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Inhibition of Interleukin-4 Production in CD4$^+$ T Cells by Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) Ligands: Involvement of Physical Association between PPAR-γ and the Nuclear Factor of Activated T Cells Transcription Factor

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ABSTRACT

Peroxisome proliferator-activated receptor-γ (PPAR-γ) has been implicated in the regulation of multiple inflammatory processes. However, little is known of PPAR-γ in the regulation of interleukin (IL)-4 expression in T cells. In this study, the effects of PPAR-γ ligands on production of IL-4, a pro-inflammatory cytokine associated with the pathophysiology of allergic diseases, were investigated. 15-Deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) and ciglitazone, two representative PPAR-γ ligands, significantly inhibited IL-4 production in both antigen-stimulated primary CD4$^+$ T cells and the phorbol 12-myristate 13-acetate (PMA)/ionomycin-activated EL-4 T cell line. 15d-PGJ_2 and ciglitazone inhibited the activation of IL-4 gene promoter in EL-4 T cells transiently transfected with IL-4 promoter/reporter constructs, and the repressive effect mapped to a region in the IL-4 promoter containing binding sites for nuclear factor of activated T cells (NF-AT). The activation of T cells by PMA/ionomycin resulted in a marked enhancement of the binding activities to the NF-AT site that was significantly inhibited by the addition of PPAR-γ ligands. In cotransfected EL-4 T cells, PPAR-γ also inhibited the activation of the IL-4 promoter at multiple NF-AT sites in a ligand-dependent manner. NF-ATc1 bound PPAR-γ both in vivo and in vitro, and the interaction interfaces involved the Rel similarity domain of NF-ATc1. In cotransfections of HeLa cells, PPAR-γ inhibited the NF-ATc1 transactivation in a ligand-dependent manner. Coexpression of p300 or AP-1 relieved the PPAR-γ ligand-mediated inhibition of the NF-AT transactivation. From these results, we propose that PPAR-γ ligand-mediated suppression of IL-4 production in CD4$^+$ T cells may involve both inhibition of the NFAT-DNA interactions and competitive recruitment of transcription integrators between NF-AT and PPAR-γ.

Interleukin (IL)-4 is a pleiotropic and multifunctional cytokine produced by activated T cells, mast cells, and basophils. IL-4 plays a critical role in regulating the outcome of an immune response by facilitating the differentiation of CD4$^+$ T cells into IL-4–producing T helper (Th) type 2 cells and suppressing the differentiation of interferon-γ (IFN-γ)-producing Th1 cells, thereby favoring humoral immune responses (Abbas et al., 1996). Regulation of IL-4 gene expression, therefore, is critically important for the differentiation of Th2 cells and Th2-dependent immune responses (Brown and Hural, 1997). Dysregulated expression of IL-4–producing cells has been linked with autoimmune and allergic diseases (Choi and Reiser, 1998).

In T cells, IL-4 gene expression is regulated at the transcriptional level by both ubiquitous and cell type-restricted factors, including nuclear factor of activated T cells (NF-AT), c-Maf, GATA-3, Stat6, JunB, and other transcription factors (Brown and Hural, 1997; Szabo et al., 1997). These factors interact with a proximal promoter region composed of multiple regulatory elements that can both positively and negatively affect transcriptional activation. IL-4 gene transcrip-

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ABBREVIATIONS: IL, interleukin; Th, T helper; IFN-γ, interferon-γ; NF-AT, nuclear factor of activated T cell; RSD, Rel similarity domain; AP-1, activator protein 1; NF-κB, nuclear factor-κB; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor; PMA, phorbol 12-myristate 13-acetate; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; KLH, keyhole limpet hemocyanin; Wy14643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid; BADGE, bisphenol A diglycidyl ether; PCR, polymerase chain reaction; LS, linker scanning; GST, glutathione *S*-transferase; DN, dominant negative; TK, thymidine kinase; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

tion is mediated by subset-specific transcription factors such as GATA-3 and c-Maf during the differentiation of naive T cells into Th2 cells (Murphy et al., 2000). A phase of short-term gene transcription, elicited by the interaction of differentiated T cells with antigen, requires the antigen-induced transcription factor NF-AT (Rao et al., 1997).

NF-AT transcription factors have a highly conserved DNA binding domain of approximately 300 amino acids that shows a distant sequence but significant structural homology with the DNA-binding domain of Rel/NF-κB factors (Wolfe et al., 1997). It is designated the Rel similarity domain (RSD). Presently, the NF-AT family consists of at least four members: NF-ATp (NF-AT1 or NF-ATc2), NF-ATc (NF-AT2 or NF-ATc1), NF-AT3 (NF-ATc4), and NF-AT4 (NF-ATx or NF-ATc3). The promoters of the human and murine IL-4 genes contain at least five NF-AT sites that control their induction in T cells (Li-Weber et al., 1998). Mutation or deletion of any one of these NF-AT sites results in a significant decrease in promoter activity. Of the NF-AT family members, NF-ATp and NF-ATc are the predominant NF-AT proteins expressed in human and murine T cells. NF-ATp negatively regulates IL-4 gene expression but NF-ATc is the dominant transactivator of the IL-4 promoter (Szabo et al., 1997).

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear hormone-receptor superfamily of ligand-activated transcription factors that are related to retinoid, steroid, and thyroid hormone receptors (Lemberger et al., 1996). When PPAR-γ is activated by ligand binding, it heterodimerizes with at least one other member of the steroid receptor superfamily, the retinoic acid receptor, and activates gene expression by binding to PPAR response elements. PPAR-γ ligands can also block both AP-1 and NF-κB-mediated gene expression (Subbaramaiah et al., 2001). PPAR-γ ligands include naturally occurring ligands such as the prostaglandin D_2 metabolite 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) (Forman et al., 1995) and synthetic ligands such as the antidiabetic thiazolidinedione agents ciglitazone and troglitazone (Lehmann et al., 1995). These ligands bind to PPAR-γ and stimulate transcription of target genes. PPAR-γ is expressed as two isoforms, PPAR-γ1 and PPAR-γ2. These isoforms differ in promoter usage, with the result that PPAR-γ2 has a 30-amino acid extension at the N-terminal domain. PPAR-γ2 is predominantly expressed in adipose tissue and can regulate adipogenesis. In addition to its expression in fat cells, PPAR-γ1 is expressed in other cells, such as macrophages, colonic epithelial cells, transformed endothelial cells, and vascular smooth muscle cells. Naive mouse T cells are known to express mRNA for PPAR-γ1 but not the isoform PPAR-γ2. When T cells are activated, the expression pattern of PPAR-γ does not change (Harris and Phipps, 2001). Recent findings indicate that PPAR-γ is a negative regulator of macrophage activation and inhibits production of inflammatory cytokines derived from monocytes (Ricote et al., 1998; Chung et al., 2000). The activation of T cells differs from that of monocytes, because it proceeds by the initial activation of transcription factors that ultimately result in the biological amplification of the immune response. Although PPAR-γ mRNA is expressed in mouse T cells, little is known of PPAR-γ in the regulation of IL-4-expression by T cells.

Herein, we report that PPAR-γ ligands significantly inhibited IL-4 production in both antigen-stimulated primary T cells and phorbol 12-myristate 13-acetate (PMA)/ionomycin-activated T cell line. The experimental results indicated that PPAR-γ ligand-mediated suppression of IL-4-production from activated T cells may involve, at least in part, both inhibition of the NF-AT–DNA interactions and direct interactions between NF-ATc1 and PPAR-γ.

Experimental Procedures

Cell Lines, Cell Culture, Mice, and Transient Transfection. Lymph node cells from BALB/c mice and EL-4 thymoma cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA). CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Female BALB/c mice were obtained from Daehan Animal Inc. (Seoul, Korea). The mice were maintained in pathogen-limited conditions and treated according to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. For transfection, the cells were maintained for 24 h in 24-well plates in DMEM supplemented with 10% FBS and transfected with the indicated plasmid in the presence of Superfectam, according to the manufacturer's protocol (QIAGEN, Hilden, Germany). After 20 h, the cells were washed and refed with DMEM or RPMI 1640 containing 10% FBS. The cells were harvested 20 h later and luciferase activity was assayed as described previously (Ausubel el al., 1995). The results were normalized to the *Lac*Z-expression.

