA and AO rats. **b** Scatter plot displays the frequency of thymic CD4+CD25+Foxp3+ nTregs in young and old rats of DA and AO strains. Representative flow cytometry dot plot indicates the gating strategy for CD25+Foxp3+ cells (upper right quadrant) within CD4+CD8– single positive (SP) thymocytes, gated as shown in Fig. 3. Linear graph depicts linear regression analyses of age-related changes in absolute number of nTregs in rats of DA and AO strains. Significance of differences in the absolute number of nTregs between DA and age-matched AO rats is shown in the linear graph (B, old). In the table below the linear graph are displayed the values for slopes of linear regression lines (indicating the steepness of the lines and thereby indirectly the kinetics of the age-related changes in the analysed parameter) and their significances in DA and AO rats, and significance of difference in the regression line slopes between the examined strains. Two-way ANOVA showed significant interactions between the effects of age and strain for the frequency ($F_{(1,20)} = 90.99$, $p < 0.001$) and number ($F_{(1,20)} = 38.41$, $p < 0.001$) of CD4+CD25+Foxp3+ thymocytes, and thymic IL-15 ($F_{(1,20)} = 455.84$, $p < 0.001$) and TGF-β ($F_{(1,20)} = 140.3$, $p < 0.001$) mRNA expression. Data are presented as mean ± standard deviation (SD) ($n = 6$ per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *$p < 0.05$; ***$p < 0.001$.

among thymocytes from DA and AO rats were found (Fig. 7b). Namely, with ageing their frequency increased ($p < 0.001$) and decreased ($p < 0.001$) in DA rats and AO rats, respectively (Fig. 7b). Thus, oppositely from young rats in whom the frequency of CD4+CD25+Foxp3+ nTregs was higher ($p < 0.001$) among thymocytes from AO rats, in old rats of this strain their frequency was lower ($p < 0.001$) compared with DA rats (Fig. 7b).

The absolute number of CD4+CD25+Foxp3+ nTregs in young rats was higher in DA rats, but this difference did not



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reach statistical significance (Fig. 7b). With ageing, their absolute number decreased (p <0.001) in thymi of DA and AO rats, but the slope of this decrease was more prominent (p <0.001) in DA rats (Fig. 7b). Consequently, the absolute number of CD4+CD25+Foxp3+ nTregs in old rats was slightly but significantly (p <0.05) less in AO rats compared with DA rats (Fig. 7b).

Age- and Strain-Related Specificities in the Phenotypic Profile of Rat T-PBLs

Age- and Strain-Related Specificities in the Frequency of RTEs and Activated/Memory T Cells

To additionally evaluate the significance of age- and rat strain-related differences in thymopoiesis, the frequency of CD90+CD45RC– cells (corresponding to RTEs, which are suggested to be thymic function surrogates) [69], among the major subpopulations of T-PBLs, was attested. As expected from the thymopoietic changes, with ageing the frequency of CD90+CD45RC– RTEs decreased (p <0.001) among both CD4+ and CD8+ T-PBLs irrespective of strain (Fig. 8a). Their frequency in young rats was higher among CD4+ T-PBLs (p <0.05) and CD8+ T-PBLs (p <0.001) from AO rats, whereas in old rats their frequency within both the major T-PBL subpopulations was comparable between AO rats and DA rats (Fig. 8a).

Besides, considering that decline in thymopoietic efficacy leads to expansion of certain T-cell clones in the periphery, and consequently the rise in the frequency of CD90–CD45RC– activated/memory T cells [78], the frequency of these cells was also examined. Age-related increase in the frequency of CD90–CD45RC– cells was found among CD4+ T-PBLs (p <0.001 and p <0.01 in DA and AO rats, respectively) and CD8+ T-PBLs (p <0.001) from DA and AO rats (Fig. 8a). In either young or old rats, there was no significant strain difference in their frequency among CD4+ T-PBLs (Fig. 8a). On the other hand, the frequency of CD90–CD45RC– cells among CD8+ T-PBLs was higher (p <0.01) in young AO rats compared with age-matched DA rats, whereas in old rats it did not differ between these two strains (Fig. 8a).

Age- and Strain-Specific Differences in the Frequency of CD28– Cells among T-PBLs

Next, considering data indicating that decline in thymopoietic efficacy leads to homeostatic oligoclonal proliferation of T cells [79] and loss of CD28 surface expression [80], the frequency of CD28– cells among CD4+ and CD8+ T-PBLs was also investigated. Age-related increase in the frequency of CD28– cells was found within CD4+ T-PBLs (p <0.001) and CD8+ T-PBLs (p <0.001 and p <0.01 in DA and AO rats, respectively) in DA and AO rats (Fig. 8b). Their frequency was higher among CD4+ T-PBLs from young (p <0.001) and old (p <0.05) AO rats compared with age-matched DA rats (Fig. 8b). Differently, there were no significant strain-specific differences in the frequency of CD28– cells among CD8+ T-PBLs in either young or old rats (Fig. 8b).

Age- and Strain-Specific Differences in the Frequency of CD4+CD8+ DP T-PBLs

Considering that CD4+CD8+ DP T cells have been identified in the peripheral blood and lymphoid tissues of numerous species [81], particularly in chronic inflammation and autoimmune disorders, and suggested to possibly be endowed with auto-reactivity due to faulty thymic selection [82], their frequency in peripheral blood was also examined. It did not significantly differ among T-PBLs from young and old DA rats, whereas in AO rats it was markedly higher (p <0.001) among T-PBLs from old compared with young animals (Fig. 8c). Consequently, in old rats, their frequency was higher (p <0.001) in AO rats compared with DA rats (Fig. 8c).

