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Peroxisome Proliferator-Activated Receptor- γ Agonist Induces Regulatory T Cells in a Murine Model of Allergic Rhinitis

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. To evaluate the effects of peroxisome proliferator-activated receptor (PPAR)- γ agonist on the induction of regulatory T cells (Tregs) in a murine model of allergic rhinitis.

Study Design. Randomized controlled trial.

Setting. Animal study.

Subjects and Methods. BALB/c mice that received ovalbumin sensitization and challenge served as the ovalbumin group ($n = 6$). Two separate groups of 6 mice received intragastric administration with PPAR- γ agonist pioglitazone (30 mg/kg/d) or pioglitazone plus PPAR- γ antagonist GW9662 (0.5 mg/d) before each ovalbumin challenge. The control group ($n = 6$) was treated with drug vehicle alone. Various allergic responses were assessed. Real-time polymerase chain reaction was performed to investigate the mRNA expression of forkhead box P3 (Foxp3), T-bet, and GATA-3. Flow cytometry was used to determine the percentage of Tregs.

Results. Mice developed typical pathophysiological allergic rhinitis features after the ovalbumin challenge. The frequencies of sneezing and scratching were significantly decreased by pioglitazone treatment ($P < .0001$). Eosinophils infiltration and the levels of interleukin-5 and interferon- γ in nasal cavity lavage fluid and sera immunoglobulin E were also markedly decreased by pioglitazone ($P < .001$). The expression of Foxp3 mRNA and the population of Tregs were significantly increased by pioglitazone ($P < .05$). Cotreatment with GW9662 reversed the anti-inflammatory effects of pioglitazone. The effects of PPAR- γ agonist on Foxp3 mRNA expression and Tregs induction were abrogated by administration of GW9662.

Conclusion. PPAR- γ agonist attenuates upper airway allergic inflammation in a PPAR- γ -dependent fashion, and the beneficial effects of pioglitazone in airway allergic inflammation may be mediated by induction of Tregs.

Keywords

allergic rhinitis, pioglitazone, peroxisome proliferator-activated receptor, regulatory T lymphocyte

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Peroxisome proliferator-activated receptors (PPARs) are members of a nuclear receptor superfamily that contains transcription factors regulating gene expression.¹ So far, 3 subtypes (PPAR- α , β/δ , and γ) have been identified. Activation of PPAR- γ can downregulate the synthesis and release of immunomodulatory cytokines from various cell types, which participate in the regulation of inflammatory processes. The expression of PPAR- γ in various leukocytes suggests that this molecule plays an important role in the regulation of immune responses. Recently, PPAR- γ has been shown to be a regulator of airway inflammation in allergic asthma, and enhanced PPAR- γ expression has been detected in the airway epithelium of patients with asthma.² In addition, PPAR- γ is expressed in human nasal mucosa and upregulated in patients with perennial allergic rhinitis (AR).³ In a murine model of experimental AR, PPAR- γ activation effectively inhibited allergic symptoms, nasal mucosa inflammation, the production of antigen-specific immunoglobulin E (IgE) and Th2-type cytokines, and suppressed the overexpression of galectin-3 induced by ovalbumin (OVA).^{4,5}

AR is one of the most common chronic diseases in the industrialized world. The symptoms of AR arise as a result of IgE-mediated nasal mucosa inflammation characterized by

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inflammatory cell infiltration, such as eosinophils and lymphocytes. It has been well established that AR and asthma are characterized by similar inflammatory pathophysiologic processes, and AR is regarded as a risk factor for asthma. It has been estimated that ~8.7% to 24.1% of the population in China is suffering from AR.⁶

Forkhead box p3 (Foxp3) is an obligatory intracellular marker of regulatory T cells (Tregs). Tregs have been shown to be an important regulator in immune responses and are critical in inhibiting autoimmune diseases.⁷ Tregs also control immune responses to infections and allergens.^{8,9} Two types of Tregs have been described: nTregs are thymically derived, and iTregs are converted in the peripheral immune system from CD4+CD25-Foxp3-Teffs.⁷ Recently, it was reported that PPAR- γ activation enhanced iTregs generation by increasing retinoic acid synthesis from murine splenic dendritic cells, and this effect was PPAR- γ dependent.¹⁰ It has been suggested that PPAR- γ is an important immune modulator in lower airway allergic inflammation; however, the role of Tregs in PPAR- γ ligand-mediated immunoregulatory effects in cases of upper airway allergic inflammation has not yet been reported. In this study, we used a murine model of AR to evaluate the effects of a PPAR- γ agonist, pioglitazone (PIO), on nasal mucosa inflammation and explore the possible molecular mechanisms by which PPAR- γ regulates allergic inflammation.

