

Peroxisome Proliferator-Activated Receptor γ Agonist Down-Regulates IL-17 Expression in a Murine Model of Allergic Airway Inflammation¹

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Peroxisome proliferator-activated receptor γ (PPAR γ) plays a critical role in the control of airway inflammation. Recently, IL-17 has been found to be implicated in many immune and inflammatory responses, including airway inflammation. However, no data are available concerning the effect of PPAR γ on IL-17 production in airway inflammatory diseases. In this study, we used a mouse model of asthma to evaluate the effect of two PPAR γ agonists, rosiglitazone or pioglitazone, on IL-17 expression in allergic airway disease. After OVA inhalation, mice developed the typical pathophysiological features of asthma, and the expression of IL-17 protein and mRNA in the lungs was increased. Administration of rosiglitazone or pioglitazone reduced the pathophysiological features of asthma and decreased the increased IL-17 protein and mRNA expression after OVA inhalation. In addition, the attenuating effect of PPAR γ agonist on allergic airway inflammation and bronchial hyperresponsiveness is abrogated by coadministration of rIL-17. This study also showed that the inhibition of IL-17 activity with anti-IL-17 Ab remarkably reduced the increased numbers of inflammatory cells of the airways, airway hyperresponsiveness, and the increased levels of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid and OVA-specific IgE in serum. In addition, we found that administration of rosiglitazone or pioglitazone decreased the increased NF- κ B activity and that a NF- κ B inhibitor, BAY 11-7085, substantially reduced the increased IL-17 protein levels in the lung tissues after OVA inhalation. These findings suggest that the therapeutic effect of PPAR γ in asthma is partly mediated by regulation of IL-17 expression via NF- κ B pathway. *The Journal of Immunology*, 2009, 183: 0000–0000.

Asthma is a chronic inflammatory disease of the airways that is characterized by reversible airway obstruction and airway hyperresponsiveness (1). Airway inflammation in asthma is orchestrated by various cell types, particularly eosinophils, lymphocytes, and mast cells. Activation of these cells leads to the release of proinflammatory cytokines and mediators, which in turn cause airway hyperresponsiveness, vascular leakage, inflammatory cell infiltration, mucus hypersecretion, and airway remodeling.

IL-17 family is a recently described group of cytokines and consists of six members, namely IL-17 (also called IL-17A), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F (2). IL-17, the most investigated member in this family, exerts a wide variety of biological activities due to the ubiquitous distribution of its receptor (2). IL-17 has been implicated in many immune and inflammatory responses primarily as a proinflammatory regulator by inducing the expression of many inflammatory mediators, which include cytokines, chemokines, adhesion molecules, and

growth factors (3–6). Increased levels of IL-17 have been closely associated with a number of diseases, such as rheumatoid arthritis, inflammatory bowel diseases, allograft rejection, and autoimmune diseases (7, 8). A previous study analyzing sputum from the patients has reported that excessive IL-17 induction contributes to airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease (9). In addition, IL-17 mRNA and/or protein expression have been shown to be increased in lung cells, bronchoalveolar lavage fluids (BALF)⁴, and peripheral blood from asthmatics (10–14). Furthermore, the levels of IL-17 expression in airways have been correlated with the severity of airway hyper-sensitivity in asthmatic patients (9). These findings have indicated a potential role of IL-17 in the pathogenesis of asthma.

Peroxisome proliferator-activated receptors (PPAR) are members of a nuclear receptor superfamily containing transcription factors regulating gene expression (15). They are ubiquitously expressed throughout the body and three subtypes, which are encoded by different genes, have been identified, namely PPAR α , PPAR β/δ , and PPAR γ . Among three PPAR subtypes, PPAR γ activation down-regulates the synthesis and release of immunomodulatory cytokines from various cell types that participate in the regulation of inflammatory processes. In lung tissues, PPAR γ is most abundant in airway epithelial cells (16). In addition, PPAR γ is also expressed in smooth muscle cells, myofibroblasts, endothelial cells of the pulmonary vasculature, and inflammatory cells such as alveolar macrophages, neutrophils, eosinophils, lymphocytes, and mast cells (17, 18). Previous studies have shown that PPAR γ is

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⁴ Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff; PPAR, peroxisome proliferator-activated receptor; Rrs, respiratory system resistance.

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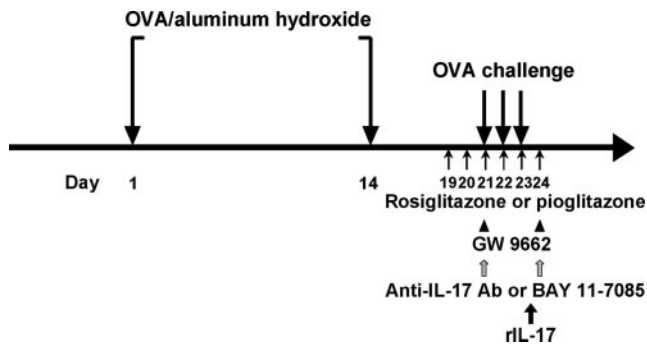


FIGURE 1. Schematic diagram of the experimental protocol. Mice were sensitized on days 1 and 14 by i.p. injection of 20 μ g of OVA emulsified in 1 mg of aluminum hydroxide. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) OVA in saline (or with saline as a control) using an ultrasonic nebulizer. Rosiglitazone or pioglitazone was administered by oral gavage six times at a 24-h interval on days 19–24, beginning 2 days before the first challenge. GW9662 was administered intratracheally twice to each animal, once on day 21 and the second time on day 24. In the case of treatment with anti-IL-17 Ab, isotype control mAb, or BAY 11-7085, it was administered i.p. twice to each animal under the same administration schedule described above. For IL-17 replacement experiments, murine rIL-17 was administered intratracheally at 12 h after the last OVA challenge in a volume of 50 μ l of sterile PBS.

involved in airway inflammation and remodeling in asthma (19–22). PPAR γ expression is increased in bronchial submucosa, airway epithelium, and smooth muscle cells of asthmatics as compared with healthy subjects (19). It has been hypothesized that the up-regulation of PPAR γ in asthma represents a self-regulatory mechanism for preventing airway inflammatory and remodeling. Recent studies have shown that activation of PPAR γ reduces expression of various cytokines, airway hyperresponsiveness, and activation of eosinophils, which are increased in asthma, suggesting therapeutic potential of PPAR γ agonists for asthma (20, 21). However, no data are available concerning the effect of PPAR γ on IL-17 production in allergic airway inflammatory disease. In this study, we used a murine model of asthma to evaluate the effect of the PPAR γ agonists rosiglitazone or pioglitazone on IL-17 expression and to investigate the molecular mechanisms by which PPAR γ regulates IL-17 expression in allergic airway inflammation.