Fig. 8. Age- and strain-specific differences in the composition of peripheral blood T-cell compartment. **a** Representative flow cytometry dot plots and scatter plots indicate the frequencies of CD90+CD45RC– recent thymic emigrants (RTEs) and activated/memory CD90–CD45RC– cells among (left) CD4+ and (right) CD8+ T-peripheral blood lymphocytes (T-PBLs) of young and old rats of DA and AO strains. RTEs and memory/activated cells in flow cytometry profiles are gated in upper left and lower left quadrants, respectively. **b** Representative flow cytometry contour plots and corresponding scatter plots indicate the frequencies of CD28– cells among (left) CD4+ and (right) CD8+ T-PBLs from young and old DA and AO rats. **c** Representative flow cytometry dot plots and corresponding scatter plot indicate the frequencies of CD4+CD8+ double positive (DP) cells among T-PBLs in rats of both ages and strains. Two-way ANOVA showed significant interactions between the effects of age and strain for the frequencies of CD90–CD45RC– cells within CD8+ T-PBLs ($F_{(1,20)}$ = 13.8, p <0.01) and DP cells within T-PBLs ($F_{(1,20)}$ = 25.33, p <0.001). Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *p <0.05; **p <0.01; ***p <0.001.

EAE Development Affected the Thymopoietic Efficacy in Old DA and AO Rats

Strain-Specific Differences in Precipitating Effects of EAE on the Thymic Weight, Thymocyte Yield, and Thymopoietic Efficacy in Old Rats

Thymic Weight and Thymocyte Yield. To assess putative strain differences in the influence of EAE development on thymus from aged rats, thymus weight and thymocyte yield were examined in old EAE-inflicted rats of DA and AO strain. Thymic weight decreased in DA (p <0.01) and AO (p <0.01) rats with EAE development, and slopes of the corresponding regression lines were similar (Fig. 9a). Thus, as in NIM control rats, in EAE-inflicted rats thymic weight was greater (p <0.001) in AO rats than in their DA counterparts (Fig. 9a).

In AO and DA rats, the thymocyte yield also decreased (p <0.001) with the disease development (Fig. 9a). Thymocyte yield was comparable between NIM DA and AO rats, but it was greater (p <0.05) in EAE-inflicted AO rats than in their DA counterparts (Fig. 9a).

Considering that proinflammatory cytokines induce/contribute to thymic atrophy/involution [24, 51, 57], the circulating levels of IL-6 and TNF-α were also determined. Indeed, in DA and AO rats inflicted with EAE the circulating levels of IL-6 and TNF-α were higher (p <0.001) than in strain-matched NIM rats (Fig. 9b). The increase in circulating level of IL-6 with EAE development was more prominent in AO than DA rats (Fig. 9b). Consequently, as in NIM rats, in EAE-inflicted rats circulating IL-6 level was higher (p <0.001) in AO rats compared with their DA counterparts (Fig. 9b). On the other hand, the increase in TNF-α circulating level was less prominent in AO rats inflicted with EAE than in their DA counterparts, so its circulating level (which did not differ between NIM AO and DA rats) was lower (p <0.05) in EAE-inflicted AO rats compared with their DA counterparts (Fig. 9b).

Frequency and Absolute Number of the Most Mature SP TCRαβ+ Thymocytes. The frequency of the most mature CD4+CD8–CD90+TCRαβ+ and CD4–CD8+CD90+TCRαβ+ cells among thymocytes from DA rats and AO rats afflicted with EAE was lower (p <0.001) compared with strain-matched NIM rats (Fig. 9c). Given that the decline in the frequency of both CD4+CD8–CD90+TCRαβ+ and CD4–CD8+CD90+TCRαβ+ cells with immunisation was more prominent in AO rats, differently from NIM rats, in EAE-inflicted rats the frequency of CD4+CD8–CD90+TCRαβ+ cells was lower (p <0.01) in AO rats, whereas that of CD4–CD8+CD90+TCRαβ+ cells was comparable between these two strains of rats (Fig. 9c).

Of note, in DA and AO EAE-inflicted rats alike, the increased (p <0.001) frequencies of CD4+CD8–CD90–TCRαβ+ and CD4–CD8+CD90–TCRαβ+ SP re-entrants, suggested to interfere with thymocyte differentiation/maturation [67, 68], were found (online suppl. Fig. 4). The absolute number of both the most mature CD4+CD8–CD90+TCRαβ+ and CD4–CD8+CD90+TCRαβ+ thymocytes in DA and AO rats decreased (p <0.001) following immunisation, but this decrease was more prominent (p <0.001 and p <0.01 for CD4+CD8–CD90+TCRαβ+ and CD4–CD8+CD90+TCRαβ+ thymocytes, respectively) in AO rats inflicted with EAE (Fig. 9c). Despite that, as in NIM rats, in EAE-inflicted rats their absolute numbers remained lower (p <0.001) in DA rats than in their AO counterparts (Fig. 9c).

