Peroxisome Proliferator-activated Receptor γ Negatively Regulates IFN-β Production in Toll-like Receptor (TLR) 3- and TLR4-stimulated Macrophages by Preventing Interferon Regulatory Factor 3 Binding to the IFN-β Promoter*

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Toll-like receptors 3 and 4 utilize adaptor TRIF to activate interferon regulatory factor 3 (IRF3), resulting in IFN-β production to mediate anti-viral and bacterial infection. Peroxisome proliferator-activated receptor (PPAR)-γ is a ligand-activated transcription factor expressed in various immune cells and acts as a transcriptional repressor to inhibit the transcription of many proinflammatory cytokines. But, the function of PPAR-γ in TLR3- and 4-mediated IFN-β production is not well elucidated. Here, we have analyzed the effect of the PPAR-γ agonists on IFN-β production in peritoneal primary macrophages in response to LPS and poly(I:C). PPAR-γ agonists inhibited LPS and poly(I:C)-induced IFN-β transcription and secretion. siRNA knockdown of PPAR-γ expression and transfection of PPAR-γ expression plasmid demonstrated that PPAR-γ agonist inhibits IFN-β production in a PPAR-γ-dependent manner. The ability of the PPAR-γ agonist to inhibit IFN-β production was confirmed in vivo as mice treated with troglitazone exhibited decreased levels of IFN-β upon LPS and poly(I:C) challenge. Chromatin immunoprecipitation (CHIP) assay and electrophoretic mobility shift assay (EMSA) demonstrated that troglitazone treatment impaired IRF3 binding to the IFN-β promoter. Furthermore, troglitazone could inhibit LPS and poly(I:C)-induced STAT1 phosphorylation and subsequent ISRE activation. These results demonstrate that PPAR-γ negatively regulates IFN-β production in TLR3- and 4-stimulated macrophages by preventing IRF3 binding to the IFN-β promoter.

The production of type I interferon (IFN-α and -β) is central to the innate immune response to eliminate viral and bacterial infection (1, 2). Several pathogen recognition receptors recognize highly conserved microbial components and activate the regulatory pathways to coordinate the production of type I IFNs. Among them, Toll-like receptor (TLR)3 and 4 use TIR domain-containing adaptor inducing IFN-β (TRIF) to activate interferon regulatory factor 3 (IRF3), resulting in IFN-β production (1–3). Although type 1 IFNs are essential for the establishment of an anti-viral and anti-bacterial state, uncontrolled expression of type 1 IFNs has manifested in diverse pathogenic autoimmune diseases, including systemic lupus erythematosus (4, 5). Thus, understanding the mechanisms that limit or down-regulate type 1 IFNs production downstream of pathogen recognition is critical for developing treatments for these diseases.

Peroxisome proliferator-activated receptors (PPAR) are members of a nuclear receptor superfamily containing PPAR-α, PPAR-β/δ, and PPAR-γ (6). PPAR-γ is expressed in various immune cells, such as primary peritoneal macrophages, dendritic cells, and T cells (7–10). Growing evidence supports an anti-inflammatory role for PPAR-γ. Activation of PPAR-γ by various ligands down-regulates the synthesis and release of proinflammatory cytokines. For example, PPAR-γ ligands inhibit the expression of iNOS and TNF-α downstream of TLRs by ligand-dependent transrepression in macrophages (8–10). Therefore, PPAR-γ ligands may be important anti-inflammatory agents to treat inflammation related diseases. Varieties of endogenous and synthetic ligands bind to PPAR-γ to modulate gene expression (11). But, the functional role of PPAR-γ on IFN-β production downstream of TLR signaling is not known.

In this study, we have analyzed the effect of the PPAR-γ agonists on IFN-β production in peritoneal primary macrophages in response to LPS and poly(I:C). PPAR-γ agonists inhibited LPS and poly(I:C)-induced IFN-β transcription and secretion in peritoneal primary macrophages in a PPAR-γ-dependent manner. Furthermore, the PPAR-γ agonist, troglitazone, inhibited IFN-β production in mice following intra-peritoneal injection of LPS and poly(I:C). Chromatin immunoprecipitation (CHIP) assay and electrophoretic mobility...
ability shift assay (EMSA) showed that troglitazone treatment impaired IRF3 binding to the IFN-β promoter. As a result, LPS and poly(I:C)-induced STAT1 phosphorylation and subsequent ISRE activation were inhibited by troglitazone. These results demonstrate that PPAR-γ activation negatively regulates IFN-β production in TL3- and 4-stimulated macrophages by preventing IRF3 binding to the IFN-β promoter. Therefore, our results provide a strategy to limit or down-regulate the production type I IFNs downstream of pathogen recognition.

