Activation of Human T Lymphocytes Is Inhibited by Peroxisome Proliferator-activated Receptor γ (PPARγ) Agonists

PPARγ CO-ASSOCIATION WITH TRANSCRIPTION FACTOR NFAT*

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Xiao Yi Yang‡, Li Huaw Wang‡, Taosheng Chen‡, David R. Hodge‡, James H. Resau§, Luis DaSilva‡, and William L. Farrar§

From the §Intramural Research Support Program, SAIC Frederick and the §Cytokine Molecular Mechanisms Section, Laboratory of Molecular Immunoregulation, NCI-Frederick Cancer Research and Development Center and the ABL-Basic Research Program, NCI, National Institutes of Health, Frederick, Maryland 21702

T lymphocyte activation is highlighted by the induction of interleukin-2 (IL-2) gene expression, which governs much of the early lymphocyte proliferation responses. Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. PPARγ mRNA expression was found in human peripheral blood T lymphocytes, raising the possibility of PPARγ involvement in the regulation of T cell function. Here we show that PPARγ ligands, troglitazone and 15-deoxy-D12,14-prostaglandin J2, but not PPARα agonist Wy14643, inhibited IL-2 production and phytotrehemagglutinin-inducible proliferation in human peripheral blood T-cells in a dose-dependent manner. This inhibitory effect on IL-2 was restricted to the PPARγ-expressing, not the PPARγ-lacking, subpopulation of transfected Jurkat cells. The activated PPARγ physically associates with transcriptional factor NFAT regulating the IL-2 promoter, blocking NFAT DNA binding and transcriptional activity. This interaction with T-cell-specific transcription factors indicates an important immunomodulatory role for PPARγ in T lymphocytes and could suggest a previously unrecognized clinical potential for PPARγ ligands as immunotherapeutic drugs to treat T-cell-mediated diseases by targeting IL-2 gene expression.

*T lymphocytes are a key step in the initiation of an immunological response. An activating stimulus initiates a complex signaling cascade, resulting in cell proliferation and secretion of cytokines that enhance the immune response (1, 2). Interleukin-2 (IL-2) plays a key role in controlling T-cell proliferation, and consequently, numerous studies have focused on understanding IL-2 gene regulation (2). Peroxisome proliferator-activated receptors (PPARs) are transcription factors and members of the nuclear receptor superfamily (3). To date, three different PPARs, named PPARα, PPARδ, and PPARγ, have been identified in mammalian cells. PPARγ is primarily involved in the regulation of genes related to lipid metabolism and plays a role in adipocyte differentiation (4, 5). However, in higher organisms, the widespread tissue distribution of PPARγ (6) suggests an involvement of the nuclear receptor in multiple biological processes. Recent findings have indicated that PPARγ is a negative regulator of macrophage activation and inhibits production of monocyte inflammatory cytokines (7, 8). It is well known that the activation of T lymphocytes is much different from monocytes because it proceeds by the initial activation of transcription factors that ultimately fulfill the biological amplification of the immune response. Although PPARγ mRNA is expressed in human peripheral blood T lymphocytes (9), nothing is known about the physiologic regulation of PPARγ in T-cell activation. Here we demonstrate that the activated PPARγ physically associates with the T-cell-specific transcriptional factor NFAT and blocks NFAT DNA binding and transcriptional activity. Ultimately, the transcription and production of the vital T-cell cytokine IL-2 is blocked.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Fresh human peripheral blood T lymphocytes obtained from normal healthy donors (10) and Jurkat T cells were maintained as described (9, 10).

**Proliferation Assays—**T cells (5 × 10^6/well) were plated in 96-well microtiter plates in the presence or absence of PHA (1 μg/ml). Cells were treated for 72 h with troglitazone (Parke-Davis), 15d-PGJ$_2$ (Calbiochem), or Wy14643 (Biomol) and pulsed for the remaining 4 h of the assay with $[^3]$H/thymidine (0.5 μCi/200 μl). The incorporation was analyzed by liquid scintillation counting.