Materials and Methods

Animals and experimental protocol

Female C57BL/6 mice, 8–10 wk of age and free of murine-specific pathogens, were obtained from the Orientbio, were housed throughout the experiments in a laminar flow cabinet, and were maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University (23). Mice were sensitized on days 1 and 14 by i.p. injection of 20 μ g of OVA (Sigma-Aldrich) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical) in a total volume of 200 μ l, as described previously (Fig. 1) (24, 25). On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12; Omron).

Administration of rosiglitazone, pioglitazone, GW9662, murine rIL-17, anti-IL-17 Ab, isotype control mAb, or BAY 11-7085

Rosiglitazone (5 mg/kg body weight/day; GlaxoSmithKline) dissolved in distilled water or pioglitazone (10 mg/kg body weight/day; Takeda Chemical Industries) dissolved in DMSO and diluted with 0.9% NaCl was administered six times by oral gavage at a 24-h interval on days 19–24, beginning 2 days before the first challenge with OVA, as previously de-

scribed with some modifications (Fig. 1) (21, 24). A selective antagonist of PPAR γ , GW9662 (0.5 mg/kg body weight/day; Cayman Chemical), dissolved in PBS, was administered intratracheally two times to each animal, once on day 21 and the second time on day 24. For IL-17 replacement experiments, 1.5 μ g of murine rIL-17 (R&D Systems) was administered intratracheally at 12 h after the last OVA challenge in a volume of 50 μ l of sterile PBS, as described elsewhere (26, 27). Anti-IL-17 Ab or isotype control mAb (5 mg/kg body weight/day; R&D Systems) was administered i.p. twice to each animal, once on day 21 and the second time on day 24. An inhibitor of NF- κ B activation, BAY 11-7085 (20 mg/kg body weight/day; BIOMOL), dissolved in DMSO and diluted with 0.9% NaCl, was administered by i.p. injection twice to each animal, once on day 21 and the second time on day 24.

Collection of blood and BAL samples

At 48 h after the last challenge, mice were sacrificed with an overdose of pentobarbital sodium (100 mg/kg body weight, administered i.p.). Blood was drawn by puncturing the vena cava and centrifuged. Serum was snap frozen in liquid nitrogen and stored at -70°C for OVA-specific IgE measurements. BAL was performed as described previously (18). Total cell numbers were counted using a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron). The smears were stained with Diff-Quik solution (Dade Diagnostics) to determine differential cell counts.

Western blot analysis

Protein expression levels were analyzed by means of Western blot analysis, as described previously (18, 21). The blots were then incubated with an anti-IL-17 Ab (R&D Systems).

RNA isolation and RT-PCR

Total RNA was isolated from lung tissues using a rapid extraction method (TRI-Reagent) as described previously (28). The primers used were as follows: IL-17, sense, 5'-TCTCATCCAGCAAGAGATCC-3', and antisense, 5'-AGTTTGGGACCCCTTTACAC-3'; PPAR γ , sense, 5'-ATGCCATTCTGGCCCACTT-3', and antisense, 5'-CCCTTG CATCCTTCACAAGCATG-3'; and GAPDH, sense, 5'-GCCATCAA CGACCCCTTCATTGAC-3', and antisense, 5'-ACGGAAGGCCAT GCCAGTGACTT-3'.

Quantitative real-time RT-PCR

Quantitative RT-PCR analysis was performed using the LightCycler Fast-Start DNA Master SYBR Green I (Roche Diagnostics). The sequences of primers used were as follows: IL-17, sense, 5'-TCTCATCCAGCAAGAG ATCC-3', and antisense, 5'-AGTTTGGGACCCCTTTACAC-3'; PPAR γ , sense, 5'-ATGCCATTCTGGCCCACTT-3', and antisense, 5'-CC CTTGCATCCTTCACAAGCATG-3'; and β -actin, sense, 5'-CAGATCAT GTTTGAGACCTTC-3', and antisense, 5'-ACTTCATGATGGAATTGA ATG-3'. Calculation of the relative mRNA levels of each sample was performed according to the manufacturer's protocol.

Measurement of Th2 cytokines

Levels of IL-4, IL-5, and IL-13 were quantified in the supernatants of BALF by enzyme immunoassays according to the manufacturer's protocol (IL-4, BioSource International; IL-5 and IL-13, R&D Systems).

Measurement of serum OVA-specific IgE

OVA-specific IgE levels were measured by ELISA according to the manufacturer's protocol, using a mouse OVA-IgE ELISA kit (MD Biosciences).

Histology

For histological examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems Nussloch), placed on glass slides, deparaffinized, and stained sequentially with H&E (Richard-Allan Scientific) or periodic acid-Schiff (PAS).

Quantitation of airway mucus expression

To quantitate the level of mucus expression in the airway, the numbers of PAS-positive and PAS-negative epithelial cells in individual bronchioles were counted as described previously (29, 30). Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchiole divided by the total number of epithelial cells of each bronchiole.

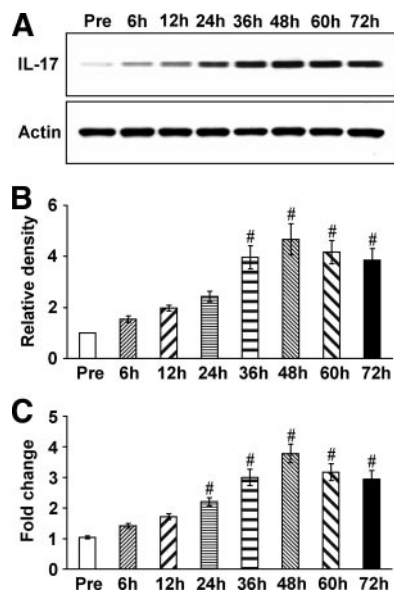


FIGURE 2. IL-17 protein levels and mRNA expression in lung tissues of OVA-sensitized and -challenged mice. Sampling of lung tissues was performed from sensitized mice challenged with OVA. *A*, Western blot analyses of IL-17 protein. *B*, Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 to actin in lung tissues before OVA inhalation is arbitrarily presented as 1. *C*, Quantitative analyses of IL-17 mRNA expression by means of real-time RT-PCR. The relative ratio of IL-17 mRNA to β -actin mRNA in lung tissues before OVA inhalation is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group. Six, 12, 24, 36, 48, 60, and 72 h are time periods after the challenge. Pre, 1 h before the first challenge; #, $p < 0.05$ vs Pre.

