Differentiated all-trans retinoic acid response of naive CD4+CD25– cells isolated from rats with collagen-induced arthritis and healthy ones under *in vitro* conditions

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Abstract

Aim o the study: *To compare the potential of CD4+CD25– cells, isolated from both healthy rats and rats with CIA (Collagen-Induced Arthritis), for differentiation into regulatory T cells in the presence of all-trans retinoic acid in order to learn more about the activation mechanisms and therapeutic potential of regulatory T cells.*

Material and methods: *Sorted CD4+CD25– cells were cultured in vitro with/without ATRA, and then the frequency of regulatory T cells and their ability to secrete IL-10 by CD4+ FOXP3+ cells was examined. Gene expression of the foxp3, rarα, rarβ, rxrβ, and ppar β/δ and protein expression of the Rarα, Rarβ, and Rxrβ in cells after stimulation with ATRA were also investigated.*

Results: *CD4+CD25– cells isolated from healthy animals or from animals with CIA are characterised by different potential of the differentiation into CD4+CD25+ FOXP3+ cells. Retinoic acid receptor Rxrβ is present in the CD4+CD25– cells isolated from rats with CIA.*

Conclusions: *We showed that although ATRA did not increase the frequency of Treg in culture, it significantly increased expression of rarβ and rxrβ only in lymphocytes taken from diseased animals and foxp3 expression only in healthy animals. Moreover, after ATRA stimulation, the frequency of Treg-produced IL-10 tended to be lower in diseased animals than in the healthy group. The results imply that the potential of nadve cell CD4 lymphocytes to differentiate into Tregs and their putative suppressive function is dependent on the donor's health status.*

Key words: *regulatory T cells, Treg, all-retinoic acid, atra, collagen-induced arthritis.*

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Introduction

Among the regulatory T lymphocytes (Treg) characterised by the CD4+CD25+FOXP3+ phenotype, three basic subpopulations are distinguished based on the place of their development. First, the thymus regulatory T cells (tTregs or nTregs), which are developed in the thymus. Second, the peripheral regulatory T cells (pTregs) produced peripherally in the spleen and lymph nodes, and gut-associated lymphoid tissue (GALT) from naive CD4+CD25– lymphocytes as a response to antigen stimulation; and third, the iTregs – cells induced under *in vitro* conditions [1].

The Treg lymphocytes are responsible for peripheral tolerance of self-antigens, and they control the immune response process against exogenous antigens [2]. Their immunosuppressive effect is conditioned by stable expression of the FOXP3 transcription factor and consists – among others – of the release of immunosuppressive cytokines such as IL-10 and TGF-β, as well as the reduction of an-

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tigen presentation by antigen-presenting cells, by blocking their costimulatory function [3]. Some Tregs can be differentiated from CD4+ in the presence of IL-10 in *in vitro* conditions. These Tregs have been named Treg 1, and their main characteristic feature is high secretion of IL-10. However, it is also possible to obtain *in vitro* Tregs that produce TGF-β (named Th3) [4].

Maynard's team demonstrated that, despite the induction of Foxp3 by TGF-β, ATRA *in vitro* in the signal path of TGF-β inhibits the secretion of IL-10 in CD4+ naive cells. Similarly, *in vivo* ATRA produced in the small intestine by dendritic cells inhibits induction of IL-10 in CD4+ cells but initiates the expression of Foxp3. In their studies, they also made the control cell cultures of CD4+ T cells in the presence of a RAR antagonist – LE540, which resulted in secretion of IL-10 at a level significantly higher than in culture only with TGF-β or TGF-β + ATRA. Same team also demonstrated that ATRA inhibits only transcription, but not translation of IL-10 [5]. However, supplementation of diet with high dose of ATRA in Balb/c mice has significantly increased secretion of IL-10 in lymphocytes isolated from lymph nodes. However, these CD4+ cells were stimulated by influenza virus [6]. The discrepancy of results achieved in the cited works can be justified by a different approach taken to antigenic stimulation.

As well as IL-10, IL-35 is also a suppressive cytokine known to have an inhibitory effect on lymphocyte proliferation and effector Th17 of CD4+ CD25+ cells [7, 8]. Kochetkov *et al.* have proven the anti-inflammatory effect of IL-35 in CIA in DBA/1 mice in the context of production of IL-10 by CD4+ CD39+ or CD4+ CD39–. Both C57BL/6 and IL-10 –/– mice were administered orally 0.75 mg IL-35 or PBS. Subsequently, CIA was induced in mice. Animals that received IL-35 did not have any characteristic clinical symptoms of CIA [9].

On the other hand, the reduced suppressive activity of Treg cells, as well as their reduced frequency researched under *in vitro* conditions, is characteristic for patients with autoimmune diseases, including type 1 diabetes, multiple sclerosis [10], and rheumatoid arthritis [11]. The emergence of effective methods of *in vitro* expansion of polyclonal Treg lymphocytes has made it possible to attempt cellular immunotherapy in animal model diseases and in humans affected by autoimmune diseases, as well as in transplantation medicine [12].

In animal models of autoimmune diseases it was found that adoptive transfer of Treg lymphocytes may effectively inhibit the progression of the disease. Morgan *et al.* were the first to demonstrate that adoptive transfer of CD4+CD25+ cells in DBA/1 mice in the first stage of CIA development improves the clinical picture of model RA [13]. Moreover, experiments where Treg cells were administered by adoptive transfer have proved successful, both in mouse model of MS [14] and in NOD mice [15]. In our previous research we demonstrated that the adoptive transfer of Treg cells induced under *in vitro* conditions reduces clinical symptoms of CIA in Wistar rats [16].

Among the many agents used for the induction of the differentiation of CD4+ lymphocytes into iTreg cells, rapamycin and all-trans-retinoic acid (ATRA) seem to be most effective. Under their influence the induced lymphocytes gain stable phenotype of Treg cells that are able to maintain its immunosuppressive function towards the effector lymphocytes. ATRA is thought to regulate the foxp3 gene expression by inducing chromatin decondensation and histone acetyltransferase recruitment in the promoter region. However, ATRA does not always increase foxp3 expression in lymphocytes in the cytokine environment, which assures the differentiation towards Treg [17], but it exerts a positive influence on the induction of other Treg cell features. Among others, it increases the expression of chemokines, assuring their targeted migration, and stabilises foxp3 expression by preventing the disadvantageous transformation into Th17 [18].

On the other hand, the process of Treg cell induction by ATRA may correlate with the expression of nuclear retinoic acid receptors as its natural cellular ligands. There are two types of nuclear retinoic acid receptors: RAR (retinoic acid receptor) and RXR (retinoid X receptor). RAR occurs in three subtypes: RARα, RARβ, and RARγ [19]. Also, three subtypes of RXR are known: α, β, and γ. In addition, multiple isoforms of RAR and RXR receptors have been described. They are either synthesised from different promotors (e.g. RARα1 and RARα2) or produced by alternative splicing. Some of them are inducible by retinoic acid [20].

The RAR family receptors are activated by both all-trans RA (ATRA) and by 9-cisRA, whereas the RXR family is activated exclusively by 9-cis RA. ATRA, however, is likely to exhibit some RXR-stimulating activity *in vitro* due to its conversion to 9-cis-RA [21]. RXR can form homo- and heterodimers. After the binding of ATRA with ligands, specific sequences (retinoic acid response sequence) of target gene promoters are activated – RARE for RAR or RXRE for RXR. Specific binding of RA to RAR and RXR homo- or heterodimers takes place in the nucleus where they are constantly present. The process of binding of ligand with protein receptor causes dissociation of repressor protein and binding of the ligand with co-activator.

Very scarce research evidence is available on the presence of RAR and RXR isoforms subtypes in the T lymphocyte and Treg cells. It has been demonstrated that retinoic acid may participate in regulation of its own activity via modulation of the expression of RARs [22].

As has been already described in the literature that blocking of the Rarα inhibits differentiation of Tregs in GALT [23-25] which could suggest an important role of this receptor in immune phenomena. In experiments conducted on Rarα –/– mice it was demonstrated that the potential of CD4+CD62+hi to differentiate into Th1 or Th17 is much smaller than the wild type strain. Similarly, the frequency of Tregs present in the small intestinal mucosa is significantly lower in Rarα –/– mice compared to the wild-type. As already described by Hall *et al.*, Rarα has been identified as an important mediator in the regulatory immune processes [26].

