



Immunopharmacology and inflammation

Beneficial effects of rosiglitazone, a peroxisome proliferator-activated receptor- γ agonist, in a mouse allergic asthma model is not associated with the recruitment or generation of Foxp3-expressing CD4⁺ regulatory T cellsTomasz Maślanka^{a,*}, Iwona Otrocka-Domagala^b, Monika Zuśka-Prot^a, Michał Gesek^b^a Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego Street 13, 10-719 Olsztyn, Poland^b Department of Pathological Anatomy, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego Street 13, 10-719 Olsztyn, Poland

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ABSTRACT

The activation of peroxisome proliferator-activated receptor γ (PPAR- γ) has been shown to attenuate allergic airway inflammation (AAI). To gain better understanding of mechanisms underlying this effect, the impact of rosiglitazone (RSG), a PPAR- γ agonist, on CD4⁺ effector (Teff) and Foxp3-expressing regulatory (Treg) T cells in a mouse model of allergic asthma was studied. Furthermore, we investigated whether the activation of PPAR- γ may directly affect IL-4, IL-10 and IL-17 production by CD4⁺ T cells. RSG attenuated but did not prevent ovalbumin (OVA)-induced AAI, and this effect was PPAR- γ -dependent. RSG reduced but did not abolish the OVA-induced increase in the count of CD4⁺ Teff cells in the mediastinal lymph nodes (MLNs) and lungs, and this effect was PPAR- γ -dependent. RSG did not affect the absolute number of Treg cells in the MLNs and lungs of OVA-immunized mice. In vitro exposure of lung lymphocytes to RSG did not influence the percentage of IL-4-, IL-10- and IL-17-producing CD4⁺ T cells. Our results indicate that the impairment of clonal expansion of CD4⁺ Teff cells in the MLNs is involved in the anti-asthmatic properties of PPAR- γ agonists. Activation of PPAR- γ did not affect the recruitment of Treg cells to the MLNs and lungs nor did it induce their local generation. This indicates that Treg cells are not involved in producing the anti-asthmatic effect of PPAR- γ agonists. The results suggest that beneficial effects of PPAR- γ agonists in asthma treatment are not mediated through a direct inhibitory effect on IL-4, IL-10 and IL-17 production by CD4⁺ Teff cells.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the superfamily of nuclear receptors. So far, three different subtypes have been described, namely PPAR- α , PPAR- β/δ and PPAR- γ . Although PPAR- γ are characterized as a regulator of adipocyte differentiation and lipid metabolism (Tontonoz et al., 1994), accumulating evidence has shown that PPAR- γ is involved in asthma pathogenesis and that ligand-induced activation of this receptor can suppress asthma development (Banno et al., 2018). Therefore, PPAR- γ agonists are considered as potential anti-asthmatic agents. Literature data indicate that PPAR- γ agonists exert anti-asthmatic effects primarily by suppressing pro-inflammatory cells and mediators involved in the pathophysiology of asthma (Banno et al., 2018). However, there are single papers suggesting that the beneficial effects of PPAR- γ agonists in allergic diseases may be partially mediated by the induction of Foxp3⁺ CD25⁺ CD4⁺ regulatory T (Treg) cells (Lee et al., 2015; Wang

et al., 2011; Yu et al., 2017). In the light of the above, we hypothesized that the anti-asthmatic effect of PPAR- γ agonists could be associated with the increased recruitment of Treg cells into the mediastinal lymph nodes (MLNs) and lungs (i.e. at the inductive and effector sites of an immune response in allergic asthma, respectively) and/or local generation of Treg cells. Therefore, the principal aim of this research has been to shed light on the involvement of Treg cells in the anti-asthmatic action of PPAR- γ agonists.

The results of previous studies demonstrated that administration of PPAR- γ agonists to OVA-immunized mice affected the production of important cytokines involved in the pathogenesis of asthma, i.e. IL-4, IL-10 and IL-17 (Hammad et al., 2004; Kim et al., 2005; Lee et al., 2015; Zhao et al., 2014). In these studies, cytokine levels were assessed in the respiratory tract tissues, while there is lack of research on effects of PPAR- γ agonists on the frequency of IL-4, IL-10 and IL-17-producing CD4⁺ T cells. CD4⁺ effector T (Teff) cells play a central role in asthma pathogenesis. Following antigen presentation by dendritic cells (DCs) to

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naïve T cells in the MLNs, specific CD4⁺ T cells are activated and differentiated into IL-4-producing T helper type 2 (Th2) cells, which migrate to the lungs and orchestrate pulmonary immune responses. IL-17-producing CD4⁺ Th17 cells are another crucial subset of Teff cells involved in the pathogenesis of asthma. IL-10 is produced by a wide variety of cell types, including Th2 cells and T regulatory type 1 (Tr1) cells (Lambrecht and Hammad, 2003).

The present study and earlier work (Hammad et al., 2004) demonstrated that rosiglitazone (RSG), a PPAR- γ agonist, reduced the clonal expansion of CD4⁺ Teff cells in the MLNs. However, it is unknown whether the aforementioned effect of PPAR- γ agonists on IL-4, IL-10 and IL-17 production was only a consequence of reduced expansion of CD4⁺ Teff, or if this effect additionally resulted from the direct influence of these agents on cytokine production by CD4⁺ T cells. Hence, another objective of our investigations was to determine whether the activation of PPAR- γ may directly affect IL-4, IL-10 and IL-17 production by CD4⁺ T cells.

2. Materials and methods

2.1. Animals

All of the procedures were approved by the Local Ethics Commission. The experiments were carried out on 6-week-old Balb/c mice. Mice were bred and maintained under standard laboratory conditions [12/12 h light/dark cycle, controlled temperature ($21 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$), and ad libitum access to autoclaved food and water] in the Animal Facility of the Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn. Mice were euthanized by asphyxiation with CO₂.

2.2. Antigen immunization, airway challenge and treatment protocol

A schematic diagram showing the design of the experiment is presented in Fig. 1. Mice were divided into four groups, namely the PBS (phosphate buffered saline) group (PBS-sensitized and -challenged mice, i.e. healthy mice/negative control), ovalbumin (OVA) group [OVA-sensitized and -challenged mice, i.e. mice with OVA-induced allergic airway inflammation (AAI) which is identified as a model of allergic asthma], OVA/RSG group [OVA-sensitized and -challenged mice treated with rosiglitazone (RSG)] and OVA/GW9662/RSG group [OVA-sensitized and -challenged mice treated with GW9662 and RSG]. Mice

were sensitized [under isoflurane anesthesia (Aerrane, Baxter, Deerfield, IL, USA)] on days 0 and 14 via intraperitoneal (i.p.) injection of 20 μg OVA (Grade V) emulsified in 2 mg aluminum hydroxide (AH) (both from Sigma-Aldrich, Schnelldorf, Germany) in total volume of 200 μl PBS. Mice were challenged intranasally (i.n.) with 100 μg of OVA in 50 μl of PBS once daily for 4 or 8 days, as indicated in Fig. 1. Mice in the negative control group (PBS group) received only AH in PBS (sensitization) or PBS alone (challenge).