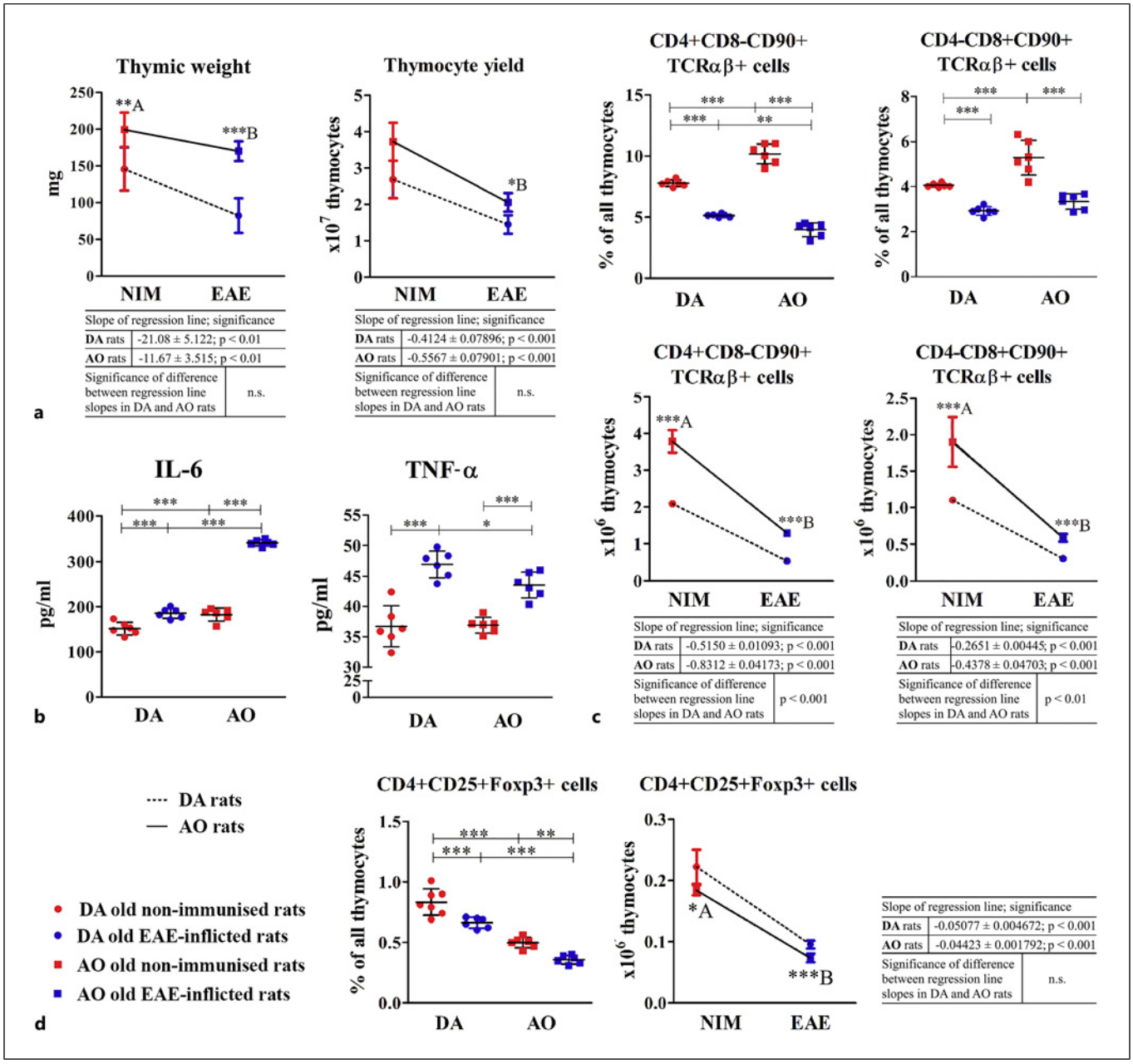
Frequency and Absolute Number of nTregs. The frequency of CD4+CD25+Foxp3+ nTregs in EAE-inflicted DA rats (p <0.001) and AO rats (p <0.01) was lower than in strain-matched NIM rats (Fig. 9d). In both NIM and EAE-inflicted rats, their frequency was lower (p <0.001) in AO rats than in DA rats (Fig. 9d).

Irrespective of rat strain, the absolute number of CD4+CD25+Foxp3+ nTregs decreased (p <0.001) with immunisation (Fig. 9d). In EAE-inflicted rats, as in NIM rats, their absolute number was lower (p <0.001) in AO rats than in DA rats (Fig. 9d).

Strain-Related Specificities in the Influence of EAE on Phenotypic Profile of Old Rat T-PBLs

EAE Influenced the Frequency of RTEs and Activated/Memory T-PBLs. As expected from analysis of thymopoietic efficacy, EAE development was associated with reduction in the frequency of CD90+CD45– RTEs among both CD4+ (p <0.01 and p <0.001 in DA and AO rats, respectively) and CD8+ (p <0.01) T-PBLs from DA and AO rats (Fig. 10a). This decrease resulted in a loss of strain difference (reflecting their higher frequency in AO rats) observed in the frequency of CD90+CD45– RTEs among CD4+ T-PBLs in NIM rats (Fig. 10a). On the other hand, the frequency of CD90+CD45– RTEs among CD8+ PBLs was higher (p <0.01) in NIM and EAE-inflicted AO rats compared with corresponding DA rats (Fig. 10a).

Next, the frequency of CD90–CD45– activated/memory cells among the major T-PBL subpopulations was determined. Their frequency was higher among CD4+ T-PBLs from DA and AO EAE-inflicted rats compared with strain-matched NIM rats, but this increase reached statistical significance (p <0.01) only in AO rats (Fig. 10a). The frequency of these cells did not statistically significantly differ between either NIM or EAE-inflicted AO and DA rats (Fig. 10a). Differently from the frequency of CD90–CD45– activated/memory cells among CD4+ T-PBLs, their frequency among CD8+ T-PBLs increased (p <0.01) in DA rats and AO rats following



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immunisation for EAE (Fig. 10a). Additionally, differently from the frequency of CD90–CD45– activated/memory cells among CD4+ T-PBLs, their frequency among CD8+ T-PBLs (which was comparable between NIM rats of DA and AO strain) was lower (p <0.01) in EAE-inflicted AO rats than in their DA counterparts (Fig. 10a).

EAE Influenced the Frequency of CD28– Cells among T-PBLs. Considering increase in the level of circulating proinflammatory cytokines [26, 27] and the greater frequency of activated/memory cells among T-PBLs following immunisation, the frequency of CD28– cells among T-PBLs was also assessed. Indeed, their frequency among both major subpopulations of T-PBLs increased (p <0.001) in EAE-inflicted DA and AO rats compared with strain-matched NIM rats (Fig. 10b). It is noteworthy that the frequency of CD28– cells among CD4+ T-PBLs was higher in NIM (p <0.001) and EAE-inflicted (p <0.05) AO rats compared with corresponding DA rats (Fig. 10b).