Monoclonal Antibodies, Cytokines, and Reagents. Anti-IL-4 mAbs 11B11 and BVD2 were obtained from M. Howard (DNAX Research Institute, Stanford, CA). Recombinant murine IL-4 was purchased from BD Pharmingen (San Diego, CA). Anti-CD8 mAb (Lyti-2.2, hybridoma 3.155) and anti-CD4 mAb (L3T4, hybridoma GK 1.5) were purified from ascitic fluids by ammonium sulfate precipitation. Hybridoma cell lines 3.155 and GK 1.5 were purchased from American Type Culture Collection (Manassas, VA). Keyhole limpet hemocyanin (KLH) was from Calbiochem (San Diego, CA). PMA, ionomycin, and Wy14643 were from Sigma-Aldrich Co. (St. Louis, MO). 15d-PGJ_2 and ciglitazone were from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Bisphenol A diglycidyl ether (BADGE) was from Fluka (Milwaukee, WI).

Plasmids. The −741/56 fragment of the murine IL-4 promoter was generated by polymerase chain reaction (PCR) from genomic DNA of DBA/2 mice. The PCR products were cloned into the *Bam*HI and *Eco*RI sites of the plasmid pGEM-7Z, followed by sequencing of both strands to verify sequence fidelity. The pGEM (IL-4/741) was subcloned into pGL3-basic luciferase vector (Promega, Madison, WI). All the deletion mutants of mIL-4 promoter were generated by PCR, using an upstream primer containing a *Bam*HI site. A linker-scanning mutant (IL-4/LS) was generated by a two-step PCR procedure with overlapping internal primers that contain mutated sequences for the NF-AT sites (P1 and P4). The GST-PPAR-γ expression vector was subcloned into *Sma*I and *Xho*I restriction sites of the pGEX4T-1 expression vector (Amersham Biosciences, Piscataway, NJ). The mNF-ATc1 cDNA was generated by reverse transcription-PCR from mRNA of BALB/c mice. The PCR products were cloned into the *Eco*RI and *Xho*I sites of the pCDNA3 expression vector (Invitrogen). All deletion mutants of mNF-ATc1 were generated by PCR using an upstream primer containing an *Eco*RI site, followed by both strands to verify sequence fidelity. The L466A/E469A PPAR-γ double mutant (PPAR-γ DN) was generated by site-directed mutagenesis of the wild-type receptor and verified by sequencing. His-NF-ATc1 was generated by PCR using the C-terminal His-tagged PCR primer. Amplified PCR products of His-NF-ATc1 were cloned into *Eco*RI and *Xho*I sites of the pCDNA3 expression vector (Invitrogen Co., San Diego, CA). The reporter constructs Gal4-TK-luc, PPAR-γ expression vector, and the transfection indicator pRSV β-gal were constructed as described previously (Chung et al., 2000).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from the cells, and the EMSA was performed as described previously (Chung et al., 2000). The sequence of the oligonucleotide used as probe was 5′-GAGCCCTAAACTCATTTTCCCTTGAAA-3′ (NF-AT).

Coimmunoprecipitation Assay. COS-7 cells were transiently transfected with 10 μg of His-NF-ATc1, PPAR-γ, and/or pCDNA3. Transfected cells were induced by PMA/ionomycin in the absence or presence of PPAR-γ ligands (15d-PGJ_2 or ciglitazone). After 36 h, nuclear extracts were purified from the transfected cells. Nuclear extracts (100 μg) were diluted 1:10 with binding buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40, 10% glycerol, and 600 mM NaCl) and incubated with Ni^{2+} agarose beads, which were equilibrated in binding buffer for 12 h. After extensive washing, the beads were boiled for 4 min in SDS-PAGE sample buffer. The eluted material was separated by SDS-PAGE and analyzed by Western blotting with a rabbit polyclonal anti-PPAR-γ mAb (Santa Cruz Biotechnology, Santa Cruz, CA).

GST Pull-Down Assay. GST fusion or GST alone were expressed in *Escherichia coli* bound to glutathione-Sepharose-4B beads (Amersham Biosciences) and incubated with labeled proteins expressed by in vitro translation by using the TNT-coupled transcription-translation system, as described by the manufacturer (Promega). The GST pull-down assay was performed as described previously (Chung et al., 2000).

In Vitro Stimulation of Lymph Node Cells. Lymph node cells were obtained from mice injected in the footpads with 100 μg of KLH absorbed to aluminum hydroxide adjuvant. Seven days later, lymph node cells were obtained from the immunized mice, and single-cell suspensions were prepared. The cells were cultured in vitro with KLH (100 μg/ml) in the absence or presence of PPAR-γ ligands. At the specified times, cell supernatants were harvested for IL-4 protein determinations and total RNA was isolated for IL-4 mRNA measurements.

In Vitro Depletion of T-Cell Subsets. For in vitro depletion of either $CD8^+$ T cells or $CD4^+$ T cells, mAbs for each T-cell subset were used as described previously (Kim et al., 2000). In brief, lymph node cells from immunized mice were incubated with anti-CD4 (L3T4) or anti-CD8 (Lyt-2.2) mAbs on ice for 30 min, followed by addition of low-toxicity rabbit complement (Pel-Freez, Rogers, AR) at 37°C for 45 min. The antibodies were titered such that the concentrations used were five times the minimal amount required to saturate the specific binding sites of lymph node cells from naive BALB/c mice, as determined by cytofluorimetric analysis of serially diluted antibodies. After depletion of the specific cell-types, the remaining cells were washed with serum-free RPMI 1640 medium, and incubated with KLH (50 μg/ml) for 4 days. Immunofluorescent analysis of lymph node cells after mAb treatment indicated that there was >95% depletion of specific T-cell subsets with no decrease in the frequency of the other subsets.

IL-4 Measurements by ELISA and Reverse Transcription-PCR. The levels of IL-4 in the culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). The mAbs for coating the plates and the biotinylated second mAbs were 11B11 and BVD6. Total RNA was prepared from the cells and reverse-transcribed into cDNA. Total RNA was isolated by the single step method using the TRI-Reagent (Sigma). The sequences of PCR primers were as follows: mouse IL-4 (sense, 5′-ATGGGTCTCAACCCCCAGCTAGT-3′; antisense, 5′-GCTCTTTACGCT TTCCAGGAAGTC-3′), and β-actin (sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; antisense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′). The PCR products for IL-4 and β-actin genes were 397 and 349 base pairs, respectively. After amplification, the PCR products were separated in 1.2% (w/v) agarose gels and stained with ethidium bromide.

Statistical Analysis. Student's *t* test and one-way analysis of variance followed by the Bonferroni method were used to determine the statistical differences between the values of various experimental and control groups. A p value of <0.05 was considered statistically significant.

Results

PPAR-γ Ligands Inhibited IL-4 Production in both Antigen-Primed Primary $CD4^+$ T Cells and the PMA/Ionomycin-Stimulated T Cell Line. We investigated the effect of PPAR-γ ligands on the production of IL-4 by primary lymph node cells from KLH-primed mice. The cells were stimulated in vitro for 4 days with KLH in the absence or presence of PPAR-γ ligands. As indicated (Fig. 1A), 15d-PGJ_2, a natural PPAR-γ ligand, and ciglitazone, a thiazolidinedione family, significantly inhibited KLH-stimulated IL-4 production in a dose-dependent manner. In contrast, Wy14643, a well known PPAR-α ligand (Forman et al., 1997), didn't repress the production of IL-4 in primary KLH-primed cells (Fig. 1A) and also in PMA/ionomycin-stimulated EL-4 T cells (Fig. 1C), suggesting that the inhibitory effects of IL-4 production was specific for PPAR-γ ligands.

To determine whether $CD4^+$ T cells were the IL-4 producing cell-type affected by KLH-treatment, either $CD4^+$ or $CD8^+$ T cells were depleted from KLH-primed lymph node cells by treatment with anti-CD4 or anti-CD8 mAbs, plus complement, followed by stimulation in vitro with KLH. The IL-4 levels in the depleted cell cultures were compared with those of cell cultures incubated with either growth medium or complement alone, followed by in vitro stimulation with KLH. As indicated (Fig. 1B), in vitro re-stimulation of KLH-primed lymph node cells with KLH-induced significant levels of IL-4, which were reduced to the background levels after depletion of $CD4^+$ T cells, indicates that $CD4^+$ T cells were the primary cell-type responsible for the production of IL-4 in KLH-activated lymph node cells.