Cytosolic or nuclear protein extractions for analyses of NF- κ B p65 and PPAR γ

Cytosolic or nuclear extractions for analyses of NF- κ B p65 and PPAR γ were performed as described previously (21, 25). The levels of NF- κ B p65 and PPAR γ were analyzed by Western blotting using NF- κ B p65 Ab (Upstate Biotechnology) or PPAR γ Ab (Santa Cruz Biotechnology).

Determination of airway responsiveness

Airway responsiveness was assessed as described elsewhere (31). The data of respiratory system resistance (Rrs) were collected as an invasive measure of the airway responsiveness to methacholine by using a computer-controlled small animal ventilator (flexiVent; SCIREQ).

Densitometric analyses and statistics

All immunoreactive signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad). Data were expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by Scheffé's test. The unpaired Student's *t* test was used to compare between two groups. Statistical significance was set at $p < 0.05$.

Results

IL-17 protein levels and mRNA expression increased in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that levels of IL-17 protein in lung tissues were increased ~ 1.5 -, 2.0 -, 2.4 -, 4.0 -, 4.7 -, 4.2 - and 3.9 -fold at 6, 12, 24, 36, 48, 60, and 72 h after OVA inhalation, respectively, compared with the prechallenge period (Fig. 2, *A* and *B*). Real-time RT-PCR analysis revealed that IL-17 mRNA expression was increased ~ 1.4 -, 1.7 -, 2.2 -, 3.0 -, 3.8 -, 3.2 -, and 2.9 -fold at 6, 12, 24, 36, 48, 60, and 72 h after OVA inhalation, respectively, compared with the prechallenge period (Fig. 2*C*).

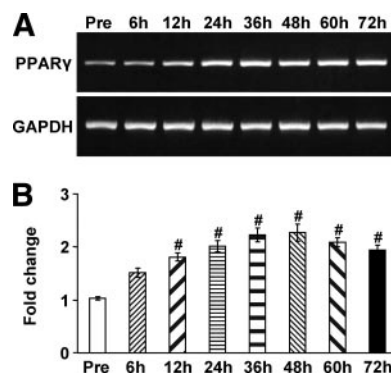


FIGURE 3. PPAR γ mRNA expression in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed in lung tissues from sensitized mice challenged with OVA. *A*, Representative RT-PCR analysis of PPAR γ mRNA expression. *B*, Quantitative analyses of PPAR γ mRNA expression by means of real-time RT-PCR. The relative ratio of PPAR γ mRNA to β -actin mRNA in lung tissues before OVA inhalation is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group. Six, 12, 24, 36, 48, 60, and 72 h are time periods after the challenge. Pre, 1 h before the first challenge; #, $p < 0.05$ vs Pre.

PPAR γ mRNA expression increased in lung tissues of OVA-sensitized and -challenged mice

Real-time RT-PCR analysis revealed that expression of PPAR γ mRNA was increased ~ 1.5 -, 1.8 -, 2.0 -, 2.2 -, 2.2 -, 2.0 -, and 1.9 -fold at 6, 12, 24, 36, 48, 60, and 72 h after OVA inhalation, respectively, compared with the prechallenge period (Fig. 3).

Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on IL-17 protein levels and mRNA expression in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that the increased levels of IL-17 protein at 48 h after OVA inhalation were decreased significantly by the administration of rosiglitazone or pioglitazone (Fig. 4, *A* and *B*). The rosiglitazone-mediated decrease of IL-17 protein level was blocked by cotreatment with a PPAR γ antagonist, GW9662. RT-PCR and real-time RT-PCR analyses showed that the increased IL-17 mRNA expression after OVA inhalation was significantly reduced by the administration of rosiglitazone or pioglitazone (Fig. 4, *C* and *D*). The inhibitory effect of rosiglitazone treatment on IL-17 mRNA expression in lung tissues was abrogated when GW9662 was administered concomitantly with the agonist. These results indicate that the effect of rosiglitazone treatment on IL-17 expression was mainly acting through PPAR γ in this model.

Effect of rosiglitazone or pioglitazone on IL-17 protein levels in BALF of OVA-sensitized and -challenged mice

Western blot analysis revealed that levels of IL-17 protein in BALF were significantly increased at 48 h after OVA inhalation compared with those in the control mice (Fig. 5). The increased IL-17 protein levels after OVA inhalation were significantly decreased by the administration of rosiglitazone or pioglitazone.

Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on cellular changes in BALF

Numbers of total cells, lymphocytes, neutrophils, and eosinophils in BALF were increased significantly at 48 h after OVA inhalation compared with the numbers after saline inhalation (Fig. 6*A*). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly reduced by the administration of

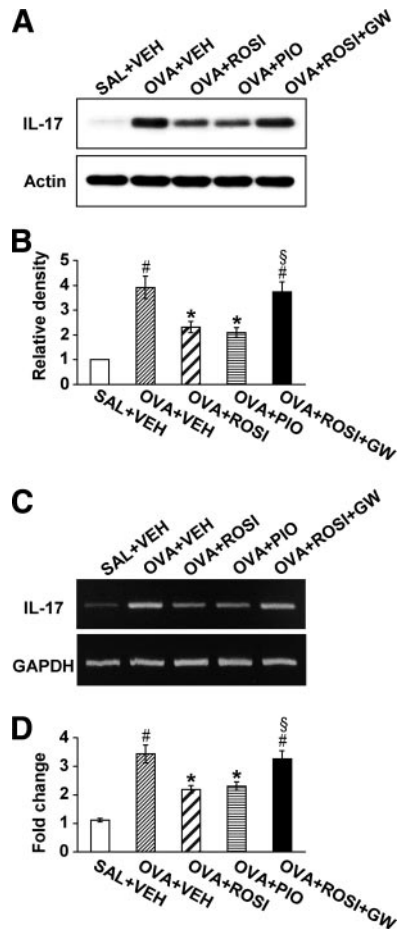


FIGURE 4. Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on IL-17 protein levels and mRNA expression in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (0.05% DMSO) (SAL+VEH), OVA-inhaled mice administered drug vehicle (0.05% DMSO) (OVA+VEH), OVA-inhaled mice administered rosiglitazone (OVA+ROSI), OVA-inhaled mice administered pioglitazone (OVA+PIO), and OVA-inhaled mice administered GW9662 plus rosiglitazone (OVA+ROSI+GW). **A**, Western blot analyses of IL-17 protein. **B**, Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 in the lung tissues of SAL+VEH is arbitrarily presented as 1. **C**, Representative RT-PCR analyses of IL-17 mRNA expression. **D**, Quantitative analyses of IL-17 mRNA expression by means of real-time RT-PCR. Data represent mean $\pm$ SEM from seven mice per group. #, $p < 0.05$ vs SAL+VEH; *, $p < 0.05$ vs OVA+VEH; §, $p < 0.05$ vs OVA+ROSI.