In our experiment, we studied the response of naive CD4+CD25– T cells isolated from healthy rats and from rats with CIA to ATRA under *in vitro* conditions. We showed that lymphocytes isolated from healthy rats responded differently to ATRA than those isolated from diseased animals. In particular, in diseased animals ATRA significantly increased the transcription of RXR beta and RAR beta in stimulated cells.

Material and methods

Animals

Twelve female, six-week-old, inbred Wistar rats weighing 150-200 g from a colony bred at Wroclaw Medical University, Department of Pathology, Poland were used. The experiment was conducted upon permission No. 60/2011 of the Local Ethical Committee II in Wroclaw (Poland). The animals received water and food *ad libitum*, and were kept at room temperature (24°C) in an animal facility in a standard microbiological regime in a dark-light cycle (12 : 12).

Model CIA

Six experimental animals were immunised twice, subcutaneously at the tail base with 1 : 1 (v/v) 200 μg bovine collagen type II (BD Bioscience, USA) and IFA (Freund's Incomplete Adjuvant) (Sigma Aldrich, USA), at a volume of 200 μl at a seven-day interval. Another six healthy rats were the control group without any immunisation. The rats were euthanised by inhalational anaesthesia with isoflurane and dislocation of the cervical vertebrae.

Clinical assessment of CIA

The evaluation of arthritis severity was restricted to the hind paws of the animals, in accordance with the modified and extended numerical scale described by Suszko and Obminska-Mrukowicz [27]. The severity of clinical signs was scored as follows: 0 points – no change; 0-20 points – swelling of the fingers (0-4 points for each of five fingers); 0-5 points – redness and swelling of the ankle; 0-5 points – redness and swelling in the paw; and 0-10 points – severe, extensive inflammation of the hind paw multiple joints involved, and/or deformation of joints (*ankylosis*) with function impairment. The arthritis score for each rat was the average score of both hind paws (the highest score for one hind paw of a rat = 40).

ELISA test

The level of anti-bovine collagen type II (anti-CII) IgG antibodies in the sera obtained from immunised and control rats was examined using an ELISA assay. Nunc Maxisorp plates were coated with 2 μg/ml bovine collagen (BD Bioscience, USA) in 0.1 M carbonate buffer, pH 9.6, by overnight incubation at 4°C. The analysed serum was diluted 500-fold in PBS buffer with 0.05% Tween 20 and incubated at 37°C for 1.5 hours. Anti-rat IgG (Jackson ImmunoResearch, USA) conjugated with horseradish peroxidase was used to assess the level of anti-CII antibodies. Supersensitive TMB reagent (Sigma-Aldrich, USA) was used as substrate (100 μl/well). Enzymatic reaction was stopped after 15 minutes with 2M H_2SO_4 (50 ml/well). The optical density of the wells' content was determined at λ = 450 nm using a BioTek μQuantum reader (BioTek, USA).

Flow cytometry

Lymphocytes were isolated from the spleen and lymph nodes by sieving into PBS. After that, 5 × 10^5 lymphocytes were suspended in a FACS buffer (40 ml PBS, 0.8 ml foetal calf serum [Life Technologies, USA], 40 mg NaN_2). Cells were stained with anti-rat CD4 FITC (e-Bioscience, USA) and anti-rat CD25-PE (e-Bioscience, USA) for 30 minutes at 4°C, washed twice with FACS buffer, fixed, and permeabilised with Fix/Perm buffer (e-Bioscience, USA) and permeabilisation buffer (e-Bioscence, USA) in accordance with the manufacturer's instructions. FOXP3 protein was stained with anti-Mouse/Rat FOXP3 Alexa Fluor 647 (e-Bioscience, USA) for 30 minutes at 4°C. The cells were washed twice with FACS buffer, fixed in 2% paraformaldehyde and analysed using a FACSCalibur flow cytometer and Weasel 2.0 software. For IL-10 intracellular staining, cells, after extracellular staining by anti-CD4 and anti-CD-25, were cultured at 1 × 10^6 cells/well, in 24-well plates coated with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 in the presence of 100 U/ml IL-2 in RPMI 1640 medium supplemented with 100 unit/ml penicillin/streptomycin, 20 mM L-glutamine, 5 ng/ml TGF-β, 10% FCS, and with 1 μl/1 × 10^6 cells Golgi Plug, 50 ng/ml PMA, and 1 μg/ml ionomycin, with or without 10 nM ATRA. After incubation the cells were washed twice with 2 ml of PSB + 2% FBS, fixed, and permeabilised with Fix/Perm buffer (e-Bioscience, USA) and permeabilisation buffer (e-Bioscence, USA) in accordance with the manufacturer's instructions. Then cells were stained with anti-rat IL-10 PE (BD Bioscience, USA) for 30 minutes at 4°C. The cells were washed twice with FACS buffer, fixed in 2% paraformaldehyde and analysed using a FACSCalibur flow cytometer and Weasel 2.0 software.

FACS sorting

Lymphocytes isolated from the spleen and the lymph nodes of healthy or CIA rats were mixed together and suspended in PBS + 2% foetal calf serum (FCS) and stained with rat anti-CD4 FITC (e-Bioscience, USA), anti-rat CD25-PE (e-Bioscience, USA) for 30 minutes at 4°C. The cells were then washed twice with PBS + 2% FCS, and disaggregated in sterile FACS tubes with a 35-μm nylon mesh cell strainer cap (BD Biosciences, USA). 50 × 10^6 cells prepared in this manner were sorted by CD4+CD25– phenotype on the BD FACSAria II at 15,000 cells/second. Following sorting, the CD4+CD25– cells were immediately centrifuged in PBS + 2% FCS.

Culture of CD4+CD25– cells

After sorting, the cells were cultured for four days at 1 × 10^6 cells/well, in 24-well plates coated with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 in the presence of 200 U/ml interleukin-2 in RPMI 1640 medium supplemented with 100 unit/ml penicillin/streptomycin, 20 mM L-glutamine, 5 ng/ml TGF-β, 10% FCS and with or without 10 nM of all-retinoic acid (Sigma Aldrich, USA). Dosage of ATRA was tested according to data from the literature [28]. The control culture had no ATRA.

Isolation of mRNA

Cells isolated from the spleen and lymph nodes from healthy or diseased rats after being sorted according to the CD4+CD25- phenotype and after culturing in the presence of or without 10 nM ATRA in a quantity of 0.5 × 10^6 were suspended in 0.5 ml TriReagent and stored at –20°C until the time of isolation (no longer than one month). The RNA isolation procedure was tested in pilot researches and the highest quality was obtained with the Qiagen Rneasy Mini Kit (50) (catalogue number 74104, Qiagen, USA); however, the procedure proposed by the producer was modified and the RNA isolation was performed in a manner described further. RW1 and RPE are buffers included in the Qiagen Rneasy Mini Kit (50). First, the samples were defrosted and 0.2 ml of chloroform was added. The sample was intensively stirred for 15 seconds and incubated for five minutes at room temperature and then centrifuged at 12,000 × g for 15 minutes at 4°C.

The RNA water phase was collected to a sterile Eppendorf tube and one volume of 70 % EtOH was added. After stirring, and the whole sample was moved to a centrifugal column centrifuged for 15 seconds at 8000 g at room temperature. Then 350 μl of the RW1 buffer was added on the centrifugal column and centrifuged for 15 seconds at 8000 g at room temperature. The next step was the digestion of genomic DNA remains. For this purpose, a solution was prepared according to the manufacturer's instructions (Dnase I, nr cat. EN0525, Fermentas, USA) in the amount of 20 μl per sample of the reaction mixture and incubated for 15 minutes at 37°C. Afterwards, 350 μl of RW1 buffer was added, centrifuged for 15 seconds at 8000 g at room temperature. 500 μl of RPE Buffet was addend and centrifuged for 15 seconds at 8000 g at room temperature, and then the column was placed in a new Eppendorf tube. 50 μl of $H_2O_{\text{RNase free}}$ was applied and centrifuged for one minute at 8000 g at room temperature. RNA concentration was determined with Nano Drop 2000.