Materials and Methods

Animals and Experimental Protocol

Female BALB/c mice, 8 to 10 weeks of age and free of murine-specific pathogens, were purchased from Shanghai SLAC Laboratory Animal Co (Shanghai, China) and maintained in a standard laboratory under a specific pathogen-free state. All experimental animals used in this study were under an approved protocol by the Institutional Animal Care and Use Committee of Tongji University. Eighteen mice were sensitized on days 0 and 10 by intraperitoneal injection of 20 μ g of OVA (grade V; Sigma, St Louis, Missouri) emulsified in 400 μ L of sterile phosphate-buffered saline (PBS) containing 2 mg of alum (Pierce Chemical Company, Rockford, Illinois). For intranasal challenge, 20 μ L of 100 mg/mL solution of OVA was instilled into both nares on days 13 to 17. Six animals were assigned to 1 of 3 groups. Six animals received PIO (30 mg/kg body weight/d; Actos, Takeda Pharmaceuticals, Osaka, Japan) by oral gavage (suspended in 0.5 mL of 1% carboxymethylcellulose) 3 hours before each OVA challenge. Six animals received PPAR- γ antagonist GW9662 (0.5 mg/d; Sigma) by oral gavage at the same time as administration of PIO. A separate group of 6 mice was treated with drug vehicle and served as a control.

Assessment of Nasal Symptoms

The amount of sneezing and nose scratching that occurred for 10 minutes after the last challenge was counted. Mice were subjected to a single-blind observation by examiners without identifying the experimental groups.

Collection of Blood and Nasal Cavity Lavage Fluid

One day after the last challenge, mice were sacrificed with an overdose intraperitoneal injection of pentobarbital sodium (100 mg/kg body weight). Blood was collected from the retroorbital sinus and centrifuged. Serum was stored at -70°C for IgE measurement. Tracheotomy was performed on mice, the nasal cavity was washed thrice with the same 0.5 mL PBS through incision of the trachea, and the nasal cavity lavage fluid was collected and centrifuged. Supernatants were stored at -70°C for cytokine measurement.

Serum OVA-Specific IgE Assay

Serum OVA-specific IgE was measured by an enzyme-linked immunosorbent assay (ELISA) kit using anti-IgE as a capture antibody and biotinylated OVA and horseradish peroxidase (HRP)-conjugated streptavidin for detection (MD Biosciences, St Paul, Minnesota). The detection limit of this ELISA kit was 3.8 ng/mL. Twofold serial dilutions were prepared for each serum (starting dilution 1:20). Antibody titers were calculated as the dilution corresponding to twice the mean absorbance value obtained at 450 nm.

Cytokine ELISA

Supernatants from nasal cavity lavage fluids were collected at the indicated time points. Quantitative ELISA for interleukin (IL)-5 and interferon (IFN)- γ were performed using commercially available kits (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. The minimum significant values of these assays were 7 pg/mL for IL-5 and 2 pg/mL for IFN- γ .

Tissue Preparation

The decapitated heads were fixed in 4% paraformaldehyde for 48 hours followed by decalcification in 10% EDTA for 3 to 4 weeks. The embedded tissues were cut into 5- μ m thin sections. Coronal nasal sections were stained with hematoxylin and eosin, and the amount of eosinophils within the mucosa and submucosa of the nasal septum was counted under a microscope at 4 fields with $\times 400$ magnification in a blinded manner.

Real-Time Polymerase Chain Reaction

Spleen was removed aseptically 24 hours after the last challenge. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, California) from splenic mononuclear cells, and the first strand was reverse-transcribed using random primer (TaKaRa, Otsu, Japan). The levels of Foxp3, T-bet, GATA-3, and β -actin mRNA expression were determined by real-time polymerase chain reaction (PCR) using the ABI PRISM 7300 Real-Time PCR System and SYBR Green PCR master mix (TaKaRa). Specific primer sequences are displayed in **Table 1**. The levels of these mRNA expressions were analyzed by the ABI 7300 Sequence Detection System. The results were normalized by β -actin expression and shown as fold increase over the expression of the control group.