**EXPERIMENTAL PROCEDURES**

**Mice and Reagent**—C57BL/6j mice were obtained from Joint Ventures Sipper BK Experimental Animals (Shanghai, China). All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Medical School of Shandong University, Jinan, Shandong Province, China. LPS (Escherichia coli, 055:B5) and poly(I:C) were purchased from Sigma; LPS was re-purified as described (12). Troglitazone and GW9662 were purchased from Sigma. Rosiglitazone and 15d-PGJ2 were from Cayman (Ann Arbor, MI). Antibodies for IRF3, STAT1, PPAR-γ, Sp1, Hsp90, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies specific to phosphorylated STAT1 (Tyr-701) and IRF3 (Ser-396) were from Cell Signaling. Their respective horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Technology (Santa Cruz, CA). The antibodies specific to phospho-PPAR-γ (Ser-112), HIF-1α, and PPAR-γ were from Santa Cruz Biotechnology. The antibodies specific to phospho-HIF-1α (Ser-533), and PPAR-γ were from Cell Signaling. Their respective horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture**—Female C57BL/6j mice (5–6 weeks old) were used for the preparation of primary mouse macrophages. Thioglycolate-elicited mouse peritoneal macrophages were prepared as described (13). The cells were cultured in endotoxin-free DMEM with 10% FCS (Invitrogen). After 1 h, non-adherent cells were removed. The next day, the cells were transfected with Geneporter 2 Transfection Reagent (GTI, San Diego, CA) according to the manufacturer’s instructions. Mouse macrophage cell line RAW 264.7 and human HEK292 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37 °C under 5% CO2 in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. LPS and poly(I:C) were used at final concentrations of 100 ng/ml and 20 μg/ml, respectively.

**Plasmid Constructs**—PPAR-γ cDNA was amplified by RT-PCR from total mRNA of RAW264.7 cells, and cloned into pcMV-FLAG expression vector to produce the PPAR-γ expression plasmid. The primers used were PPAR-γ F, 5'-ATAAGAATGCGGCCGCACTGGTGGACACAGAGATGCC-3' and PPAR-γ R, 5'-GCGGTACCCATATAAAGTCTCTTGTAGATCTCC-3'. IFN-β and IRF3 reporter plasmids were kind gifts from Dr. Cao Xue Tao (14). The IRF3 reporter plasmids are composed of two plasmids, p-55UASGLuc and p-EF-GALA/IRF3 expression plasmids, which were first described by Dr. Yoneyama (15). TRIF and IRF3 expression plasmids were from Dr. Zhengfan Jiang (Peking University, China). ISRE reporter plasmid was a kind gift from Dr. Hongbing Shu (Wuhan University, China).

**Treatment of Animals and Isolation of Peritoneal Lavage Fluid and Serum**—Female C57BL/6j mice (5–6 weeks old) were intraperitoneally injected with thioglycolate to elicit peritoneal macrophages. After 3 days, the mice were treated with DMSO or 5 mg/kg of troglitazone administrated intraperitoneally for 40 min, followed by PBS, 1.8 mg/kg of LPS or 1 mg/kg of poly(I:C) intraperitoneal administration for 1 h. Mice were anesthetized with halothane and exsanguinated; the contents of the peritoneal cavity were sampled by peritoneal lavage. To obtain samples for cytokine assessment, 1 ml of PBS was injected intraperitoneally, the abdominal area was massaged to distribute the fluid, the skin over the peritoneal cavity (but not the peritoneal lining) was removed to allow visualization of the fluid, and a sample (0.7 ml) from the peritoneal cavity was removed using a needle (25 g) and syringe. After centrifugation (300 × g for 5 min), the cell pellet was saved, and the supernatant was stored at −20 °C until needed for cytokine assay. If analysis of cells was to be part of the experiment, an additional 7 ml of PBS was injected into the peritoneal cavity, and the steps outlined above were repeated to obtain the remaining cells. The cell pellets from the 1- and 7-lavages were pooled and used for subsequent analysis.

**RNA Interfering**—PPAR-γ siRNA, small interfering RNA targeting PPAR-γ, were 5’-AAUAUGACCCUGAGCUCCAGAAAUAG-3’ (PPAR-γ RNAi 1) and 5’-UAUUCUGGAGCUUCAGGUAUAUUAU-3’ (PPAR-γ RNAi 2). The control siRNA sequence was 5’-UUUCGAGCGUCUGUGACCCGUU-3’. For 1 × 106 cells, 0.4 nmol of PPAR-γ siRNA was mixed with 15 μl of Geneporter 2 Transfection Reagent and transfected into the cells. After 6 h, the supernatant was removed and fresh medium was added. The cells were cultured for another 36 h before additional experiments.

