

# Involvement of IL-10 in Peroxisome Proliferator-Activated Receptor $\gamma$ -Mediated Anti-Inflammatory Response in Asthma

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## ABSTRACT

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) plays an important role in controlling immune and inflammatory responses. Recent studies have demonstrated that activation of PPAR $\gamma$  reduces airway hyper-responsiveness and activation of eosinophils that are increased by induction of asthma. We have used a mouse model of asthma to determine the role of PPAR $\gamma$  in the regulation of the pulmonary immune response, more specifically in the involvement of immunoregulatory cytokine interleukin (IL)-10. Administration of PPAR $\gamma$  agonists or adenovirus carrying PPAR $\gamma$  cDNA (AdPPAR $\gamma$ ) reduced eosinophilic airway inflammation and airway hyper-responsiveness. Expression of PPAR $\gamma$  was increased by ovalbumin inhalation, and the

increase was further enhanced by the administration of PPAR $\gamma$  agonists or AdPPAR $\gamma$ . The increased IL-10 levels in lung tissues after ovalbumin inhalation were further increased by the administration of rosiglitazone, pioglitazone, or AdPPAR $\gamma$ . Levels of IL-4, IL-5, and ovalbumin-specific IgE were also increased after ovalbumin inhalation, and the increased levels were significantly reduced by the administration of the PPAR $\gamma$  agonists or AdPPAR $\gamma$ . The results also showed that inhibition of IL-10 activity with anti-IL-10 receptor antibody partially restored the inflammation. These findings suggest that a protective role of PPAR $\gamma$  in the pathogenesis of the asthma is partly mediated through an IL-10-dependent mechanism.

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily of ligand-activated transcriptional factors (Mangelsdorf et al., 1995). Among them, PPAR $\gamma$  was originally characterized as a regulator of adipocyte differentiation and lipid metabolism (Tontonoz et al., 1994). However, accumulating evidence indicates that PPAR $\gamma$  affects cell cycle, differentiation, and apoptosis (Chinetti et al., 1998). In addition, PPAR $\gamma$  activation down-regulates synthesis and release of immunomodulatory cytokines from various cell types (Chinetti et al., 1998; Ricote et al., 1998; Gelman et al., 1999). Previous studies have demonstrated that activation of PPAR $\gamma$  reduces airway hyper-responsiveness and activation of eosinophils that are increased by induction of asthma (Woerly et al., 2003; Honda et al., 2004; Lee et al., 2005).

Asthma is a chronic inflammatory disorder of the airways

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in which many cell types play a role (Bousquet et al., 2000). Eosinophil response seems to be a critical feature in asthma (Frigas and Gleich, 1986). Eosinophils play an effector role by release of proinflammatory mediators, cytotoxic mediators, and cytokines, resulting in vascular leakage, hypersecretion of mucus, smooth muscle contraction, epithelial shedding, and bronchial hyper-responsiveness. These cells are also involved in the regulation of the airway inflammation and in the initiation of the remodeling process by the release of cytokines and growth factors. IL-10 is a regulatory cytokine produced by several cell types and exhibits antiallergic inflammatory properties (Hobbs et al., 1998; Moore et al., 2001). IL-10 down-regulates IL-4 and IL-5 expression by T-helper type 2 cell lymphocytes and inhibits eosinophil survival and IgE synthesis (Del Prete et al., 1993; Punnonen et al., 1993; Takanashi et al., 1994). However, inter-relationship between PPAR $\gamma$  and IL-10 in regulation of anti-inflammatory function in asthma is not clearly understood.

In the present study, we have used a murine model for asthma to determine the role of PPAR $\gamma$  in the regulation of the pulmonary immune response, more specifically in the involvement of IL-10.

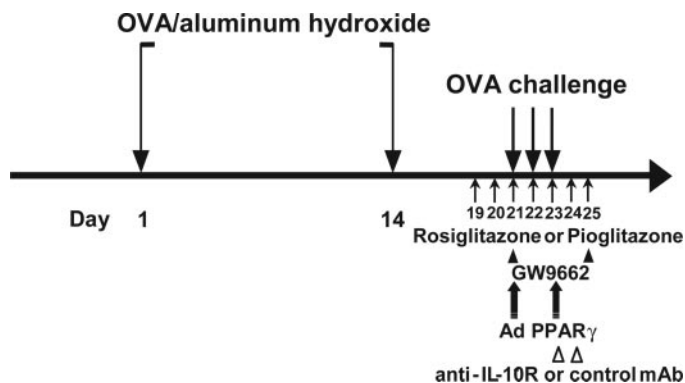
**ABBREVIATIONS:** PPAR, peroxisome proliferator-activated receptor; IL, interleukin; BAL, bronchoalveolar lavage; GW9662; mAb, monoclonal antibody; HRP, horseradish peroxidase; TBST, Tris-buffered saline/Tween 20; R<sub>L</sub>, airway resistance; AdPPAR $\gamma$ , adenovirus carrying PPAR $\gamma$  cDNA.

## Materials and Methods

**Animals and Experimental Protocol.** Female BALB/c mice, 8 to 10 weeks of age and free of murine-specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejeon, Korea). The mice 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 Medical School. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20  $\mu$ g ovalbumin (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200  $\mu$ l, as described previously (Fig. 1) (Kwak et al., 2003; Lee et al., 2004). On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) ovalbumin in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron Corp., Tokyo, Japan).

Bronchoalveolar lavage (BAL) was performed at 60 h after the last challenge. At the time of lavage, the mice (six mice in each group) were sacrificed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg of body weight, administered intraperitoneally). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4°C. Part of each pool was then centrifuged and the supernatants were kept at -70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytospin (Thermo Electron, Waltham, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics, Aguada, Puerto Rico) to examine the cell differentials. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Interinvestigator variation was <5%. The mean number from the two investigators was used to estimate the cell differentials.