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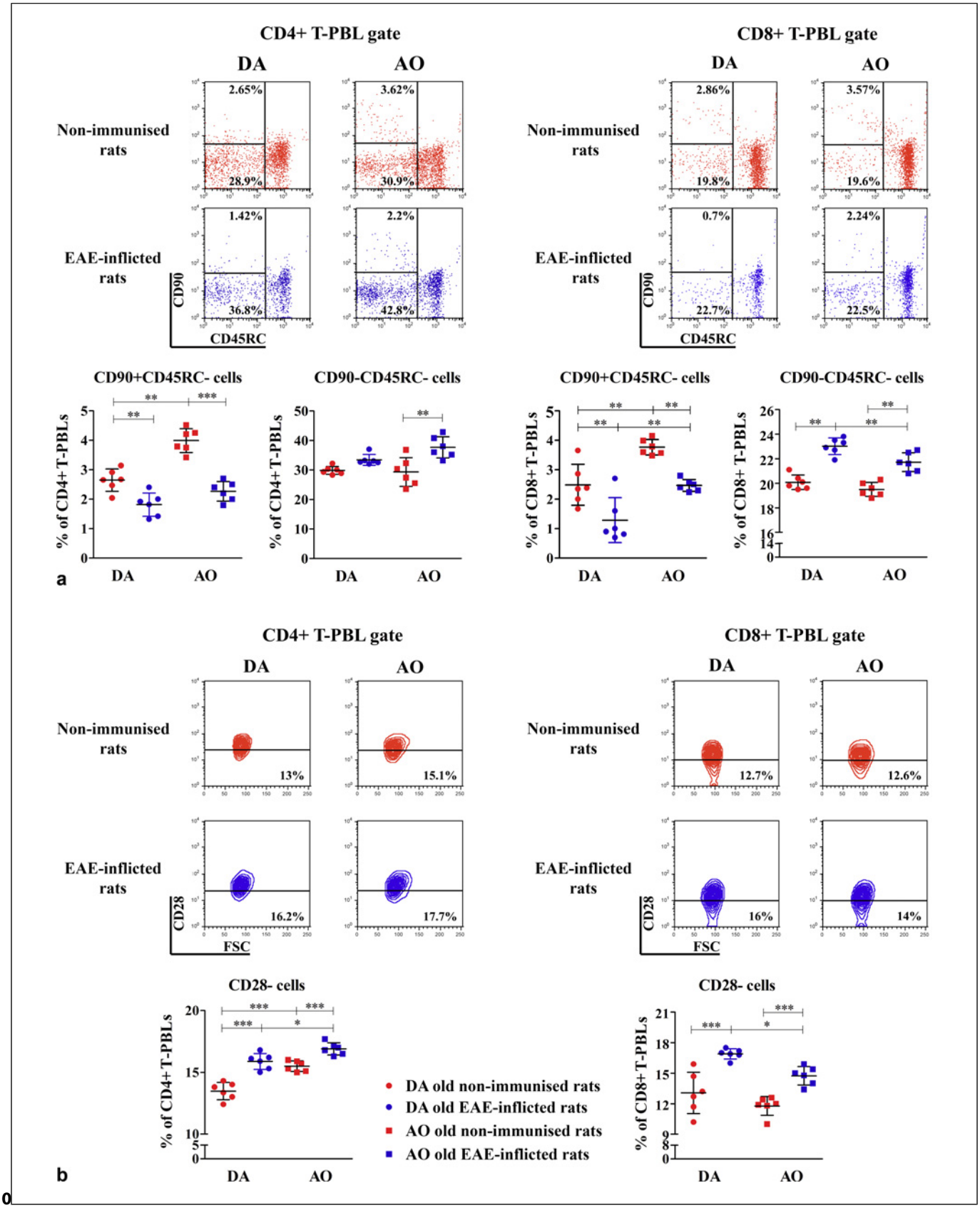
DOI: 10.1159/000535150

On the other hand, although the frequency of CD28– cells among CD8+ T-PBLs was comparable between NIM rats, it was slightly, but significantly lower (p <0.05) among EAE-inflicted AO rats compared with their DA counterparts (Fig. 10b).

Discussion

This study extended our previous research on the influence of age-related thymic changes on the increased susceptibility of old AO rats to develop clinically manifested EAE [10] by comparative analyses of age-related changes in the overall efficacy of thymopoiesis and particularly mechanisms controlling central tolerance and generation of CD8+ T cells prone to differentiate into IL-17-producing ones in DA rats (shown to be less prone to EAE induction with ageing), in order to identify putative strain-based thymus-related specificities that could be associated with the opposing effect of ageing on the susceptibility of these two strains to EAE. Differently from DA rats, in AO rats the surface expression of CD90, the molecule associated with negative regulation of thymocyte selection threshold and "fine-tuning" of T-cell repertoire [35], on DP thymocytes undergoing lineage commitment was upregulated, whereas the generation of nTregs was impaired partly reflecting the differences in thymic expression of cytokines supporting their development. Collectively, these findings pointed to mechanisms, which could make old AO rats, differently from DA rats, more prone to induction of the clinically manifested EAE, which, also differently from that in DA rats, has prolonged duration. In the same vein was the hereby presented finding indicating that ageing, differently from DA rats, in AO rats augments thymic generation of CD8+ T cells prone to differentiate into IL-17-producing cells, which are important for development of clinically manifested EAE in old rats of this strain [20–22]. Furthermore, this study showed that development of EAE leads not only in AO rats, but also in DA rats (in whom thymic involution developed at a greater pace), to precipitation of thymic involution progression and, in the periphery, to the increased generation of highly proinflammatory and cytotoxic CD28– T cells, which, in turn, may contribute to maintenance/progression of the disease [10, 25]. Of note, it also revealed a more prominent accumulation of CD28– cells within CD4+ T-PBLs in EAE-inflicted AO rats compared with their DA counterparts. Additionally, less efficient generation of nTregs in EAE-inflicted AO rats compared with their DA counterparts could also contribute to a prolonged duration of the disease in these rats.