Rosiglitazone (BRL-49653; RSG), a thiazolidinedione (TZD), was used to activate PPAR- γ . RSG is a selective agonist of PPAR- γ and has no PPAR- α -binding action. PPAR- γ -independent effects have been found for the TZD family of PPAR- γ agonists (Chawla et al., 2001; Koeffler, 2003). Therefore, to exclude a PPAR- γ -independent effect, an additional group of mice was pretreated with GW9662, a selective PPAR- γ antagonist that blocks RSG binding (Knouff and Auwerx, 2004; Pershadsingh, 2004). Both agents were purchased from Sigma-Aldrich. RSG and GW9662 were solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich) as a stock solution (1 mg/150 μl) and diluted into a working volume in 0.5% carboxymethylcellulose in PBS (CMC; Sigma-Aldrich). Mice from the OVA/GW9662/RSG group were treated with i.p. injection of GW9662 (1 mg/kg/day), while mice in the remaining groups received vehicle (VEHICLE 1), i.e. an equivalent volume of DMSO/CMC alone. One hour later, mice from the OVA/RSG and OVA/GW9662/RSG groups were administered an i.p. injection of RSG (5 mg/kg/day), whereas the animals from the PBS and OVA groups were treated with vehicle (VEHICLE 2), i.e. the equivalent volume of DMSO/CMC alone. Treatment was started 48 h prior to i.n. challenge and continued for 6 and 10 days; RSG or VEH 2 were given 3 h before challenge (Fig. 1). RSG and GW9662 doses were chosen according to our preliminary studies and relevant published reports (Cuzzocrea et al., 2004; De Backer et al., 2009; El-Naa et al., 2015; Lee et al., 2006).

2.3. Lung histology

Lungs taken during necropsy were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Lung histological Sections (3–4 μm) were stained with haematoxylin and eosin (H&E) (Merck, Germany) for detection of inflammatory infiltrates and with periodic acid-Schiff (PAS) (Sigma-Aldrich) for goblet cells and mucus production visualization. Sections were analyzed using a light microscope (Olympus BX51, Japan) with a digital camera (ColorView, Olympus, Japan) and Cell B software (Olympus, Japan). Airway inflammation

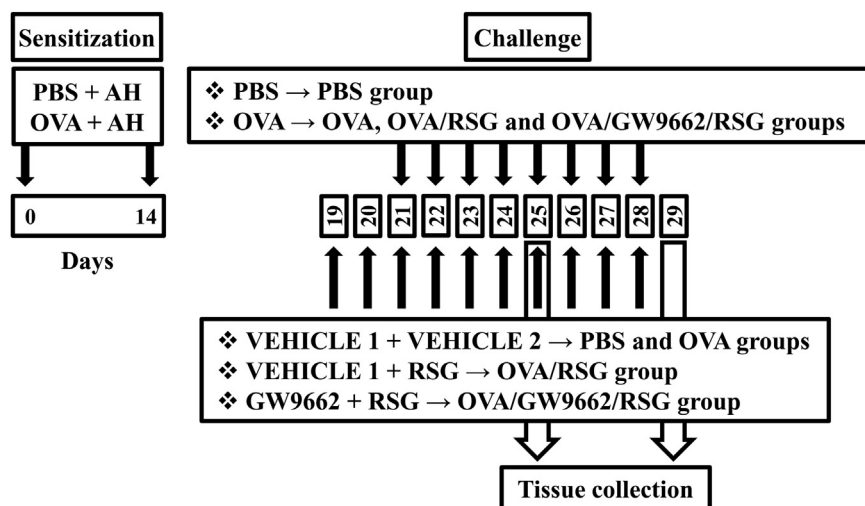


Fig. 1. Experimental study design. Mice were divided into four groups, namely the PBS group (PBS-sensitized and -challenged mice, i.e. healthy mice/negative control), OVA group [ovalbumin (OVA)-sensitized and -challenged mice, i.e. mice with OVA-induced model of allergic asthma/positive control], OVA/RSG group [OVA-sensitized and -challenged mice treated with rosiglitazone (RSG)] and OVA/GW9662/RSG group [OVA-sensitized and -challenged mice treated with GW9662 and RSG]. Mice were sensitized to OVA by two intraperitoneal (i.p.) injections on days 0 and 14 with OVA absorbed on aluminum hydroxide (AH). Subsequently, mice were challenged intranasally with OVA (OVA, OVA/RSG and OVA/GW9662/RSG groups) once daily on days 21–28. Mice in the negative control group (PBS group) received only aluminum hydroxide in PBS (sensitization) or PBS alone (challenge). GW9662, RSG, VEHICLE 1 (vehicle for GW9662) or VEHICLE 2 (vehicle for RSG) administration was started 48 h prior to i.n. challenge (i.e. on day 19 after the initial sensitization) and continued for 6 or 10 consecutive days. On 4 and 8 days postchallenge, mice were sacrificed (24 h after the last administration of the RSG or VEHICLE 2) and the mediastinal lymph nodes and lungs were harvested.

Table 1

The scoring system used for evaluation of airway inflammation (Wittke et al., 2004).

Category	Criterion of evaluation	Points assigned per criterion
Inflammation	No inflammation	0
	Minimal: inflammatory cells present	1
	Mild: a few (< 3) loci of inflammation	2
	Moderate: multiple (≥ 3) loci of inflammation	3
	Severe: inflammatory cells throughout the lung	4
Epithelial thickening	Normal	0
	Epithelial thickening visible	1
	Loci of thickened airway	2
	Airways nearly completely obstructed by epithelial thickening	3
	Normal structure not present, little to no obstructed airway	4
Mucous scores	No mucous	0
	A few cells secreting mucous	1
	Many cells secreting mucous	2
	Extensive mucous production	3

was quantified in the peribronchial region of 5 different medium-sized bronchi (diameter range of 150–250 μm ; 40x magnification) per slide on the basis of a scoring system (Table 1) previously described and used in similar studies (Wittke et al., 2004). The results were averaged and totaled for each mouse, and thereafter, the mean [$\pm$ standard deviation (S.D.)] score per group was calculated. Epithelial thickness (as the area between the luminal cell membrane and the basement membrane) was measured at 4 sites for 5 different medium-sized bronchi per slide. All measurements were averaged, giving the mean ($\pm$ S.D.) epithelial thickness per group.