**Vectors.** The E1/E3-deleted replication-deficient recombinant adenovirus was made using the AdEasy system (Quantum Biotech,



**Fig. 1.** Schematic diagram of the experimental protocol. Mice were sensitized on days 1 and 14 by intraperitoneal injection of ovalbumin 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) ovalbumin in saline (or with saline as a control) using an ultrasonic nebulizer. In the case of treatment with rosiglitazone or pioglitazone, it was given by oral gavage seven times at 24 h-intervals on days 19 to 25, beginning 2 days before the first challenge. In the case of GW9662 treatment, GW9662 was administered intratracheally two times to each animal, once on day 21 and the second time on day 25. Adenoviral vector was administered intratracheally two times to each animal, once on day 21 (1 h before the first airway challenge with ovalbumin) and the second time on day 23 (3 h after the last airway challenge with ovalbumin). Anti-IL-10 receptor antibody (Anti-IL-10R) or isotype control mAb were administered intraperitoneally two times to each animal, once on day 23 (1 h after the last airway challenge with ovalbumin) and the second time on day 24 (24 h after the last airway challenge with ovalbumin).

Montreal, Canada) described by He et al. (1998). KpnI-XhoI restriction fragments from pcDNA3/wild-type PPAR $\gamma$  cDNA were ligated into KpnI-XhoI-digested pShuttleCMV. To create AdLacZ, a SalI-NotI restriction fragment from pcDNA3.1/LacZ (Invitrogen, San Diego, CA) was ligated to SalI-NotI-digested pShuttleCMV. Recombination into the pAdEasy-1 viral backbone was accomplished in bacteria (*Escherichia coli*, strain BJ5183, recA-deficient) according to the manufacturer's instructions. The recombination was verified, and the adenoviral recombinant DNA was transferred to a regular strain *E. coli* (DH5 $\alpha$ ) that generated far greater yields of DNA. Recombinant pAdEasy plasmids containing CMV-cDNA inserts were purified over QIAGEN columns (Qiagen Inc., Valencia, CA), and 5  $\mu$ g of PacI-digested DNA was used to transfect QBI-293A cells using the calcium phosphate method (Promega, Madison, WI). Cells were seeded at  $2 \times 10^6$  cells per 150-mm culture dish at 24 h before transfection. Lysis of transfected cells indicating adenoviral growth occurred within 4 days. After amplification, lysates containing clonal recombinant adenovirus were prepared from 150-mm culture dishes and purified by CsCl gradient centrifugation. Recovered virus was aliquoted and stored at -20°C in 5 mM Tris buffer, pH 8.0, containing 50 mM NaCl, 0.05% bovine serum albumin, and 25% glycerol. Virus was titrated by serial dilution infection of QBI-293A cells, and plaques were counted under an overlay of 0.3% agarose, 10% fetal bovine serum, and  $1 \times$  Dulbecco's modified Eagle's medium.

**Administration of Rosiglitazone, Pioglitazone, GW9662, Ad Vectors, Anti-IL-10 Receptor Antibody, and Isotype Control Monoclonal Antibody.** Rosiglitazone (5 mg/kg body weight/day; GlaxoSmithKline, Uxbridge, Middlesex, UK) dissolved in distilled water or pioglitazone (10 mg/kg body weight/day; Takeda Chemical Industries Ltd., Osaka, Japan) dissolved in dimethyl sulfoxide and diluted with 0.9% NaCl, was administered seven times by oral gavage at 24-h intervals on days 19 to 25, beginning 2 days before the first challenge (Fig. 1). GW9662 (0.5 mg/kg body weight/day; Cayman Chemical, Ann Arbor, MI) dissolved in phosphate-buffered saline, was administered intratracheally to each animal twice, once on day 21 and the second time on day 25 (Fig. 1). Anti-IL-10 receptor antibody or isotype control mAb (12.5 mg/kg body weight/day; BD Pharmingen, San Diego, CA) was administered intraperitoneally to each animal twice, once on day 23 (1 h after the last airway challenge with ovalbumin) and the second time on day 24 (24 h after the last airway challenge with ovalbumin) (Fig. 1). Adenoviral vectors were administered intratracheally ( $10^9$  plaque-forming units) to each animal twice, once on day 21 (1 h before the first airway challenge with ovalbumin) and the second time on day 23 (3 h after the last airway challenge with ovalbumin) (Fig. 1).

**Measurement of Cytokines.** Levels of IL-4 and IL-5 were quantified in the supernatants of BAL fluids by enzyme immunoassays according to the manufacturer's protocol (Pierce Endogen, Rockford, IL). Sensitivities for IL-4 and IL-5 assays were 5 pg/ml.

**Analysis of Ovalbumin-Specific IgE.** Ovalbumin-specific IgE levels were measured by capture enzyme-linked immunosorbent assay as described previously (MacLean et al., 2000). Microtiter plates were coated with a purified anti-mouse IgE mAb (BD Pharmingen). To detect ovalbumin-specific IgE, diluted serum samples were added to each well, and the plates were incubated for 2 h at room temperature. After washing five times, biotinylated ovalbumin (10  $\mu$ g/ml) and horseradish peroxidase (HRP)-conjugated streptavidin were added to each well. Plates were washed, and 3,3',5,5'-tetramethylbenzidine substrate was added. After incubation for 30 min in the dark at room temperature, the plates were read at 450 nm using a microplate reader (Molecular Dynamics, Sunnyvale, CA).