To investigate whether the inhibitory effect of PPAR-γ ligands on IL-4 production also occurred in T cells, EL-4 T cells were stimulated in vitro for 30 h with PMA/ionomycin in the absence or presence of PPAR-γ ligands. As indicated (Fig. 1C), PPAR-γ ligands significantly inhibited IL-4 production by PMA/ionomycin-stimulated EL-4 cells in a dose-dependent manner. 15d-PGJ_2 and ciglitazone were not toxic to the cells at concentrations of <10 and <75 μM, respectively, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (data not shown).

We next analyzed IL-4 mRNA levels in KLH-primed lymph node cells and PMA/ionomycin-activated EL-4 cells in the presence of PPAR-γ ligands, to determine whether changes in IL-4 production were accompanied by changes in the expression of IL-4 mRNA. As shown in Fig. 2, 15d-PGJ_2 and ciglitazone significantly inhibited IL-4 mRNA levels in both KLH-primed lymph node cells and PMA/ionomycin-activated EL-4 T cells, indicating that the changes in IL-4 production with PPAR-γ ligands were present at the transcriptional level. In contrast, treatment with 15d-PGJ_2 and ciglitazone did not affect β-actin mRNA expression by KLH-primed lymph node cells or PMA/ionomycin-activated EL-4 T cells, suggesting that the inhibitory effect of IL-4 production by 15d-PGJ_2 and ciglitazone was not the result of a generalized inhibition of cellular activation.

PPAR-γ Ligands Inhibited NF-AT–Mediated Activation of the IL-4 Gene Promoter by PMA/Ionomycin. The IL-4 gene promoter contains multiple binding sites for mem-

bers of the NF-AT family of transcription factors, termed P elements P0 to P4 (Tara et al., 1993). To identify the region involved in actions of PPAR-γ ligands, we generated a series of luciferase reporter constructs containing the IL-4 gene promoter sequences from positions −741, −251, and −46 to + 71 relative to the transcription initiation site (Fig. 3A). EL-4 cells were transfected with each of these constructs and stimulated with PMA/ionomycin in the absence or presence of PPAR-γ ligands, and the luciferase activity was determined. As shown in Fig. 3B, each of these constructs showed strong stimulation with PMA/ionomycin in the absence of 15d-PGJ_2 or ciglitazone but impaired stimulation in the presence of 15d-PGJ_2 and ciglitazone. In particular, deleting sequences to −251 (IL-4/251) did not diminish the PMA/ionomycin-dependent promoter activities, and the inhibitory effect of PPAR-γ ligands was still observed, suggesting that the target site for PPAR-γ ligands resided within this region. However, addition of 15d-PGJ_2 and ciglitazone to PMA/ionomycin-stimulated cells did not repress luciferase activity of the IL-4/46 deletion mutant, indicating that the inhibitory effect of PPAR-γ ligands on IL-4 production was mediated through NF-AT sites.

To directly test the role of NF-AT sites found between −46 and −251 of the IL-4 gene promoter in the PPARγ ligand-mediated inhibitory actions, we introduced linker scanning mutations into two NF-AT sites (P1 and P4) within the context of the −741/+71 construct (IL-4/LS). Although all P elements to some extent contribute to IL-4 gene control, the major positive regulatory P elements seem to be P1 and P4. The PMA/ionomycin-dependent promoter activation was still observed with IL-4/LS promoter construct although significantly reduced (Fig. 2B). However, addition of PPAR-γ ligands to PMA/ionomycin-stimulated cells did not have any repressive effects with IL-4/LS construct, clearly indicating that the inhibitory effect of PPAR-γ ligands on IL-4 production was mediated through the NF-AT sites.

Direct Interaction between PPAR-γ and NF-AT. With the precedent of direct interaction of human NF-AT with human PPAR-γ on the human IL-2 promoter (Yang et al., 2000), we hypothesized that the association of mouse NF-ATc1 with mouse PPAR-γ1 may have led to the NF-AT-mediated inhibitory action of 15d-PGJ_2 and ciglitazone. We investigated whether NF-ATc1 could associate with PPAR-γ in the cellular context. HeLa cells were transfected with vectors expressing His-tagged NF-ATc1, PPAR-γ, or both. Nuclear extracts from these cells were subjected to precipitation with nickel-linked beads to capture the His-tagged protein and then immunoblotted for PPAR-γ. As shown in Fig. 4A, PPAR-γ and NF-ATc1 form a complex when these two factors are cotransfected into HeLa cells. To localize the interaction region, GST alone and GST fusion to PPAR-γ1 were expressed, purified, and tested for interaction with in vitro translated NF-ATc1. Schematic representations of NF-ATc1 and their deletion constructs are presented in Fig. 4B. Radiolabeled NF-ATc1 interacted with GST fusions to the PPAR-γ1 but not with GST alone. Furthermore, the NF-ATc1

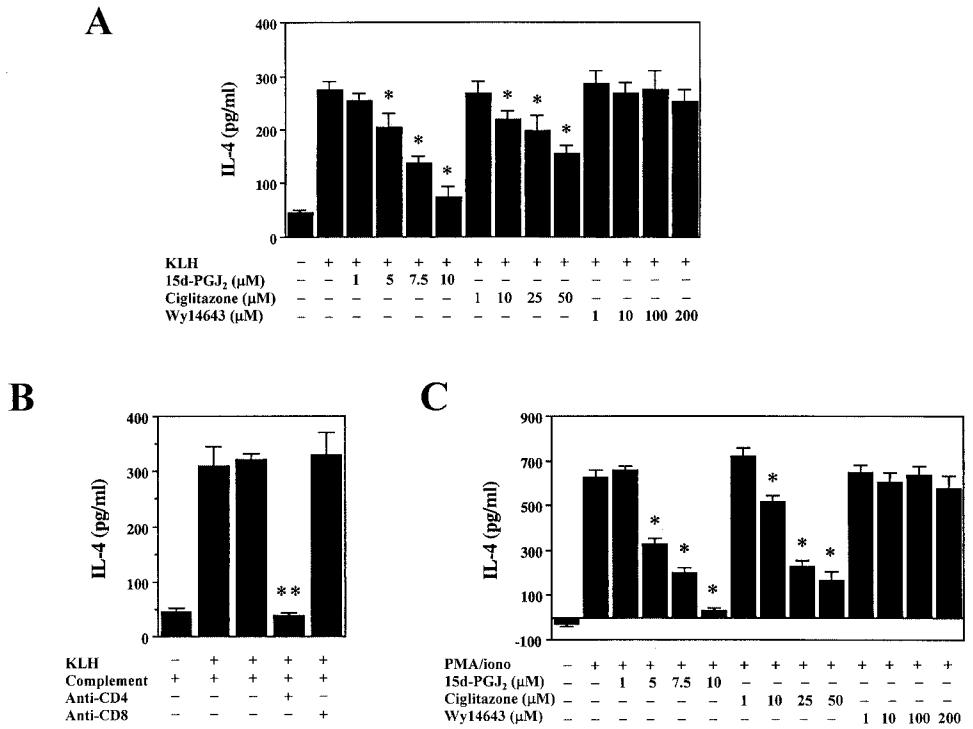

Fig. 1. Inhibition of IL-4 production in activated primary $CD4^+$ T cells and EL-4 cell-line by PPAR-γ ligands. A, 15d-PGJ_2 and ciglitazone inhibited KLH-stimulated IL-4 production by primary lymph node cells. Mice were injected into the footpad with KLH in alum. After 7 days, regional lymph node cells were collected and stimulated in vitro with KLH (100 μg/ml), in the presence of varying doses of 15d-PGJ_2, ciglitazone or Wy14643 for 4 days. The cell culture-supernatants were harvested and assayed for IL-4 levels by ELISA. B, $CD4^+$ T cells were the major cell type for IL-4 production induced by KLH. Lymph node cells were incubated for 30 min with anti-CD8 or anti-CD4 mAbs on ice, followed by incubation with complement at 37°C for 45 min. After washing, the cells were stimulated with KLH (50 μg/ml) for 4 days and the levels of IL-4 in the supernatants were determined. C, EL-4 cells were stimulated for 30 h with PMA (5 ng/ml) and ionomycin (100 nM) in the absence or presence of 15d-PGJ_2, ciglitazone or Wy14643, and the IL-4 levels were determined. The data represent the mean ± S.E. (n = 4). *, $p < 0.01$ versus KLH- or PMA/ionomycin-stimulated cells without PPAR-γ ligand treatment. **, $p < 0.001$ versus KLH-stimulated cells treated with complement only.

did not bind with a partial NF-ATc1 that lacks the RSD region (Fig. 4C), suggesting that PPAR-γ1 directly associates with NF-AT through a subregion of NF-ATc1 containing RSD.