Fig. 9. Significance of genetic background for the effects of EAE on thymopoietic efficacy in old rats. **a** Linear graphs depict linear regression analyses of EAE-related changes in (left) thymic weight and (right) thymocyte yield in old non-immunised (NIM) and EAE-inflicted rats of DA and AO strains. Significances of differences in the thymic weight and thymocyte yield between old DA NIM/EAE rats and age-matched AO rats are shown in the linear graphs (A, NIM; B, EAE). In the tables below the linear graphs are displayed the values for slopes of linear regression lines (indicating the steepness of the lines and thereby indirectly the kinetics of the EAE-related changes in the analysed parameters) and their significances in DA and AO rats, and significance of difference in the regression line slopes between the examined strains. **b** Scatter plots display plasma concentrations of (left) IL-6 and (right) TNF-α in old NIM and EAE rats of DA and AO strains. **c** Scatter plots display the frequencies of (left) CD4+CD8– and (right) CD4–CD8+ single positive (SP) CD90+TCRαβ+ cells retrieved from thymi of old NIM and EAE rats of DA and AO strains. Linear graphs depict linear regression analyses of EAE-related changes in the absolute numbers of (left) CD4+CD8– and (right) CD4–CD8+ SP CD90+TCRαβ+ cells in old NIM and EAE rats of DA and AO strains. Significances of differences in the absolute numbers of CD4+CD8– and CD4–CD8+ SP CD90+TCRαβ+ cells between old DA NIM/EAE rats and age-matched AO rats are shown in the linear graphs (A, NIM; B, EAE). In the tables below the linear graphs are displayed the values for slopes of linear regression lines (indicating the steepness of the lines and thereby indirectly the kinetics of the EAE-related changes in the analysed parameters) and their significances in DA and AO rats, and significance of difference in the regression line slopes between the examined strains. **d** Scatter plot displays the frequency of CD4+CD25+Foxp3+ natural regulatory T cells (nTregs) in thymi of old NIM and EAE rats of DA and AO strains. Linear graph depicts linear regression analyses of EAE-related changes in the absolute numbers of nTregs in old NIM and EAE rats of DA and AO strains. Significances of differences in the absolute numbers of nTregs between old DA NIM/EAE rats and age-matched AO rats are shown in the linear graph (A, NIM; B, EAE). In the table next to the linear graph are displayed the values for slopes of linear regression lines (indicating the steepness of the lines and thereby indirectly the kinetics of the EAE-related changes in the analysed parameters) and their significances in DA and AO rats, and significance of difference in the regression line slopes between the examined strains. Two-way ANOVA showed significant interactions between the effects of strain and EAE development for thymic weight ($F_{(1,20)}$ = 19.104, p <0.01), circulating levels of IL-6 ($F_{(1,20)}$ = 166.4, p <0.001), the frequencies of CD4+CD8–CD90+TCRαβ+ ($F_{(1,20)}$ = 69.64, p <0.01) and CD4–CD8+CD90+TCRαβ+ ($F_{(1,20)}$ = 5.152, p <0.05) cells, and the number of CD4+CD8–CD90+TCRαβ+ cells ($F_{(1,20)}$ = 15.1, p <0.001). Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *p <0.05; **p <0.01; ***p <0.001; n.s. – non-significant.



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Age-Related Thymic Alterations Contributing to the Opposite Changes in the Susceptibility of DA and AO Rats to EAE Induction

In accordance with data indicating that the initial (determined in young animals) thymocyte yield does not necessarily correlate with that at older age [49], the decrease of thymocyte yield was found in DA and AO rats, but in AO rats (initially exhibiting greater thymocyte absolute number) it was milder than in DA rats. In accordance with age-related decline in the thymocyte yield, in DA and AO rats was found the rise in circulating level of IL-6, cytokine associated with thymic atrophy/involution [24, 51], and in thymic expression of its transcript. Concordantly, in rats of both strains, age-associated decline in the expression of TEC-related molecules, i.e., Foxn1, a key regulator of TEC development/maintenance, and IL-7, a cytokine with a critical role in survival and proliferation of the most immature thymocytes [61], was found. Additionally, with ageing the expression of mRNA for IL-21, a cytokine significantly expanding positively selected DP thymocytes without affecting the diversity of the intrathymic or peripheral TCR repertoire (as it affects post-selection thymocyte expansion), when administered to old rats, decreased in the thymus of DA and AO rats [83]. Indeed, in accordance with these findings, the frequency of DP thymocytes decreased in DA and AO rats with ageing. There were some strain differences in the expression of mRNAs for aforementioned cytokines, which could be related to strain differences in the pace of thymic involution (judging not only by thymocyte yield, but also by thymic weight). Namely, although ageing downregulated thymic expression of IL-7 mRNA in rats of both strains, its expression was greater in thymi from old AO rats compared with age-matched DA rats. The lower amount of mRNA for TGF-β, the cytokine inhibiting proliferative action of IL-7 on thymocytes, in thymi from old AO rats (oppositely to old DA rats) [84, 85], could suggest even a less prominent decrease inhibitory effect of IL-7 on thymocyte proliferation in old AO rats compared with age-matched DA rats. Indeed, greater overall thymocyte proliferation was found in old AO compared with age-matched DA rats. On the other hand, irrespective of greater amount of IL-7 mRNA in thymi of old AO rats compared with age-matched DA rats, the overall frequency of apoptotic cells was higher among thymocytes from old AO rats. This could be partly ascribed to higher level of circulating IL-6 in old AO rats compared with their DA counterparts, as it has been shown to inhibit apoptosis of some other cells [86]. However, it is noteworthy that the expression of mRNA does not necessarily correlate with the expression level of the protein, and that thymic cell sensitivity to action of these molecules was not examined. Given that strain differences in age-related changes in these parameters are not easy to directly correlate with strain differences in age-related changes in thymocyte yield (less prominent decrease in AO rats), as the total thymocyte yield may also be influenced by alterations in the rate of blood-borne thymocyte precursor ingress into thymus and/or egress of the mature cells from the thymus into the periphery. At present, there are no data to support either of these two options.

The analysis of thymocyte subset composition showed that ageing in both strains of rats examined in this study led to relative expansion of the subset of the least mature DN thymocytes, the phenomenon less prominent in AO rats. Considering the changes in the expression of cytokines influencing directly or indirectly DN thymocyte survival and expansion (IL-7, IL-6, and TGF-β) [24, 51, 61], and IL-21, as it is also shown to influence DN thymocyte expansion, but to a lesser extent than DP ones [83], it may be hypothesised that either increased ingress of thymocyte precursors and/or decelerated transition to the DP stage contributed to age-related expansion of DN thymocyte subset registered in old rats of both strains. In favour of the latter option was the age-related decrease in the frequency of DP cells, which was particularly pronounced in AO rats. However, before accepting the previous assumption, it should be considered that the decrease in

Fig. 10. Strain-specific influence of immunisation for EAE on the composition of peripheral blood T-cell compartment in old rats. **a** Representative flow cytometry dot plots and scatter plots indicate the frequencies of CD90+CD45− recent thymic emigrants (RTEs) and activated/memory CD90−CD45− cells among (left) CD4+ and (right) CD8+ T-peripheral blood lymphocytes (T-PBLs) of old non-immunised (NIM) and EAE-inflicted rats of DA and AO strains. RTEs and memory/activated cells in flow cytometry profiles are gated in upper left and lower left quadrants, respectively. **b** Representative flow cytometry contour plots and corresponding scatter plots indicate the frequencies of CD28− cells among (left) CD4+ and (right) CD8+ T-PBLs from old NIM and EAE rats of DA and AO strains. Two-way ANOVA showed significant interactions between the effects of strain and EAE development for the frequencies of CD90+CD45− cells ($F_{(1,20)}$ = 8.250, p <0.01) and CD28− cells ($F_{(1,20)}$ = 4.486, p <0.05) within CD4+ T-PBLs. Data are presented as mean ± standard deviation (SD) (n = 6 per group). Symbols in scatter plots represent individual data values, and wide bars indicate means. *p <0.05; **p <0.01; ***p <0.001.