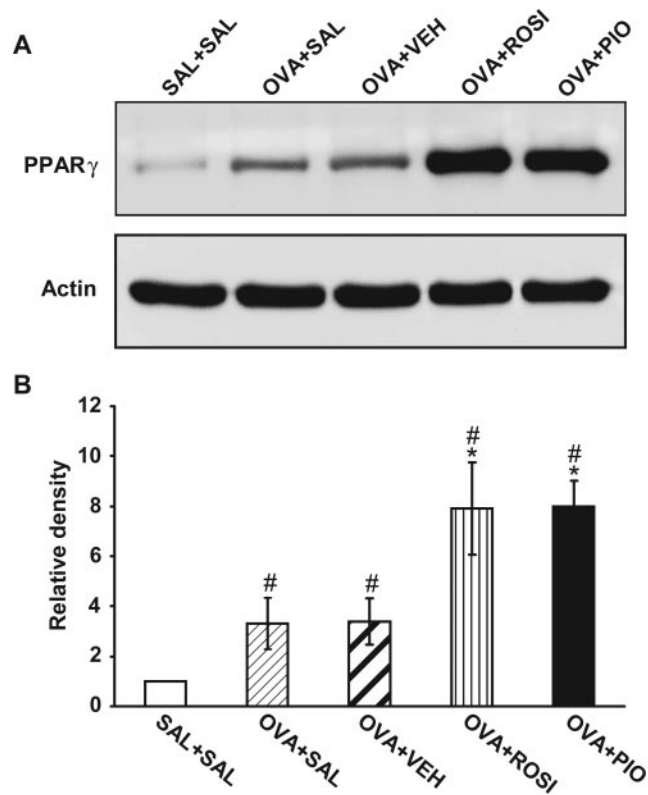
**Western Blot Analysis.** Lung tissues were homogenized in the presence of protease inhibitors to obtain extracts of proteins. Protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA). Samples (30  $\mu$ g of protein per lane) were loaded on a 12% SDS-polyacrylamide gel electrophoresis gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chal-

font, Buckinghamshire, UK) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween 20 (TBST; 25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h, and the blots were then incubated with an anti-IL-10 antibody (MBL International Corporation, Woburn, MA), anti-IL-4 antibody (Serotec Ltd., Oxford, UK), or anti-IL-5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4°C. Anti-rabbit or anti-mouse HRP conjugated IgG was used to detect binding of the antibody. The membranes were stripped and reblotted with anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibody was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

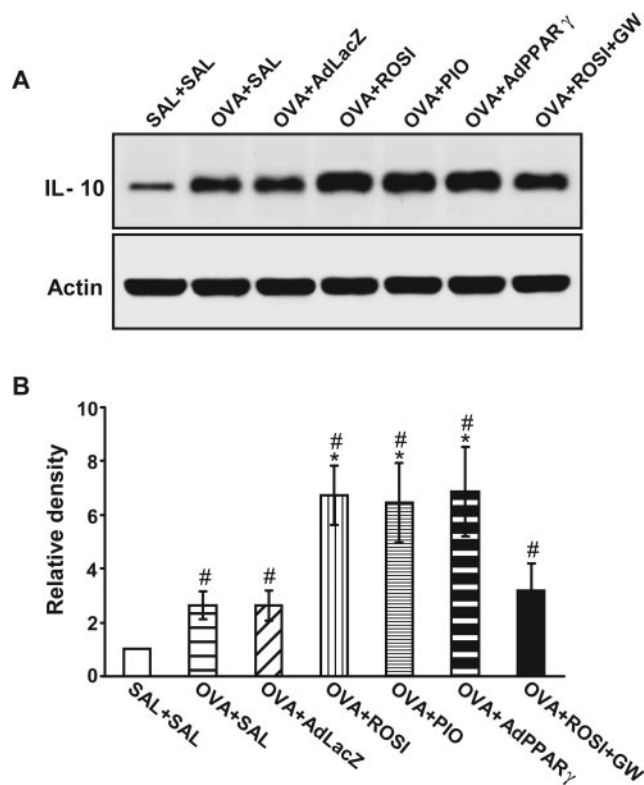
**Determination of Airway Responsiveness.** Airway responsiveness was also assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere (Takeda et al., 1997; Eum et al., 2003). Anesthesia was achieved with 80 mg/kg of pentobarbital sodium injected intraperitoneally. The trachea was exposed through midcervical incision, tracheostomy was performed, and an 18-gauge metal needle was inserted. Mice were connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, Canada). The mouse was quasi-sinusoidally ventilated with nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm of H<sub>2</sub>O to achieve a mean lung volume close to that

during spontaneous breathing. This was achieved by connecting the expiratory port of the ventilator to water column. Methacholine aerosol was generated with an in-line nebulizer and administered directly through the ventilator. To determine the differences in airway response to methacholine, each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/ml in saline). After each methacholine challenge, the data of airway resistance ( $R_L$ ) was continuously collected. Maximum values of  $R_L$  were selected to express changes in airway function, which was represented as a percentage change from baseline after saline aerosol.

**Histology.** At 60 h after the last challenge, lungs were removed from the mice after sacrifice. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin and 4% acetic acid) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4- $\mu$ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). Three independent blinded investigators graded inflammation score. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as



**Fig. 2.** Effect of rosiglitazone or pioglitazone on PPAR $\gamma$  protein expression in lung tissues of ovalbumin-sensitized and -challenged mice. A, Western blotting of PPAR $\gamma$ . PPAR $\gamma$  protein expression was measured at 60 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered rosiglitazone (OVA+ROSI), and ovalbumin-inhaled mice administered pioglitazone (OVA+PIO). B, densitometric analyses are presented as the relative ratio of PPAR $\gamma$  to actin. The relative ratio of PPAR $\gamma$  in the lung tissues of SAL+SAL is arbitrarily presented as 1. Results are represented as mean  $\pm$  S.E.M. from six mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.



**Fig. 3.** Effect of rosiglitazone, pioglitazone, AdPPAR $\gamma$ , or GW9662 plus rosiglitazone on IL-10 protein in lung tissues of ovalbumin-sensitized and -challenged mice. A, Western blotting of IL-10. IL-10 protein expression was measured at 60 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), ovalbumin-inhaled mice administered rosiglitazone (OVA+ROSI), ovalbumin-inhaled mice administered pioglitazone (OVA+PIO), ovalbumin-inhaled mice administered AdPPAR $\gamma$  (OVA+AdPPAR $\gamma$ ), and ovalbumin-inhaled mice administered GW9662 plus rosiglitazone (OVA+ROSI+GW). B, densitometric analyses are presented as the relative ratio of IL-10 to actin. The relative ratio of IL-10 in the lung tissues of SAL+SAL is arbitrarily presented as 1. Results are represented as mean  $\pm$  S.E.M. from six mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL.



described elsewhere (Tournoy et al., 2000). A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by a thin layer (one to five cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells.