2.4. Isolation of inductive and effector tissue lymphocytes

2.4.1. MLNs

MLNs were removed and subjected to dounce homogenization. The resulting cell suspensions were filtered through nitex fabric (Fairview Fabrics, Hercules, USA), washed with FACS (fluorescence-activated cell-sorting) buffer [FB; Dulbecco's PBS devoid of Ca^{2+} and Mg^{2+} with 2% (v/v) heat-inactivated FBS (both from Sigma-Aldrich)], and centrifuged (300 $\times g$ for 5 min at 5 $^{\circ}\text{C}$; the same parameters were used for all cell-washing procedures). Cells were re-suspended in FB, counted and stained for flow cytometric analysis.

2.4.2. Lungs

Lungs obtained from two mice were combined into one sample. Lungs were minced, subjected to dounce homogenization, and washed in incomplete medium (RPMI-1640 + 10 mM HEPES buffer + 10 U/ml penicillin/streptomycin; all from Sigma-Aldrich). Subsequently, lung tissues were digested with 50 U/ml (25 ml per sample) collagenase type IV (Sigma-Aldrich) solution containing DNase I (50 U per ml Sigma-Aldrich) in a 50-ml glass flask with a magnetic stir bar. Following rapid agitation during digestion at 37 $^{\circ}\text{C}$ for 90 min, the resulting cell supernatant was removed and filtered through nitex fabric, and the cells were washed in complete medium [CM; RPMI-1640 + 10% heat-inactivated fetal bovine serum + 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer + 10 mM non-essential amino acids + 10 mM sodium pyruvate + 10 U/ml penicillin/streptomycin; all from Sigma-Aldrich]. Isolated cells were re-suspended in a 40% percoll solution (100% percoll solution = 90% of percoll + 10% of 10x Hanks' Balanced Salt Solution; both from Sigma-Aldrich; dilutions of percoll solution were prepared using CM), and then they were layered over a 60% percoll solution and subjected to gradient centrifugation (400 $\times g$ for 20 min at 20 $^{\circ}\text{C}$). Mononuclear cells were removed from the interface layer, washed and then re-suspended in FB (for flow cytometric analysis) or CM (for cell culture) and counted.

2.5. Culture conditions

In order to establish whether in mice with the OVA-induced model of allergic asthma the activation of PPAR- γ directly or indirectly affects IL-4, IL-10 and IL-17 production by CD4^{+} T cells, lung lymphocytes were exposed to RSG in concentrations reflecting its plasma levels achieved in vivo at a typical dose (Avandia, 2014) and in ten-fold lower concentration. Briefly, lung mononuclear cells were aseptically isolated from the lungs of untreated OVA-immunized mice as described above. To eliminate the influence of individual differences between animals, the same cells were used as both control and treated cells. Therefore, the cells isolated from three mice were pooled to obtain sufficient number of cells for seeding. The cells were adjusted to 2×10^6 cells/ml in CM and seeded in 24-well plates in 1 ml aliquots. They were pre-incubated for 12 h without (control) or with two concentrations of RSG (10^{-6} and 10^{-7} M) followed by 6 h stimulation with 50 ng/ml of phorbol-12-myristate-13-acetate (PMA) and 1 $\mu\text{g}/\text{ml}$ of ionomycin (both from Sigma-Aldrich) in the presence of 1 $\mu\text{l}/\text{ml}$ of Protein transport inhibitor (containing brefeldin A; BD Biosciences, San Jose, USA) during the last 4 h to raise intracellular cytokine stores by inhibiting their secretion. RSG was dissolved in DMSO, thus the same amount of DMSO was added to control wells. Each experiment included 5 wells of cells for each condition tested. All experiments were repeated independently three times (overall $n = 15$). The plates were incubated at 37 $^{\circ}\text{C}$ in an atmosphere of a humidified incubator with 5% CO_2 and 95% air.

2.6. Flow cytometry

2.6.1. Extracellular staining

Cells prepared as described above were stained for surface antigens with fluorochrome conjugated monoclonal antibodies (mAbs): FITC rat anti-mouse CD4 (clone H129.19, IgG2a, κ), APC-Cy7 rat anti-mouse CD8a (clone 53-6.7, IgG2a, κ), PE-Cy7 rat anti-mouse CD25 (clone PC61, IgG1, λ ; all from BD Biosciences); cells were stained for CD8 expression to distinguish $\text{CD4}^{+}\text{CD8}^{-}$ T cells from double positive (i.e. $\text{CD4}^{+}\text{CD8}^{+}$) T cells.

2.6.2. Intracellular staining for Foxp3-expressing cells

Following surface staining for CD4, CD8 and CD25, cells were washed, fixed and permeabilized using a mouse Foxp3 buffer set (BD Biosciences) according to the manufacturer's protocol. Subsequently, cells were labeled with PE-conjugated rat anti-mouse Foxp3 mAb (clone: MF23, IgG2b, BD Biosciences).

2.6.3. Intracellular staining for IL-4-, IL-10-, IL-17-producing cells

Following surface staining for CD4, cells were washed, fixed and permeabilized using Cytofix/Cytoperm solution and Perm/Wash buffer