the relative size of DP thymocyte subset in rats of both strains was followed by the increase in the frequency of SP thymocytes over the same period of life. To fully appreciate significance of age-related increase in the frequency of CD4+CD8− and CD4−CD8+ SP thymocytes for the size of DP subset, it should be considered that SP thymocyte subsets encompass, apart from the most mature CD90+TCRαβ+ thymocytes developing from DP cells, recirculating T cells [68] and immature SP TCRαβ− thymocytes [87, 88]. Accordingly, the frequency of the most mature SP CD90+TCRαβ+ thymocytes was attested in thymi from DA and AO rats. Indeed, with ageing the frequencies of CD4+CD8− SP CD90+TCRαβ+ thymocytes and both CD4+CD8− and CD4−CD8+ SP CD90+TCRαβ+ thymocytes increased in DA and AO rats, respectively. The effect of ageing on CD4+CD8− SP CD90+TCRαβ+ thymocytes was more prominent in AO rats. However, it is noteworthy that the increased frequency of the most mature SP thymocytes could reflect accelerated differentiation/maturation of positively selected DP TCRαβ^{hi} cells and/or impaired thymocyte-negative selection. The analysis of the positively selected DP TCRαβ^{hi} cells undergoing lineage commitment identified by downregulation of one of the two coreceptor molecules [66] showed that with ageing the frequencies of CD4^{hi}CD8^{lo}TCRαβ^{hi} cells and CD4^{lo}CD8^{hi}TCRαβ^{hi} cells among DP TCRαβ+ thymocytes from AO rats increased and decreased, respectively. On the other hand, their frequencies remained unaltered in DA rats. With ageing, the surface density of CD90, the molecule whose density increases on DP TCRαβ+ thymocytes in Wistar rats [16], prominently increased on both CD4^{hi}CD8^{lo}TCRαβ^{hi} and CD4^{lo}CD8^{hi}TCRαβ^{hi} thymocytes, which are shown to be direct precedents of the most mature SP cells, in AO rats [65]. On the other hand, its density on these cells remained unaffected in DA rats. Given that increased CD90 surface density is associated with downregulation of TCRαβ signalling and an inappropriate negative selection [64], accelerated differentiation/maturation could also not be excluded, particularly in the development of CD4−CD8+CD90+TCRαβ+ SP thymocytes in old AO rats. On the other hand, to age-related increase in the frequency of CD4+CD8−CD90+TCRαβ+ SP thymocytes in DA rats most likely contributed their accelerated differentiation/maturation. In favour of this assumption is age-related decrease in the frequency of TCRαβ− cells which passed β-selection [65], followed by the increase in that of TCRαβ+ cells, which arranged α chain, within DP thymocyte subset in old DA rats. Additionally, it is noteworthy that CD90 surface density increased with ageing on TCRαβ+ thymocytes from both DA and AO rats, the phenomenon particularly prominent in AO rats, suggesting an increased positive selection/diminished negative selection in the early DP developmental stage [16]. Collectively, the previous findings may indicate that a prominent age-related impairment of the negative selection, most likely, at least partly, related to the age-related upregulation of CD90 expression, contributed to breach of self-tolerance in old AO rats immunised for EAE and the clinical disease development. The previous findings are consistent with herein presented age-related decrease in thymic expression of TGF-β, a cytokine shown to inhibit CD90 gene and protein expression in/on some other cell types [89], specifically in AO rats, as its expression in DA ones (oppositely to AO rats) increased with ageing.

The analysis of the age-related changes in the other component of central tolerance, i.e., the frequency of CD4+CD25+Foxp3+ nTregs in thymi of DA and AO rats, showed that ageing exerted the opposing effect on their frequency in DA and AO rats. Namely, with ageing the frequency CD4+CD25+Foxp3+ Tregs increased in DA rats, but decreased in AO rats, suggesting that this mechanism of central tolerance in AO rats was also negatively affected by ageing. This finding could be mainly related to the opposite effects (increase and decrease in DA and AO rats, respectively) of ageing on the expression of mRNA for TGF-β, a cytokine required for the induction of Foxp3 in Treg precursors, and consequent nTreg cell generation [77], in DA and AO rats. This difference seems to be consistent with impaired thymocyte-negative selection even in early DP developmental stage in old AO compared with DA rats [77, 90]. In the same vein was the opposite effects of ageing (viz., age-related increase and decrease in DA and AO rats, respectively) on the expression of IL-15, a cytokine also involved in intrathymic nTreg development [91] in AO and DA rats. Moreover, it is noteworthy that in DA rats age-related decline in the total thymocyte yield was steeper than in AO rats whereas on the contrary, age-related decrease in the overall absolute number of nTregs was steeper in AO rats compared with DA ones, thereby additionally supporting their putative role in the increased propensity of old AO rats for development of clinically manifested disease. Besides, it also pointed to a mechanism that could contribute to lower susceptibility of old DA rats to the development of EAE when compared with young ones.