**Preparation of Mouse Lung Nuclei for Analysis of PPAR $\gamma$ .** Lungs were removed and homogenized in 8 volumes of a lysis buffer containing 1.3 M sucrose, 1.0 mM MgCl<sub>2</sub>, and 10 mM potassium phosphate buffer, pH 7.2. The homogenate was filtered through four layers of gauze and centrifuged at 1000g for 15 min. The resulting pellets were carefully recovered and resuspended in 2.4 M sucrose, 1.0 mM MgCl<sub>2</sub>, and 10 mM potassium phosphate buffer, pH 7.2, to maintain a final 2.2 M sucrose concentration and centrifuged at 100,000g for 1 h. The resulting nuclear pellets were washed once with a solution containing 0.25 M sucrose, 0.5 mM MgCl<sub>2</sub>, and 20 mM Tris-HCl, pH 7.2, and centrifuged at 1000g for 10 min. The pellets were solubilized with a solution containing 50 mM Tris-HCl, pH 7.2, 0.3 M sucrose, 150 mM NaCl, 2 mM EDTA, 20% glycerol, 2% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and protein inhibitor cocktails. The mixture was kept on ice for 2 h with gentle stirring and centrifuged at 12,000g for 30 min. The resulting supernatant was used as solubilized nuclear proteins for detection of PPAR $\gamma$ .

For Western analysis, samples (30  $\mu$ g of protein per lane) were loaded on a 10% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% nonfat dry milk in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h, and the blots were then incubated with antibody against PPAR $\gamma$  (Santa Cruz Biotechnology), overnight at 4°C. Anti-mouse HRP conjugated IgG was used to detect binding of the antibody. The membranes were stripped and reblotted with anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibody was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (GE Healthcare).

**Densitometric Analyses and Statistics.** All immunoreactive and phosphorylation signals were analyzed by densitometric scan-

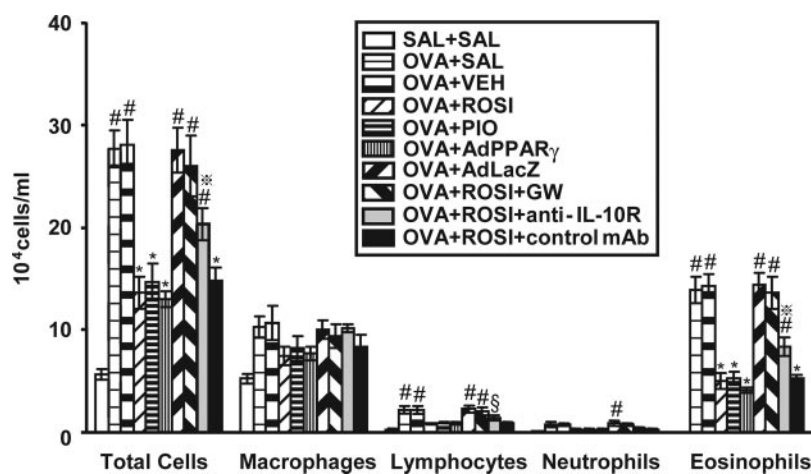
ning (Gel Doc XR; Bio-Rad Laboratories). Data were expressed as mean  $\pm$  S.E.M. Statistical comparisons were performed using one-way analysis of variance followed by Fisher's test. Significant differences between groups were determined using the unpaired Student's *t* test. Statistical significance was set at *p* < 0.05.

## Results

**PPAR $\gamma$  Agonists Increase PPAR $\gamma$  Levels in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice.** Western blot analysis revealed that PPAR $\gamma$  levels were increased significantly at 60 h after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 2). The increased PPAR $\gamma$  levels in lung tissues at 60 h after ovalbumin inhalation were further increased by the administration of rosiglitazone or pioglitazone.

**Effect of Rosiglitazone, Pioglitazone, Adenovirus Carrying PPAR $\gamma$  cDNA, or GW9662 Plus Rosiglitazone on IL-10 Protein in Lung Tissues of Ovalbumin-Sensitized and -Challenged Mice.** Western blot analysis showed that IL-10 levels were increased significantly at 60 h after ovalbumin inhalation compared with the levels after saline inhalation (Fig. 3). The increased IL-10 levels in lung tissues at 60 h after ovalbumin inhalation were further increased by the administration of rosiglitazone, pioglitazone, or AdPPAR $\gamma$ , whereas the rosiglitazone-mediated increase of IL-10 level was blocked by a PPAR $\gamma$  antagonist, GW9662 (Fig. 3).

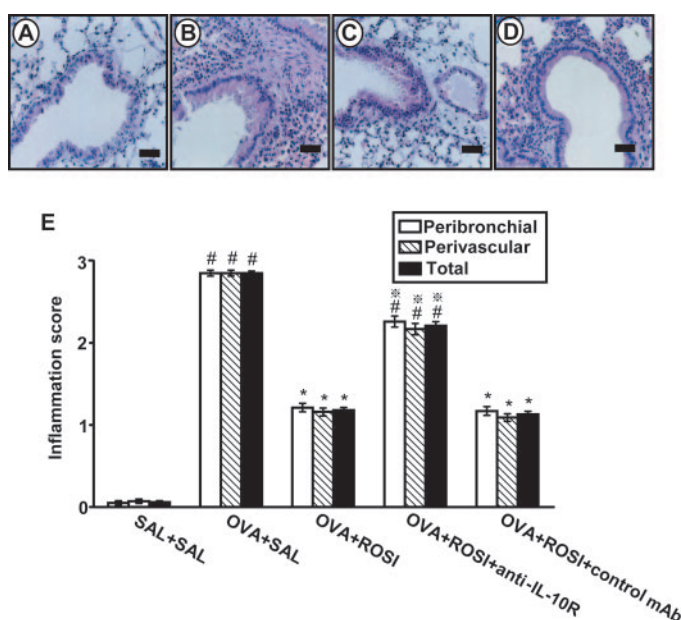
**Effect of Rosiglitazone, Pioglitazone, AdPPAR $\gamma$ , GW9662 Plus Rosiglitazone, or Anti-IL-10 Receptor Antibody with Rosiglitazone on Cellular Changes in BAL Fluids.** Numbers of total cells and eosinophils were increased significantly at 60 h after the last ovalbumin challenge compared with levels after saline inhalation (Fig. 4). The increased numbers of total cells and eosinophils in BAL fluids at 60 h after the last ovalbumin challenge were significantly reduced by the administration of rosiglitazone, pio-