RT-PCR

The reversed transcription reaction (RT-PCR) was conducted with the use of a RT-PCR device – High Capacity cDNA Reverse Transcription Kit (catalogue no. 4368814, Applied Biosystems, USA) in the MJ Mini Personal Thermal Cycler BIORAD apparatus. The RT-PCR reaction was conducted at the RNA matrix, 2 μg per reaction. RNA solution was added in the amount of 10 μl to 10 μl of the reaction mixture. Samples were cooled in ice, and reversed transcription reaction was conducted for five minutes at 25°C, then for 120 minutes in 37°C, and five seconds at 85°C.

Real-time PCR

The expression of *foxp3*, *rar*α, *rar*β, and *rxr*β genes was determined by the Real-time PCR method in the Applied Biosystems 7300/7500 Real Time PCR System apparatus with a relative method, with β-*act* as a reference gene. The reaction mixture consisted of 10 μl 2 × TaqMan Gene expression Master Mix, 1 μl of TaqMan probe, 8 μl $H_2O_{\text{RNase free}}$, and 1 μl cDNA. Reaction program: preliminary 50°C, one cycle, two minutes; denaturation 95°C, one cycle, 10 minutes, 40 cycles: denaturation 95°C 0:15 minutes, elongation 60°C one minute. A formula was used for the calculations (Livak and Schmittgen, 2001). TaqMan probes made by Life Technologies company: β-act (Rn00667869_m1), foxp3 (Rn01525092_m1), rarα (Rn00580551_m1), rarβ (Rn01537837_m1), rxrβ (Rn01399560_m1) were used. Real Time PCR System SDS Version 1.4 was the computer software used.

BCA

A Bicinchoninic Acid Protein Assay Kit (BCA kit) (Sigma Aldrich, USA) was used for the determination of protein concentration in the prepared samples. For this purpose, a 1 : 50 mixture of copper sulphate solution (II) and bicinchoninic acid solution was prepared. 25 μl of the examined protein was added to 200 μl of the mixture, and incubated for 30 minutes at 37°C. Next, the absorbency of the research sample against control sample was measured at wavelength λ = 550 nm. The protein concentration was determined based on a standard curve of bovine serum albumin in a concentration of 0.1-1 mg/ml.

Western blotting

Freshly isolated from all the groups being examined (*n* = 6) and sorted CD4+CD25– cells were washed twice with ice-cold PBS and homogenised in RIPA buffer (Sigma Aldrich, Poland) supplemented with Protease Inhibitor Cocktail III (Merck Millipore, Germany). Then the samples were incubated for 30 minutes on ice and centrifuged twice at 8000 g for 10 minutes to collect the supernatant, which was frozen at –80°C and stored until later use. The total protein concentration was determined using Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich, Poland) according to the manufacturer's instructions. Cell lysates were mixed with 4xTris-glycine-SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 5% mercaptoethanol, 0.015% bromophenol blue) and boiled for five minutes. Samples (25 µg per well) were then separated on 12% SDS-polyacrylamide gels and transblotted (350 mA, 90 minutes, cold) onto nitrocellulose membranes (0.45 µm pore size). The membranes were blocked overnight with 5% non-fat dry milk in Tris-buffered saline (pH 7.4) at 4°C with gentle shaking. After washing three times with TBS with 0.2% Triton X-100, the blots were incubated for 90 minutes at RT with mouse monoclonal antibody against RXRβ (sc-742, Santa Cruz, Dallas, USA), rabbit polyclonal antibody against RARβ (sc-552, Santa Cruz, Dallas, USA), and rabbit polyclonal antibody against RARα (sc-551, Santa Cruz, Dallas, USA). All antibodies were used at a 1:500 dilution in TBS with 0.2% Triton X-100 and non-fat dry milk. After washing three times with TBS with 0.2% Triton X-100 and non-fat dry milk, the membranes were incubated for one hour at RT with a goat polyclonal secondary antibody against rabbit IgG conjugated with HRP (Santa Cruz, Dallas, USA) and rabbit polyclonal secondary antibody against mouse IgG conjugated with HRP (Santa Cruz, Dallas, USA). Secondary antibodies were used at a dilution of 1 : 100 in TBS with 0.2% Triton X-100 and non-fat dry milk. After incubation the membranes were washed twice with TBS with 0.2% Triton X-100. Detection was performed using a WesternBright Quantum HRP (Advansta, USA) and images were captured using a GBOX imaging system (Amersham Pharmacia, Piscataway, New Jersey, USA). Densitometric analysis was performed with ImageJ software (NIH). Membranes were normalised with a rabbit monoclonal β-Actin (Cell Signaling, USA).

Tissue material

Peripheral blood for serological examination was collected from the hearts of the anaesthetised animals before euthanasia. Spleen (SPL), popliteal (PN), mesenteric (MN), inguinal (IN), and axillary lymph nodes (AXN) were dissected from the euthanised animals. Then the hind paws were dissected and fixed in buffered 4% formalin in PBS.

X-ray

X-ray examination was performed after euthanasia of rats, using a Gierth HF 90/20 X-ray unit and conventional X-ray film (Aqua Mamoray HD). Each animal underwent examination of both distal hind limbs in a dorso-plantar plane. The following radiographic abnormalities were considered: a – soft tissue swelling; b – narrowing/widening of joint space; c – subchondral bone erosions according to modified Larsen classification: 0 – normal; 1 – distinct focal osteolysis in some joint areas; 2 – highly visible osteolysis in some joint areas; 3 – total bone erosion in a joint area, D – joint degeneration, N – normal ankle joint.

Histology

Formalin-fixed sections of joints were embedded in paraffin blocks, sliced and stained with Delafield's haematoxylin–water eosin. Preparations were viewed and evaluated using a BX53 Olympus microscope. Digital photos were taken with an Olympus Colorview IIIu camera.

Statistical analysis

Research was performed in one experiment and there were six rats in every group, constituting an independent experimental system. Results were analysed using Statistica software, version 10.0, by analysis of variance (ANOVA) and Duncan's post-hoc test for statistical significance, with significance levels of $p < 0.05$ and $p < 0.01$. Groups were compared between healthy or CIA group and control or ATRA option of *in vitro* cell culture analysis.

Results

Clinical, serological, and histological evaluation of rats with CIA

Six-week old female Wistar rats were divided into two groups (healthy and with CIA) of 6 animals per group. The stage of CIA was evaluated by an algorithm with a scale from 0 to 40 points. On the 21st day of observation, when the animals were euthanised, the average number of points according to the adopted evaluation scale for rats with CIA was 30 ±6 (left hind limb) and 32 ±6 (right hind limb) (Fig. 1A). No macroscopic changes were found among healthy rats (Fig. 1B).

Both the healthy rats and those with CIA were examined for the level of specificity of the antibody response to the type-II bovine collagen (anti-CII), with which the animals were immunised in order to trigger CIA. In rats with CIA it was 0.8 ±0.23 level OD units on the 21st day. Healthy rats had no anti-CII antibodies (Fig. 2).

Histological analysis of rats with CIA has shown an increase in the number of blood vessels in the subependymal layer and inflammatory infiltrates of mononuclear cells were observed, localised mainly around vessels (++), mainly lymphocytic (also some plasmatic cells and macrophages), cells proliferation of the subependymal layer (synoviocytes), and focal villi proliferation (Fig. 3B). Normal