**Detection of Cytokines and NO Production**—2 × 105 mouse peritoneal macrophages were seeded into 24-well plates and incubated overnight. Cells were treated with troglitazone, rosiglitazone, or 15d-PGJ2 for 40 min, and stimulated with LPS or poly(I:C) for the indicated time periods. The concentrations of IFN-β, TNF-α, IL-6, and RANTES in culture supernatants were measured by ELISA kits (R&D Systems, Minneapolis, MN). NO was detected by the Griess reaction. For cytokine quantification from peritoneal lavage fluid and serum, peritoneal lavage fluid and serum were prepared as above, and IFN-β and RANTES were measured by ELISA kits (R&D Systems).

**Nuclear Extract Preparation**—Monolayers of peritoneal macrophages were washed with phosphate-buffered saline and harvested by scraping into cold phosphate-buffered saline. The cell pellet obtained by centrifugation was suspended in buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride; then 10% Nonidet P-40 was added and vortexed briefly, and the nuclei were pelleted by centrifugation. The nuclear proteins were extracted with buffer containing 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1.0 mM DTT, and 1.0 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 14,000 × g, and the supernatant containing the nuclear proteins was stored at −80 °C until use.
RNA Quantitation and Immunoblot Analysis—Total RNA was extracted with TRIzol reagent according to the manufacturer’s instructions (Invitrogen). A LightCycler (ABI PRISM® 7000) and a SYBR RT-PCR kit (Takara) were used for quantitative real-time RT-PCR analysis. Specific primers used for RT-PCR assays were 5'-ATGAGTGGTGTGCAGCC-3' (sense), 5'-TGACCTTTAATGGTAGTTC-3' (antisense) for IFN-β and 5'-TGTTACCAACTGGGACAGCA-3' (sense), 5'-CTGGGTGATCCTTCTACGCT-3' (antisense) for β-actin. Data were normalized to β-actin expression in each sample. For immunoblot analysis, cells were lysed with M-PER Protein Extraction Reagent (Pierce) supplemented with a protease inhibitor “mixture,” then protein concentrations in the extracts were measured with a bicinchoninic acid assay (Pierce) and were made equal with extraction reagent. Equal amounts of extracts were separated by SDS-PAGE, then transferred onto nitrocellulose membranes for immunoblot analysis as described previously (12).

Assay of Luciferase Reporter Gene Expression—RAW264.7 macrophages, or HEK293 cells were cotransfected with the mixture of the indicated luciferase reporter plasmid, pRL-TK-Renilla-luciferase plasmid, and the indicated amounts of PPAR-γ, TRIF, or IRF3 constructs using Jet-PEI transfection reagent (Polyplus). Total amounts of plasmid DNA were equalized via empty control vector. After 24 h, in selected instances, the cells were treated with TLR agonists. Luciferase activities were measured with a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

Electrophoretic Mobility Shift Assays (EMSA)—1 × 10⁶ mouse peritoneal macrophages were seeded into 6-well plates and incubated overnight. Cells were treated with DMSO or troglitazone for 40 min, and stimulated with poly(I:C) for the indicated time periods. Nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Synthetic complementary oligonucleotides were 3’-biotinylated using the biotin 3’-end DNA labeling kit (Pierce) according to the manufacturer’s instructions and annealed for 2 h at room temperature. The sequences of the oligonucleotides used were 5-GAAAACTGAAGGGAGAATGAAAA-3’ (nt –142 to nt –119) for the IRF3. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/μl of poly(dI-dC), 0.05% Nonidet P-40, 5 mM MgCl₂, 10 mM EDTA, and 2.5% glycerol in 1× binding buffer (LightShift™ chemiluminescent EMSA kit, Pierce) using 20 fmol of biotin end-labeled target DNA and 4 μg of nuclear extract. Assays were loaded onto native 7% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (Hybond™, N⁺) in 0.5× Tris borate/EDTA at 100 V for 30 min. Transferred DNAs were cross-linked to the membrane at 120 ml/cm² and detected using horseradish peroxidase-conjugated streptavidin (LightShift™ chemiluminescent EMSA kit) according to the manufacturer’s instructions. In competitive binding assays, unlabeled oligonucleotides were added at a 100-fold molar excess. In noncompetitive assays, unlabeled EBNA oligonucleotides were used. Supershift assays were performed by preincubating nuclear extracts with IRF3 antibody.