Table 1. Primer Sequences

Foxp3	Forward: 5'-GAAAGCGGATACCAAATGA-3' Reverse: 5'-CTGTGAGGACTACCGAGCC-3'
T-bet	Forward: 5'-GCCAGGGAACCGCTTATA-3' Reverse: 5'-CCTTGTTGTTGGTGAGCTTTA-3'
GATA-3	Forward: 5'-CTGGATGGCGGCAAAGC-3' Reverse: 5'-GTGGGCGGGAAGGTGAA-3'
β-actin	Forward: 5'-CCCATCTACGAGGGCTAT-3' Reverse: 5'-TGTCACGCACGATTTCC-3'

Abbreviation: Foxp3, forkhead box P3.

Flow Cytometry

For cell surface staining, 10⁶ splenic mononuclear cells were incubated with FITC-conjugated antimouse CD4 (GK1.5) antibody (eBioscience, San Diego, California). For intracellular staining, cells stained with CD4 were incubated with fixation/permeabilization working solution, and Fc receptors were blocked with excess mouse Fc block. Cells were then stained with PE-Cy5-conjugated antimouse Foxp3 (FJK-16s) antibody (eBioscience). CD4⁺Foxp3⁺ T cells were analyzed using FACS-Calibur (Becton Dickinson, San Jose, California).

Statistical Analysis

Data were reported as mean ± SD. One-way analysis of variance (ANOVA) and Mann-Whitney *U* test were employed to determine the statistical significance of the values obtained, and *P* < .05 was considered statistically significant.

Results

PIO Inhibits Nasal Allergic Symptoms by PPAR-γ Activation

The frequency of sneezing and nose scratching for 10 minutes after the last challenge was 52.00 ± 6.53 and 72.83 ± 5.07, significantly higher than the control group at 8.17 ± 1.01 and 22.67 ± 3.93, respectively, *P* < .0001. In OVA-exposed mice, PIO treatment significantly reduced the frequency of sneezing and scratching to 12.50 ± 1.78 and 42.17 ± 5.59, *P* < .0001 (**Figure 1A**); the inhibitory effect of PIO treatment on nasal symptoms was abrogated when a PPAR-γ antagonist, GW9662, was administered simultaneously, and the frequency of sneezing and scratching was increased to 64.00 ± 6.03 and 62.67 ± 5.14, respectively (*P* < .0001; **Figure 1A**).

We further found that a wealth of eosinophils (80.67 ± 5.64) migrated into the nasal tissue of OVA-exposed mice compared with control (1.67 ± 1.42), *P* < .0001. Interestingly, the infiltration of eosinophils was also significantly decreased to 38.83 ± 4.47 by PIO treatment, *P* < .001. The number of eosinophils that migrated into the nasal mucosa was increased to 62.50 ± 3.68 when GW9662 was administered, *P* < .001, compared with PIO treatment (**Figure 1B**).

Serum OVA-Specific IgE

Administration of PIO to OVA-exposed mice significantly decreased OVA-specific IgE levels from 61.81 ± 2.45 ng/mL to 28.73 ± 1.63 ng/mL, *P* = .023 (**Table 2**). Cotreatment with GW9662 reversed the PIO effect and increased OVA-specific IgE levels to near OVA group levels (56.36 ± 3.49 ng/mL, *P* < .05) compared with the OVA/PIO group.

IL-5 and IFN-γ in Nasal Cavity Lavage Fluid

Enhanced IgE production and inflammatory response in rhinitis are due to the predominant production of Th2 cytokines, such as IL-4, IL-5, and IL-13, and the allergic symptoms can be alleviated by inhibiting these Th2 responses. Based on the idea of counterbalance between Th1 and Th2 cells,¹¹ several adjuvants that preferentially induce Th1 response have been tested for their inhibitory activity to allergic response. We next investigated cytokine production from Th1 and Th2 cells in nasal cavity lavage fluid. The concentration of IL-5, a cytokine associated with eosinophils, was significantly increased in OVA-exposed mice (40.13 ± 2.45 ng/mL, *P* < .0001). PIO treatment decreased IL-5 production to 9.03 ± 0.41 ng/mL, *P* < .0001, versus OVA mice. GW9662 cotreatment partly reversed the PIO inhibitory effect on IL-5 production to 15.56 ± 1.14, *P* = .001, compared with OVA/PIO mice (**Table 2**).