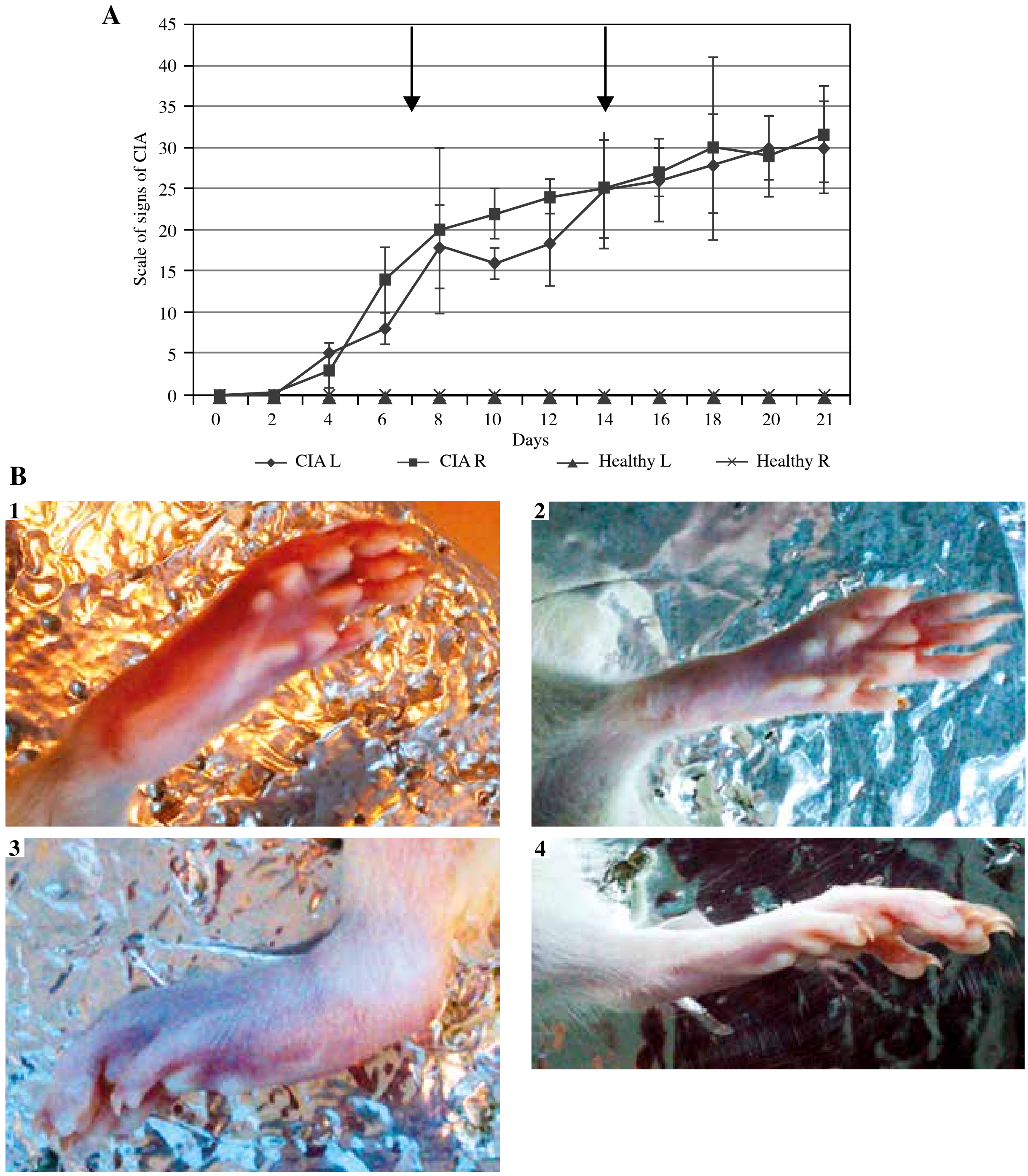

Fig. 1. A) Development of CIA symptoms in healthy rats (Healthy) and in rats with CIA (CIA). The evaluation of arthritis severity was restricted to the hind paws – left (L) and right (R) – of the animals, in accordance with the modified and extended numerical scale described by Suszko and Obminska-Mrukowicz [30]. Arrows are marking days of immunization. Rats from the group H did not show any symptoms of the disease (data not shown). Arrows indicate days on which the rats were immunized. B) Images of hind limbs of healthy rats (2 and 4), and rats with CIA (1 and 3)

rats, however, did not show any histopathological changes (Fig. 3A).

Radiographic examination of animals with CIA showed changes typical for rheumatoid arthritis with osteolysis – swelling of the soft tissue surrounding the joint, thinned and blurred image of the tarsal bones in the joint, and osteolysis of the tarsal bones (Fig. 4B). No lesions were observed in healthy animals (Fig. 4A).

Popliteal, inguinal, axillary lymph nodes, and spleen were isolated from rats after their euthanasia. Among isolated lymphoid organs, popliteal lymph nodes stood out macroscopically – they were larger in animals with CIA than in healthy animals (data not shown).

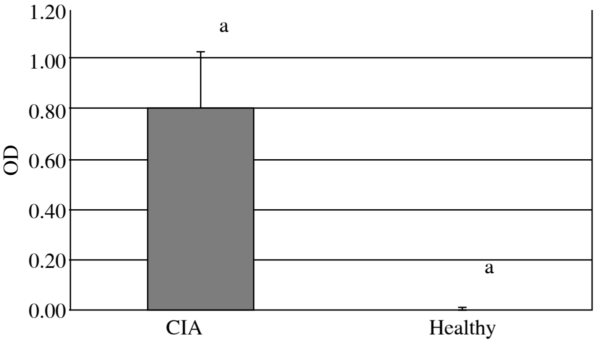

Fig. 2. The level of anti-CII antibodies in healthy rats and rats with CIA. $N = 6$, the same letters indicate statistically significant differences – lower case letters indicate significant differences at $p < 0.01$ (OD – optical density)

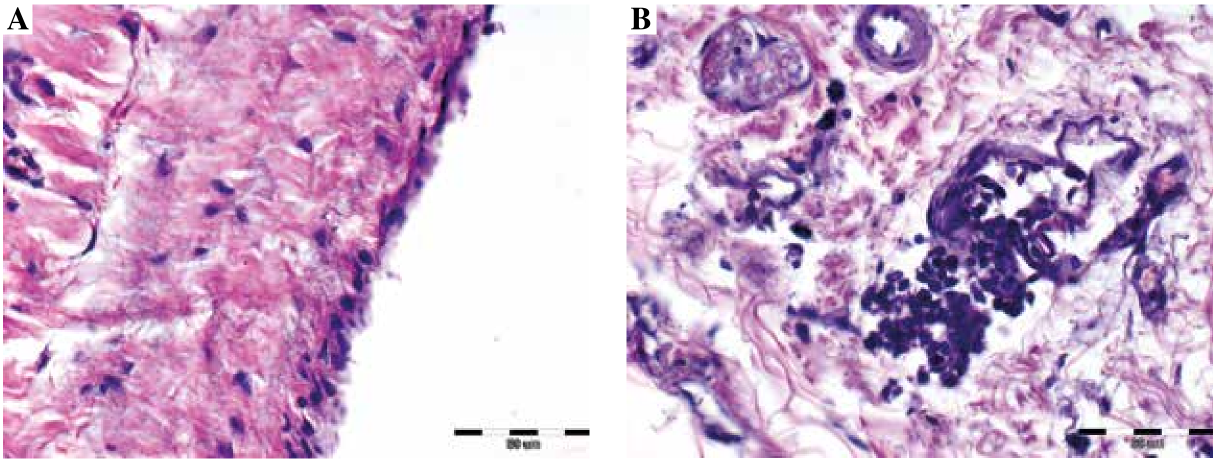

Fig. 3. Representative images from histological analysis of periarticular tissues in healthy rats (A) and rats with CIA (B). In rats with CIA has been investigated an increase in the number of blood vessels in the subependymal layer and inflammatory infiltrates of mononuclear cells were observed (B). However normal rats, did not show any histopathological changes (A)

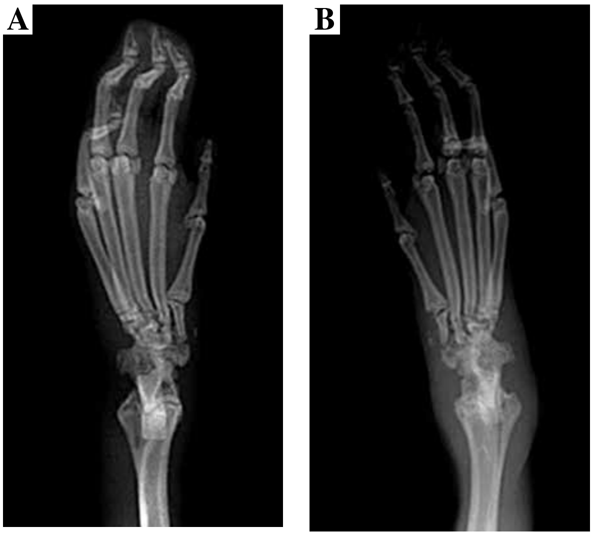

Fig. 4. Representative X-ray images of joints from healthy rats (A) with normal ankle joint and rats suffering from collagen-induced arthritis (CIA) with total bone erosion in a joint area