NF-AT Binding to the NF-AT Site Was Inhibited by 15d-PGJ$_2$ and Ciglitazone. Steroid receptors inhibit NF-AT binding to NF-AT sites in a ligand-dependent manner (Takeuchi et al., 1998; Towers and Freedman, 1998). To determine whether PPAR-γ ligand-mediated inhibition of the NF-AT transactivation also involved similar mechanisms, we analyzed NF-AT DNA binding activity in nuclear extracts of unstimulated or PMA/ionomycin-stimulated T cells in the presence of 15d-PGJ$_2$ or ciglitazone. As shown in Fig. 5A, nuclear extracts of PMA/ionomycin-activated T cells exhibited strong NF-AT binding activity in the electrophoretic mobility shift assay using a labeled oligonucleotide containing a consensus NF-AT site. The binding was specific because it was competed with an unlabeled, identical oligonucleotide, but not with unrelated, nonspecific oligonucleotide, and it was absent with nuclear extracts from unstimulated cells. Nuclear extracts from EL-4 cells stimulated by PMA/ionomycin in the presence of PPAR-γ ligands revealed diminished NF-AT DNA binding activity (Fig. 5A). To rule out the possibility that these inhibitory actions of PPAR-γ ligands were the results of PPAR-γ-directed gene expression of a third component, PPAR-γ ligands were directly added to the binding reactions, along with nuclear extracts from PMA/ionomycin-stimulated EL-4 T cells. In these experiments, the NF-AT DNA binding activities decreased in a PPAR-γ ligand-dose-dependent manner, suggesting that the PPAR-γ ligand-bound receptor may directly modulate the NF-AT-DNA interactions by forming a complex with NF-AT that is unable to bind NF-AT sites (Fig. 5B). Furthermore, to determine whether the NF-AT DNA binding activity was dependent of the binding of either ligands to the endogenous PPAR-γ, PPAR- γ was depleted in nuclear extract of PMA/ionomycin-stimulated EL-4 T cells by immunoprecipitation and the NF-AT DNA binding activity was determined in the absence or presence of PPAR- γ ligands. 15d-PGJ$_2$ partially inhibited the NF-AT DNA binding activity at 10 μM; ciglitazone did not (Fig. 5C).

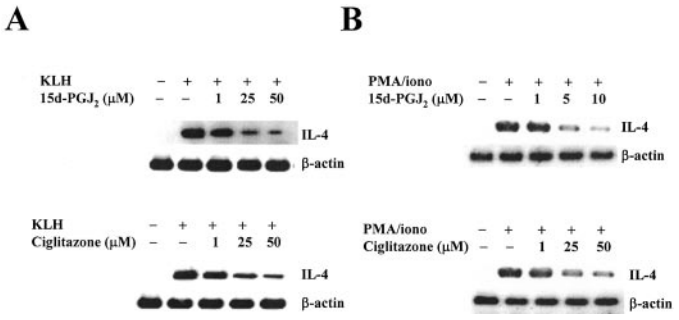

Fig. 2. Inhibition of IL-4 mRNA expression by PPAR-γ ligands. KLH-primed lymph node cells (A) and EL-4 T cells (B) were stimulated in vitro with KLH (100 μg/ml) and PMA (5 ng/ml)/ionomycin (100 nM), respectively, in the presence of varying amounts of 15d-PGJ$_2$ (1, 5, or 10 μM) or ciglitazone (1, 25, or 50 μM). Afterward, total RNA was prepared from the cells. Reverse transcription PCR was conducted with the respective primer pairs and analyzed in 1.2% agarose gels. The data are representative of three independent experiments.

Inhibitory Effects of PPAR-γ Overexpression on NF-AT–Mediated Activation of the IL-4 Promoter by PMA/Ionomycin. To determine whether over-expression of PPAR-γ affected the activation of the IL-4 promoter, EL-4 cells were transfected with each of the IL-4 promoter constructs alone or in combination with an expression vector containing PPAR-γ cDNA. Afterward, the transfected cells were stimulated with PMA/ionomycin in the absence or presence of 15d-PGJ$_2$ (5 μM) or ciglitazone (25 μM). As shown in Fig. 6, the transfected cells with PPAR-γ cDNA showed reduced activity of IL-4 promoter activity (IL-4/741). Furthermore, over-expression of PPAR-γ in EL-4 cells enhanced the down-regulation of IL-4 promoter activity by 15d-PGJ$_2$ or ciglitazone. However, over-expression of PPAR-γ did not reveal repressive effects in PMA/ionomycin-stimulated cells transfected with IL-4/46 deletion mutant, indicating that the inhibitory effects of 15d-PGJ$_2$ or ciglitazone on IL-4 production were mediated through NF-AT sites.

The NF-AT–Inhibitory Actions of 15d-PGJ$_2$ and Ciglitazone. Because the PPAR-γ binding sites involved the RSD (DNA binding site) of NF-ATc1 (Fig. 4), we tested whether the NF-AT-inhibitory actions of 15d-PGJ$_2$ and ciglitazone required binding at the NF-AT site. Gal4 fusion to

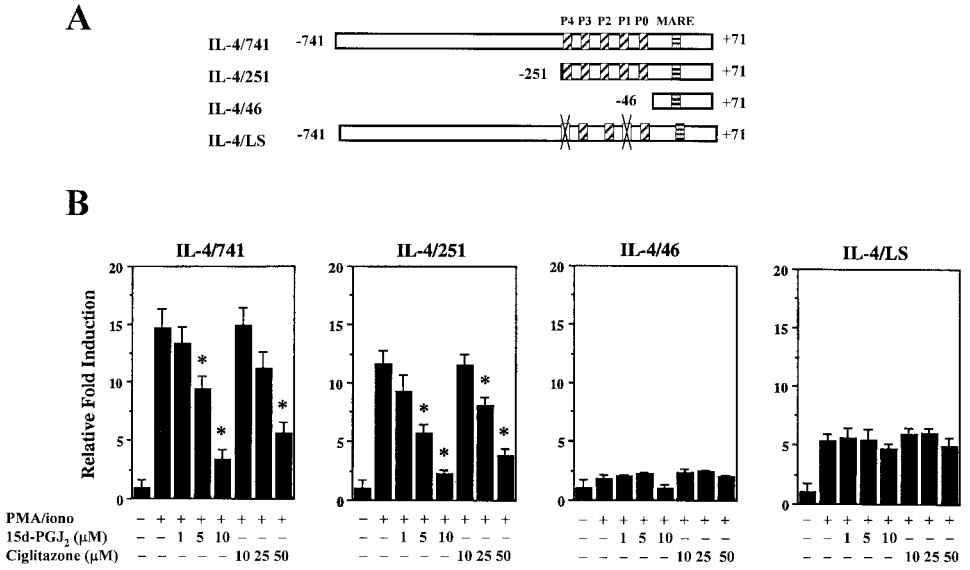

Fig. 3. Inhibition of IL-4 promoter-activation by PPAR-γ ligands. A, schematic representation of the mouse IL-4 gene promoter constructs as well as a linker-scanning mutant for NF-AT sites. P and MARE represent NF-AT binding sites and a c-Maf binding site, respectively. The nucleotide sequence numbers for each construct are indicated. B, 15d-PGJ$_2$ and ciglitazone inhibited transcriptional activity IL-4 promoter activated by PMA and ionomycin. EL-4 T cells were transiently transfected with the IL-4 promoter constructs, followed by stimulation with 5 ng/ml PMA and 100 nM ionomycin in the presence of various amounts of 15d-PGJ$_2$ or ciglitazone. The data represent the mean ± S.D. of triplicate determinations. The data are representative of four independent experiments. *, $p < 0.05$ versus a group of cells stimulated with PMA/ionomycin without 15d-PGJ$_2$ or ciglitazone.