A low level of IFN-γ, one of the principal Th1-type cytokines, was observed in OVA-exposed mice (577.59 ± 61.28 ng/mL, *P* = .001) compared with the control group. However, administration of PIO further decreased the production of IFN-γ to 417.97 ± 38.99 ng/mL. GW9662 cotreatment reversed the inhibitory effect of PIO on IFN-γ production (759.64 ± 62.58 ng/mL, *P* < .0001). Although GW9662 treatment increased IFN-γ production, this apparent increase trend was not significant compared with the control group (689.77 ± 33.78 ng/mL) by 1-way ANOVA (*P* > .05; **Table 2**).

Pathologic Changes of Nasal Mucosa

Histologic analysis of nasal mucosa from the OVA group revealed typical pathologic features of AR in the submucosa, which were infiltrated with numerous inflammatory cells. A marked reduction in the infiltration of inflammatory cells was observed in mice treated with PIO, similar to the control group. GW9662 cotreatment reversed the anti-inflammatory effects of PIO, showing inflammation similar to the OVA group (**Figure 2**).

Expression of GATA-3, T-bet, and Foxp3 mRNAs in Splenic Mononuclear Cells

The transcription factors T-bet and GATA-3 play a critical role in the differentiation of T cells into Th1 and Th2.^{12,13} In vitro studies indicated that enhanced induction of Tregs by PPAR-γ ligands was via PPAR-γ-dependent and PPAR-γ-independent mechanisms.^{10,14,15} There is a possibility that PPAR-γ ligands modulate the expression of these transcription