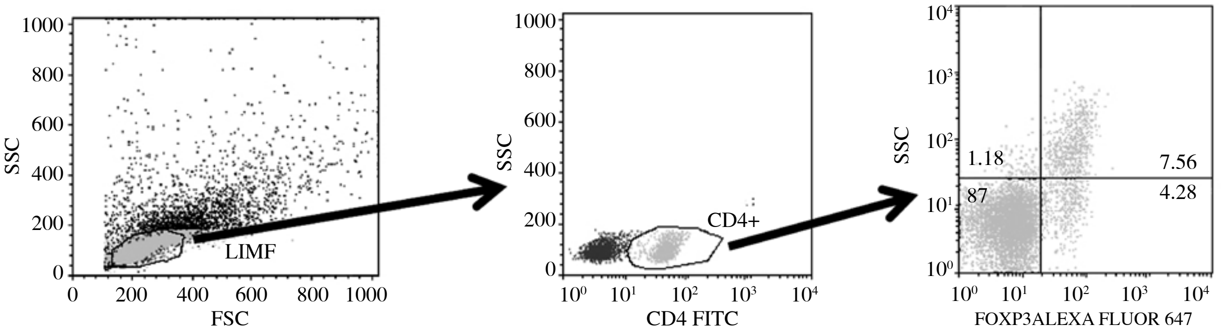

Fig. 5. The method of analysis of the CD4+CD25+FOXP3+ cells frequency

Flow cytometric analysis of the CD4+CD25+FOXP3+ cells after culture in the presence of ATRA

The frequency of regulatory T cells characterised by the CD4+CD25+FOXP3+ phenotype were analysed according to the scheme presented in Fig. 5. Cells were analysed by a lymphocyte gate on an SSC/FSC diagram and subsequently by an SSC/CD4 gate. The percentage of CD25+FOXP3+ cells was determined in the selected population of CD4+ cells.

CD4+CD25– cells isolated from animals and subjected to culture under *in vitro* conditions in the presence of ATRA or without ATRA (Control) in a group of animals with CIA differentiated more efficiently ($p < 0.05$) into regulatory T cells with the CD4+CD25+FOXP3+ phenotype (47.19 ±17.42% in the ATRA group and 45.23 ±13.39% in the control group), than cells isolated from healthy animals (27.22 ±7.61% in the ATRA group and 27.23 ±10.63% in the control group) (Fig. 6).

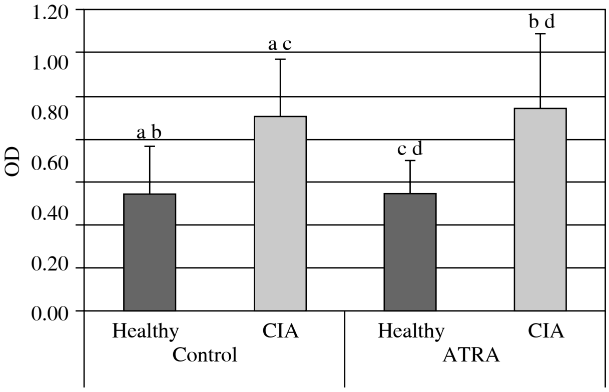

Fig. 6. Frequency of CD4+CD25+FOXP3+ lymphocytes in the population of CD4+ cells after the culture of CD4+CD25– isolated from normal rats (Healthy group) and rats with CIA (CIA group), in the presence of ATRA (ATRA) and without ATRA (K); $n = 6$, the same letters indicate statistically significant differences at $p < 0.05$

Determination of the capacity of CD4+CD25– cells to differentiate towards the CD4+FOXP3+IL-10+ phenotype after sorting and culturing in the presence of ATRA or in control culture

Cells isolated from the lymph nodes and spleens of healthy animals as well as from those with CIA were subjected to sorting in order to obtain a CD4+CD25– population, with an average purity of population of 93.05 ±3.81%.The frequency of regulatory T cells with the CD4+FOXP3+IL-10+ phenotype was analyzed according to the scheme presented in Fig. 7.

The highest capacity of differentiation into the CD4+-FOXP3+IL-10+ phenotype was exhibited by the cells isolated from healthy animals cultured *in vitro* without the addition of ATRA (15.91 ±8.45%). However, a significant difference ($p < 0.05$) was observed only in comparison to cells isolated from rats with CIA and cultured with ATRA (4.70 ±2.49%) (Fig. 8).

Determination of the gene expression of foxp3, rarα, rarβ, and rxrβ genes in CD4+CD25– cells after ATRA stimulation

The gene expression clearly changed after adding CD4+CD25– cells to the culture with 10 nM ATRA. The highest expression of *foxp3* gene was observed in cells isolated from healthy rats and cultured in the presence of 10 nM ATRA. The expression of *foxp3* in those cells was significantly higher than that in the CD4+CD25 cells examined directly after sorting, as well as after culture without ATRA ($p < 0.05$), but not in cells isolated from CIA animals cultured in the presence of ATRA (Fig. 9A).

The relative *rarα* expression clearly and significantly (at $p < 0.01$) increased in culture of the CD4+CD25– cells in the presence of ATRA both in healthy animals and those with CIA in comparison to other examined groups of cells (i.e. directly after sorting and cultured

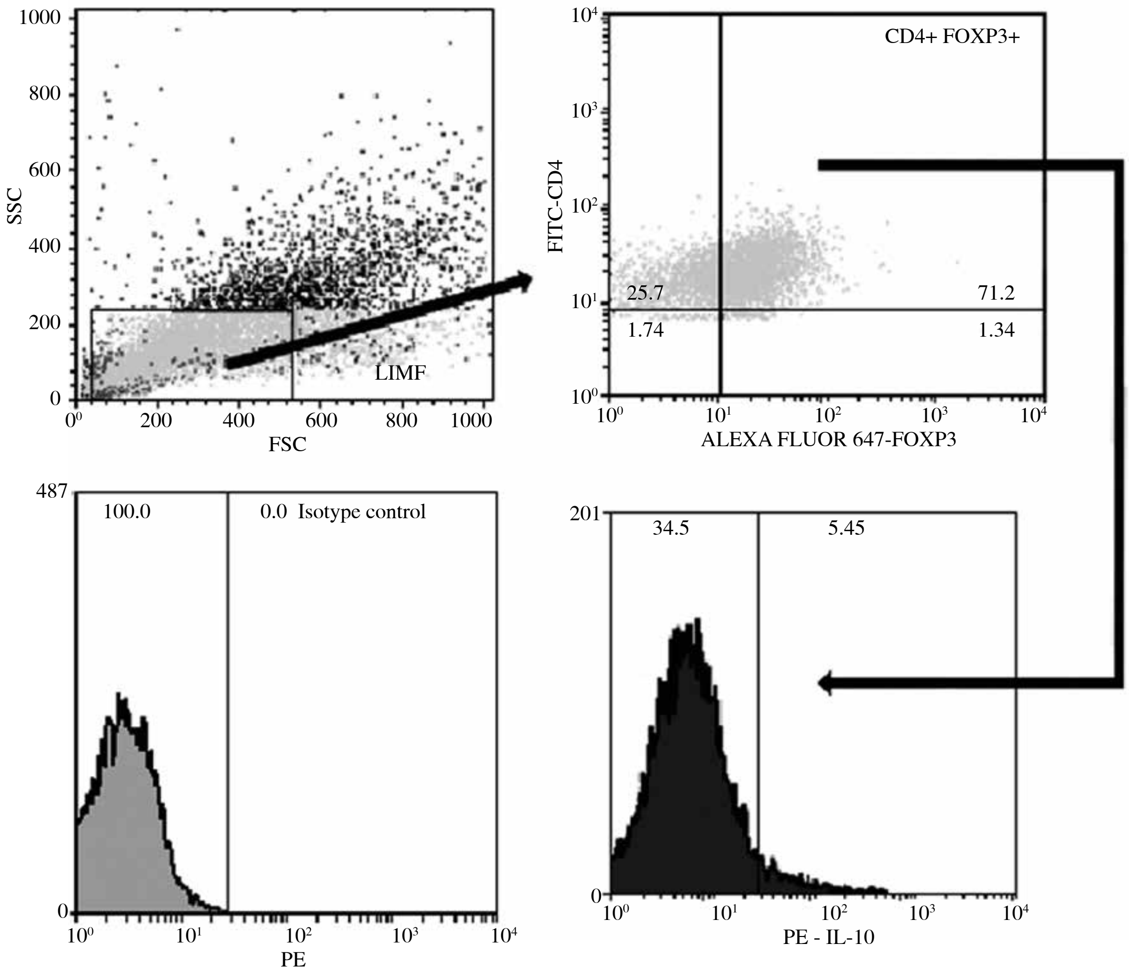

Fig. 7. Method of analysis of the CD4+ FOXP3+IL-10+ cells frequency. Cells were analyzed using a lymphocyte gate on a SSC/FSC graph, and using a SSC/CD4 gate in next step. Then CD4+ cells were analyzed using a CD4+FOXP3+ gate and CD4+FOXP3+ cells were gated and analyzed in the last stage in a histogram, where IL-10+ cells were gated

w/o ATRA). No statistically significant differences were observed between CD4+CD25– cells cultured *in vitro* in the presence of ATRA isolated from healthy animals and those isolated from animals with CIA (Fig. 9B).