**Fig. 4.** Effect of rosiglitazone, pioglitazone, AdPPAR $\gamma$ , GW9662 plus rosiglitazone, or anti-IL-10 receptor antibody plus rosiglitazone on total cells, macrophage, lymphocytes, neutrophils, and eosinophils in BAL fluids of ovalbumin-sensitized and -challenged mice. The numbers of total cells and eosinophils of BAL from saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered rosiglitazone (OVA+ROSI), ovalbumin-inhaled mice administered pioglitazone (OVA+PIO), ovalbumin-inhaled mice administered AdPPAR $\gamma$  (OVA+AdPPAR $\gamma$ ), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), ovalbumin-inhaled mice administered anti-IL-10 receptor antibody plus rosiglitazone (OVA+ROSI+anti-IL-10R), ovalbumin-inhaled mice administered GW9662 plus rosiglitazone (OVA+ROSI+GW), and ovalbumin-inhaled mice administered isotype control mAb plus rosiglitazone (OVA+ROSI+control mAb) were counted at 60 h after the last challenge. Results are represented as mean  $\pm$  S.E.M. from six mice per group. #, *p* < 0.05 versus SAL+SAL; \*, *p* < 0.05 versus OVA+SAL; × with dots, *p* < 0.05 versus OVA+ROSI and OVA+ROSI+ control mAb; §, *p* < 0.05 versus OVA+ROSI.

glitazone, or AdPPAR $\gamma$ . The inhibitory effect of rosiglitazone treatment on numbers of eosinophils in BAL fluids was abrogated when a PPAR $\gamma$  antagonist, GW9662, was administered concomitantly with the agonist. These results indicate that rosiglitazone was mainly acting through PPAR $\gamma$  in this model. The inhibitory effect of rosiglitazone treatment significantly restored the BAL eosinophilia when anti-IL-10 receptor antibody was administered concomitantly with rosiglitazone, but the restoration of the eosinophilia was not observed after the administration of isotype control mAb with rosiglitazone, suggesting that the effects induced by PPAR $\gamma$  agonist were partly mediated through an IL-10-dependent mechanism.

**Effect of Rosiglitazone or Anti-IL-10 Receptor Antibody Plus Rosiglitazone on Pathological Changes of Ovalbumin-Induced Asthma.** Histologic analyses revealed typical pathologic features of asthma in the ovalbumin-exposed mice. Numerous inflammatory cells, including eosinophils, infiltrated around the bronchioles, and the airway epithelium was thickened (Fig. 5B) compared with the control (Fig. 5A). Mice treated with rosiglitazone (Fig. 5C), showed marked reductions in the thickening of airway epithelium, in the infiltration of inflammatory cells in the peribronchiolar region, and in the number of inflammatory cells. The inhibitory effect of rosiglitazone treatment on typical

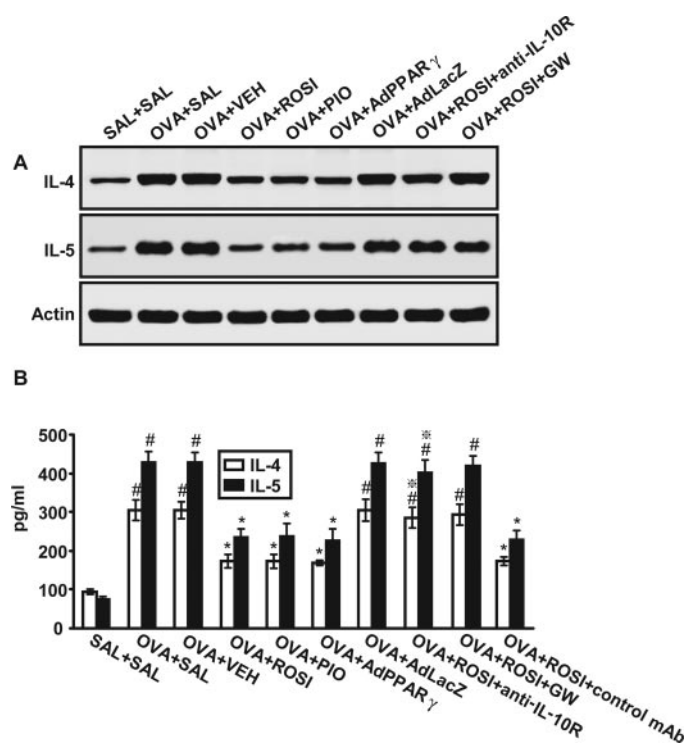


**Fig. 5.** Effect of rosiglitazone or anti-IL-10 receptor antibody plus rosiglitazone in lung tissues of ovalbumin-sensitized and -challenged mice. Representative hematoxylin/eosin-stained sections of the lungs. Sampling was performed at 60 h after the last challenge in saline-inhaled mice administered saline (A), ovalbumin-inhaled mice administered saline (B), ovalbumin-inhaled mice administered rosiglitazone (C), and ovalbumin-inhaled mice administered anti-IL-10 receptor antibody plus rosiglitazone (D). Scale bars, 50  $\mu$ m. Peribronchial and perivascular lung inflammation were measured at 60 h after the last challenge in saline-inhaled mice administered saline (SAL+Sal), ovalbumin-inhaled mice administered saline (OVA+Sal), ovalbumin-inhaled mice administered rosiglitazone (OVA+ROSI), ovalbumin-inhaled mice administered anti-IL-10 receptor antibody plus rosiglitazone (OVA+ROSI+anti-IL-10R), and ovalbumin-inhaled mice administered isotype control mAb plus rosiglitazone (OVA+ROSI+control mAb) (E). Results are represented as mean  $\pm$  S.E.M. from six mice per group. #,  $p < 0.05$  versus SAL+Sal; \*,  $p < 0.05$  versus OVA+Sal; × with dots,  $p < 0.05$  versus OVA+ROSI and OVA+ROSI+control mAb.

pathologic features of asthma in the ovalbumin-exposed mice was partially abrogated when anti-IL-10 receptor antibody was administered concomitantly with rosiglitazone (Fig. 5D).