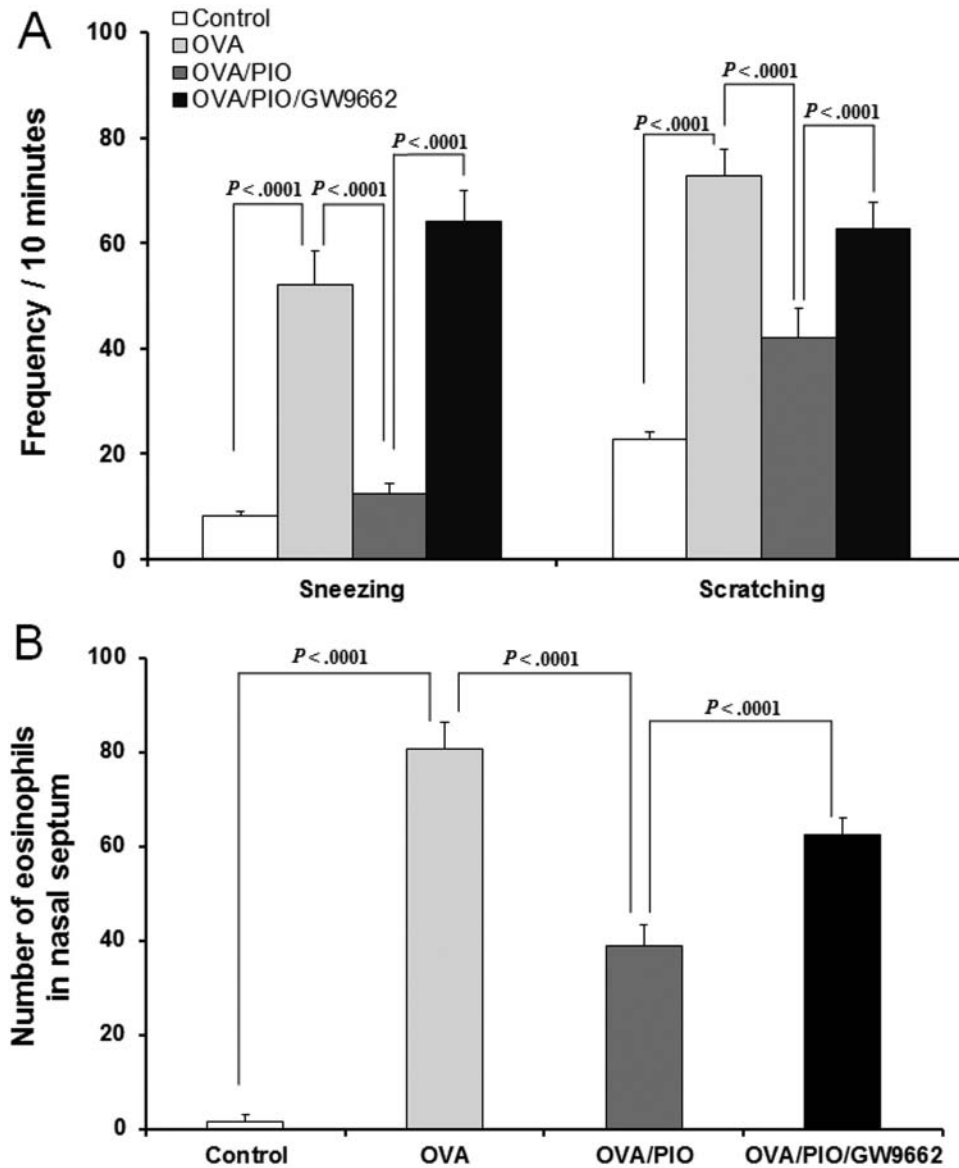


Figure 1. (A) Frequency of sneezing and scratching for 10 minutes after the last ovalbumin (OVA) challenge in mice. (B) Number of eosinophils in nasal septum mucosa. PIO, pioglitazone.

Table 2. Concentration of IgE and Cytokines (ng/mL)

	Control	OVA	OVA/PIO	OVA/PIO/GW9662
IgE	23.45 ± 1.42	61.81 ± 2.45*	28.73 ± 1.63 [†]	56.36 ± 3.49* [‡]
IL-5	9.11 ± 0.20	40.13 ± 2.45*	9.03 ± 0.41 [†]	15.56 ± 1.14* [‡]
INF- γ	689.77 ± 33.78	577.59 ± 61.28*	417.97 ± 38.99* [†]	759.64 ± 62.58* [‡]

The concentrations of IgE, IL-5, and INF- γ were measured by enzyme-linked immunosorbent assay. Data represent mean $\pm$ SD from 6 mice per group.

Abbreviations: IgE, immunoglobulin E; IL-5, interleukin-5; INF- γ , interferon-gamma; OVA, ovalbumin; PIO, pioglitazone.

* $P < .0001$ compared with control. [†] $P < .0001$ compared with OVA; [‡] $P < .0001$ compared with OVA/PIO.

factors during challenge with OVA and skew the differentiation of naive CD4⁺ T cells into Tregs. After OVA sensitization and challenge, the level of GATA-3 expression was increased by

3.18-fold ($P < .05$); the levels of T-bet and Foxp3 expression were decreased below the control level, 0.83 and 0.77, respectively, but this trend did not reach statistical significance ($P = .15$).