Furthermore, the present study confirmed increased generation of IL-17-producing CD8+ T cells in thymi from old AO rats compared with strain-matched young

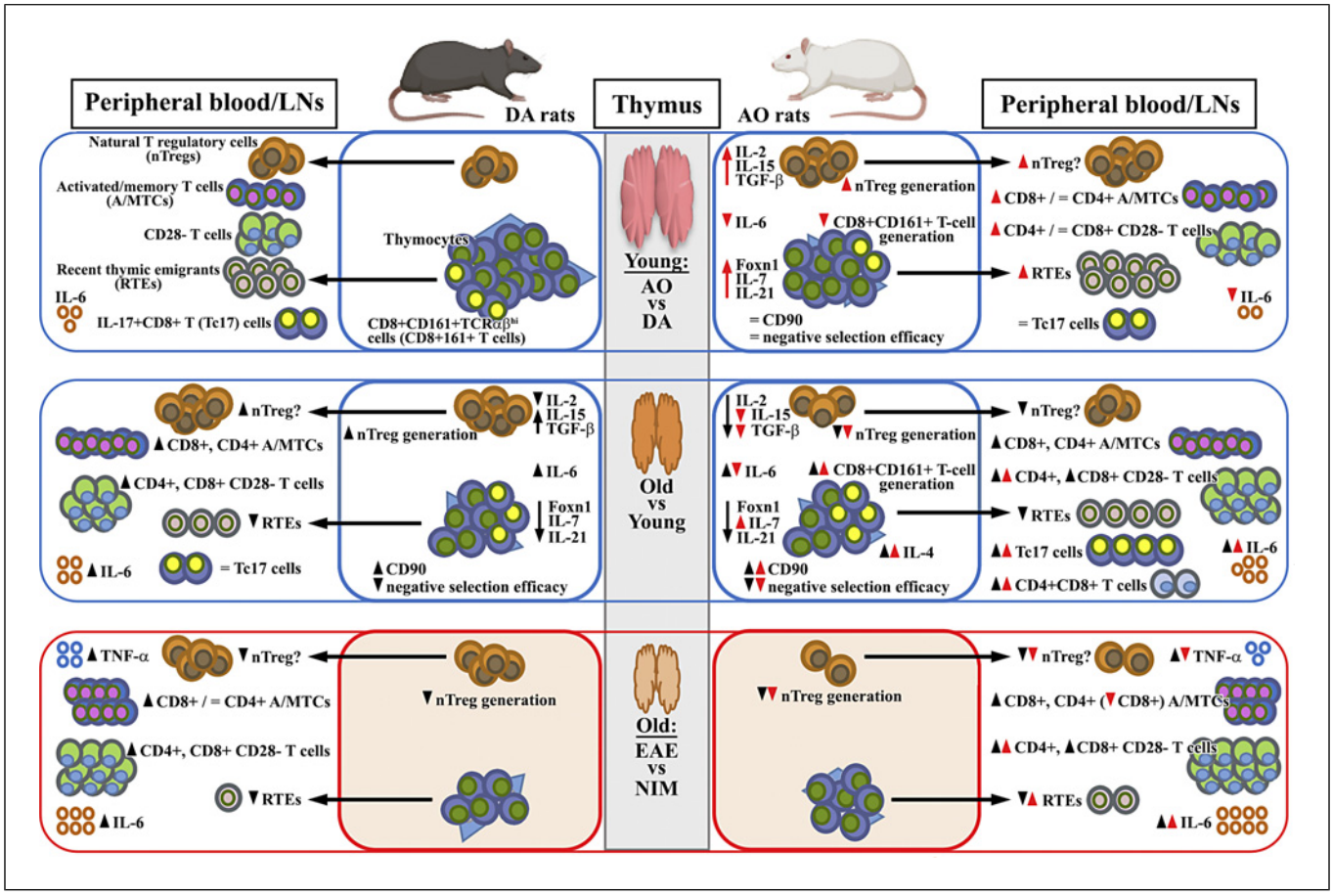

Fig. 11. Illustrated overview of the key effects of ageing and experimental autoimmune encephalomyelitis (EAE) in old rats on thymopoiesis and phenotypic profile of peripheral T-cell compartment in Dark Agouti (DA) and Albino Oxford (AO) rats showing decrease and increase in susceptibility to EAE with ageing, respectively. In the upper rectangle with blue frame is displayed greater efficiency of (i) overall thymopoiesis (mirrored in higher frequency of recent thymic emigrants [RTEs]) followed by a less efficient generation of CD8+ T cells prone to synthesise IL-17 (CD8+CD161+TCRαβ^{hi}) and (ii) central tolerance, judging by greater generation of natural regulatory T cells (nTregs) in young AO rats compared with age-matched DA rats. Additionally, strain differences in some underlying mechanisms, viz., circulating levels of IL-6 (negatively affecting thymopoietic efficacy and CD28 expression on T cells in the periphery), and thymic expression of thymosuppressive/thymostimulatory factors (IL-6, IL-7, IL-21, Foxn1) and cytokines supporting nTreg development (IL-2, IL-15, TGF-β) are also displayed. In the lower rectangle with blue frame are shown the key ageing-related changes in the thymopoietic events of putative significance for altered susceptibility to EAE of old DA and AO rats. It is indicated that ageing is associated with (i) more prominent decrease in the efficacy of negative selection (reflecting the greater density of CD90, negative regulator of thymocyte selection threshold) on CD4+CD8+ double positive (DP) thymocytes undergoing selection and the generation of nTreg (partly reflecting differences in the expression of cytokines supporting their development) and (ii) greater thymic generation of CD8+ T cells prone to synthesise IL-17, of significant importance for EAE development (partly reflecting augmented thymic IL-4 expression) in old AO rats compared with age-matched DA rats. In the same line, compared with DA rats, in peripheral blood of old AO rats is shown (1) greater frequency of possibly autoreactive CD4+CD8+ T cells (possibly escaping from thymus) and (2) proinflammatory and cytotoxic CD4+CD28– T cells (reflecting higher levels of circulating IL-6) followed by greater frequency of IL-17-producing CD8+ T cells (Tc17) in lymph nodes (LNs). In the rectangle with red frame is shown that EAE development followed by the increased levels of circulating proinflammatory cytokines in DA rats and AO rats, in turn, potentiates thymic changes, as it decreases the generation of nTregs (crucial for the control of the disease development), the effect more prominent in AO rats. Of note, this could be partly associated with prolonged duration of the disease in old AO rats. Additionally, compared with EAE-inflicted DA rats, in their AO counterparts is shown the greater frequency of proinflammatory and highly cytotoxic CD4+CD28– T cells, which most likely reflects greater circulating levels of IL-6 (as circulating TNF-α level is even slightly lower, whereas, judging by RTEs, thymocyte output is greater in AO rats), the phenomenon which could also contribute to prolonged course of the disease in these rats. Black arrows/arrowheads indicate age-related or EAE-induced changes in DA and AO rats; red arrows/arrowheads indicate the differences observed in AO rats, relative to the corresponding DA rats; EAE, experimental autoimmune encephalomyelitis-inflicted old rats; NIM, non-immunised old rats.

rats, the phenomenon related to the augmented thymic expression of IL-4 [10], and showed that it is specific for old rats of AO strain. Thus, these findings additionally corroborated their role in development of clinically manifested EAE in old AO rats [20]. To additionally support the generation of these cells, whose development is dependent on IL-4 expression [70, 71], was the age-related rise in the frequency of CD4+CD8+ DP cells within T-PBLs exclusively in AO rats, as it has been shown that they encompass, apart from DP thymocytes that have prematurely escaped from the thymus [92, 93], also CD4+ cells that re-express CD8 after activation or exposure to cytokines such as IL-4 [94–96].