The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 60 h after ovalbumin inhalation compared with scores after saline inhalation. The increased peribronchial, perivascular, and total lung inflammation after ovalbumin inhalation were significantly decreased by the administration of rosiglitazone. The inhibitory effect of rosiglitazone treatment on peribronchial, perivascular, and total lung inflammation was significantly abrogated when anti-IL-10 receptor antibody was administered concomitantly with rosiglitazone (Fig. 5E).

**Effect of Rosiglitazone, Pioglitazone, GW9662 Plus Rosiglitazone, Anti-IL-10 Receptor Antibody Plus Rosiglitazone, or AdPPAR $\gamma$  on IL-4 and IL-5 in Lung Tissues and BAL Fluids of Ovalbumin-Sensitized and -Challenged Mice.** Western blot analysis revealed that IL-4 and IL-5 protein levels in lung tissues were increased significantly at 60 h after ovalbumin inhalation compared with the



**Fig. 6.** Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, anti-IL-10 receptor antibody plus rosiglitazone, or AdPPAR $\gamma$  on IL-4 and IL-5 levels in lung tissues and BAL fluids of ovalbumin-sensitized and -challenged mice. A, Western blotting of IL-4 and IL-5 in lung tissues. B, enzyme immunoassay of IL-4 and IL-5 in BAL fluids. Sampling was performed at 60 h after the last challenge in saline-inhaled mice administered saline (SAL+Sal), ovalbumin-inhaled mice administered saline (OVA+Sal), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered rosiglitazone (OVA+ROSI), ovalbumin-inhaled mice administered pioglitazone (OVA+PIO), ovalbumin-inhaled mice administered AdPPAR $\gamma$  (OVA+AdPPAR $\gamma$ ), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), ovalbumin-inhaled mice administered GW9662 plus rosiglitazone (OVA+ROSI+GW), ovalbumin-inhaled mice administered anti-IL-10 receptor antibody plus rosiglitazone (OVA+ROSI+anti-IL-10R), and ovalbumin-inhaled mice administered isotype control mAb plus rosiglitazone (OVA+ROSI+control mAb). Results are represented as mean  $\pm$  S.E.M. from six mice per group. #,  $p < 0.05$  versus SAL+Sal; \*,  $p < 0.05$  versus OVA+Sal; × with dots,  $p < 0.05$  versus OVA+ROSI and OVA+ROSI+control mAb.

levels after saline inhalation (Fig. 6A). The administration of rosiglitazone, pioglitazone, or AdPPAR $\gamma$  reduced significantly the increased IL-4 and IL-5 levels at 60 h after ovalbumin inhalation, whereas GW9662 plus rosiglitazone or anti-IL-10 receptor antibody plus rosiglitazone did not. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays showed that levels of IL-4 and IL-5 in BAL fluids were increased significantly at 60 h after ovalbumin inhalation compared with the levels after saline inhalation. The administration of rosiglitazone, pioglitazone, or AdPPAR $\gamma$  reduced significantly the increased IL-4 and IL-5 levels at 60 h after ovalbumin inhalation, whereas GW9662 plus rosiglitazone or anti-IL-10 receptor antibody plus rosiglitazone did not (Fig. 6B).

**Effect of Rosiglitazone, Pioglitazone, GW9662 Plus Rosiglitazone, Anti-IL-10 Receptor Antibody Plus Rosiglitazone, or AdPPAR $\gamma$  on Ovalbumin-Specific IgE Levels in Sera of Ovalbumin-Sensitized and -Challenged Mice.** Enzyme immunoassays revealed that levels of ovalbumin-specific IgE in sera were increased significantly at 60 h after ovalbumin inhalation compared with the levels after saline inhalation. The administration of rosiglitazone, pioglitazone, or AdPPAR $\gamma$  reduced significantly the increased ovalbumin-specific IgE levels at 60 h after ovalbumin inhalation, whereas GW9662 plus rosiglitazone or anti-IL-10 receptor antibody plus rosiglitazone did not (Fig. 7).

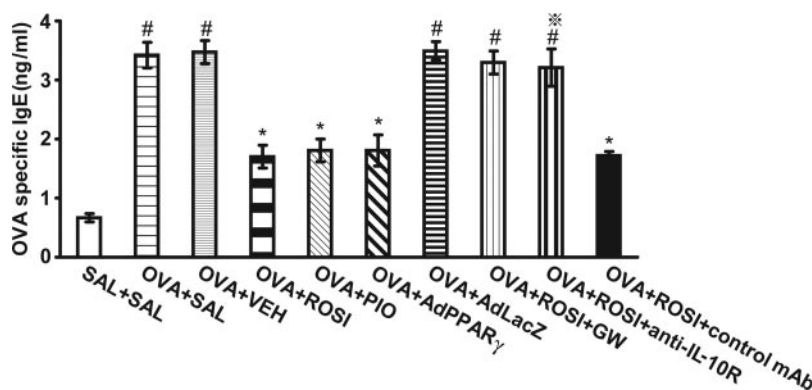
**Effect of Rosiglitazone, Pioglitazone, GW9662 Plus Rosiglitazone, AdPPAR $\gamma$ , or Anti-IL-10 Receptor Antibody on Airway Hyper-Responsiveness.** Airway responsiveness was assessed as a percentage increase of  $R_L$  in response to increasing doses of methacholine. In ovalbumin-sensitized and -challenged mice, the dose-response curve of  $R_L$  shifted to the left compared with that of control mice (Fig. 8A). In addition, the  $R_L$  produced by methacholine administration (at doses from 2.5 to 50 mg/ml) increased significantly in the ovalbumin-sensitized and -challenged mice compared with the control mice. Ovalbumin-sensitized and -challenged mice treated with rosiglitazone, pioglitazone, or AdPPAR $\gamma$  showed a dose-response curve of  $R_L$  that shifted to the right compared with that of untreated mice or ovalbumin-sensi-

tized and -challenged mice treated with AdLacZ. These results indicate that rosiglitazone, pioglitazone, or AdPPAR $\gamma$  treatment reduces ovalbumin-induced airway hyper-responsiveness. The inhibitory effect of rosiglitazone treatment on airway hyper-responsiveness was abrogated when GW9662 was administered concomitantly with the agonist.