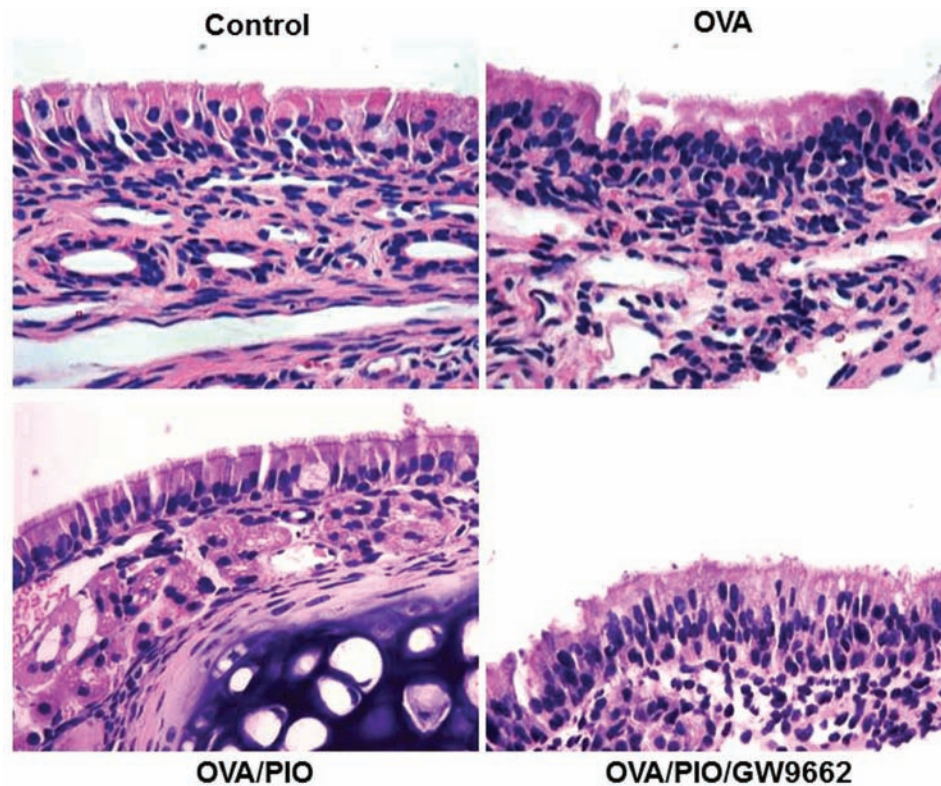


Figure 2. Effect of pioglitazone in mouse nasal tissues. Representative sections of the nasal tissues are seen at an original magnification of $\times 400$ after hematoxylin and eosin staining. OVA, ovalbumin; PIO, pioglitazone.

Administration of PIO resulted in the induction of Foxp3 expression by 2.15-fold ($P < .05$) and inhibition of GATA-3 (1.22) and T-bet (0.72) expression ($P < .05$) compared with OVA mice. GW9662 administration reversed the PIO effects on

the expression of GATA-3 (2.81) and Foxp3 (0.88) ($P < .05$). GW9662 cotreatment increased the expression of T-bet by 1.26, but this did not reach statistical significance compared with control (**Figure 3**).

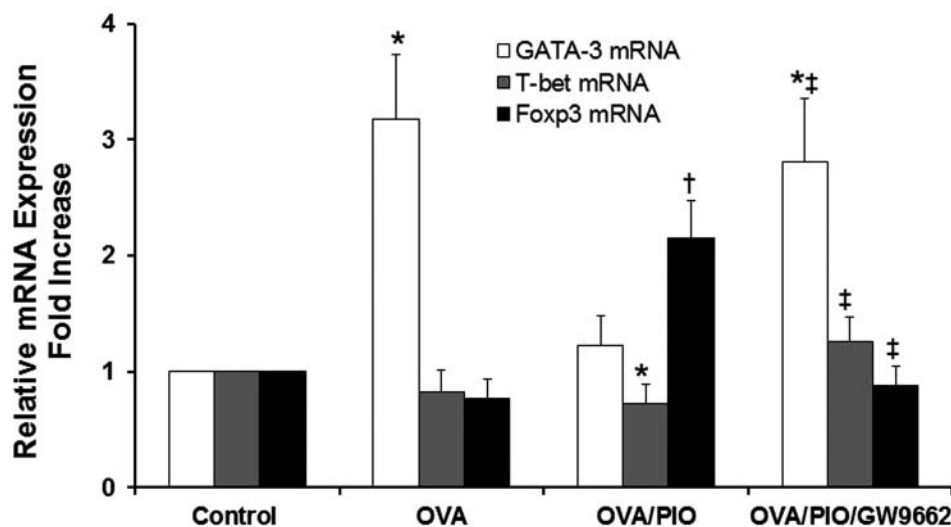


Figure 3. Quantitative analyses of GATA-3, T-bet, and Foxp3 mRNAs expression by real-time PCR. The results were normalized by β -actin expression. * $P < .05$ versus control; † $P < .05$ versus OVA; ‡ $P < .05$ versus OVA/PIO. Foxp3, forkhead box p3; OVA, ovalbumin; PIO, pioglitazone.