**IL-2 Measured by ELISA—**T cells were grown to 2.5 × 10^6 cells/ml and treated with PHA/PHA in the presence or absence of the different ligands. Cell supernatants were collected and assayed for human IL-2 using Endogen kits (Wolburn).

**Electrophoretic Mobility Shift Assay (EMSA)—**The sequences of the oligonucleotides (5’ to 3’) used as probes were AATGGAGGGAAAAAAAAAGCTGTTTATACAGAGGCGT (NFATwt), AATTGGAGGAAAAAC- TTACATACAGAGGCGT (NFATmt), and ATTGGAGGAAAAAC- TTACATACAGAGGCGT (NFATmt). The EMSA was performed as described (11).

**Ribonuclease Protection Assays—**Total RNA was isolated using TriZOL (Life Technologies, Inc.) and a RNase protection assay was performed as described (11).

**Flow Cytometry and Sterile Cell Sorting—**Cell-sorting for green fluorescent protein (GFP) and propidium iodide fluorescence were performed (12) using a FACStar Plus (Becton Dickinson, San Jose, CA)

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**To whom correspondence should be addressed: National Cancer Institute, P.O. Box B, Bldg. 560, Rm. 31-76, Frederick, MD 21702. Tel.: 301-846-1503; Fax: 301-846-6019; E-mail: farrar@mail.ncifcrf.gov.**

†To whom correspondence should be addressed: National Cancer Institute, P.O. Box B, Bldg. 560, Rm. 31-76, Frederick, MD 21702. Tel.: 301-846-1503; Fax: 301-846-6019; E-mail: farrar@mail.ncifcrf.gov.
with INNOVA 70-4 argon laser.

Luciferase Assays—The transfected cells were pelleted, lysed, and measured by a luminometer (Monolight 3010, Pharmingen) according to the manufacturer’s instruction.

Co-immunoprecipitation Assays—Cells were lysed in 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.5% Nonidet P-40. Immunoprecipitation was carried out using polyclonal anti-PPARγ antibodies (Santa Cruz). Western blot was performed as described (11).

RESULTS AND DISCUSSION

To determine whether PPARγ interferes with T lymphocyte activation, we investigated the effect of the PPARγ ligands on PHA-mediated T-cell proliferation and IL-2 production. Human peripheral blood T cells were stimulated by PHA and cultured with various concentrations of different ligands for 72 h. As shown in Fig. 1A, the specific PPARγ agonist of the thiazolidinedione family, troglitazone (13), completely abolished PHA-induced [³H]thymidine incorporation in a dose-dependent manner.

![FIG. 1](image1)

**Fig. 1.** Inhibition of PHA-induced proliferation and IL-2 production by PPARγ ligands in human T cells. A, PHA-induced cell proliferation is inhibited by troglitazone (○) or 15d-PGJ₂ (●), but not by Wy14643 (▲), in human peripheral blood T cells in dose-dependent manner. The incorporation of [³H]thymidine was plotted on the abscissa expressed as total counts/min (n = 6). B, troglitazone and 15d-PGJ₂, but not Wy14643, inhibit PHA/PMA-mediated induction of IL-2 in human T cells. Freshly prepared human T cells were incubated in medium containing PHA/PMA and ligands for 12 h. The concentration of IL-2 released into the medium was determined by ELISA. Error bars show mean ± S.D. of the three determinations. ○, + PHA/PMA; ▲, + PHA/PMA C, PPARγ ligands inhibit PHA/PMA-inducible IL-2 mRNA expression in human T cells. Cells were stimulated by PHA/PMA and treated with troglitazone (25 µM), 15d-PGJ₂ (10 µM), or dimethyl sulfoxide. Total RNA was isolated and subjected to analysis by RNase protection assay for expression of human IL-2 or L32 control genes using ³²P-labeled probe.