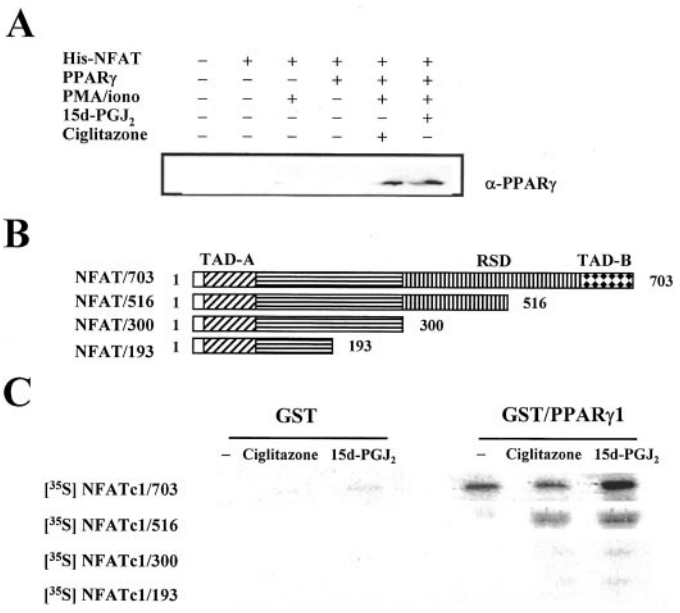

Fig. 4. Interactions of PPAR-γ with NF-ATc1 in vivo and in vitro. A, PPAR-γ associates with NF-ATc1 in cells. HeLa cells were transfected with PPAR-γ and NF-ATc1 containing an N-terminal His tag. Nuclear extracts from trasfected cells induced by PMA/Iono and/or ligands (5 μM 15-PGJ_2 or 25 μM ciglitazone) were incubated with beads containing Ni^{2+} ion. After extensive washing, the bound proteins were eluted and separated by SDS-PAGE, followed by Western blotting using an anti-PPAR-γ antibody. B, the schematic shows the structure of the NF-ATc1 factor and three NF-ATc1 fragments. C, PPAR-γ directly interacts with NF-ATc1. The wild-type and deletion mutants NF-ATc1 were labeled with [^{35}S]methionine by in vitro translation and incubated with glutathione beads containing GST alone or GST fusions to PPAR-γ1 in the presence of 5 μM 15-PGJ_2 (or 25 μM ciglitazone), as indicated. Beads were washed, and specifically bound material was eluted with reduced glutathione and resolved by SDS-polyacrylamide gel electrophoresis. Typically, approximately 10 to 20% of total input was retained.

NF-ATc1 (Gal4/NF-ATc1) was expressed in HeLa cells, along with a reporter-construct controlled by upstream Gal4 sites. As shown in Fig. 7, Gal4/NF-AT directed a strong activation of the reporter gene. Cotransfection of increasing amounts of the PPAR-γ expression vector had no significant effects in the absence of 15d-PGJ_2 or ciglitazone. In contrast, PPAR-γ in the presence of 15d-PGJ_2 or ciglitazone resulted in inhibition of Gal4/NF-AT transactivation in a PPAR-γ dose-dependent manner (Fig. 7A). Next, transient transfections were performed to elucidate further the effects of PPAR-γ and PPAR-γ ligands on NF-AT transactivation. As shown in Fig. 7B, the transactivation activity of NF-AT was inhibited by PPAR-γ in the presence of PPAR-γ ligands. Interestingly, the cotransfection of HeLa cells with PPAR-γ DN, a dominant-negative mutant antagonizing PPAR-γ signaling, moderately enhanced the reporter gene expression in the absence of PPAR-γ ligands, whereas the suppressive effects of 15d-PGJ_2 and ciglitazone on NF-AT transactivation were blocked by over-expressing a dominant-negative form of PPAR-γ (Fig. 7B). These results suggested that the inhibitory actions of 15d-PGJ_2 or ciglitazone could also occur through the cross-talk between NF-AT and ligand-activated PPAR-γ.

Inhibitory Effects of PPAR-γ in Interaction between NF-AT and p300 or AP-1. Because it was reported that competition for common coactivators could be a mechanism of gene repression by nuclear receptors (Lee et al., 2001), the association of p300, a coactivator that has been shown to interact with PPAR-γ and NF-AT (Garcia-Rodriguez and Rao, 1998), was investigated in the repression of NF-AT by ligand-activated PPAR-γ. The p300 was cotransfected into HeLa cells along with a reporter construct Gal4-Tk-luc and Gal4/NF-AT. As shown in Fig. 8A, increasing amounts of cotransfected p300 enhanced the transactivation of this reporter in a dose-dependent manner. Interestingly, cotransfection with PPAR-γ in the absence of PPAR-γ ligands inhib-

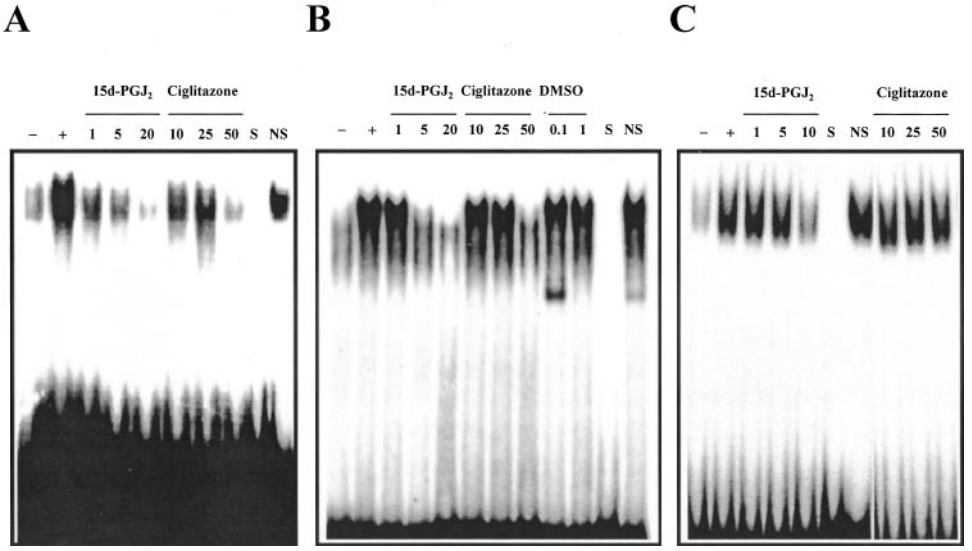

Fig. 5. PPAR-γ ligand-mediated inhibition of DNA-binding by NF-AT. A, 5d-PGJ_2 and ciglitazone decreased DNA binding of NF-AT. Nuclear extracts from EL-4 cells stimulated by PMA and ionomycin in the presence of 15d-PGJ_2 (1, 5, or 10 μM) and ciglitazone (10, 25, or 50 μM) were examined for NF-AT DNA binding activity in the EMSA, using a labeled oligonucleotide containing a NF-AT site of IL-4 promoter, as indicated. B, 15d-PGJ or ciglitazone were directly added to nuclear extracts prepared from EL-4 cells stimulated by PMA and ionomycin in the absence of 15d-PGJ_2 or ciglitazone, and NF-AT binding activity was examined in the EMSA. Increasing amounts of 15d-PGJ_2, ciglitazone, or carrier [dimethyl sulfoxide (DMSO); 0.1 and 1%] was used as indicated. C, 15d-PGJ or ciglitazone were added to nuclear extracts from PMA/ionomycin-activated EL-4 cells immunodepleted of PPAR-γ, and NF-AT binding activity was examined in the EMSA. S and The presence of unlabeled identical (S) and nonspecific (NS) oligonucleotides is indicated. The specific NF-AT complexes are indicated.

ited the enhanced transactivation of reporter by p300. However, the enhanced transactivation of reporter was blocked by ligand-activated PPAR-γ. Indeed, the inhibitory effects of NF-AT by ligand-activated PPAR-γ were largely relieved upon addition of increasing amounts of p300 expression vector, and the relieved effect was saturated at >500 ng of p300 (Fig. 8B). Thus, competition for limiting amounts of p300 could account for the mutual inhibitions between NF-AT and ligand-activated PPAR-γ.