rosiglitazone or pioglitazone. The inhibitory effect of rosiglitazone treatment on numbers of total cells, lymphocytes, neutrophils, and eosinophils in BALF was abrogated when a PPAR γ antagonist, GW9662, was administered concomitantly with the agonist.

Effect of rosiglitazone or pioglitazone on pathological changes of OVA-induced asthma

Histological analyses revealed typical pathologic features of asthma in the OVA-exposed mice. Numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles and mucus and debris had accumulated in the lumens of bronchioles (Fig. 6C) as compared with the control (Fig. 6B). Mice treated with rosiglitazone (Fig. 6D) or pioglitazone (Fig. 6E) showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar region and in the amount of debris in the airway lumens.

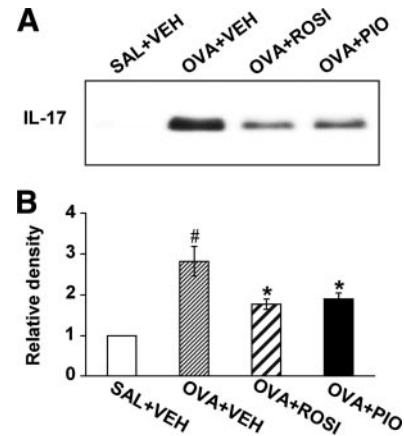


FIGURE 5. Effect of rosiglitazone or pioglitazone on IL-17 protein levels in BALF of OVA-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered rosiglitazone (OVA+ROSI), and OVA-inhaled mice administered pioglitazone (OVA+PIO). **A**, Western blotting of IL-17. **B**, Densitometric analyses are presented as the relative ratio of IL-17 levels in OVA+VEH, OVA+ROSI, or OVA+PIO to those in SAL+VEH. The IL-17 protein level in BALF of SAL+VEH is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group. #, $p < 0.05$ vs SAL+VEH; *, $p < 0.05$ vs OVA+VEH.

Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on airway hyperresponsiveness

Airway responsiveness was assessed as a percent increase of Rrs in response to increasing doses of methacholine. In OVA-sensitized and -challenged mice, the dose-response curve of percent Rrs shifted to the left compared with that of the control mice (Fig. 6F). In addition, the percent Rrs produced by methacholine administration (at dose of 50 mg/ml) increased significantly in the OVA-inhaled mice compared with the control mice. OVA-sensitized and -challenged mice treated with rosiglitazone or pioglitazone showed the dose-response curve of percent Rrs that shifted to the right and a significant reduction in the percent Rrs produced by methacholine at 50 mg/ml dose compared with those of untreated mice. The inhibitory effect of rosiglitazone treatment on airway hyperresponsiveness was abrogated by cotreatment with GW9662.

Effect of rosiglitazone or pioglitazone on levels of IL-4, IL-5, and IL-13 in BALF of OVA-sensitized and -challenged mice

Enzyme immunoassays showed that levels of IL-4, IL-5, and IL-13 in BALF were significantly increased at 48 h after OVA inhalation compared with those in the control mice (Fig. 6G). The increased levels of these cytokines in BALF after OVA inhalation were significantly reduced by the administration of rosiglitazone or pioglitazone.

Effect of rosiglitazone or pioglitazone on OVA-specific IgE levels in serum of OVA-sensitized and -challenged mice

Administration of rosiglitazone or pioglitazone to OVA-sensitized and -challenged mice significantly decreased OVA-specific IgE levels in serum compared with the levels in OVA-inhaled mice administered drug vehicle (Fig. 6H).

Effect of rosiglitazone or pioglitazone on airway mucus expression of OVA-sensitized and -challenged mice

Percentage of airway epithelium, which stained positively with PAS in OVA-sensitized and -challenged mice (Fig. 7, B and E),