Chromatin Immunoprecipitation (ChIP) Assay—Mouse peritoneal macrophages were pretreated with DMSO, 30 μM troglitazone for 40 min and then stimulated with 20 μg/ml of poly(I:C) for the indicated time periods or left unstimulated. Chromatin from macrophages was fixed and immunoprecipitated using the ChIP assay kit as recommended by the manufacturer (Upstate Biotechnology, Inc.). The purified chromatin was immunoprecipitated using 2 μg of anti-IRF3, 2 μg of anti-PPAR-γ, or 2 μg of irrelevant antibody (anti-actin) antibodies. The input fraction corresponded to 0.1 and 0.05% of the chromatin solution before immunoprecipitation. After DNA purification, the presence of the selected DNA sequence was assessed by PCR. The primers were 5’-CCAGGAGCTTGAAATAGAA-3’, 5’-TGCAATGGAATGATCTTCTT-3’ for IFN-β promoter (−200 to −41) and 5’-GCTAC-TCTGCTGGCTTTTCA-3’, 5’-TACAGTTTCACCAATTGCTGGAG-3’ for the IFN-β promoter (−391 to −261). The PCR program was: 94 °C × 3 min; followed by 94 °C × 30 s, 55 °C × 30 s, and 72 °C × 30 s for a total of 40 cycles; and then 72 °C × 10 min. PCR products were resolved in 10% acrylamide gels. The average size of the sonicated DNA fragments subjected to immunoprecipitation was 500 bp as determined by ethidium bromide gel electrophoresis.

Statistical Analysis—All data are presented as mean ± S.E. of three or four experiments. Analysis was performed using a Student’s t test. Values of p < 0.05 were considered significant.

RESULTS

PPAR-γ Agonists Negatively Regulate LPS and Poly(I:C)-induced IFN-β Production in Macrophages—To investigate the roles of PPAR-γ on TLR-stimulated IFN-β production, primary peritoneal macrophages were stimulated with LPS (TLR4 ligand) or poly(I:C) (TLR3 ligand) in the presence of PPAR-γ agonists, troglitazone. IFN-β transcripts and protein secretion were then measured by quantitative RT-PCR and ELISA, respectively. As expected, stimulation with both LPS and poly(I:C) greatly increased IFN-β protein secretion into the culture medium (Fig. 1, A and B). Treatment with troglitazone significantly decreased LPS and poly(I:C)-induced IFN-β secretion (Fig. 1, A and B, p < 0.01). Similarly, the poly(I:C)-induced IFN-β steady state mRNA level was greatly decreased in the presence of troglitazone (Fig. 1C). Furthermore, troglitazone inhibited poly(I:C)-induced IFN-β secretion in a dose-dependent manner, a concentration as low as 5 μM could significantly inhibit IFN-β secretion (Fig. 1D). Rosiglitazone and 15d-PGJ₂, two other synthetic and natural PPAR-γ agonists, had a similar inhibitory effect on LPS and poly(I:C)-induced IFN-β production (Fig. 1E). As a control, LPS and poly(I:C)-induced TNF-α and IL-6 production were not inhibited by troglitazone treatment, whereas nitric oxide (NO) production was significantly inhibited by troglitazone (Fig. 1F). Collectively, these results suggest that PPAR-γ agonists inhibit TLR3- and -4-induced IFN-β transcription and protein expression in primary peritoneal macrophages.
Troglitazone Inhibits IFN-β Production in a PPAR-γ-dependent Manner—It has been reported that PPAR-γ agonists can use both PPAR-γ-dependent and PPAR-γ-independent mechanisms to regulate inflammatory responses (14). To determine whether PPAR-γ is involved in troglitazone-mediated inhibition of TLR-induced IFN-β production in macrophages, a series of experiments were performed. First, IFN-β promoter luciferase reporter and PPAR-γ expression plasmids were co-transfected into RAW264.7 cells. RAW264.7 macrophages have a very low level expression of PPAR-γ compared with that of primary peritoneal macrophages. LPS and poly(I:C) stimulation greatly increased IFN-β promoter activity in the presence and absence of PPAR-γ. Without transfection of PPAR-γ expression plasmid, troglitazone treatment alone could not decrease LPS- and poly(I:C)-induced IFN-β promoter activity in RAW264.7 cells (Fig. 2A, p > 0.05). In contrast, LPS- and poly(I:C)-induced IFN-β promoter activity was decreased ~70% and ~75%, respectively, in the presence of troglitazone and the PPAR-γ expression plasmid (Fig. 2A, p < 0.01).