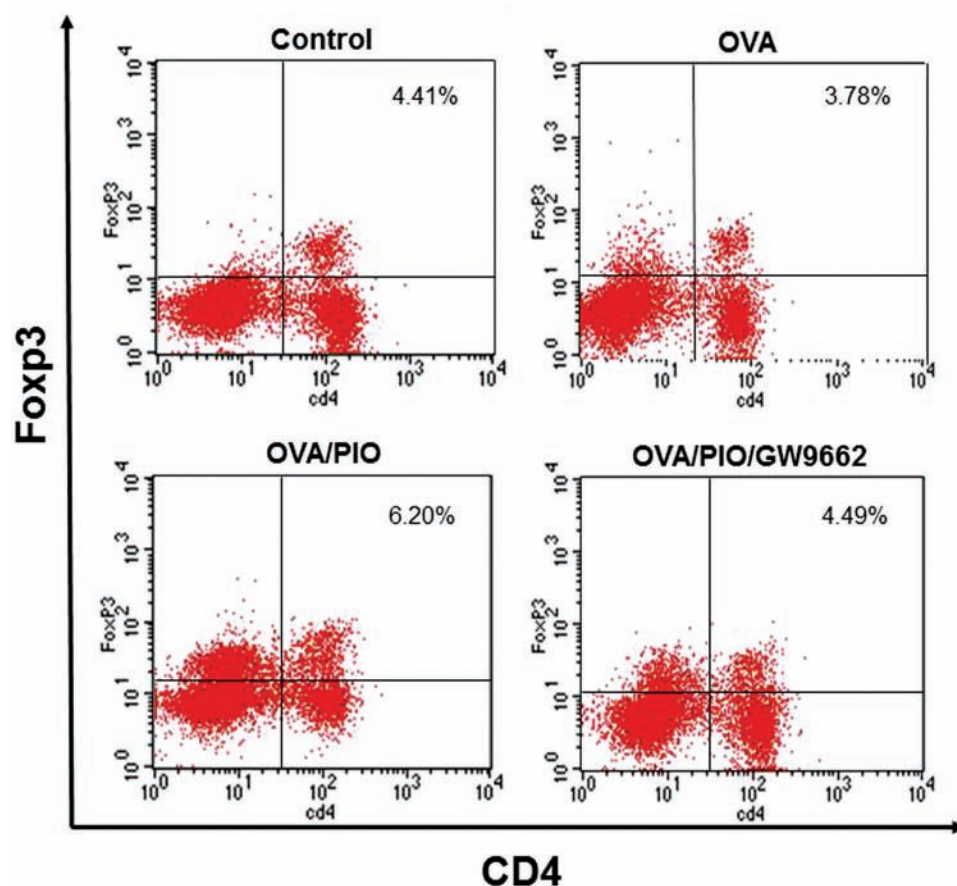


Figure 4. Effect of pioglitazone (PIO) on the induction of regulatory T cells. Representative data from 1 of 6 experiments for each group. Foxp3, forkhead box p3; OVA, ovalbumin.

Effect of PIO on Tregs Induction

We next examined the proportion of Tregs from whole spleen cells by flow cytometry analysis. After sensitization and challenge with OVA, there was a decrease in the percentage of Tregs ($3.93\% \pm 0.16\%$) compared with control ($4.38\% \pm 0.11\%$), $P = .012$. PIO treatment significantly increased the percentage of Tregs by $6.27\% \pm 0.17\%$, $P < .001$. After GW9662 cotreatment, Tregs counts were decreased to $4.53\% \pm 0.25\%$, $P < .001$, compared with the OVA/PIO group. **Figure 4** shows the representative flow cytometry analysis of Tregs.

Discussion

We studied the effects of PPAR- γ agonist on upper airway allergic inflammation and examined the role of the PPAR- γ pathway in a mouse model of AR. PIO attenuated the nasal allergic symptoms and eosinophils infiltration induced upon OVA challenge, inhibited the production of key Th1 and Th2 cytokines, and increased the Tregs population. Our findings suggest a role of the PPAR- γ agonist for not only modulating airway allergic inflammation but also inducing Tregs. The anti-inflammatory effects of the PPAR- γ agonist, rosiglitazone, have been previously demonstrated in models of allergic inflammation, which focused on suppressing the overexpression of galectin-3.^{4,5} However, to our knowledge, the present

study is the first to document the beneficial effects of PIO on upper airway allergic inflammation in a PPAR- γ -dependent manner.

Beyond regulating insulin and glucose metabolism for the correction of metabolic disturbances in type II diabetes, the pleiotropic effects of PPAR- γ agonists may also be due to downregulating most cells of the innate and adaptive immune system.¹⁶⁻²² Lee et al²³ have shown that activation of PPAR- γ by PIO enhanced the expression of PPAR- γ , resulting in reduction of eosinophils inflammation and airway hyperresponsiveness. We speculate that the PPAR- γ pathway may play an important role in the anti-inflammatory effects of PIO. In the present study, we showed not only that PIO reduced upper airway allergic inflammation and eosinophils infiltration in nasal mucosa but also that GW9662, a selective antagonist of PPAR- γ , in large part reversed the beneficial effects of PIO. Although we cannot entirely exclude additional PPAR- γ -independent inhibition, the data support a major role for direct activation of the PPAR- γ pathway. In our findings, PIO also inhibited the production of important Th1 and Th2 cytokines, and GW9662 cotreatment reversed the PIO effects with respect to IL-5 and IFN- γ in nasal cavity lavage fluid.