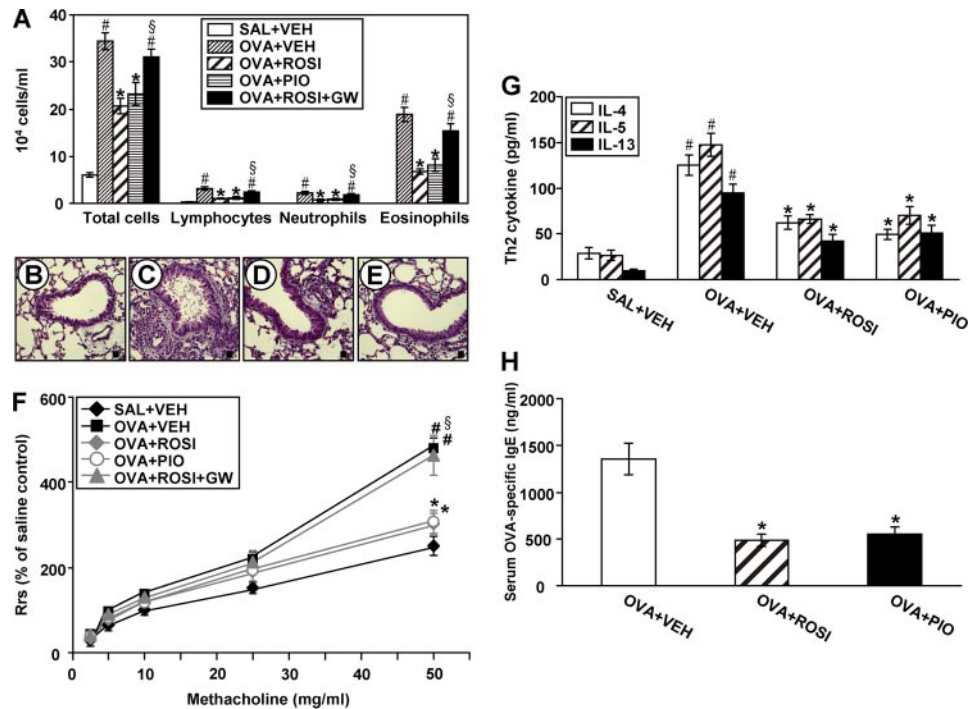


FIGURE 6. A, Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on total and differential cell counts in BALF of OVA-sensitized and -challenged mice. Differential cell counts in BALF from saline-inhaled mice administered drug vehicle (SAL + VEH), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered rosiglitazone (OVA + ROSI), OVA-inhaled mice administered pioglitazone (OVA + PIO), and OVA-inhaled mice administered GW9662 plus rosiglitazone (OVA + ROSI + GW). B–E, Effect of rosiglitazone or pioglitazone in lung tissues of OVA-sensitized and -challenged mice. Representative H&E-stained sections of the lungs. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (B), OVA-inhaled mice administered drug vehicle (C), OVA-inhaled mice administered rosiglitazone (D), and OVA-inhaled mice administered pioglitazone (E). Bars indicate scale of 20 μ m. F, Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on airway responsiveness in OVA-sensitized and -challenged mice. Airway responsiveness was measured at 48 h after the last challenge in SAL + VEH, OVA + VEH, OVA + ROSI, OVA + PIO, and OVA + ROSI + GW. G, Effect of rosiglitazone or pioglitazone on levels of Th2 cytokines in BALF of OVA-sensitized and -challenged mice. Enzyme immunoassay of IL-4, IL-5, and IL-13 in BALF. H, Effect of rosiglitazone or pioglitazone on OVA-specific IgE levels in serum of OVA-sensitized and -challenged mice. Levels of OVA-specific IgE in serum were analyzed using ELISA. Sampling was performed at 48 h after the last challenge in OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered rosiglitazone (OVA + ROSI), and OVA-inhaled mice administered pioglitazone (OVA + PIO). Data represent mean $\pm$ SEM from seven mice per group. #, $p < 0.05$ vs SAL + VEH; *, $p < 0.05$ vs OVA + VEH; §, $p < 0.05$ vs OVA + ROSI.

was significantly greater than that in the control mice (Fig. 7, A and E). Administration of rosiglitazone (Fig. 7, C and E) or pioglitazone (Fig. 7, D and E) to OVA-sensitized and -challenged mice

significantly reduced the percentage of airway epithelium staining positively with PAS compared with that of OVA-inhaled mice administered drug vehicle (Fig. 7, B and E).

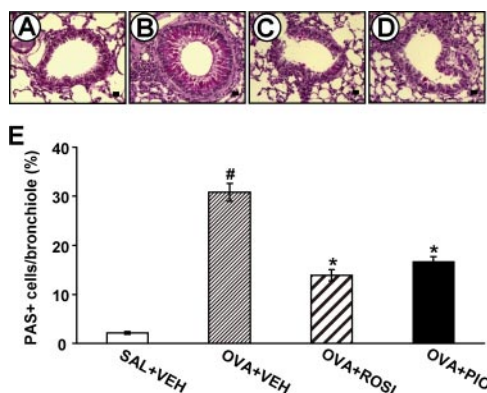


FIGURE 7. Effect of rosiglitazone or pioglitazone on airway mucus expression in OVA-sensitized and -challenged mice. A–D, Representative PAS-stained sections of the lungs. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (A), OVA-inhaled mice administered drug vehicle (B), OVA-inhaled mice administered rosiglitazone (C), and OVA-inhaled mice administered pioglitazone (D). The violet color indicates PAS-positive mucus expression. Bars indicate 20 μ m. E, Quantitation of airway mucus expression. Bars represent mean $\pm$ SEM from seven mice per group. #, $p < 0.05$ vs SAL + VEH; *, $p < 0.05$ vs OVA + VEH.

Effect of rIL-17 plus pioglitazone on cellular changes in BALF and airway hyperresponsiveness

To verify that PPAR γ -induced inhibition of IL-17 expression is important for attenuation of the allergic airway response, we performed the replacement experiments using rIL-17. The inhibitory effect of pioglitazone on the increase in numbers of total cells, lymphocytes, neutrophils, and eosinophils in BALF was abrogated when rIL-17 was administered to OVA-sensitized and -challenged mice treated with pioglitazone (Fig. 8A). In addition, administration of rIL-17 to OVA-inhaled mice treated with pioglitazone shifted a dose-response curve of percent Rrs to the left and increased significantly the percent Rrs produced by methacholine administration (at dose of 50 mg/ml), as compared with those in OVA-inhaled mice treated with pioglitazone plus PBS (Fig. 8B). These results indicate that exogenous IL-17 treatment abrogates the inhibitory effect of pioglitazone on airway inflammation and bronchial hyperresponsiveness.

Effect of anti-IL-17 Ab on cellular changes in BALF

Cellular changes were examined after administering anti-IL-17 Ab to investigate whether inhibition of IL-17 activity reduces airway inflammation. The administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice significantly decreased the increased

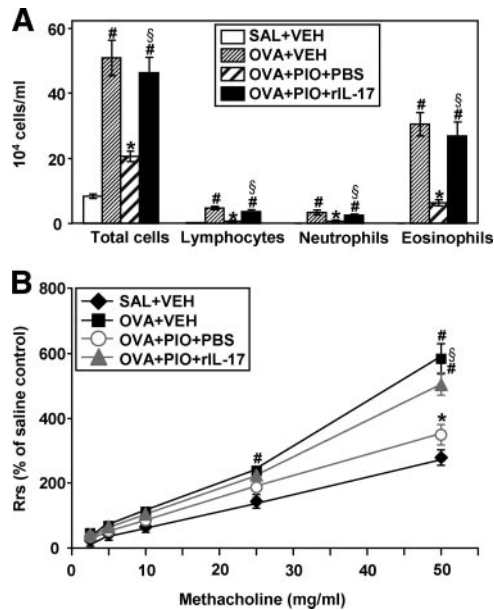


FIGURE 8. Effect of rIL-17 plus pioglitazone on total and differential cell counts in BAL fluids and airway responsiveness of OVA-sensitized and -challenged mice. **A**, Differential cell counts in BALF from saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered pioglitazone plus PBS (OVA+PIO+PBS), and OVA-inhaled mice administered pioglitazone plus rIL-17 (OVA+PIO+rIL-17) were determined at 48 h after the last challenge. **B**, Airway responsiveness was measured at 48 h after the last challenge in SAL+VEH, OVA+VEH, OVA+PIO+PBS, and OVA+PIO+rIL-17. Data represent mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs SAL+VEH; *, $p < 0.05$ vs OVA+VEH; §, $p < 0.05$ vs OVA+PIO+PBS.

numbers of total cells, lymphocytes, neutrophils, and eosinophils compared with the numbers in OVA-inhaled mice administered isotype control mAb (Fig. 9A).