As a result of GST pull-down experiments (Fig. 4C), PPAR-γ physically interacts with the C-terminal region of NF-AT; this region also associates with the AP-1 family, which is well known as a collaborator of NF-AT (Gonzalez et al., 2001). Accordingly, to delineate whether the effects of PPAR-γ and PPAR-γ ligands are involved in the transcriptional cross-talk between NF-AT and AP-1, transfection experiments were performed using a Gal4/NF-AT in the absence or presence of c-Fos (a component of AP-1) and PPAR-γ. As shown in Fig. 9, cotransfection of HeLa cells with c-Fos enhanced the transactivation of reporter in the absence of PPAR-γ and PPAR-γ ligand. Importantly, PPAR-γ remarkably suppressed the enhanced NF-AT transactivation in the presence of 15d-PGJ_2 or ciglitazone.

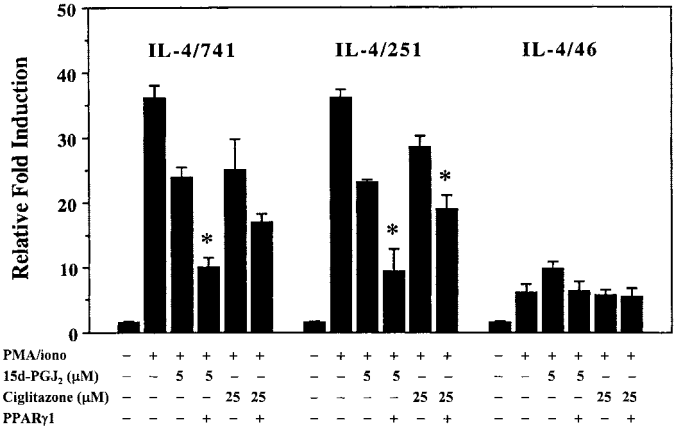

Fig. 6. Effects of PPAR-γ overexpression on down-regulated IL-4 promoter activity by PPAR-γ ligands. EL-4 cells were transfected with IL-4 promoter constructs or IL-4 promoter constructs plus PPAR-γ1 cDNA (250 ng) in the absence or presence of 15d-PGJ_2 or ciglitazone, followed by stimulation with PMA/ionomycin. The total amount of DNA in each reaction was kept constant at 1.0 μg by adding empty vectors. The results are expressed as an induction (*n*-fold) over the value obtained with the unstimulated EL-4 cells transfected with the IL-4 constructs, which was assigned an arbitrary value of 1. The data represent the mean ± S.D. of triplicate determinations. The data are representative of three independent experiments. *, $p < 0.005$ versus PMA/ionomycin-stimulated cells in the presence of PPAR-γ ligand but without PPAR-γ1 cotransfection.

Effect of a PPAR-γ Antagonist, BADGE, on Suppressed IL-4 Production by 15d-PGJ_2 or Ciglitazone. A number of novel PPAR-γ agonists and antagonists were identified (Houseknecht et al., 2002). BADGE was reported to act as a relatively selective antagonist for PPAR-γ (Wright et al., 2000). If the suppression of IL-4 expression by PPAR-γ ligands were mediated via PPAR-γ, we would expect that the PPAR-γ-specific antagonist would restore the inhibitory effect of PPAR-γ ligands on IL-4 production. To test this hypothesis, KLH-primed lymph node cells were pretreated with BADGE at 25 μM for 1 h before the addition of 15d-PGJ_2 or ciglitazone. Afterward, they were stimulated for 4 days with KLH (100 μg/ml). As indicated (Fig. 10A), the suppression of KLH-induced IL-4 production by ciglitazone was abrogated by BADGE, whereas the inhibitory effect of 15d-PGJ_2 on IL-4 production was partially restored by BADGE. Similar results were found in EL-4 cells (data not shown). These results suggest that ciglitazone and 15d-PGJ_2 might inhibit IL-4 production in T-lymphocytes in different ways, respectively.

Discussion

In this study, we have demonstrated that the PPAR-γ ligands 15d-PGJ_2 and ciglitazone inhibited IL-4 production in both antigen-primed primary $CD4^+$ T cells and the PMA/ionomycin-activated EL-4 T cell line in a dose-dependent manner. The inhibitory effect was caused, at least in part, by down-regulation of NF-AT activation and binding to the NF-AT sites by interactions of PPAR-γ and NF-AT. Our

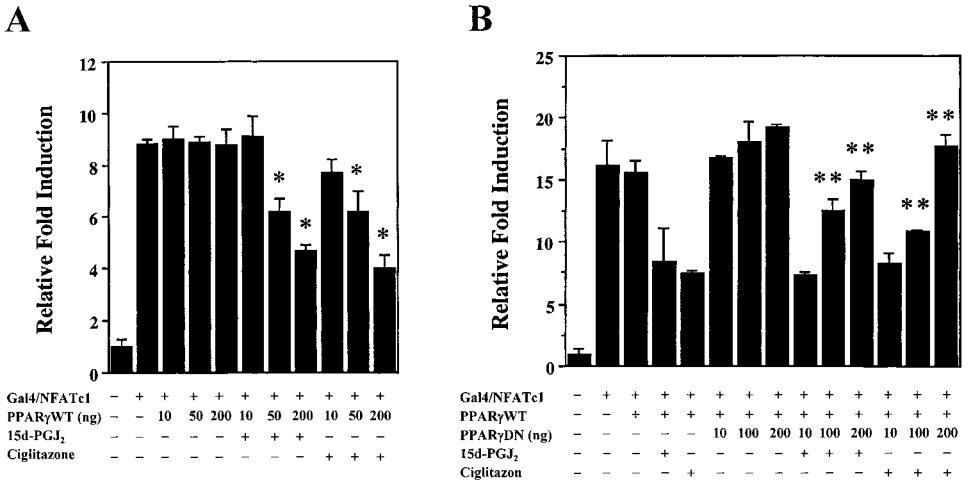

Fig. 7. 15d-PGJ_2 and ciglitazone-mediated transrepression of NF-ATc1 in the absence of NF-AT DNA binding site. HeLa cells were transfected with a reporter gene (Gal4-TK-Luc) and Gal4/NF-ATc1 (100 ng) expression vector, along with PPAR-γ1 (10, 50, or 200 ng) expression vectors (A) and PPAR-γ DN (10, 100, or 200 ng) expression vector (B). After transfection for 24 h, cells were treated with media, 15d-PGJ_2 (5 μM), or ciglitazone (25 μM), as indicated. Normalized luciferase expressions from triplicate samples are presented relative to *LacZ* expression, and standard deviations are less than 5%. The data represent the mean ± S.D. of triplicate determinations. The data are representative of three independent experiments. *, $p < 0.005$ versus a group of cells cotransfected with Gal4/NFATc1 and PPAR-γ wild type (WT) but without PPAR-γ ligand treatment. **, $p < 0.01$ versus a group of cells cotransfected with Gal4/NFATc1, PPAR-γ WT, and PPAR-γ DN but without PPAR-γ ligand treatment.

present finding that PPAR-γ ligands affect IL-4 production by $CD4^+$ T cells is of special interest, because IL-4 mediates important pro-inflammatory functions in asthma, including the induction of the IgE isotype switch, expression of vascular cell adhesion molecule-1, promotion of eosinophil transmigration across endothelium and the induction of Th2-mediated immune responses (Hikidia et al., 1999; Fukushi et al., 2000; Avila et al., 2002).

PPAR-γ and its ligands have now been implicated in the pathology and/or treatment of numerous diseases including obesity, diabetes, atherosclerosis and cancer (Houseknecht et al., 2002). Although PPAR-γ-expression was initially considered to be restricted to tissues such as liver and fat, recent work has demonstrated PPAR-γ expression in vascular cells, such as monocytes/macrophages, epithelial cells, and smooth muscle cells. Ricote et al. (1998) reported that 15d-PGJ_2 and synthetic PPAR-γ ligands inhibited the expression of a number of genes that were up-regulated during macrophage activation. These included inducible nitric-oxide synthase, metallanoproteinase-9, and cytokines, such as tumor necrosis factor-α, IL-1, and IL-6. Recent studies also documented PPAR-γ expression in murine and human T cells (Yang et al., 2000). The expression and function of PPAR-γ in T cells significantly expands the immunoregulatory role of PPAR-γ. Marx et al. (2002) reported that activation of PPAR-γ in human T cells limited the expression of such proinflammatory cytokines as IFN-γ, yielding potential therapeutic benefits in pathological processes (e.g., atherosclerosis and transplantation-associated arteriosclerosis). It is now clear that PPAR-γ–mediated immunoregulation can be mediated at the level of both macrophages and T cells.