This finding was further confirmed in HEK293 cells. Both TLR3 and TLR4 utilize adaptor TRIF to induce IFN-β production. Transfection of the TRIF expression plasmid can lead to IFN-β transcription in HEK293 cells. F, mouse peritoneal macrophages were stimulated with 100 ng/ml of LPS or 20 μg/ml of poly(I:C) for 8 h in the presence of DMSO or troglitazone. The production of TNF-α and IL-6 were detected by ELISA. NO was detected by the Griess reaction. Data are shown as mean ± S.D. (n = 3) of one representative experiment. **, p < 0.01, ▲, p > 0.05.
agonist inhibits IFN-\(\beta\) production in a PPAR-\(\gamma\)-dependent manner.

To further demonstrate that the PPAR-\(\gamma\) agonist inhibits IFN-\(\beta\) production in a PPAR-\(\gamma\)-dependent manner, PPAR-\(\gamma\)-specific siRNA was transfected into primary peritoneal macrophages. PPAR-\(\gamma\) protein was decreased \(\sim 60\%\) and \(\sim 90\%\), respectively, by transfection of PPAR-\(\gamma\) RNAi 1 and RNAi 2 as measured by immunoblotting (Fig. 2C). Similar results were obtained in three independent experiments. D, 1.5 \(\times\) 10\(^6\) mouse peritoneal macrophages were transfected with control small RNA (Ctrl RNAi) or PPAR-\(\gamma\) siRNA (PPAR-\(\gamma\) RNAi). After 36 h, PPAR-\(\gamma\) expression in the cells was detected by immunoblot. PPAR-\(\gamma\) expression was decreased by \(\sim 60\%\) and \(\sim 90\%\), respectively, by transfection of PPAR-\(\gamma\) RNAi 1 and RNAi 2 as measured by immunoblotting (Fig. 2C). Similar results were obtained in three independent experiments. D, 1.5 \(\times\) 10\(^6\) mouse peritoneal macrophages were transfected with control small RNA (Ctrl RNAi) or PPAR-\(\gamma\) siRNA (PPAR-\(\gamma\) RNAi). After 36 h, PPAR-\(\gamma\) expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments. D, 1.5 \(\times\) 10\(^6\) mouse peritoneal macrophages were transfected with control small RNA (Ctrl RNAi) or PPAR-\(\gamma\) siRNA (PPAR-\(\gamma\) RNAi). After 36 h, PPAR-\(\gamma\) expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments. D, 1.5 \(\times\) 10\(^6\) mouse peritoneal macrophages were transfected with control small RNA (Ctrl RNAi) or PPAR-\(\gamma\) siRNA (PPAR-\(\gamma\) RNAi). After 36 h, PPAR-\(\gamma\) expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments.

Finally, GW9662, a PPAR-\(\gamma\) antagonist, was used in troglitazone treatments. Troglitazone-mediated inhibition of LPS and poly(I:C)-induced IFN-\(\beta\) production was completely abrogated by GW9662 (Fig. 2E). Taken together, these data indicate that PPAR-\(\gamma\) agonists inhibit TLR3- and -4-induced IFN-\(\beta\) production in a PPAR-\(\gamma\)-dependent manner.

**FIGURE 2. Troglitazone inhibits IFN-\(\beta\) production in a PPAR-\(\gamma\)-dependent manner.** A, RAW264.7 cells in 96-well plates (2.5 \(\times\) 10\(^4\)/well) were transiently co-transfected with 100 ng of IFN-\(\beta\) reporter plasmid and 10 ng of pTK-Renilla plasmid, together with 100 ng of PPAR-\(\gamma\) or control plasmid. After 24 h, cells were pretreated with DMSO, 30 \(\mu\)M troglitazone for 40 min and then stimulated with 100 ng/ml of LPS or 20 \(\mu\)g/ml of poly(I:C) for 6 h. Luciferase activity was measured and normalized by Renilla luciferase activity. Data are shown as mean \(\pm\) S.D. (n = 6) of one representative experiment. B, HEK293 cells were transfected with 100 ng of IFN-\(\beta\) luciferase reporter plasmid, 10 ng of pTK-Renilla plasmid, 100 ng of TRIF expressing plasmid, together with 100 ng of PPAR-\(\gamma\) or control plasmid. After 8 h of culture, cells were treated and luciferase activity was measured as above. C, mouse peritoneal macrophages were transfected with control small RNA (Ctrl RNAi) or PPAR-\(\gamma\) siRNA (PPAR-\(\gamma\) RNAi). After 36 h, PPAR-\(\gamma\) expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments. D, 1.5 \(\times\) 10\(^6\) mouse peritoneal macrophages were transfected with control small RNA (Ctrl RNAi) or PPAR-\(\gamma\) siRNA (PPAR-\(\gamma\) RNAi). After 36 h, PPAR-\(\gamma\) expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments. D, 1.5 \(\times\) 10\(^6\) mouse peritoneal macrophages were transfected with control small RNA (Ctrl RNAi) or PPAR-\(\gamma\) siRNA (PPAR-\(\gamma\) RNAi). After 36 h, PPAR-\(\gamma\) expression in the cells was detected by immunoblot. Similar results were obtained in three independent experiments.