Moreover, as expected [8, 10], in both AO and DA rats with ageing the efficiency of generation of new CD4+ and CD8+ T cells was reduced, mirrored in the decreased frequency of RTEs, suggested to be the surrogate marker for thymic output, among both the major subpopulations of T-PBLs. This was followed by the accumulation of activated/memory cells and proliferatively exhausted CD28– (null) cells, which acquire expression of several receptors commonly associated with natural killer cells, secrete large amounts of proinflammatory cytokines, and express cytotoxic properties in the elderly and old experimental animals, contributing to the development of chronic inflammatory diseases in old age [9, 97, 98]. Considering less prominent age-associated decline in the number of both subsets of the most mature CD90+TCRαβ+ SP thymocytes in AO compared with DA rats leading to their greater absolute numbers in old AO rats compared with age-matched DA rats, and comparable frequency of RTEs among peripheral CD4+ and CD8+ T-PBLs from old AO rats compared with age-matched DA rats, suggested an decelerated egress of mature SP cells from the thymus into the periphery in old AO rats. The frequency of activated/memory cells within the major subpopulations of T-PBLs also did not differ between old AO and old DA rats. However, the frequency of CD28– cells was higher among CD4+ PBLs from old AO rats compared with age-matched DA rats. This could be related to the higher level of circulatory IL-6 in old AO rats [26, 27]. Additionally, this finding may suggest a different sensitivity of CD4+ and CD8+ T-PBLs to IL-6 action on CD28 expression [26, 27].

Development of EAE in DA and AO Rats Contributed to the Progression in Thymic Involution

As expected from previous study [10], development of CNS autoimmunity precipitated thymic involution. Although the circulating levels of proinflammatory IL-6 cytokine were elevated in old AO rats compared with DA counterparts, the steepness of the slope line describing the decline in thymic weight and thymocyte yield was similar in DA and AO rats, possibly suggesting strain differences in the sensitivity to its action. Of note, accordingly, the values of these parameters were higher in AO rats inflicted with EAE compared with their DA counterparts. The absolute number of the most mature SP CD90+TCRαβ+ thymocytes also decreased in rats of both strains. To fully appreciate significance of the decrease in the absolute number of the most mature SP CD90+TCRαβ+ thymocytes observed in these rats, the decline in the frequency of RTEs among CD4+ and CD8+ T-PBLs was examined. Differently from the frequency of RTEs among CD8+ T-PBLs, which decline to approximately the same extent in DA and AO rats following immunisation, the frequency of RTEs among CD4+ T-PBLs decreased to a greater extent in EAE-inflicted AO rats compared with their DA counterparts, suggesting a decelerated egress of the most mature CD4+CD8– SP CD90+ TCRαβ+ thymocytes into the periphery. Accordingly, the frequency of activated/memory cells increased among CD4+ T-PBLs in EAE-inflicted rats compared to strain-matched NIM rats. Additionally, the frequency of CD28– cells was higher among CD4+ T-PBLs from AO rats inflicted with EAE compared with DA rats inflicted with EAE. Thus, prolonged duration of EAE in AO compared to DA rats could be partly ascribed to the higher frequency of these proinflammatory cytotoxic CD4+ cells in AO compared with EAE-inflicted DA rats [24, 25].

To prolonged duration of EAE in AO rats inflicted with EAE could also contribute the reduced absolute number of nTregs in AO rats compared with their DA counterparts. This could reflect a genetically determined decreased generation of these cells in AO rats compared with DA ones as shown herein and in our previous study [25]. To corroborate the previous notion are data indicating that prevalence of newly generated naive nTregs is critical for Treg suppressive function, so thymectomy inhibits spontaneous remission in rat EAE [99] whereas newly generated nTregs are critical for Treg-mediated suppression of autoimmune response in MS [100].

In conclusion, our results indicate that when comparing the effects of ageing/EAE development on T-cell immune compartment across animals of different genetic backgrounds it must be considered that parameters of thymopoietic efficacy and quality significantly differ between them (Fig. 11). Accordingly, they corroborate previously stated notion [38] that caution is necessary when designing pharmacological or other interventions aimed at modulating thymic activity in order to modulate immune response in genetically diverse populations, such as humans, and interpreting their outcomes.

Furthermore, they indicate that autoimmune pathology of CNS may additionally worsen thymic involution and potentiate thymic-involution-dependent immune changes, the phenomenon requiring further research.

Statement of Ethics

Experiments within the study were performed in accordance with 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 relevant national legislative (Animal Welfare Act, "Official Gazette of RS," No. 41/2009). This study protocol was reviewed and 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 care staff and a veterinarian monitored animal health status on a daily basis. The study was performed in accordance with ARRIVE guidelines for reporting in vivo animal experiments.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

This research was funded by the Ministry of Science, Technological Development and Innovation, Republic of Serbia through Grant Agreement with University of Belgrade – Faculty of Pharmacy No: 451-03-47/2023-01/200161, Immunology Research Centre "Branislav Janković," Institute of Virology, Vaccines and Sera "Torlak" No: 451-03-47/2023-01/200177, and Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, University of Belgrade No: 451-03-47/2023-01/200007.

Author Contributions

Study conception and design: G.L., Z.S.V., and M.P.; experimental work, analysis and interpretation of data, and final approval: G.L., Z.S.V., I.P., and M.P.; acquisition of data: M.P. and I.P.; drafting of manuscript: G.L. and M.P.; critical revision: G.L., Z.S.V., and I.P.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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