Effect of anti-IL-17 Ab on airway hyperresponsiveness

Administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice shifted a dose-response curve of percent Rrs to the right and decreased significantly the percent Rrs produced by methacholine administration (at dose of 50 mg/ml), as compared with those in OVA-inhaled mice administered isotype control mAb (Fig. 9B). These results indicate that anti-IL-17 Ab treatment reduces OVA-induced airway hyperresponsiveness.

Effect of anti-IL-17 Ab on IL-4, IL-5, and IL-13 levels in BALF

Administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice significantly decreased levels of IL-4, IL-5, and IL-13 in BALF compared with the levels in OVA-inhaled mice administered isotype control mAb (Fig. 9C).

Effect of anti-IL-17 Ab on OVA-specific IgE levels in serum

Administration of anti-IL-17 Ab to OVA-sensitized and -challenged mice significantly decreased OVA-specific IgE levels in serum compared with the levels in OVA-inhaled mice administered isotype control mAb (Fig. 9D).

Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on NF- κ B p65 protein levels in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that levels of NF- κ B p65 in nuclear protein extracts from lung tissues were increased at 48 h after

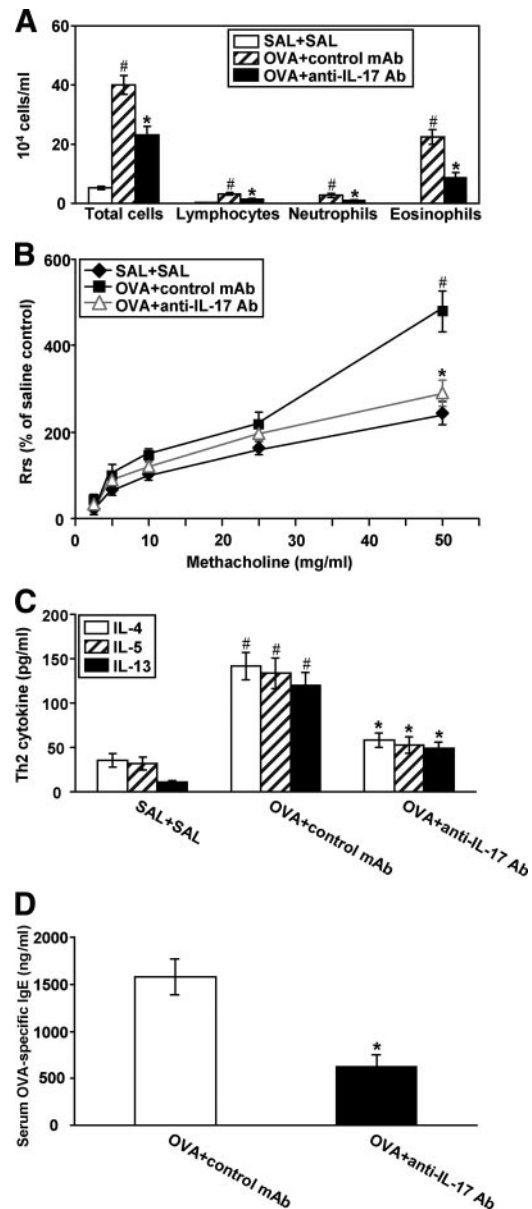


FIGURE 9. Effect of anti-IL-17 Ab on total and differential cell counts in BALF (**A**), on airway responsiveness (**B**), on Th2 cytokine levels in BALF (**C**), and on OVA-specific IgE levels in serum (**D**) of OVA-sensitized and -challenged mice. **A**, Differential cell counts in BALF from saline-inhaled mice administered saline (SAL+SAL), OVA-inhaled mice administered isotype control mAb (OVA+control mAb), and OVA-inhaled mice administered anti-IL-17 Ab (OVA+anti-IL-17 Ab). **B**, Airway responsiveness was measured at 48 h after the last challenge in SAL+SAL, OVA+anti-IL-17 Ab, and OVA+control mAb. **C**, Enzyme immunoassay of IL-4, IL-5, and IL-13 in BALF. **D**, The levels of OVA-specific IgE in serum were analyzed using ELISA. Sampling was performed at 48 h after the last challenge in OVA-inhaled mice administered isotype control mAb (OVA+control mAb) and OVA-inhaled mice administered anti-IL-17 Ab (OVA+anti-IL-17 Ab). Data represent mean $\pm$ SEM from five mice per group. #, $p < 0.05$ vs SAL+SAL; *, $p < 0.05$ vs OVA+control mAb.

OVA inhalation compared with the levels in the control mice administered saline (Fig. 10). The increased NF- κ B p65 levels in nuclear protein extracts after OVA inhalation were significantly decreased by the administration of rosiglitazone or pioglitazone. In contrast, the levels of NF- κ B p65 in cytosolic protein fractions from lung tissues were decreased after OVA inhalation compared with the levels in the control mice administered saline. The decreased

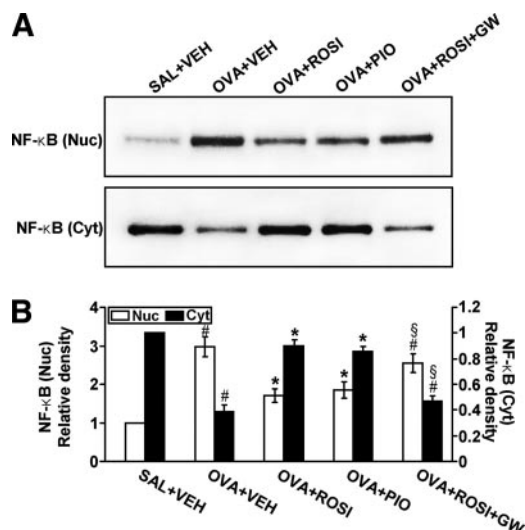


FIGURE 10. Effect of rosiglitazone, pioglitazone, or GW9662 plus rosiglitazone on NF- κ B p65 protein level in lung tissues of OVA-sensitized and -challenged mice. Levels of NF- κ B p65 were measured at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered rosiglitazone (OVA+ROSI), OVA-inhaled mice administered pioglitazone (OVA+PIO), and OVA-inhaled mice administered GW9662 plus rosiglitazone (OVA+ROSI+GW). A, Western blot analyses of NF- κ B p65 levels in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. B, Densitometric analyses are presented as the relative ratio of NF- κ B p65 levels in OVA+VEH, OVA+ROSI, OVA+PIO, or OVA+ROSI+GW to those in SAL+VEH. The relative ratio of NF- κ B in nuclear protein extracts from the lung tissues of SAL+VEH is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group. #, $p < 0.05$ vs SAL+VEH; *, $p < 0.05$ vs OVA+VEH; §, $p < 0.05$ vs OVA+ROSI.