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In contrast, in the presence of troglitazone and the PPAR-γ expression plasmid, LPS- and poly(I:C)-induced IRF3 transcriptional activity was decreased ~50 and 80%, respectively (Fig. 3A, p < 0.01). Without transfection of the PPAR-γ expression plasmid, troglitazone treatment alone could not decrease LPS- and poly(I:C)-induced IRF3 transcriptional activity in RAW264.7 cells (Fig. 3A, p < 0.05).

To further confirm the inhibitory role of troglitazone in IRF3 transcriptional activity, PPAR-γ expression plasmid, TRIF expression plasmid, and IRF3 cis-reporting plasmids were co-transfected into HEK293 cells. As shown in Fig. 3B, TRIF transfection greatly increased IRF3 transcriptional activation. After transfection of the PPAR-γ expression plasmid, TRIF-induced IRF3 transcriptional activation in HEK293 cells was decreased ~80% in the presence of troglitazone (p < 0.01). Without transfection of the PPAR-γ expression plasmid, troglitazone treatment alone did not decrease TRIF-induced IRF3 transcriptional activation (p > 0.05).

RANTES is another cytokine that can be activated by IRF3 through binding to the ISRE element in the promoter (17). Consistent with the results that PPAR-γ inhibits IRF3 transcriptional activation, LPS and poly(I:C)-induced RANTES secretion from mouse peritoneal macrophages was greatly decreased using troglitazone treatment (Fig. 3C, p < 0.01). These data suggest that TLR3- and TLR4-induced IRF3 transcriptional activation is greatly attenuated in the presence of troglitazone.

PPAR-γ Prevents IRF3 Binding to IFN-β Promoter—To investigate the inhibitory mechanism of troglitazone in IRF3 transcriptional activity, first, IRF3 Ser-396 phosphorylation and IRF3 nuclear translocation were measured in poly(I:C)-stimulated primary macrophages in the presence of troglitazone. As shown in Fig. 4A, the level of poly(I:C)-induced Ser-396 phosphorylation was not affected by troglitazone. Similarly, poly(I:C)-induced IRF3 nuclear translocation were not affected by troglitazone as measured by Western blotting with nuclear proteins (Fig. 4B). Together these data suggest that IRF3 activation is not impaired in the presence of troglitazone.

IRF3 binding to the IFN-β promoter was measured by EMSA and CHIP assays. IRF3 binds to PRDIII-I of the IFN-β promoter (18). EMSA was performed using a biotin-labeled 24-nt fragment (nt −142 to −119) containing the PRDIII-I of the IFN-β promoter. Nuclear protein was isolated from control and poly(I:C)-treated primary macrophages in the presence or absence of troglitazone. As shown in Fig. 4A, the level of poly(I:C)-induced Ser-396 phosphorylation was not affected by troglitazone. Similarly, poly(I:C)-induced IRF3 nuclear translocation were not affected by troglitazone as measured by Western blotting with nuclear proteins (Fig. 4B). Together these data suggest that IRF3 activation is not impaired in the presence of troglitazone.
ficity of IRF3 binding was confirmed by unlabeled specific, nonspecific probes, and IRF3 antibodies with nuclear extracts from poly(I:C)-stimulated cells in the absence of troglitazone (Fig. 4D).

ChIP assays were then performed to confirm the in vivo binding of IRF3. PCR analysis showed that IRF3 antibody precipitated the IFN-β promoter region (nt −200 to −41) from peritoneal primary macrophages activated with poly(I:C) for 1 and 2 h (Fig. 4E), whereas unstimulated controls did not demonstrate this DNA binding. Using troglitazone treatment, IRF3 DNA binding to the IFN-β promoter was greatly decreased in poly(I:C)-stimulated primary macrophages (Fig. 4E). At the same time, PPAR-γ binding to this promoter region was greatly enhanced by troglitazone treatment (Fig. 4F).

Control antibodies (anti-actin antibody) did not exhibit this DNA binding activity (data not shown). None of the antibodies precipitated the IFN-β promoter region (nt −391 to −260) from peritoneal primary macrophages activated with poly(I:C) for 2 h, indicating specific binding of IRF3 and PPAR-γ to the promoter region from nt −200 to −41. Collectively, these data suggest that troglitazone-activated PPAR-γ inhibits IFN-β transcription by preventing IRF3 binding to the IFN-β promoter and not by inhibition of IRF3 activation.