The inhibitory effects of PPAR-γ ligands on IL-4 production were mediated through the transcription factor NF-AT. In our study, the inhibitory effects of PPAR-γ ligands on the PMA/ionomycin-activated IL-4 gene promoter disappeared if the transcription factor NF-AT sites in the promoter were deleted. Addition of PPAR-γ ligands to PMA/ionomycin-stimulated cells didn't have any repressive effects with IL-4 gene promoter construct (IL-4/LS) with linker scanning mutations at two major NF-AT sites (P1 and P4) (Fig. 3). Furthermore, treatment with PPAR-γ ligands suppressed the NF-AT DNA binding activity in PMA/ionomycin-activated T cells (Fig. 5). The transcription factor NF-AT is known to play an essential role in the inducible transcription of the IL-4 gene during T cell-activation, because human and murine IL-4 gene promoters contain at least four NF-AT sites that control their induction in T cells (Li-Weber et al., 1998). High levels of IL-4 production in atopic Th2 cells are known to closely associate with selective reduction of suppressive NF-AT1 at the IL-4 P0 element (Pu-b_A) (Wierenga et al., 1999).

As one might expect, the influence of PPAR-γ ligands on cytokine gene expression is complex and context-dependent. In monocytes/macrophages, 15d-PGJ_2 itself can induce IL-8 gene expression, whereas the expression of monocytic chemoattractant protein-1 is suppressed. Exposure of resting monocytes/macrophages to PPAR-γ ligands led to increased expression of cytokines, such as IL-1 and IL-12 (Uyemura et al., 1996), whereas PPAR-γ ligands suppressed these cytokines in activated macrophages (Ricote et al., 1998). Interestingly, treatment with the macrophage-activation cytokine IFN-γ prevented PPAR-γ ligands from suppressing the pro-inflammatory cytokines, indicating that activation conditions affected PPAR-γ–mediated anti-inflammatory activity (Alleva et al., 2002).

In addition to macrophages, differential regulation of cytokine gene expression was reported in T cells. Pretreatment of human T cells with PPAR-γ ligands reduced anti-CD3-induced IFN-γ and IL-2 secretion (Marx et al., 2002). PPAR-γ attenuated the activation of T cells by inhibiting cytokine gene-expression and was considered a negative regulator of the inflammatory activation of T cells via inhibition of the transcription factors AP-1 and NF-κB (Wang et al., 2001). In

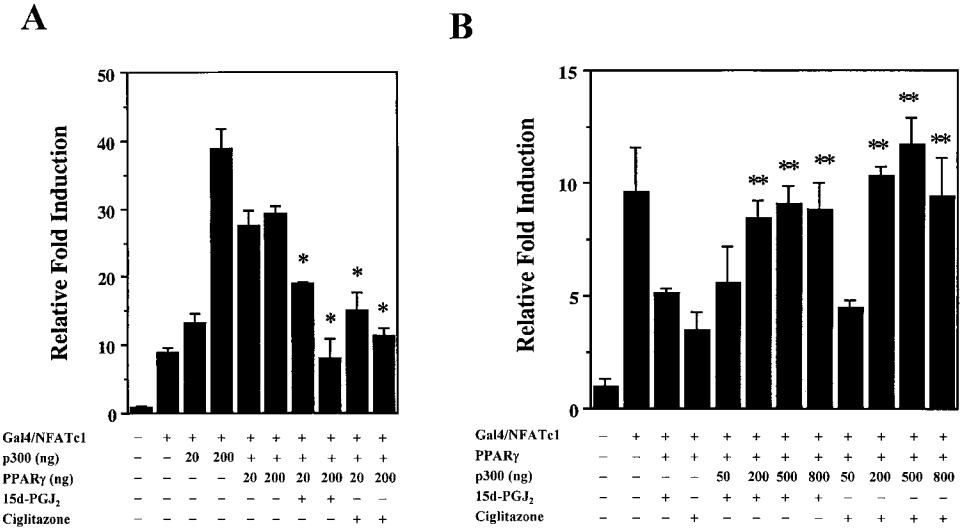

Fig. 8. Inhibitory effects of ligand-activated PPAR-γ in the transactivation between NF-AT and p300. A, HeLa cells were transfected with a reporter gene (Gal4-TK-Luc) and Gal4/NF-ATc1 (100 ng) expression vector, either in the absence or presence of p300 (20 or 200 ng), along with PPAR-γ1 (20 or 200 ng) expression vectors. B, HeLa cells were transfected with Gal4/NF-AT (100 ng) and PPAR-γ1 (200 ng) expression vectors in the absence or presence of varying amounts of p300 (50, 200, 500, or 800 ng). After 24 h, the cells were treated with media, 15d-PGJ_2 (5 μM), or ciglitazone (25 μM), as indicated. Normalized luciferase expressions from triplicate samples are presented relative to *Lac*Z expression, and standard deviations are less than 5%. The data represent the mean ± S.D. of triplicate determinations. The data are representative of four independent experiments. *, $p < 0.001$ versus a group of cells cotransfected with Gal4/NF-ATc1, p300, and PPAR-γ but without PPAR-γ ligand treatment. **, $p < 0.001$ versus a group of cells cotransfected with Gal4/NF-ATc1 and PPAR-γ in the presence of PPAR-γ ligand but without p300 cotransfection.

contrast, 15d-PGJ_2 induced a significant increase in both IL-8 mRNA and protein from anti-CD3 and anti-CD28-stimulated human T lymphocytes via a MAPK and NF-κB pathway, whereas 15d-PGJ_2 treatment of unstimulated T cells induced cell death, suggesting that 15d-PGJ_2 can act as a potent proinflammatory mediator in activated human T cells by inducing the production of IL-8 (Harris et al., 2002). In our study, 15d-PGJ_2 significantly suppressed IL-4 production in both antigen-stimulated primary $CD4^+$ T cells and the PMA/ionomycin-activated EL-4 T cell line. Thus, the effects of PPAR-γ ligands on cytokine gene expression were dependent on the activation state of the cell, and PPAR-γ ligands regulated T cells by possessing both pro- and anti-inflammatory properties.

15d-PGJ_2 and ciglitazone may inhibit IL-4 production in activated T cells via different binding regions of PPAR-γ. Our data indicated that the inhibitory effect of ciglitazone on IL-4 expression was completely abrogated by BADGE, a PPAR-γ antagonist, whereas the diminished production of IL-4 in the presence of 15d-PGJ_2 was partially restored by BADGE treatment (Fig. 10A). These results suggest that ciglitazone and 15d-PGJ_2 might inhibit, at least in part, IL-4 production by T-lymphocytes in different ways. Furthermore, the effects of 15d-PGJ_2 on IL-4 production were mediated via both mechanisms, which are dependent and independent of endogenous PPAR-γ. 15d-PGJ_2 partially inhibited NF-AT binding activity in nuclear extract immuno-depleted of PPAR-g (Fig. 5C); ciglitazone did not. Several studies have reported that 15d-PGJ_2 can have profound effects on gene expression and production of inflammatory mediators by mechanisms that are independent of PPAR-γ (Straus et al., 2000). In addition, Gonzalez et al. (2002) reported that the addition of BADGE was unable to modify diminished nitrate/nitrite levels induced by 15d-PGJ_2. Furthermore, the ligand binding domain of PPAR-γ was broadly specific, which may explain the diversity of ligands for PPAR-γ. For example, rosiglitazone occupies roughly 40% of the ligand-binding site in the ternary complex (Nolte et al., 1998).