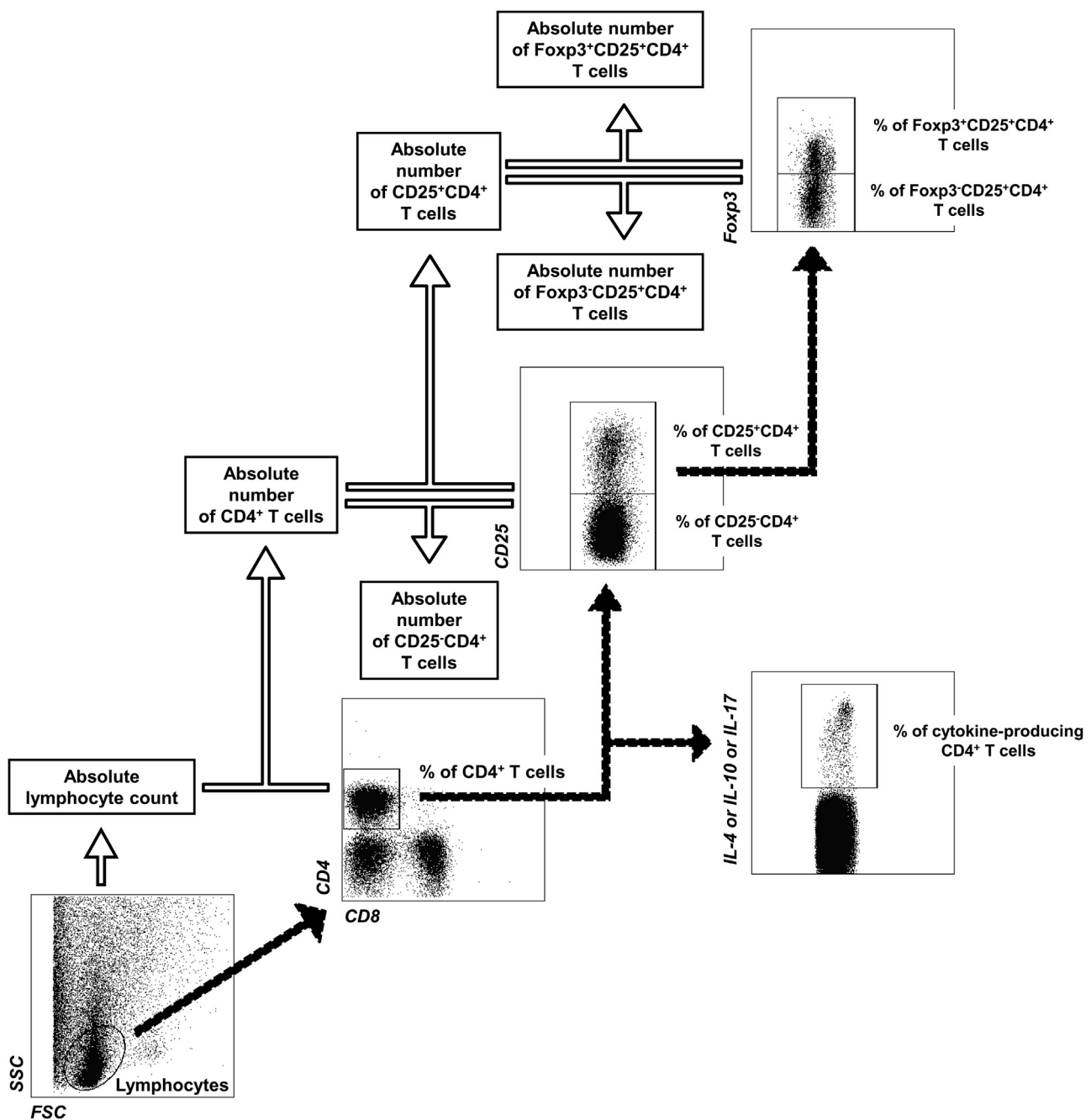


Fig. 2. Gating strategy for the flow cytometric data analysis and calculation of the absolute cell counts of lymphocyte subsets. Lymphocytes were identified based on forward and side scatter (FSC/SSC) properties and then gated for the expression of CD4 surface receptors. Relative to the presence of CD25 expression, the CD4⁺ T cell population was subdivided into CD25⁺ and CD25⁺ cell subsets. Subsequently, CD25⁺CD4⁺ T cells were analyzed for Foxp3 expression. Absolute cell counts of lymphocyte subsets (i.e. number of cells from particular subpopulations per tissue sample) were calculated using the dual platform method, as shown above. Furthermore, under in vitro conditions, the CD4⁺ T cell population was analyzed for cytokine (IL-4, IL-10 and IL-17) production.

(both from BD Biosciences) according to the manufacturer's protocol. Subsequently, cells were stained with PE-CF594 rat anti-mouse IL-4 (clone 11B11, IgG1), APC rat anti-mouse IL-10 (clone JES5-16E3, IgG2b) and PerCP-Cy5.5 anti-mouse IL-17 (clone TC11-18H10, IgG1, λ ; all from BD Biosciences).

2.7. FACS acquisition and analysis

Flow cytometry analysis was performed using a FACSCanto II cytometer (BD Biosciences). The data were acquired by FACS Diva version 6.1.3 software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc., Stanford, USA). Absolute cell counts of cell subsets (i.e.

number of cells from a particular subpopulation per tissue sample) were calculated using the dual platform method, i.e. the absolute cell count was determined by calculating the data obtained from a cell counting chamber by the percentage of particular cell subsets. Fig. 2 presents a gating strategy for flow cytometric data analysis and specifies how the absolute count was calculated for particular cell subsets.

2.8. Statistical analyses

Data were expressed as the mean ($\pm$ S.D.) for 8 (in vivo experiments) or 15 (in vitro experiments) mice/samples per group. Statistical analysis was performed using one-way analysis of variance (ANOVA)