The highest expression of *rar*β gene was observed in cells isolated from rats with CIA cultured in the presence of ATRA in comparison with the expression in cells cultured without ATRA addition, both in healthy and diseased rats and in comparison with cells examined directly after sorting and isolated from healthy animals ($p < 0.05$) (Fig. 9C).

The expression of *rxr*β gene was similar to the expression profile of the *rα r*β gene. The highest expression was observed in cells isolated from rats with CIA cultured under *in vitro* conditions in the presence of ATRA, which indicated statistically significant differences at $p < 0.05$ compared to control cultures without ATRA, both for healthy and diseased rats and against cells isolated from healthy animals after sorting (Fig. 9D).

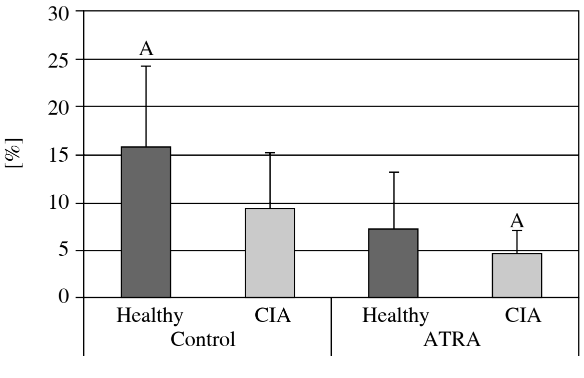

Fig. 8. Frequency of CD4+FOXP3+IL-10+ cells differentiated from CD4+CD25– cells (isolated from the spleen, popliteal, inguinal, mesenteric, and subbrachial lymph nodes, sampled from healthy rats [Healthy group] and rats with CiA [CIA group]) *in vitro* and in the presence of 10 nM ATRA (ATRA) and without ATRA (Control); $n = 6$, the same letters indicate statistically significant differences at $p < 0.05$

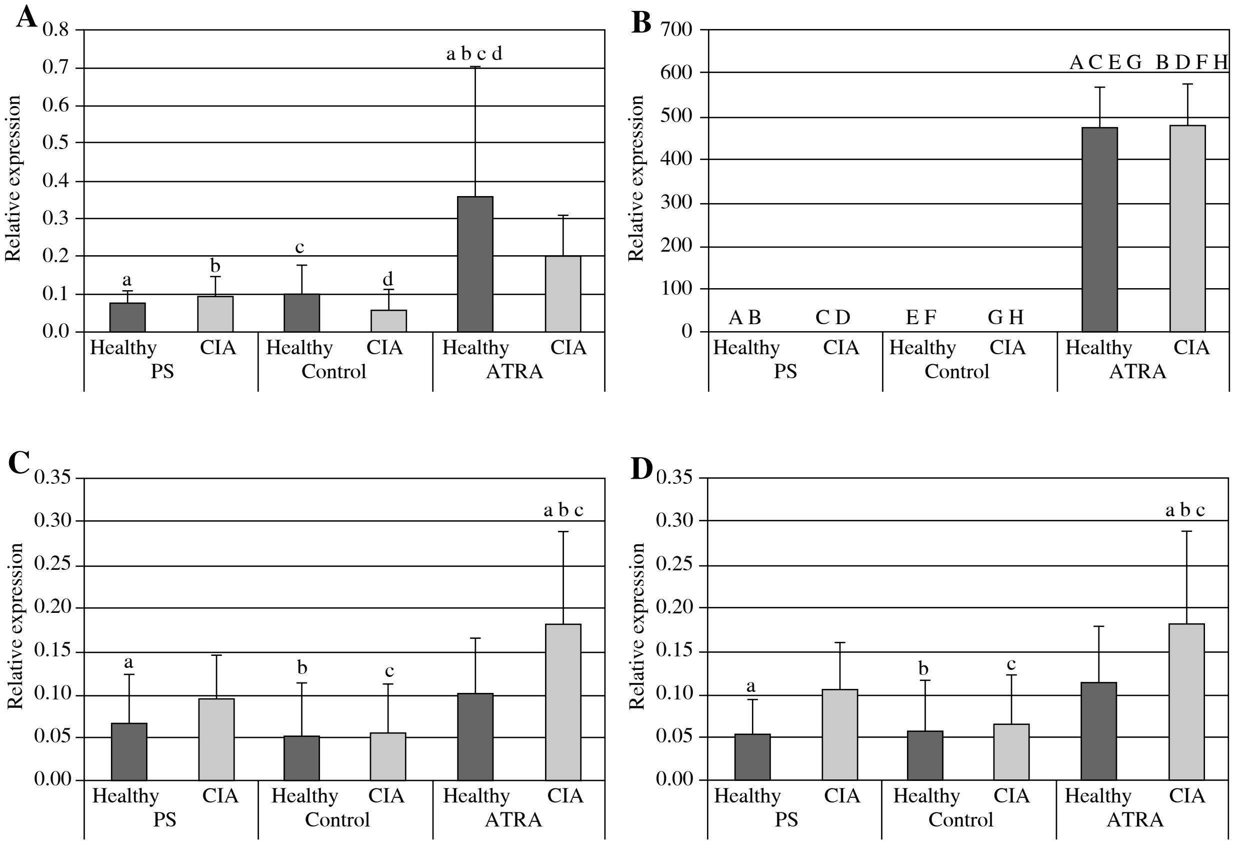

Fig. 9. Analysis of changes in the expression of Foxp3 (A), rarα (B), rarβ (C), rxrβ (D) genes in comparison to β-actin reference gene, in healthy rats and rats with CIA after sorting the CD4+CD25– (PS) cells from the lymph nodes and spleen after cell culture in RPMI medium with IL-2 and TGF-β without the addition of ATRA (K) and after cell culture in RPMI medium with IL-2 and TGF-β with the addition of 10 nM ATRA (ATRA). Gene expression was determined by real-time PCR (TaqMan). Capital letters indicate significance at $p < 0.01$ and lowercase letters indicate significance at $p < 0.05$. The same letters indicate significance between pairs of results

The expression of Rarα, Rarβ, and Rxrβ protein

Due to the fact that a high number of cells are needed for protein expression examination, and taking into account the efficiency of cell sorting, differentiation rates, and animal care regulations, the investigations on Rarα, Rarβ, and Rxrβ protein expression were restricted only to CD4+CD25– cells sorted from lymph nodes and spleen in healthy animals and in animals with CIA. Protein expressions were examined against β-actin as the reference protein (Fig. 10).

When comparing the expression of the retinoic acid receptors in the CD4+CD25– cells isolated from healthy rats and rats CIA, a statistically significant difference was observed for the expression of Rxrβ ($p = 0.003$). Rxrβ expression was not observed in healthy animals, while in animals with CIA the ratio of β-actin to Rxrβ was 0.96 ±0.18. As for Rarα, higher expression of this protein was observed in animals with CIA than in healthy animals (1.43 ±0.73 β-actin to Rarα ratio in animals with CIA and 0.88 ±0.18 β-actin to Rarα ratio in healthy animals).

On the other hand, a higher Rarβ expression was observed in healthy animals (2.53 ±1.76 β-actin ratio) when compared to animals with CIA (1.08 ±0.10 of β-actin ratio). No statistically significant differences in Rarα and Rarβ expression were observed between healthy animals and those with CIA; nevertheless, comparing obtained results between the groups reveal varying tendency towards the expression of examined retinoic acid receptors within examined groups (Fig. 11).