NF- κ B p65 levels in the cytosolic protein fractions were substantially increased by the administration of rosiglitazone or pioglitazone. However, no significant changes were observed in OVA-sensitized and -challenged mice treated with GW9662 plus rosiglitazone.

Effect of BAY 11-7085 on IL-17 protein levels in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis showed that the increased IL-17 protein levels after OVA inhalation were significantly reduced by the administration of BAY 11-7085, an inhibitor of NF- κ B activation (Fig. 11).

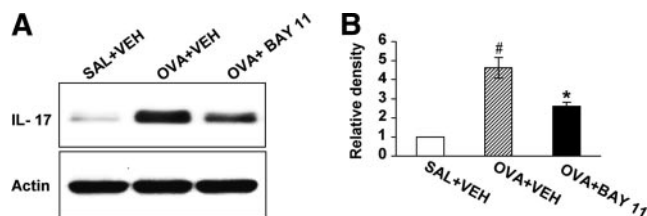


FIGURE 11. Effect of BAY 11-7085 on IL-17 protein levels in lung tissues of OVA-sensitized and -challenged mice. Sampling was performed at 48 h after the last challenge in saline-inhaled mice administered drug vehicle (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), and OVA-inhaled mice administered BAY 11-7085 (OVA+BAY 11). A, Western blotting of IL-17. B, Densitometric analyses are presented as the relative ratio of IL-17 to actin. The relative ratio of IL-17 in the lung tissues of SAL+VEH is arbitrarily presented as 1. Data represent mean $\pm$ SEM from seven mice per group. #, $p < 0.05$ vs SAL+VEH; *, $p < 0.05$ vs OVA+VEH.

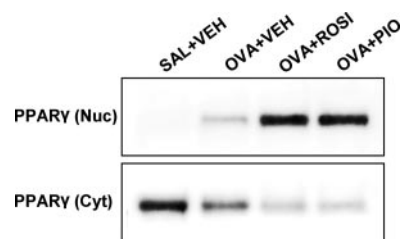


FIGURE 12. Effect of rosiglitazone or pioglitazone on PPAR γ protein expression in lung tissues of OVA-sensitized and -challenged mice. Western blotting of PPAR γ in nuclear (Nuc) and cytosolic (Cyt) protein extracts from lung tissues. PPAR γ protein expression was measured at 48 h after the last challenge in saline-inhaled mice administered saline (SAL+VEH), OVA-inhaled mice administered drug vehicle (OVA+VEH), OVA-inhaled mice administered rosiglitazone (OVA+ROSI), and OVA-inhaled mice administered pioglitazone (OVA+PIO). Results were similar in seven mice per group.

Effect of rosiglitazone or pioglitazone on PPAR γ protein expression in lung tissues of OVA-sensitized and -challenged mice

Western blot analysis revealed that PPAR γ expression in nuclear protein extracts from lung tissues were increased at 48 h after OVA inhalation compared with the levels in the control mice administered saline (Fig. 12). The increased PPAR γ expression in nuclear protein extracts was further increased by the administration of rosiglitazone or pioglitazone. In contrast, PPAR γ expression in cytosolic protein fractions from lung tissues was decreased after OVA inhalation compared with that in the control mice administered saline. The decreased PPAR γ expression in cytosolic protein fractions was further decreased by the administration of rosiglitazone or pioglitazone.

Discussion

PPAR γ activation down-regulates the synthesis and release of many cytokines from various cell types that participate in the inflammatory processes (32, 33). Reports indicating a therapeutic effect of PPAR γ agonists in asthma have accumulated over the years (20, 21). However, the effect of PPAR γ on the expression of IL-17 in airway inflammatory disease has not been clarified. In this study, an OVA-induced model of asthma revealed that numbers of inflammatory cells of the airways, airway hyperresponsiveness, airway mucus expression, and levels of Th2 cytokines in BALF and of OVA-specific IgE in serum, as well as the expression of IL-17 protein and mRNA in the lungs are increased after OVA inhalation. Administration of the PPAR γ agonists rosiglitazone or pioglitazone reduced the pathophysiological signs of asthma and decreased the increased IL-17 protein and mRNA expression after OVA inhalation. In addition, the attenuating effect of PPAR γ agonist on allergic airway inflammation and bronchial hyperresponsiveness is abrogated by coadministration of rIL-17. Our results also showed that inhibiting IL-17 activity with anti-IL-17 Ab remarkably reduces airway inflammation, airway hyperresponsiveness, and the increased levels of Th2 cytokines in BALF and OVA-specific IgE in serum. In addition, we found that the increased NF- κ B activity after OVA inhalation is decreased by the administration of the PPAR γ agonists and that the inhibition of NF- κ B activation reduces the increase of IL-17 protein levels in lung tissues. These results suggest that the therapeutic effect of PPAR γ in asthma is partly mediated by down-regulating IL-17 expression via the modulation of NF- κ B activity. To the best of our knowledge, this is the first study to clarify the effect of PPAR γ on IL-17 expression in allergic airway inflammation.