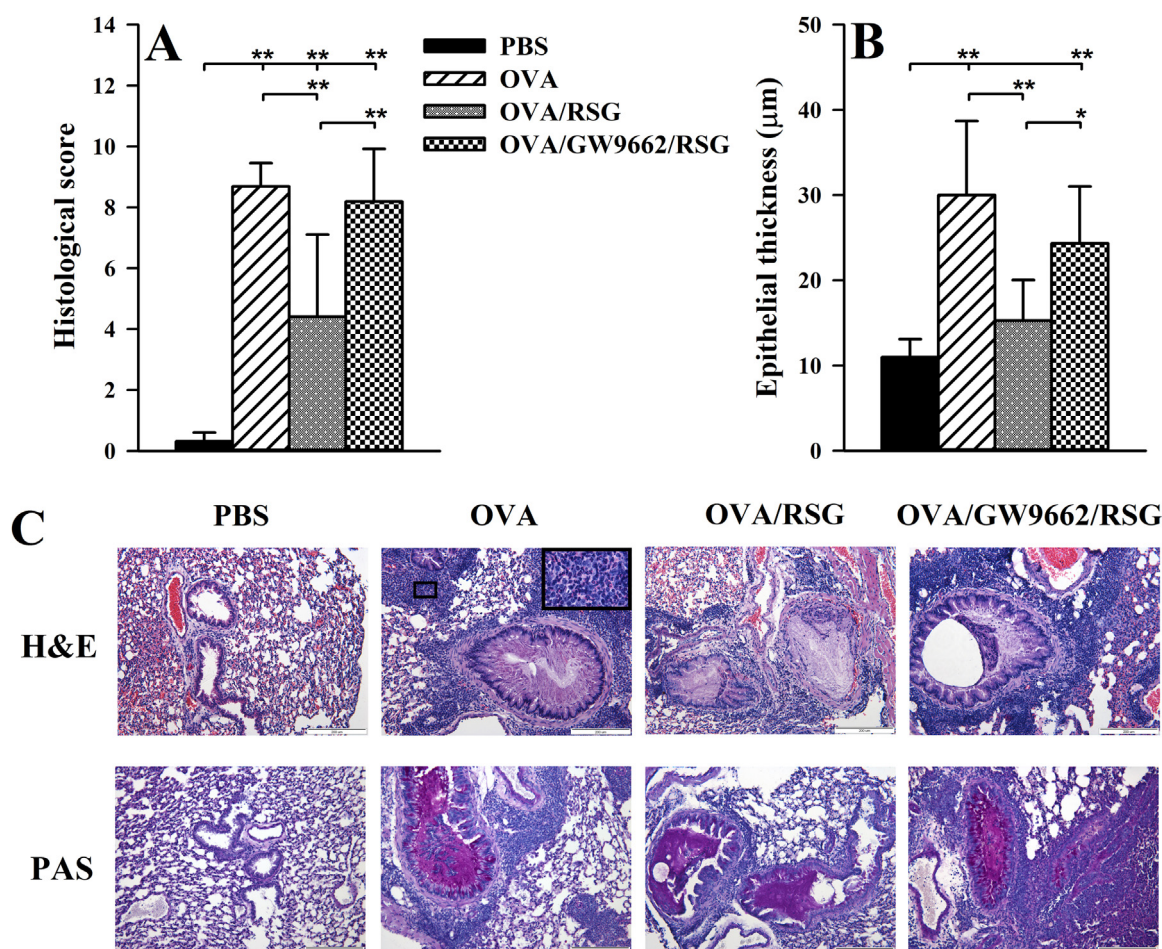


Fig. 3. Quantitative assessment of allergic airway inflammation and treatment efficacy. Mean histology scores of airway inflammation (A) and mean epithelial thickness (B) were assessed in the lungs of non-immunized mice (PBS), OVA-immunized mice (OVA), RSG-treated, OVA-immunized mice (OVA/RSG) and GW9662- and RSG-treated, OVA-immunized mice (OVA/GW9662/RSG). Results are expressed as the mean ($\pm$ S.D.) of two independent experiments with 4 animals per group ($n = 8$ per group, $**P < 0.001$, $*P < 0.05$). Examples of photomicrographs of tissue sections stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) (C). Densely infiltrated inflammatory cells (enlargement of the rectangle in the upper photograph) with mucous hypersecretion are visible in lung sections of ovalbumin-immunized mouse (OVA). Marked reduction in the infiltration of inflammatory cells was observed in RSG-treated, OVA-immunized mice (OVA/RSG), while a pretreatment with a PPAR- γ antagonist reversed the protective action of RSG (OVA/GW9662/RSG). The treatment with RSG and GW9662 was started 48 h prior to OVA challenge and was continued daily for the next 8 days. One day after the last treatment and challenge, mice were sacrificed, and lung samples were individually collected for histopathological examination. Magnification: 20-fold; 100-fold for the insert.

followed by the Bonferroni's *post hoc* test. Differences were deemed significant when the *p* values were < 0.05 .

3. Results

3.1. RSG attenuates, but does not prevent, airway inflammation and this effect is exerted in a PPAR- γ -dependent fashion

All mice in the OVA group developed allergic inflammation typical of OVA-induced AAI, including airway epithelium thickening and infiltration of inflammatory cells around the bronchi and bronchioles. The infiltrates were composed of eosinophils, neutrophils and lymphocytes (Fig. 3C, H&E). Goblet cell hyperplasia and mucus hypersecretion were observed (Fig. 3C, PAS). Administration of RSG attenuated, but did not resolve, the OVA-induced infiltration of inflammatory cells (Fig. 3C, H&E) and mucus production in the airways (Fig. 3C, PAS), while a pretreatment with GW9662 reversed the protective action of RSG (Fig. 3C).

The histopathological findings were quantitatively expressed using an appropriate scoring system. The mean scores for the OVA group were significantly higher compared to the PBS and OVA/RSG groups, but did not differ significantly from those achieved by the OVA/

GW9662/RSG group (Fig. 3A). However, the mean scores for the OVA/RSG group were considerably greater than the corresponding values obtained in the PBS group (Fig. 3A). Quantitative assessment of the epithelial thickness demonstrated a significant increase with regard to this parameter in the OVA group compared to the PBS and OVA/RSG groups (Fig. 3B). The epithelial thickness did not differ significantly between the PBS and OVA/RSG groups, or between the OVA and OVA/GW9662/RSG groups (Fig. 3B). Thus, the quantitative evaluation confirmed that RSG attenuated airway inflammation and this effect was abolished by the co-administration of GW9662.

3.2. RSG does not affect the number of Foxp3-expressing CD4⁺ regulatory T cells in the MLNs and lungs of OVA-immunized mice & RSG reduces but does not prevent the OVA-induced increase in the count of activated CD4⁺ T effector cells in the MLNs and lungs and this effect is exerted via a PPAR- γ -dependent mechanism

At both time points, the absolute count of Foxp3⁺CD25⁺CD4⁺ T cells was considerably increased in the MLNs of mice from the OVA, OVA/RSG and OVA/GW9662/RSG groups compared to the PBS group (Fig. 4A). No differences were demonstrated in terms of this parameter between the OVA, OVA/RSG and OVA/GW9662/RSG groups (Fig. 4A).