The reduced value of  $R_L$  after the administration of rosiglitazone was increased significantly when anti-IL-10 receptor antibody was administered concomitantly with rosiglitazone (Fig. 8B). These findings suggest that the effect induced PPAR $\gamma$  agonist on airway hyper-responsiveness were partly mediated through an IL-10-dependent mechanism.

## Discussion

PPARs are transcriptional factors belonging to the ligand-activated nuclear receptor superfamily which upon heterodimerization with the retinoic X receptor, to recognize PPAR response elements, located in the promoter of target genes (Issemann et al., 1993). Protein interactions play an important role in the actions of PPARs. In the inactivated state, nuclear receptors such as PPARs are considered complexes bound with corepressor proteins. Upon ligand activation, PPARs dissociate from corepressors and recruit coactivators, including the PPAR-ligand protein and the steroid receptor coactivator-1, which can translocate from the cytoplasm to the nucleus (Zhu et al., 1996, 1997; Bishop-Bailey and Hla, 1999). Among PPARs, PPAR $\gamma$  plays an important role in anti-inflammatory responses (Chinetti et al., 1998; Jiang et al., 1998; Ricote et al., 1998; Gelman et al., 1999). Recent studies have demonstrated that activation of PPAR $\gamma$  reduces expression of various cytokines, airway hyper-responsiveness, and activation of eosinophils, which are all increased by induction of asthma (Woerly et al., 2003; Honda et al., 2004; Lee et al., 2005). IL-10 is an anti-inflammatory cytokine that down-regulates cellular immunity and allergic inflammation. However, inter-relationship between these proteins in the pathogenesis of the asthma has not been clarified. Our present study with ovalbumin-induced murine model of asthma has revealed that activation of PPAR $\gamma$  with



**Fig. 7.** Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, anti-IL-10 receptor antibody plus rosiglitazone, or AdPPAR $\gamma$  on ovalbumin-specific IgE levels in sera of ovalbumin-sensitized and -challenged mice. Sampling was performed at 60 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered rosiglitazone (OVA+ROSI), ovalbumin-inhaled mice administered pioglitazone (OVA+PIO), ovalbumin-inhaled mice administered AdPPAR $\gamma$  (OVA+AdPPAR $\gamma$ ), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), ovalbumin-inhaled mice administered GW9662 plus rosiglitazone (OVA+ROSI+GW), ovalbumin-inhaled mice administered anti-IL-10 receptor antibody plus rosiglitazone (OVA+ROSI+anti-IL-10R), and ovalbumin-inhaled mice administered isotype control mAb plus rosiglitazone (OVA+ROSI+control mAb). Results are represented as mean  $\pm$  S.E.M. from six mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL; × with dots,  $p < 0.05$  versus OVA+ROSI and OVA+ROSI+control mAb.

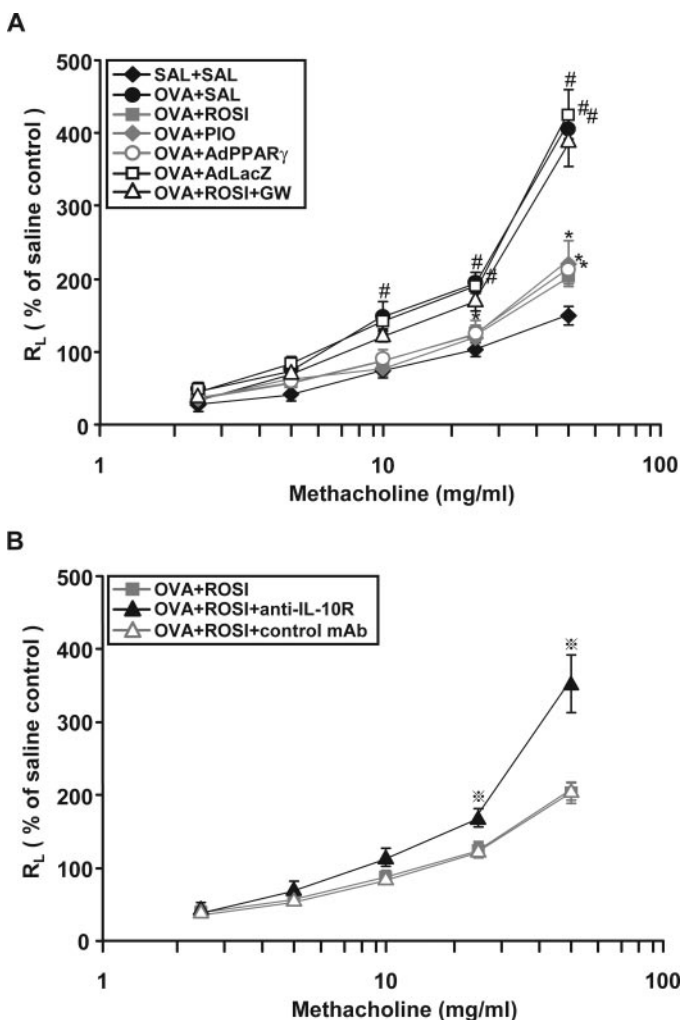


the agonists enhances expression of PPAR $\gamma$ , resulting in reduction of eosinophilic inflammation and airway hyper-responsiveness. The increased IL-10 levels in lung tissues after ovalbumin inhalation are further increased by the administration of rosiglitazone, pioglitazone, or AdPPAR $\gamma$ . In addition, inhibition of IL-10 activity with anti-IL-10 receptor antibody partially restores the inflammation. These findings suggest that a protective role of PPAR $\gamma$  in the pathogenesis of

the asthma is partly mediated through an IL-10-dependent mechanism.