IL-17 is a comparatively recently discovered cytokine, which has attracted the attention of many scientists because of its involvement in immune and inflammatory responses (5, 34). IL-17

in the airways is most likely produced by a unique Th lineage called Th17 cells (35). IL-17 is also expressed in eosinophils, neutrophils, and monocytes (10, 34). The biological function of IL-17 is associated neutrophil-dominated inflammation, as a promoter of granulopoiesis, neutrophil accumulation, and neutrophil activation in the lung (5, 34). A previous study on a murine model of allergic asthma has shown that IL-17 expression in airways is up-regulated upon allergen inhalation, concomitant with the induction of bronchial neutrophilic influx (36). Accordingly, it has been proposed that IL-17 orchestrates the neutrophilic influx into airways in asthma. The effect of IL-17 on neutrophilic recruitment may be caused by the induction of neutrophil-directed chemokines and cytokines such as CXCL1, CXCL8, CCL4, G-CSF, GM-CSF, and ICAM-1 (37–39). Interestingly, a recent study (40) has demonstrated that IL-17R gene-deficient mice show a reduced recruitment of not only neutrophils but also eosinophils into airways upon Ag challenge. In addition, eosinophil peroxidase activities in lung tissues and OVA-specific serum IgE concentrations were reduced in the absence of IL-17R signaling (40). Furthermore, IL-17 has also been reported to be involved in the activation of allergen-specific T cells (41). Both IL-17-deficient and IL-17R-deficient mice revealed the decreased levels of Th2 cytokines, which are associated with reduced airway hypersensitivity (40, 41). Consistent with these observations, the present study showed that the expression of IL-17 protein and mRNA is up-regulated in OVA-induced allergic airway disease. To clarify the role of IL-17 in pathogenesis of allergic airway inflammation, in this current study, we performed an experiment using an anti-IL-17-blocking Ab. Our data showed that the inhibition of IL-17 activity with the anti-IL-17 Ab remarkably reduced Ag-induced airway infiltration of inflammatory cells, including eosinophils and neutrophils, airway hyperresponsiveness, and the increased levels of Th2 cytokines in BALF and OVA-specific IgE in serum. Moreover, to support these mechanistic insights for the effects of IL-17 on allergic airway disease, we examined whether the attenuation of asthmatic phenotypes (i.e., the increase of airway inflammatory cells recruited to lungs and bronchial hyperresponsiveness) is restored by exogenous IL-17 administration to OVA-inhaled mice treated with PPAR γ agonist pioglitazone. The results showed that the administration of rIL-17 abrogates the inhibitory effect of pioglitazone on airway inflammation and bronchial hyperresponsiveness. Taken together, IL-17 may contribute to allergic airway inflammation through the following mechanisms: the recruitment of airway inflammatory cells, Th2-mediated immune responses, and altering the levels of allergen-specific Ab such as IgE.

PPAR γ has been suggested to be a regulator of inflammatory and immune responses (32). Recent studies have reported that PPAR γ activation by administration of PPAR γ agonists or adenovirus carrying PPAR γ cDNA decreases Ag-induced airway inflammation, airway hyperresponsiveness, and the expression of various cytokines as well as airway remodeling, which is characterized by thickening of the smooth muscle layer, subepithelial collagen deposition, and increased airway mucus production (20–22, 42). Consistent with these observations, our results showed that administration of the PPAR γ agonists substantially ameliorate the pathophysiological features of asthma, including airway inflammation, airway hyperresponsiveness, and the increased levels of Th2 cytokines and serum OVA-specific IgE. Therefore, the beneficial effect of PPAR γ agonists on most of the pathophysiological changes associated with asthma has suggested their possible use for treating asthma. However, the mechanisms that underlie the therapeutic activity of PPAR γ in asthma remain to be elucidated. The present study found that administration of PPAR γ agonists substantially decreased the increased IL-17 protein and mRNA

expression after OVA inhalation. Supporting our results, a study has demonstrated that treatment of rosiglitazone significantly decreases expression of IL-17 in an animal model of inflammatory bowel disease (43). Moreover, we showed that exogenous IL-17 treatment abrogates the inhibitory effect of PPAR γ agonist on airway inflammation and bronchial hyperresponsiveness. Accordingly, these findings suggest that the therapeutic effect of PPAR γ in asthma is exerted by down-regulating IL-17 expression, providing a piece of evidence for a specific interaction between PPAR γ signaling and IL-17 expression in allergic airway inflammation.

NF- κ B plays an essential role in immune and inflammatory responses, including asthma (44, 45). In this study, as was expected, NF- κ B protein levels in nuclear extracts were found to be substantially increased in our murine OVA-induced asthma model, indicating that NF- κ B was activated. Activation of this transcription factor results in the induction of many inflammatory genes encoding cytokines (TNF- α , IL-4, IL-5, IL-6, IL-9, and IL-13), chemokines (RANTES and eotaxin), adhesion molecules (ICAM-1 and VCAM-1), growth factors, and receptors, which are potentially relevant to the pathogenesis of asthma (46, 47). In addition, recent studies (6, 48–51) have shown that inhibiting NF- κ B activation reduces IL-17 production both in vitro and in vivo. As for modulation of IL-17 expression by NF- κ B, among various regulatory factors for IL-17 expression, some genes are also induced by the activation of NF- κ B. For example, NF- κ B activation has been found to promote the expression of TCR, IL-6, and IL-15 (52, 53). These findings have suggested that the NF- κ B pathway plays a critical role in the regulation of IL-17 expression through multiple mechanisms in allergic airway inflammation. This study showed that administration of rosiglitazone or pioglitazone results in a significant reduction in NF- κ B activity as well as in expression of IL-17 in lung tissues. In addition, the increased IL-17 protein levels after OVA inhalation were decreased by administration of an inhibitor of NF- κ B activation, BAY 11-7085. Taken together, these findings suggest that the inhibition of NF- κ B signal transduction pathway by the action of PPAR γ agonists is involved in the attenuation of IL-17 expression as well as other proinflammatory molecules in allergic airway inflammation.

In conclusion, our results have demonstrated that PPAR γ agonists reverse the pathophysiological features of asthma examined and suppress IL-17 expression in lungs. In addition, the attenuating effect of PPAR γ agonist on allergic airway inflammation and bronchial hyperresponsiveness is abrogated by administration of rIL-17. Inhibition of IL-17 activity also markedly reduces airway inflammation, airway hyperresponsiveness, and the increased levels of Th2 cytokines and serum OVA-specific IgE. These findings suggest that the therapeutic effect of PPAR γ in asthma is exerted via the down-regulation of IL-17 expression. We have also found that PPAR γ agonists inhibit NF- κ B activity and that NF- κ B inhibition reduces the increased IL-17 expression. On the basis of these observations, we suggest that NF- κ B pathway can be one of regulatory mechanisms underlying PPAR γ -mediated IL-17 regulation in allergic airway inflammation. Thus, our findings provide an important mechanism for the use of PPAR γ agonists for the prevention and/or treatment of asthma and other allergic airway diseases.

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Disclosures

The authors have no financial conflict of interest.

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