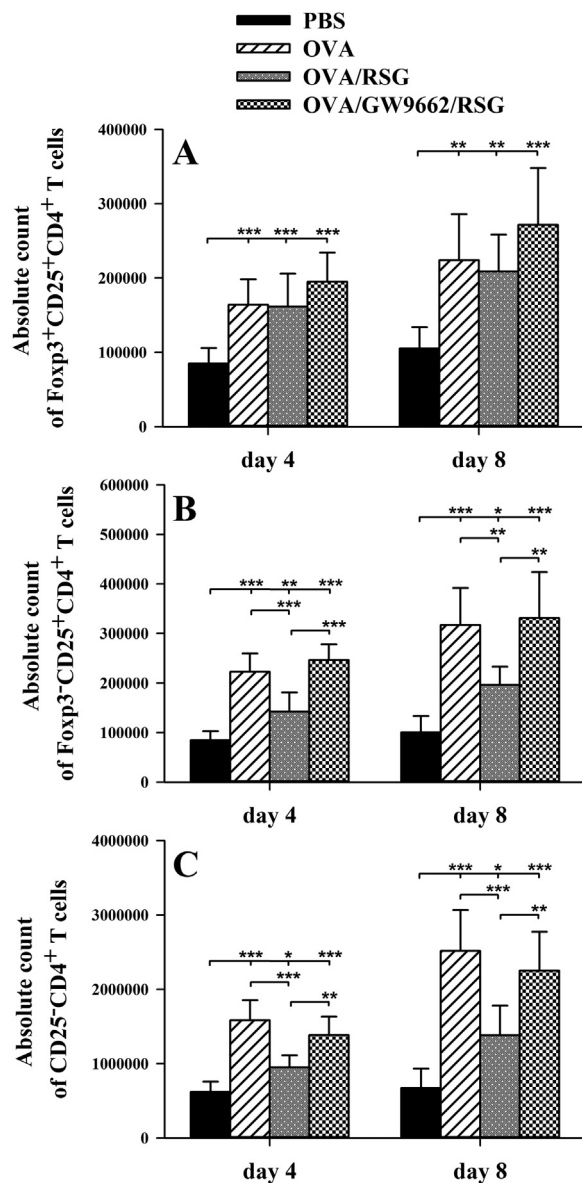


Fig. 4. Effect of rosiglitazone (RSG) on the number of CD4⁺ T cells in the mediastinal lymph nodes (MLNs) of ovalbumin (OVA)-immunized mice. The absolute counts of Foxp3⁺CD25⁺CD4⁺ (A) Foxp3⁺CD25⁺CD4⁺ (B) and CD25⁺CD4⁺ (C) T cells were determined in the MLNs of non-immunized mice (PBS), OVA-immunized mice (OVA), RSG-treated, OVA-immunized mice (OVA/RSG) and GW9662- and RSG-treated, OVA-immunized mice (OVA/GW9662/RSG). RSG and GW9662 administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed, and MLN samples were individually collected. The absolute count represents the number of cells of each subset per MLN sample. Results are expressed as the mean ($\pm$ S.D.) of two independent experiments with 4 mice per group ($n = 8$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Similarly to the observations in the MLNs, at both time points, the absolute number of Foxp3⁺CD25⁺CD4⁺ T cells was considerably elevated in the lungs of both untreated OVA-immunized and GW9662/RSG-treated OVA-immunized mice (Fig. 5A). At the first time point, the absolute number of these cells was significantly lower in the RSG/OVA group compared to the OVA and OVA/GW9662/RSG groups and did not differ significantly from that in non-immunized mice (Fig. 5A). However, at the second time point, the absolute number of Foxp3⁺CD25⁺CD4⁺ T cells in the lungs of mice from the OVA/RSG group did not differ significantly from the values of this parameter obtained in the OVA and OVA/GW9662/RSG groups, while being

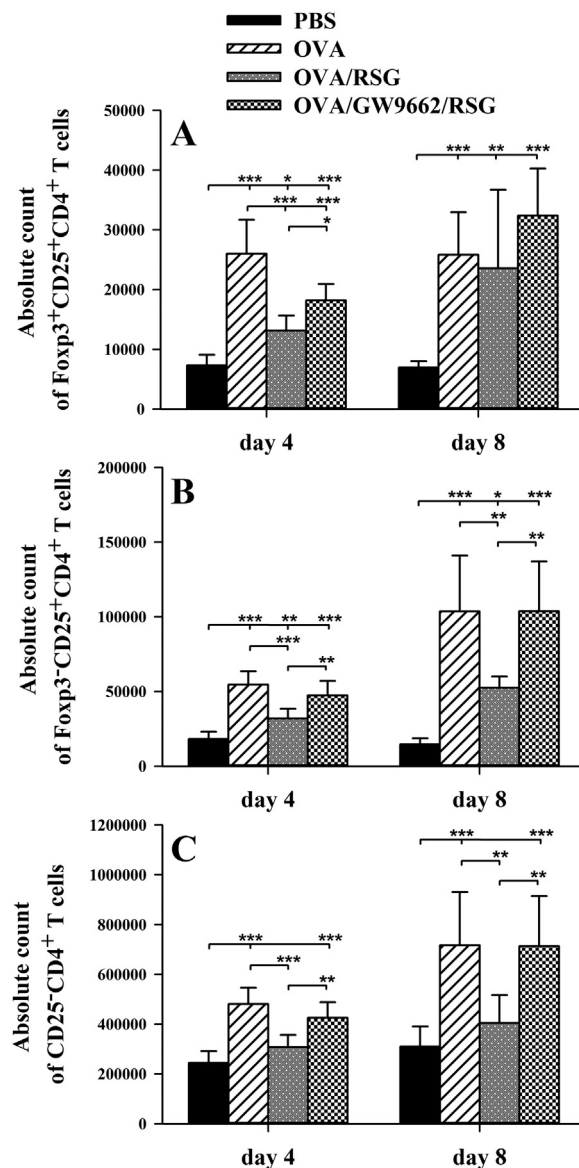


Fig. 5. Effect of rosiglitazone (RSG) on the number of CD4⁺ T cells in lungs of ovalbumin (OVA)-immunized mice. The absolute counts of Foxp3⁺CD25⁺CD4⁺ (A) Foxp3⁺CD25⁺CD4⁺ (B) and CD25⁺CD4⁺ (C) T cells were determined in the lungs of non-immunized mice (PBS), OVA-immunized mice (OVA), RSG-treated, OVA-immunized mice (OVA/RSG) and GW9662- and RSG-treated, OVA-immunized mice (OVA/GW9662/RSG). RSG and GW9662 administration was started 48 h prior to OVA challenge. At the indicated number of days after challenge, mice were sacrificed and lung samples were collected. The absolute count represents the number of cells of each subset per lung sample pooled from two animals. Results are expressed as the mean ($\pm$ S.D.) of two independent experiments involving 4 lung samples per group ($n = 8$ per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

considerably higher than in the PBS group (Fig. 5A). Thus, the administration of RSG did not prevent or reduce the OVA-induced increase in the count of Foxp3⁺CD25⁺CD4⁺ T cells in the MLNs and lungs, although the agent delayed this effect in the latter tissue.