**Troglitazone Inhibits IFN-β and RANTES Production in Vivo**—To further confirm that troglitazone inhibits IFN-β production in vivo, IFN-β secretion in blood and peritoneal fluid was measured in mice by intraperitoneal injection of LPS and poly(I:C). Female C57BL/6J mice (4 weeks old) were first injected with thioglycollate to elicit peritoneal macrophages. After 3 days, the mice were treated with PBS or LPS (1.8 mg/kg) or poly(I:C) (1 mg/kg) by intraperitoneal administration. For LPS/poly(I:C) and troglitazone treatments, mice were first treated by troglitazone (5 mg/kg) for 40 min, followed by LPS or poly(I:C) challenge. Four hours after LPS and poly(I:C) in-
traperitoneal injection, blood and peritoneal fluid were obtained, and IFN-β was measured by ELISA. As shown in Fig. 5A, IFN-β in peritoneal fluid was greatly increased after LPS or poly(I:C) injection, compared with control mice that received PBS. In contrast, when compared with mice that only received LPS or poly(I:C), the level of IFN-β was decreased ~40 and ~45%, respectively (p < 0.01), in mice administered troglitazone and challenged with PBS or poly(I:C) (Fig. 5A). These finding demonstrate that troglitazone inhibits IFN-β production in peritoneal macrophages in vivo.

Serum IFN-β level was also greatly increased in LPS and poly(I:C)-challenged mice compared with control mice that received PBS. Mice administered troglitazone and challenged with LPS or poly(I:C) exhibited a significant decrease in the level of IFN-β in the serum (Fig. 5C, p < 0.05), indicating that troglitazone can inhibit IFN-β production not only in macrophages but also in other cells following LPS stimulation.

Similarly, RANTES secretion in both blood and peritoneal fluid was greatly increased in mice administered troglitazone and challenged with LPS or poly(I:C), as compared with mice that only received LPS or poly(I:C) (Fig. 5, B and D, p < 0.01). Collectively, these findings demonstrate that administration of troglitazone can inhibit IRF3 transcriptional activation and subsequent IFN-β and RANTES production in vivo upon LPS or poly(I:C) challenge.

**PPAR-γ Attenuates TLR-induced IFN-β/STAT Signaling**—TLR-induced IFN-β production can activate transcription factor STAT1. Phosphorylated STAT1 conjugates with STAT2 and IRFs to generate the ISGF3 transcription complex. To confirm the function of PPAR-γ-mediated inhibition of IFN-β production, STAT1 phosphorylation (Tyr-701) was measured by immunoblotting in poly(I:C) and LPS-stimulated primary peritoneal macrophages in the presence of troglitazone. As reported, both poly(I:C) and LPS could induce STAT1 phosphorylation within 30 min. In contrast, poly(I:C) and LPS-induced STAT1 phosphorylation was greatly decreased by the treatment of troglitazone (Fig. 6A and B).

The ability of troglitazone to inhibit IFN-β production is dependent on PPAR-γ. PPAR-γ-specific siRNA was transfected into peritoneal primary macrophages, then poly(I:C)-induced STAT1 phosphorylation was measured by immunoblotting in the presence of troglitazone. As shown in Fig. 6C, poly(I:C)-induced STAT1 phosphorylation was greatly increased in PPAR-γ-specific siRNA-transfected macrophages compared with that in control siRNA-transfected cells.

Finally, poly(I:C) and LPS-induced ISRE-dependent activity was measured by transfecting ISRE reporting luciferase plasmid into RAW264.7 cells using the troglitazone treatment. Poly(I:C) and LPS stimulation induced 3- and 4-fold increases in ISRE activity, respectively (Fig. 6D). Troglitazone treatment and transfection of PPAR-γ expression plasmid significantly decreased poly(I:C) and LPS-induced ISRE-dependent activity by ~50 and ~50%, respectively (Fig. 6D, p < 0.01). Consistent with IFN-β expression data, without transfection of the PPAR-γ expression plasmid, troglitazone treatment alone could not decrease LPS and poly(I:C)-induced ISRE-dependent activity in RAW264.7 cells (Fig. 6D). Collectively, these data indicate that PPAR-γ activation by troglitazone attenuates TLR3- and -4-induced IFN-β production and subsequent STAT1 phosphorylation and ISRE activation.