Discussion

The presence of ATRA in the milieu of CD4+CD25– cells isolated from animals with CIA and from healthy animals differentially affects Treg cell frequency and foxp3 gene expression.

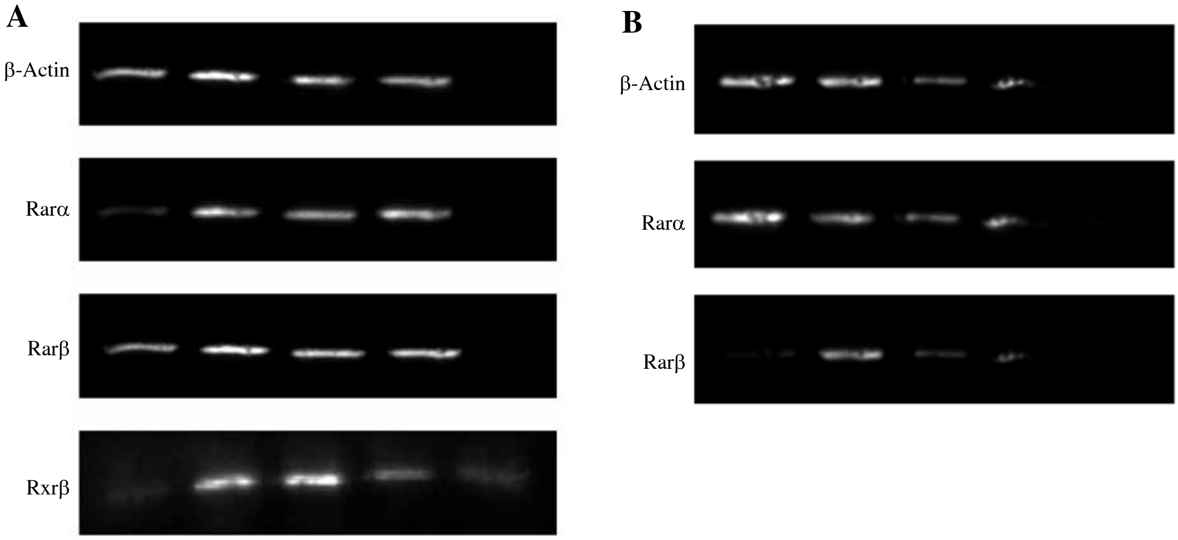

Fig. 10. The results of the Rarα, Rarβ and Rxrβ proteins expression determined by Western blotting against β-actin as reference protein in healthy animals and animals with CIA. Proteins expression was determined in cells isolated from the spleens and lymph nodes (popliteal, inguinal, mesenteric, and axillary) from normal rats and rats with the CIA. A) rats with CIA; B) healthy rats

In the course of model RA, an increase in the population of Treg cells may be obtained by intraperitoneal infusion of ATRA, which results in the alleviation of disease symptoms [29]. Moreover, CD4+CD25– cells isolated from healthy mice and cultured *in vitro* in the presence of ATRA differentiate into CD4+CD25+FOXP3+ cells. These observations indicate that ATRA induces the differentiation of cells into Treg lymphocytes both in healthy animals and in the course of developing model RA. It also supports Treg lymphocytes phenotypic and functional stability in the presence of proinflammatory cytokines [30]. The results of our research indicated that *foxp3* gene expression was strongly induced in CD4+CD25– cells under the influence of 10 nM ATRA stimulation in healthy rats only.

Retinoic acid has a profound impact on cell proliferation, metabolism and differentiation. ATRA, as mentioned above, is also a potent stimulator of Treg differentiation [17]. On the other hand, its effectiveness as the stimulator of Treg differentiation may be related to the initial metabolic status of progenitor cells used for Treg cells expansion under *in vitro* conditions. It has been demonstrated that severe metabolic modifications and cell senescence are involved in the pathogenesis of autoimmune diseases involving rheumatoid arthritis [31].

However, in our experiment we did not observe any supportive role of ATRA in differentiation of CD4+CD25– into Treg cells under *in vitro* conditions both in healthy animals and in rats with CIA. On the other hand, these cells were significantly prone to develop into Treg cells in rats with CIA. The mechanism of this phenomenon is not clear. However, the differential metabolic states of healthy and diseased animals should be taken into consideration. It was showed by Yang *et al.* that glycolytic activity of naive T lymphocytes in patients with rheumatoid arthritis is lower than in a healthy control group [31].

Moreover, in chronic inflammatory diseases like in rheumatoid arthritis, insulin resistance is observed and cellular glucose uptake is inhibited [32]. On the other hand, contrary to T effector cells where glycolysis promotes their development and differentiation T regulatory cell induction is independent of glycolysis and relies on lipid oxidation [33]. Therefore, paradoxically, the metabolic state of diseased animals may promote differentiation of naive CD4+ lymphocytes into Treg.

CD4+CD25– cells cultured *in vitro* in the presence of ATRA indicate different potential to propagate towards CD4+FOXP3+IL-10+ phenotype cells

Interleukin 10 is a suppressive cytokine. Treg cells produce IL-10, assuring the immunosuppressive mechanism of attenuation of autoimmune phenomena, including RA [34].

In our research we observed that in *in vitro* cultures of CD4+CD25– cells isolated from healthy animals and conducted in the presence of ATRA, the frequency of CD4+-FOXP3+IL-10+ cells was statistically significantly higher than in cultures of cells isolated from animals with CIA.

In view of the available literature, the correlation between the release of IL-10 by iTregs differentiated from

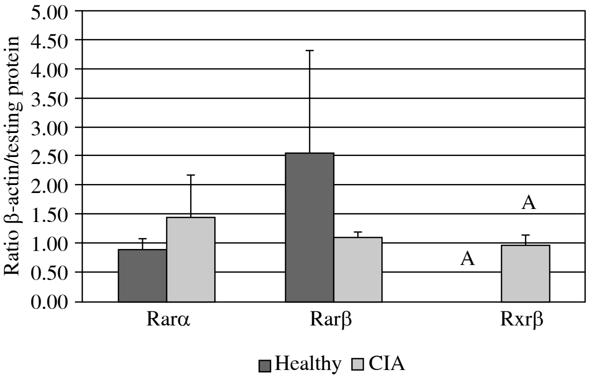

Fig. 11. Comparison of Rarα, Rarβ and Rxrβ protein expression in healthy animals and animals with the CIA. Proteins were determined in cells isolated from the spleens and lymph nodes (popliteal, inguinal, mesenteric, and subbrachial) from normal rats and rats with the CIA. *N* = 4; These same letters indicate significance between pairs of results. Significance at $p = 0.003$

CD4+CD25– cells subjected to culture under *in vitro* conditions in the presence of ATRA and those cultured without the addition of ATRA, is not clear. CD4+ lymphocytes cultured in the presence of LE540 (RAR antagonist) significantly increases the release of IL-10. The results of our research, where the highest release of IL-10 was from cells isolated from healthy animals cultured *in vitro* with no ATRA added, confirm the inhibitory effect of ATRA on the synthesis of IL-10 [35]. Therefore, an assumption can be made that the observed immunosuppressive effects of iTreg cells in the course of CIA may be the result of triggering suppressive mechanisms independent from IL-10.

Rarα expression is increased in induced Treg cells in the presence of ATRA

Little is known about the differences in the expression of retinoic acid receptors on the gene and protein levels between healthy and diseased individuals. So far, the role of rarα in promyelocytic leukaemia (APL) has not been described in detail. However, it was demonstrated that in the pathomechanism of APL, translocation of the arms of chromosomes 15 and 17 takes place, which results in the fusion of PML/RARα transcripts. PML/RARα protein may behave like a transcription factor dependent on ATRA, which leads to the development of an effective pharmacotherapy, where an oral supply of ATRA decreases pathophysiology of APL [36].

Moreover, in lymphocytes the regulation of retinoic acid receptor expression under the influence of their natural ligand- ATRA is complicated because of differential constitutive expression of their isoforms and accessibility of functional RARE in promoter regions of their genes [37].