In the MLNs of mice from the OVA/RSG group, the absolute count of Foxp3⁺CD25⁺CD4⁺ and CD25⁺CD4⁺ T cells was considerably lower than in the OVA and OVA/GW9662/RSG groups, but still higher than in healthy controls (Fig. 4B and C). The same results were obtained with respect to Foxp3⁺CD25⁺CD4⁺ T cells in the lungs (Fig. 5B). Thus, RSG only reduced but did not prevent the OVA-induced increase in the count of Foxp3⁺CD25⁺CD4⁺ and CD25⁺CD4⁺ T cells in the MLNs and Foxp3⁺

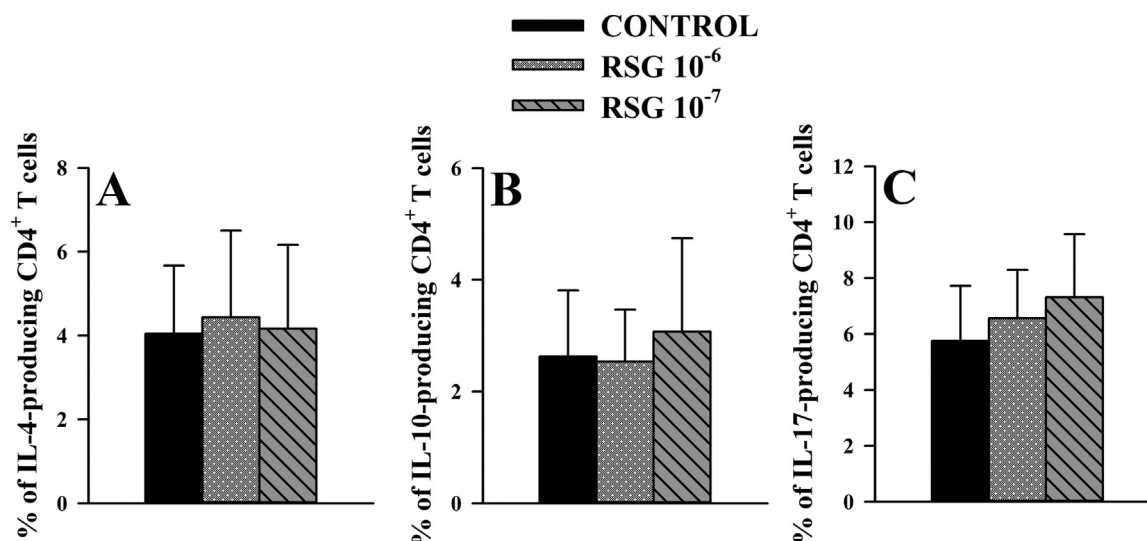


Fig. 6. Effect of rosiglitazone (RSG) on IL-4 (A), IL-10 (B) and IL-17 (C) production by lung CD4⁺ T cells. Cytokine production was investigated in lung mononuclear cells isolated from untreated, OVA-immunized mice. The cells were cultured in the absence (CONTROL) or presence of RSG followed by stimulation with phorbol-12-myristate-13-acetate and ionomycin in the presence of protein transport inhibitor. Results are expressed as a percentage of IL-4-, IL-10- and IL-17-producing cells within the CD4⁺ T cell subset. Results are the mean (± S.D.) of three independent experiments with five wells per experiment (n = 15).

CD25⁺CD4⁺ cells in the lungs. Interestingly, the administration of RSG prevented an OVA-induced increase in absolute counts of CD25⁺CD4⁺ T cells in the lungs, while the pretreatment with GW9662 abolished this action (Fig. 5C). The results indicate that the RSG-induced effects were mediated by PPAR-γ dependent mechanisms.

3.3. Exposure to RSG does not affect IL-4, IL-10 and IL-17 production by CD4⁺ T cells

The effect of PPAR-γ activation on production of the selected cytokines was assessed in lung mononuclear cells incubated in the absence or presence of RSG with the accompanying PMA/ionomycin stimulation in the presence of a protein transport inhibitor. The exposure of cells to either concentration of RSG did not affect significantly the percentage of IL-4-, IL-10- and IL-17-producing cells within CD4⁺ T cell subset (Fig. 6).

4. Discussion

The present study demonstrated that treatment with RSG only attenuated but did not prevent or abolish OVA-induced AAI, and this effect was PPAR-γ-dependent. This is in line with the results of other authors who found that administration of RSG reduced but did not abolish airway inflammation in a mouse model of neutrophilic (Zhao et al., 2014) and eosinophilic asthma (Yu et al., 2017). Furthermore, these results are in compliance with the ones obtained in short-term randomized controlled trials on asthma (Richards et al., 2010; Spears et al., 2009). The cited researchers found that rosiglitazone had modest beneficial effects in the treatment of asthma. Therefore, it was concluded that PPAR-γ agonist monotherapy is unlikely to represent a clinically useful intervention in human asthma (Richards et al., 2010). The current results are concordant with this conclusion.

The present research aimed at verifying the involvement of Foxp3⁺CD25⁺CD4⁺ Treg cells in the anti-asthmatic action of PPAR-γ agonists. CD25 is a marker of recent T cell activation (Waldmann, 1991), although it is also expressed on naturally occurring (nTreg) and adaptive/inducible (iTreg) Foxp3-expressing Treg cells (Chatenoud, 2011). In turn, Foxp3 is a unique marker and a “master” regulator of the development and suppressive function of Treg cells (Fontenot et al., 2003). Therefore, with a certain measure of simplification, it can be stated that cells of the phenotypes Foxp3⁺CD25⁺CD4⁺ and Foxp3⁺

CD25⁺CD4⁺ represent Treg and activated Teff cells, respectively. In turn, the CD25⁺CD4⁺ T cell subset should be considered primarily as a pool of resting T (Trest) cells.

The present study has demonstrated that the amount of activated CD4⁺ Teff cells in the MLNs and lungs of mice with OVA-induced model of allergic asthma was significantly higher compared to healthy control mice. The treatment with RSG reduced but did not prevent or abolish this effect. Co-treatment with GW9662 abolished the RSG-induced reduction in the count of activated CD4⁺ Teff cells, which confirms that this effect was exerted via a PPAR-γ-dependent mechanism. Similar results were achieved for Trest cells. Thus, our results indicate that the impairment of the activation and clonal expansion of CD4⁺ Teff cells in the MLNs is involved in the anti-asthmatic action of PPAR-γ agonists, which is consistent with results from earlier studies (Hammad et al., 2004; Yu et al., 2017; Zhao et al., 2014). However, taking into account the latest findings, it is questionable whether the activation of PPAR-γ may reduce directly the CD4⁺ Teff cell response in AAI. Recently, Nobs et al. (2017) have reported that PPAR-γ plays a key role in both T cells and DCs for development of Th2 immune responses. In the light of this finding, the PPAR-γ agonist-induced attenuation of CD4⁺ Teff cell response in a mouse model of allergic asthma cannot be exerted by direct inhibitory action on DCs and/or CD4⁺ Teff cells, but is rather mediated through some indirect mechanism/s. Such mechanisms could include elevated recruitment of nTreg cells into the MLNs and lungs and/or local generation of iTreg cells, which should lead to the suppression of CD4⁺ Teff cell responses in these tissues. This assumption was supported by previous studies suggesting that the beneficial effects of PPAR-γ agonists in allergic diseases may be partially mediated by an increase in the amount of Treg cells (Lee et al., 2015; Wang et al., 2011; Yu et al., 2017). Treg cells constitute a unique lineage of CD4⁺ T cells which can suppress CD4⁺ Teff cells, and therefore they are often referred to as “professional” suppressor cells (Shevach, 2001). Treg cells can suppress Th2 and Th17 cell-mediated inflammation and prevent allergic asthma both in asthmatics and in animal models (Tao et al., 2015; Tian et al., 2014).