**DISCUSSION**

Growing evidence support an anti-inflammatory role for PPAR-γ. Activation of PPAR-γ by various ligands down-regu-
Our results suggest that PPAR-γ agonists are expressed in both murine CD4 and CD8 T cells, and PPAR-γ agonists impair LPS and poly(I:C)-induced IRF3 transcription and protein secretion in vitro and in vivo. In particular, the PPAR-γ agonist, troglitazone, impaired LPS and poly(I:C)-induced IRF3 binding to the IFN-β promoter. As a result, LPS and poly(I:C)-induced STAT1 phosphorylation and subsequent ISRE activation are inhibited by troglitazone. Considering the involvement of IFN-β in several pathogenic autoimmune diseases, our results suggest that PPAR-γ agonists may have therapeutic potential to cure these diseases. In accordance with our results, recent studies have shown that PPAR-γ agonists could ameliorate murine lupus in a mouse model of SLE (22, 23).

The production of type I IFNs, including IFN-α and -β, is central for the innate immune responses to eliminate viral and bacterial infection. At the same time, uncontrolled expression of type I IFNs has been found in diverse pathogenic autoimmune diseases, including systemic lupus erythematosus (4, 5). Several pathogen recognition receptors, such as TLR3, TLR4, RIG-I, and MDA-5, recognize highly conserved microbial components and activate the regulatory pathways to coordinate the production of type I IFNs (24). TLR3 and TLR4 can use TRIF to activate IRF3, resulting in IFN-β production (3, 25). RIG-I and MDA-5 use the adapter called MAVS (also known as Cardif, IPS-1, or VISA) to activate IRF3, leading to IFN-β production (26–29). IRF3, as an essential transcription factor for IFN-β production, requires strict regulation. IRF3 degradation mediated by polyubiquitination to negatively regulate IFN-β production has been reported in several studies (30–33). Our studies show that IRF3 binding was impaired by troglitazone, a ligand for PPAR-γ. Therefore, IRF3 can be regulated at different levels.

A variety of PPAR-γ agonists have been demonstrated to have anti-inflammatory functions, including endogenous ligands such as 15d-PGJ2 and synthetic ligands such as troglitazone and rosiglitazone. Consistently, our data show that these three PPAR-γ ligands could inhibit LPS and poly(I:C)-induced IFN-β production in macrophages, indicating that these three ligands may use the same mechanism to inhibit IFN-β production. PPAR-γ agonists can use both PPAR-γ-dependent and PPAR-γ-independent mechanisms to regulate inflammatory responses (16). It has been reported that 15d-PGJ2 can inhibit NF-κB-mediated transcriptional activation by a PPAR-γ-dependent transrepression mechanism and by PPAR-γ-independent mechanisms, including inhibition of IkB kinase (IKK) activity and inhibition of NF-κB DNA binding (34, 35). Our siRNA knockdown and overexpression of PPAR-γ strongly demonstrated the PPAR-γ agonist inhibiting IFN-β production is dependent on the presence of PPAR-γ.

Ligand-activated PPAR-γ uses different mechanisms to regulate gene expression. A detailed mechanism by which
**Negative Regulation of IFN-β Production by PPAR-γ**

PPAR-γ agonists repress LPS-induced iNOS expression has been characterized in macrophages (36). After LPS stimulation, the corepressor NCOR complex was cleared from the iNOS promoter by ubiquitination-mediated degradation, then p65-p50 and another coactivator complex were recruited to initiate transcription of iNOS. In the presence of PPAR-γ ligands, PPAR-γ is SUMOylated. Ligand-bound and SUMOylated PPAR-γ binds to the corepressor NCOR complex to block ubiquitination and degradation of this complex and subsequent p65-p50 binding to iNOS promoter. NF-κB activation and nuclear translocation are not impaired. Consistent with this model, we found that IRF3 phosphorylation of IRF3 dimerization and nuclear translocation were not altered following troglitazone treatment. However, IRF3 DNA binding was impaired as measured by EMSA and CHIP assays. Importantly, PPAR-γ was impaired as measured by EMSA and CHIP assays. Consequently, PPAR-γ inhibition of iNOS expression may be the same. Alternatively, ligand-bound PPAR-γ may bind directly to the IRF3 element in IFN-β promoter and prevent the binding of IRF3. It has been reported that K-bZIP from Kaposi sarcoma-associated herpesvirus is capable of binding the binding of IRF3. It has been reported that K-bZIP from macrophages by preventing IRF3 binding to the IFN-β promoter. NF-κB activation and nuclear translocation are not impaired. In the presence of PPAR-γ agonists repress LPS-induced iNOS expression downstream of pathogen recognition and suggest that PPAR-γ agonists may have therapeutic potential in autoimmune diseases with uncontrolled IFN-β production.

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