Our research have shown a significant increase in rarα gene expression in CD4+CD25– cells cultured *in vitro* in the presence of 10 nM ATRA, in both healthy and diseased animals. However, no differences in rarα expression between healthy and diseased animals were observed. The TaqMan sequence we used in our experiment did not allow us to indicate which isoform of RARalpaha (1 or 2) is expressed. However, as indicated by Takayema *et al.* in rat embryos [38] and Ballow *et al.* [37] in human lymphocytes, only RAR alpha 2 isoform is upregulated after ATRA treatment, which indicates the presence of functional RARE in the promoter sequence of this gene. Nevertheless, it is worth noting that ATRA increases RAR alpha in rat's Treg cells, which is beneficial for stable expression of FOXP3 and may inhibit undesirable conversion of Treg into Th17 cells [39].

After culturing in the presence of ATRA, rarβ and rxrβ expression is higher in diseased animals

On the contrary, we demonstrated varying expression of rarβ and rxrβ receptor genes in CD4+CD25– cells isolated from healthy animals and those with CIA cultured *in vitro* in the presence of ATRA. The expression of rarβ and rxrβ genes was induced under the influence of ATRA in cells isolated from animals with CIA, and was statistically significantly higher ($p < 0.01$) than in CD4+CD25– cells obtained by sorting from lymph nodes and spleen, as well as in comparison to control cultures with no ATRA added.

It has been shown that the expression of *rarα, rarβ*, and *rxrβ* genes in the synovial membrane isolated from humans suffering from osteoarthritis (OA) is variable. *Rarα* and *rxrβ* expression in humans with OA is low, whereas the expression of *rarβ* is high [40]. The results of research conducted by Collins-Racie *et al.* on the expression of *rarα, rarβ*, and *rxrβ* are different from those of our research; we did not observe any differences in *rarβ* expression between CD4+CD25– cells isolated from rats with CIA and those from healthy animals. Our research was conducted with different biological material; CD4+CD25– lymphocytes were isolated from lymph nodes and spleen, while Collins-Racie *et al.* examined gene expression of retinoic acid receptors in the synovial membrane. The presence of the individual retinoic acid receptors correlates with the type of tissue – *rarβ* is present in brain and limb tissues [41], whereas rarα and rarγ are typical for haematopoietic cells [42]. In CD4+CD25–CD44^{lo}FOXP3– cells, *rarα, rarβ*, and *rarγ* show minimal expression while the expression of *rarα* is very high [43].

It also seemed that CD4+CD25– cells isolated from rats showed constitutive expression of rarβ, which is different from the retinoic receptor expression pattern observed in human T lymphocytes, thymocytes and T-cell hybridoma, which constitutively expressed rar-α and rar-γ but not rar-β. On the other hand, for the first time the presence of an auto-regulated response element and dependent on ATRA was demonstrated in rar-β promoter [44].

Differences in the increase of rxrβ gene expression in CD4+CD25– cells after their isolation, sorting, and *in vitro* culturing in the presence of ATRA may be justified by induction of the aging process of T lymphocytes in rheumatoid arthritis [45]. Brtko *et al.* showed that oral supply of 0.5 mg/kg of body mass of 13-cis retinoic acid in elderly patients suffering from RA induces an increase of rxrβ expression in mononuclear cells of peripheral blood [46]. In conclusion, the increase in rxrβ gene expression in CD4+CD25– cells may indirectly indicate the acceleration of the aging process of T lymphocytes during the development of model RA.

It is interesting that in CD4+CD25– cells obtained by sorting from lymph nodes and spleen, no significant differences in the expression of Rarβ on protein level was observed between healthy animals and those with CIA, whereas the Rxrβ protein was present only in cells isolated from animals with CIA.

It may indicate changes in the cells' metabolism in the course of disease development, which affects the expression management of retinoic acid receptors. Metabolic changes occurring during the progress of the disease concern – among others – inborn errors of metabolism (IEMs), which mainly result from genetic dysfunctions of metabolic signalling pathways [47].

RXRb was not detected *in vivo* in healthy animals

In isolated CD4+CD25– cells either from diseased or healthy animals there were no significant differences in the level of transcriptional expression of all examined retinoic acid nuclear receptors. At the protein level it is worth noticing that in healthy animals RXRb was not detected.

So far, little is known about the epigenetic agents that influence the development of RA [48]. Nevertheless, it has been demonstrated that epigenetic agents influence the development and pathophysiology of autoimmune diseases. Especially important are: DNA methylation, histone deacetylation, and the expression of non-coding micro-RNA [49]. Potential hormonal control of gene expression [50] affects the expression of transcript factors, which play an important role in the pathogenesis of autoimmune diseases [51].

In our results it was shown that the expression of retinoic acid receptors in CD4+CD25– cells isolated from healthy rats and rats with CIA on the protein level examined, a statistically significant difference in expression of Rxrβ ($p = 0.003$) was demonstrated. In literature, there is no information about the changes in Rxr expression in CIA or RA, but detailed research on osteoarthritis in humans demonstrated numerous essential differences in the expression of transcription factor genes in comparison with healthy human individuals. Reduced expression of rxrβ gene in articular cartilage in humans with OA was observed, whereas our research on model RA indicate an increase in rxrβ expression, both on the gene and protein level in CD4+CD25– cells isolated from rats with CIA in comparison to those isolated from healthy rats. In our studies the CD4+DC25– cells were cultured under *in vitro* conditions in the presence of 10 nM ATRA, which is a ligand for the RAR/RXR heterodimers. The differentiation of rxrβ expression between RA and OA may occur by different epigenetic mechanisms of these diseases.

All-trans retinoic acid, as a ligand for RAR and RXR transcription factors (retinoic acid receptors), is important not only in gut immunity [52] but also in attenuation of inflammatory conditions in the course of RA development [29]. Rxr proteins are an atypical group of retinoic acid X receptors because they may form heterodimers with other receptors, such as: VDR (vitamin D receptor), LXR (liver X receptor), or PPARs (peroxisome proliferation-activated receptors) [53].

RXR proteins play a special role in shaping the expression of Foxp3 and the differentiation of CD4+ cells towards Th17 or Treg cells. It has been demonstrated that culturing CD4+ cells in the presence of AM80 and PA024 induces the expression of Foxp3. However, the presence of an antagonist for RAR LE540 eliminates this effect, which indicates that the interaction between RAR and RXR is necessary for the induction of Foxp3 in CD4+ cells [54].

The negative role of the influence of RXR on LRX was indirectly shown on model CIA rheumatoid arthritis in DBA/1 mice, in which an increased pro-inflammatory effect was observed after oral administration of LXR agonists. In human leukocytes *in vitro,* LXR agonists have induced increased production of pro-inflammatory IL-1β, IL-6, and IL-17 cytokines [55]. It was also demonstrated that oral supply of LXRα and LXRβ (GW3965) agonists in C57BL/6 mice, in which the CIA was triggered, causes the exacerbation of clinical symptoms characteristic of CIA [56]. Thus, the modulation of LXR activity does not seem to be an appropriate target for therapeutic interventions in cases of osteoarthritis.

Summary

In our work we have demonstrated differential response of naive T CD4+ lymphocytes isolated from rats with CIA and from healthy animals differentiated into Tregs under *in vitro* conditions in the presence of ATRA. We showed that although ATRA does not increase the frequency of Treg in culture, it significantly increases expression of rarβ and rxrβ only in lymphocytes taken from diseased animals and foxp3 expression only in healthy animals. Moreover, after ATRA stimulation the frequency of Treg-produced IL-10 tended to be lower in diseased animals than in a healthy group. The results imply that the potential of naïve cell CD4 lymphocytes to differentiate into Tregs and their putative suppressive function is dependent on the donor's health status.

Executive summary – summary points

- ATRA did not increase the frequency of Treg in culture; it significantly increased expression of rarβ and rxrβ only in lymphocytes taken from diseased animals and foxp3 expression only in healthy animals.
- After ATRA stimulation the frequency of Treg-produced IL-10 tended to be lower in diseased animals than in the healthy group. The results imply that the potential of naive cell CD4 lymphocytes to differentiate into Tregs and their putative suppressive function is dependent on the donor's health status.
- Due to the varying immunological potential of CD4+CD25– cells isolated from healthy rats and from those with model RA, one should consider supplementing the screening of patients qualified for immunomodulatory therapies based on the immunosuppressive potential of ATRA-induced iTreg cells.
- Detailed diagnostics of the expression of retinoic acid receptors on gene and protein levels in each patient could increase the effectiveness of immunotherapeutic therapy.

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The authors declare no conflict of interest.

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