Our study demonstrated that activation of PPAR-γ with RSG neither increased the recruitment of nTreg cells to the MLNs and lungs nor did it induce local generation of iTreg cells. Although the absolute number of Treg cells in the OVA/RSG group was elevated compared to the PBS group, and the same effect occurred in the OVA and OVA/GW9662/RSG groups. The fact that the co-administration of a PPAR-γ blocker did

not affect the absolute number of Treg cells proves that the increase in the amount of these cells in RSG alone treated mice was not caused by RSG, but resulted exclusively from immunization with OVA.

Taking all of the above information into consideration, it can be stated that the current results definitely do not confirm the hypothesis that Treg cells mediate PPAR- γ activation-induced down-regulation of CD4⁺ Teff cell response in allergic asthma and that these cells are involved at all in producing the anti-asthmatic effect of PPAR- γ agonists. This conclusion is not in accord with results of other studies, which supported the above hypothesis (Lee et al., 2015; Yu et al., 2017; Wang et al., 2011). In our opinion, this discrepancy arises from the fact that in the cited research it was the percentage rather than the absolute count of Treg cells that was used as a parameter to assess the count of Treg cells. Moreover, the assessment was not made in the inductive and effector tissues of the immune response associated with the analyzed disease model, as it was done in our study, but in the spleen (Lee et al., 2015; Wang et al., 2011) or blood (Yu et al., 2017). Determination of an absolute count of some cell subset provides the information on the actual number of cells in this subset, while percentage data do not reflect it; instead, they only show mutual percent proportions of particular cell subsets. Proliferation, recruitment and redistribution of cells from various CD4⁺ T cell subsets occurring in the course of allergic asthma cause such drastic changes in their number that these changes cannot be properly captured and measured with the help of an analysis based on percentages of cells. Therefore, in our opinion, the present results provide more precise and relevant data on the effect of PPAR- γ agonist on the amount of Tregs in the course of AAI.

The literature indicates that administration of PPAR- γ agonists to OVA-immunized subjects affected levels of IL-4, IL-17 and IL-10 in the respiratory tract tissues (Hammad et al., 2004; Kim et al., 2005; Lee et al., 2015; Zhao et al., 2014). Because there is no research on effects of PPAR- γ agonists on the frequency of IL-4⁺CD4⁺, IL-10⁺CD4⁺ and IL-17⁺CD4⁺ T cells (i.e. on cell subsets which largely reflect Th2, Tr1 and Th17 cells, respectively), it was unknown whether these effects were only a consequence of reduced expansion of CD4⁺ Teff or this effect additionally resulted from direct influence of these agents on cytokine production by CD4⁺ T cells. In order to resolve this question, an effect of RSG on percentages of cytokine-producing CD4⁺ T cells was investigated under in vitro conditions. The reason why this parameter was chosen lay in the essence of the undertaken research, which was to determine whether activation of PPAR- γ could induce or abolish the capacity of CD4⁺ T cells to produce IL-4, IL-10 and IL-17. An in vivo assessment of the absolute count of cytokine-producing CD4⁺ T cells could not yield a correct answer to the set question because in that case the result would additionally comprise the influence that had no connection with the ability to induce or inhibit production of cytokines by CD4⁺ T cells (i.e. RSG-induced reduction of absolute count of CD4⁺ Teff cells in the lungs).

Exposure of lymphocytes isolated from the lungs of untreated mice with OVA-induced AAI to RSG did not affect the percentages of IL-4⁺, IL-10⁺ and IL-17⁺ cells within the CD4⁺ T cell subset. These results strongly suggest that activation of PPAR- γ does not induce the capacity of producing IL-4, IL-10 and IL-17 by CD4⁺ T cells, nor does it abolish such capacity. However, this does not mean that the results prove that activation of PPAR- γ leaves the IL-4, IL-10 and IL-17 unaffected because assessment of the influence on the total production of cytokines in the respiratory system is a completely different matter. This study as well as the earlier investigations proved that PPAR- γ agonists reduced clonal expansion of CD4⁺ Teff cells in the MLNs (of which a natural consequence is the decreased infiltration of the lungs by these cells), it was clear that this effect would translate into reduced abundance of the key producers of IL-4, IL-10 and IL-17, and therefore would lead to reduced levels of these cytokines in the respiratory tract. This line of reasoning agrees with the reports indicating that PPAR- γ agonists reduced production of IL-4 (Hammad et al., 2004; Kim et al., 2005), IL-10 (Lee et al., 2015) and IL-17 (Zhao et al., 2014) in respiratory tract tissues.

Recapitulating, the results achieved in this study strongly suggest that PPAR- γ agonists neither induce nor abolish the production of IL-4, IL-10 and IL-17 by CD4⁺ T cells as such, although they can reduce the production of these cytokines by limiting the formation of their potential producers in the MLNs. In other words, the results implicate that the influence of PPAR- γ agonists on production of IL-4, IL-10 and IL-17 by CD4⁺ T cells has an indirect character.

5. Conclusions

Treatment with RSG only attenuated but did not prevent or abolish OVA-induced AAI, and this effect was PPAR- γ -dependent. Our results confirm previous findings that the impairment of the activation and clonal expansion of CD4⁺ Teff cells in the MLNs is involved in the anti-asthmatic action of PPAR- γ agonists. Activation of PPAR- γ with RSG neither increased the recruitment of nTreg cells to the MLNs and lungs nor induced local generation of iTreg cells. This indicates that Treg cells are not involved in producing the anti-asthmatic effect of PPAR- γ agonists. Moreover, our data strongly suggest that beneficial effects of PPAR- γ agonists in asthma treatment are not mediated through a direct inhibitory effect on the production of IL-4, IL-10 and IL-17 by CD4⁺ Teff cells. However, it should be concluded that PPAR- γ agonists can reduce the production of IL-4, IL-10 and IL-17, but in an indirect fashion, through the reduction of proliferation of their producers in the MLNs.

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Conflict of interest statement

The authors declare that they have no conflict of interests.

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