Thiazolidinediones such as rosiglitazone and pioglitazone are high-affinity ligands for PPAR $\gamma$  and are used as insulin-sensitizing drugs in type 2 diabetes mellitus (Lehmann et al., 1995). PPAR $\gamma$  is originally known to regulate adipocyte differentiation and lipid metabolism (Tontonoz et al., 1994). Previous studies have demonstrated that PPAR $\gamma$  may be involved in airway inflammation and airway hyper-responsiveness in asthma (Woerly et al., 2003; Honda et al., 2004; Lee et al., 2005). Consistent with these observations, our results have shown that administration of the PPAR $\gamma$  agonists or AdPPAR $\gamma$  substantially inhibits expression of cytokines (IL-4 and IL-5), airway hyper-responsiveness, and eosinophilic inflammation. Moreover, induction of asthma through ovalbumin challenge increases expression of PPAR $\gamma$  itself and administration of the agonists further enhances the receptor expression. Up-regulation of PPAR $\gamma$  expression has also been observed in human asthmatic airways (Benayoun et al., 2001). Over-expression of PPAR $\gamma$  by administration of AdPPAR $\gamma$  results in reduction of all asthmatic features in our ovalbumin-induced murine model of asthma. These findings indicate that PPAR $\gamma$  is associated with anti-inflammatory responses in asthma.

The role of IL-10 as an anti-inflammatory cytokine in allergic inflammation has been reported (Borish, 1998). Administration of IL-10 has been shown to abrogate allergen-induced airway inflammation (Zuany-Amorim et al., 1995), and IL-10 down-regulates IL-4 and IL-5 expression by T-helper type 2 lymphocytes (Takanashi et al., 1994). Consistent with the observations, IL-10 knockout mice develop a lethal form of allergic bronchopulmonary aspergillosis with markedly elevated expression of IL-4 and IL-5 (Grunig et al., 1997). A modulating role of IL-10 in human allergic diseases is also observed (Punnonen et al., 1993; Takanashi et al., 1994). IL-10 inhibits eosinophil survival and IgE synthesis. Although regulation mechanism of IL-10 synthesis has not been clarified, previous studies have shown that production of IL-10 is increased by treatment with a selective PPAR $\gamma$  agonist or tetradecylthioacetic acid, known as a PPAR ligand (Aukrust et al., 2003; Hammad et al., 2004). Hammad et al. (2004) suggest that PPAR $\gamma$  agonist-treated dendritic cells can induce the generation of a population of IL-10-producing T cells that could suppress some of the features of asthma. Consistent with these observations, our studies with the ovalbumin-induced asthma model show that enhanced levels of IL-10 in lung tissues after ovalbumin inhalation are further increased by the administration of the PPAR $\gamma$  agonists (rosiglitazone and pioglitazone) or AdPPAR $\gamma$ . We have also found that the administration of the PPAR $\gamma$  agonists or AdPPAR $\gamma$  reduces significantly the increased levels of IL-4 and IL-5, including the levels of ovalbumin-specific IgE synthesized by the ovalbumin inhalation. In addition, it seems that production of IL-10 induced by ovalbumin inhalation is physiologically relevant as an inhibitory signaling of asthma, because the blocking antibody, anti-IL-10 receptor antibody, partially restores inflammatory features of asthma. However, Lytle et al. (2005) have shown that rosiglitazone has anti-inflammatory effect independent of IL-10 in a animal model of inflammatory bowel disease. Although the reason is not clearly understood, the discrepancy may be due to the



**Fig. 8.** Effect of rosiglitazone, pioglitazone, AdPPAR $\gamma$ , GW9662 plus rosiglitazone, or anti-IL-10 receptor antibody plus rosiglitazone on airway responsiveness in ovalbumin-sensitized and -challenged mice. Airway responsiveness was measured at 60 h after the last challenge in saline-inhaled mice administered saline (SAL+SAL), ovalbumin-inhaled mice administered saline (OVA+SAL), ovalbumin-inhaled mice administered drug vehicle (OVA+VEH), ovalbumin-inhaled mice administered rosiglitazone (OVA+ROSI), and ovalbumin-inhaled mice administered pioglitazone (OVA+PIO), ovalbumin-inhaled mice administered AdPPAR $\gamma$  (OVA+AdPPAR $\gamma$ ), ovalbumin-inhaled mice administered AdLacZ (OVA+AdLacZ), ovalbumin-inhaled mice administered GW9662 plus rosiglitazone (OVA+ROSI+GW), ovalbumin-inhaled mice administered anti-IL-10 receptor antibody plus rosiglitazone (OVA+ROSI+anti-IL-10R), and ovalbumin-inhaled mice administered isotype control mAb plus rosiglitazone (OVA+ROSI+control mAb). A, effect of rosiglitazone, pioglitazone, AdPPAR $\gamma$ , GW9662 plus rosiglitazone on airway responsiveness. B, effect of anti-IL-10 receptor antibody plus rosiglitazone on airway responsiveness. R<sub>L</sub> values were obtained in response to increasing doses (2.5 to 50 mg/ml) of methacholine as described under *Materials and Methods*. Results are represented as mean  $\pm$  S.E.M. from six mice per group. #,  $p < 0.05$  versus SAL+SAL; \*,  $p < 0.05$  versus OVA+SAL;  $\times$  with dots,  $p < 0.05$  versus OVA+ROSI and OVA+ROSI+control mAb.

disease model of animal (inflammatory bowel disease versus asthma).

In conclusion, our results have demonstrated that administration of the PPAR $\gamma$  agonists or AdPPAR $\gamma$  may improve the asthmatic features via regulation of IL-10 expression/IL-10 receptor activation. Hence, PPAR $\gamma$  agonist may have therapeutic potential for the treatment of airway inflammation and hyper-responsiveness.

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