The regulation of cytokine gene-expression via direct interaction between nuclear receptors and transcription factors has been previously reported. Steroid receptors, including glucocorticoid, progesterone, and retinoid X receptors, were found to inhibit NF-κB activity and to interact with NF-κB in vitro (McKay and Cidlowski, 1999). We demonstrated that these interactions might be involved in the regulation of IL-12 gene expression (Na et al., 1999). Dexamethasone was shown to inhibit the IL-2 promoter by interference with AP-1. The current model of dexamethasone inhibition demonstrated interaction between the corticosteroid receptor and NF-κB. In the case of PPAR-γ, a nonsteroid nuclear receptor, it was reported that the NF-ATc1 coprecipitated with PPAR-γ in extracts of human peripheral blood T cells induced by PMA/phytohemagglutinin and 15d-PGJ_2 or troglitazone (Yang et al., 2000). We also found that PPAR-γ li-

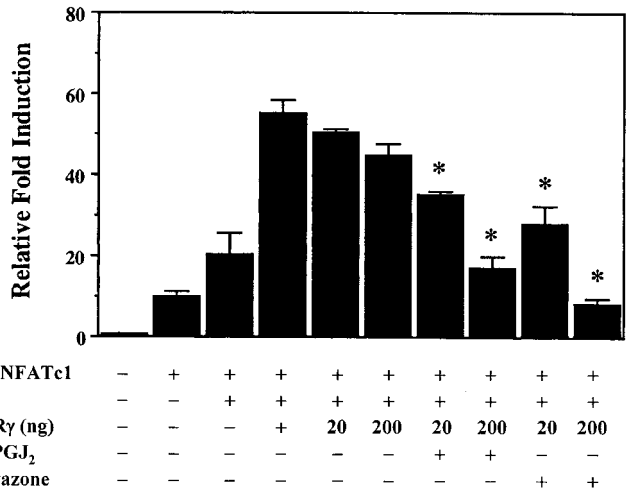

Fig. 9. Repression of ligand-activated PPAR-γ in the interaction between NF-AT and AP-1. HeLa cells were transfected with Gal4/NF-AT (100 ng), PPAR-γ (20 or 200 ng), and c-Fos (200 ng) expression vectors, along with Gal4-TK-Luc. Normalized luciferase expressions from triplicate samples are presented relative to *Lac*Z expression, and standard deviations are less than 5%. The data represent the mean ± S.D. of triplicate determinations. *, $p < 0.01$ versus a group of cells cotransfected with Gal4/NF-ATc1, c-Fos, and PPAR-γ without PPAR-γ ligand treatment.

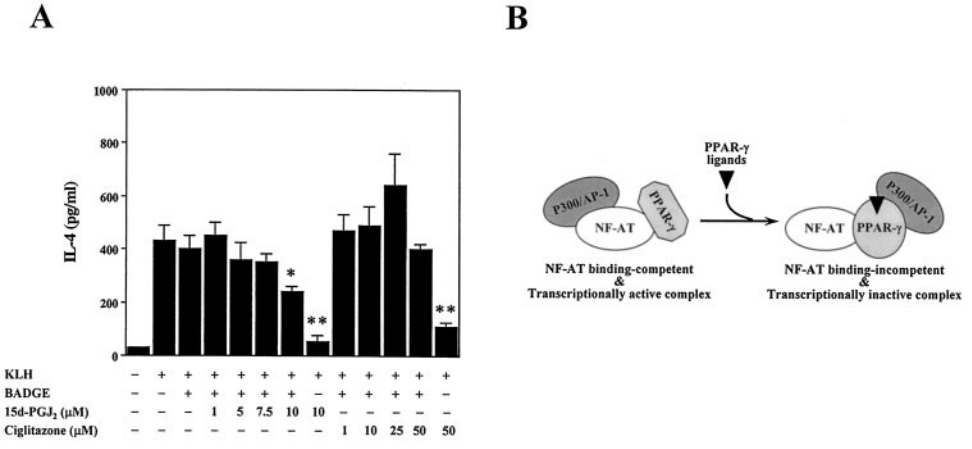

Fig. 10. Effect of the PPAR-γ antagonist BADGE on suppressed IL-4 production by PPAR-γ ligands. A, BADGE reversed the inhibition of IL-4 production stimulated by ciglitazone in KLH-activated lymph node cells. KLH-primed lymph node cells were collected and pretreated for 1 h with BADGE (25 μM). After washing, the cells were stimulated in vitro for 4 days with KLH (100 μg/ml) in the presence of varying amounts of 15d-PGJ_2 (1, 5, 7.5, and 10 μM) or ciglitazone (1, 10, 25, and 50 μM). The cell culture supernatants were harvested and assayed for IL-4 levels by ELISA. The data represent the mean ± S.E.M. ($n = 4$). *, $p < 0.01$ versus KLH-stimulated control. **, $p < 0.005$ versus KLH-stimulated group without BADGE treatment. B, a model for the NF-AT and PPAR-γ interactions. NF-AT may constitutively bind PPAR-γ through the Rel similarity domain of NF-AT. PPAR-γ ligand binding may induce significant conformational change with PPAR-γ, leading to inhibition of the NF-AT–DNA binding site interactions. p300 and AP-1 may constitutively bind NF-AT but recognize PPAR-γ only in the presence of ligand.

gands, such as oxidized low-density lipoprotein and ciglitazone, significantly inhibited IL-12 production in mouse macrophages via interaction of PPAR-γ and NF-κB (Chung et al., 2000).

As was the case with glucocorticoids and dexamethasone, this inhibition also mapped to the distal NF-AT sites in the IL-4 promoter and may involve direct interactions of PPAR-γ with NF-AT (Fig. 4) (Li-Weber et al., 1998). Although the ligand-activated PPAR-γ physically interacted with the C-terminal region of NF-AT, we cannot completely exclude any possible roles of the N-terminal region in binding with PPAR-γ because the deletion of RSD region could disrupt secondary and/or tertiary structures of the NF-AT molecule. Furthermore, NF-AT constitutively interacted with PPAR-γ (Fig. 4), whereas the inhibitory actions were absolutely PPAR-γ ligand-dependent (Figs. 1–3). Thus, NF-AT may exist constitutively associated with PPAR-γ in vivo, and this complex becomes transcriptionally inactive upon addition of PPAR-γ ligands. In addition, transcription coactivators, such as p300/cAMP response element binding protein and SRC-1, may play important roles because these cofactors are known to directly interact with PPAR-γ and can regulate the transcriptional activities (Qi et al., 2000). Indeed, increasing amounts of p300 expression vector relieved the inhibitory activity of ligands-activated PPAR-γ on NF-AT transactivation (Fig. 8). In this study, 15d-PGJ_2 and ciglitazone also inhibited the NF-AT binding activities of NF-AT in vitro (Fig. 5), suggesting that the PPAR-γ/NF-AT complex was unable to recognize NF-AT sites. However, why the PPAR-γ/NF-AT complex loses its ability to bind NF-AT sites is unknown. Conceivably, a conformational change induced by the addition of 15d-PGJ_2 and ciglitazone becomes propagated to the Rel similarity domain of NF-AT, resulting in inability to bind NF-AT sites. The inhibitory actions of 15d-PGJ_2 and ciglitazone can also operate by a novel mechanism that involves protein-protein interactions. Although we focused on the interaction between NF-AT and PPAR-γ, we cannot exclude the possibility that PPAR-γ interaction with AP-1 may also play an important role in regulating IL-4 gene transcription. AP-1 is known to be necessary for the full activity of the NF-AT DNA binding to the NF-AT sites (Li-Weber et al., 1998). Our study showed that coexpression of increasing amounts of AP-1 strongly transactivated NF-AT activity in the absence of ligand-activated PPAR-γ but impaired transactivation with ligand-activated PPAR-γ (Fig. 9). As summarized in the model (Fig. 10B), on T cell activation, cytosolic NF-AT is translocated into nucleus and associates with other factors, such as AP-1, p300/cAMP response element binding protein, and PPAR-γ, leading to NF-AT transactivation involved in IL-4 production. However, in the presence of PPAR-γ ligands, the NF-AT complex was changed into a transcriptionally inactive or less active form for IL-4 production.

In conclusion, we found that PPAR-γ forms a transcriptionally inhibitory complex with NF-AT. With the NF-AT transactivation, in particular, this PPAR-γ ligand-mediated inhibitory action seemed to involve inhibition of the NF-AT–DNA interactions as well as competitive recruitment of transcription integrators between NF-AT and PPAR-γ. This transrepression between NF-AT and PPAR-γ could play an important role in a number of different biological processes and suggests that PPAR-γ may be an anti-inflammatory target in the treatment of allergic diseases.

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