In previous murine AR models studies, the levels of IL-5 and IFN- γ were measured using bronchoalveolar lavage.^{4,24}

To fully understand the pathogenesis of allergic inflammation at a given site, it is useful to study the local changes contributing to this process. In the present study, we did a tracheotomy on mice before collecting nasal cavity lavage fluid. Thus, the nasal cavity could be thoroughly washed with PBS through the incision of the trachea, avoiding contamination from the oral cavity and lower airway.

PIO significantly decreased IL-5 and IFN- γ concentrations in nasal cavity lavage fluid, whereas these cytokines play a central role in Th1 and Th2 inflammation. It has been shown that the development of Th1 and Th2 cells is regulated primarily by expression of transcription factors T-bet and GATA-3, respectively,²⁵ and the expression of T-bet in naive CD4⁺ T cells is induced by IFN- γ .²⁶ IFN- γ promotes the differentiation of Th1 cells and inhibits the generation of Th2 cells. Because IL-5 is known to cooperate with eotaxin in eosinophils recruitment, it is possible that the decreased local production of this Th2 cytokine can inhibit eosinophils infiltration in nasal mucosa. IL-4 maintains the allergic response via effects on T and B lymphocytes and IgE.²⁷ IL-4 production was significantly decreased by rosiglitazone in AR mice.⁵ Therefore, the decrease of Th2 responses in nasal mucosa modulates the mast cell IgE-mediated nasal symptoms, in concert with the decreased production of anti-OVA IgE.

It has been reported that PPAR- γ agonists could enhance Tregs through a PPAR- γ -dependent or PPAR- γ -independent mechanism,^{10,14,15} and this effect was mediated by increasing retinoic acid synthesis from murine splenic dendritic cells.¹⁰ Foxp3 is an obligatory intracellular marker of Tregs rather than CD25.²⁸ Our findings showed that PIO was capable of reducing GATA-3 and T-bet expression and inducing Foxp3 expression in CD4⁺ T cells after challenge with OVA. The changes of GATA-3 and T-bet expression by PIO treatment displayed the same pattern of IL-5 and IFN- γ and reversal with GW9662 cotreatment. Furthermore, administration of PIO was capable of increasing the percentage of Tregs in splenic CD4⁺ T cells. Tregs can skew toward a decreased Th1 and Th2 population.²⁹ This probably is key that the expression of Foxp3 induced by PIO results in a simultaneous decrease in expression of GATA-3 and T-bet and that PIO treatment decreases IL-5 and IFN- γ concentrations in nasal cavity lavage fluid. Induced Tregs by PIO probably could recruit the peripheral immune system and suppress the allergic responses upon OVA challenge.

This study has several important limitations. First, the PIO dose we chose was based on similar dosing in the literature,¹⁵ which is much higher than that prescribed for humans. However, the dose is within the range of PIO commonly employed in investigations of murine models of various diseases.³⁰ Second, although the PPAR- γ agonist can attenuate allergic inflammation and induce Tregs, the functional study of the induced Tregs is beyond the scope of our experiments. Future studies are needed to determine the main mechanism of PPAR- γ , in which using transgenic animals and adoptive immunity experiments may confirm the role of the induced Tregs.

Overall, the present study demonstrates that the PPAR- γ agonist PIO attenuates allergic upper airway inflammation, reverses the AR pathophysiological features, and inhibits the

production or release of key Th1 and Th2 cytokines in a PPAR- γ -dependent fashion. Induction of Tregs by PPAR- γ activation may be one of the possible mechanisms by which PIO suppresses allergic inflammation. PIO or other PPAR- γ agonists may be beneficial for the treatment of AR, and results of ongoing clinical trials will be of high interest.

Author Contributions

Weihua Wang, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published; **Zhenghua Zhu**, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published; **Bingwei Zhu**, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published; **Zhaoxin Ma**, substantial contributions to conception and design, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, final approval of the version to be published.

Disclosures

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