![FIG. 2](image2)

**Fig. 2.** Troglitazone and 15d-PGJ₂ inhibit transcription and production of IL-2 in transfected Jurkat T cells in a PPARγ-dependent manner. A, Jurkat cells were transfected with either control vector (pSG5) or PPARγ wild type expression vector (pSG5hPPARγ) and co-transfected with pEGFP-C1 plasmid. Transfected target cells were sorted by FACS and then assayed for IL-2 production. B, upper panel, Jurkat cells were co-transfected with a PPARγ-expression plasmid (+PPARγ) or control DNA (−PPARγ) and a PPRE reporter gene (AOX-TK). Cells were treated with 15d-PGJ₂ or troglitazone for an additional 24 h. The luciferase activity was determined. Lower panel, Jurkat cells were co-transfected with an IL-2 promoter-luciferase reporter plasmid and a PPARγ expression plasmid (+PPARγ) or control DNA (−PPARγ). Cells were treated with ligands, stimulated by PHA/PMA, and collected for analysis of reporter gene activity 24 h later.
which is a natural PPARγ ligand. By contrast, the PPARγ-specific ligand Wy14643 did not inhibit PHA-mediated T-cell proliferation. Furthermore, PHA/PMA-induced IL-2 synthesis assayed by ELISA was also restricted by the co-addition of troglitazone or 15d-PGJ2 in a dose-dependent manner (Fig. 1B). Because the induction of IL-2 gene expression is a pivotal event in early T lymphocyte activation, we next performed RNase protection assays of human peripheral blood T cells stimulated with PHA/PMA in the presence or absence of troglitazone or 15d-PGJ2. Total RNA was isolated and probed for IL-2 mRNA. As shown in Fig. 1C, IL-2 mRNA steady-state levels were detected as early as 6 h and strongly increased after 12 h of PHA/PMA stimulation. Treatment with troglitazone or 15d-PGJ2 totally abrogated expression of IL-2 mRNA. The quantification of decreased IL-2 mRNA levels was performed by normalizing densitometric traces against L32 mRNA. The IL-2 transcript was reduced by greater than 90% as compared with controls, suggesting that PPARγ ligands blocked transcription of the IL-2 gene.

Because transfection efficiency of circulating human T cells is very poor, we chose to use the human T cell line Jurkat that expresses little detectable PPARγ mRNA (9) as a model system to directly assess the actual role of PPARγ expression in the PPARγ ligand-induced inhibitory effects on T lymphocyte activation. The Jurkat cells were transfected with PPARγ2 wild type expression vector (pSG5hPPARγ2) (15) or control pSG5 plasmid. For this study, we used GFP as a cotransfected biomarker to simplify the selection of transfected target cells (13) by FACS sorting and assay for IL-2 production. As shown in Fig. 2A, PPARγ ligands troglitazone and 15d-PGJ2 inhibited IL-2 production only in the PPARγ2-expressing, but not in PPARγ2-nonexpressing subpopulation of transfected Jurkat cells. Interestingly, Wy14643 did not also show this inhibitory effect. Thus, from the data presented, it is clear that such inhibitory effects on T-cell activation are mediated by PPARγ.

To verify that the human PPARγ2 cDNA is capable of activating gene transcription through a PPRE in human T cells, we

![Fig. 3. Troglitazone and 15d-PGJ2 inhibit DNA binding and transcriptional activation of NFAT. A and B, DNA binding of transcription factors NFAT (A) and Sp-1 (B) induced by PHA/PMA in human peripheral blood T cells as demonstrated by EMSA. Cells were treated with dimethyl sulfoxide-control or troglitazone or 15d-PGJ2 and incubated with medium (−) or PHA/PMA (+) for 2 h at 37 °C. Nuclear extracts (5 μg) were incubated with a 32P-labeled probe. Arrow indicates migrational location of each DNA complex. Excess amounts of unlabeled oligonucleotides containing wild type (wt) or mutated (mt) NFAT sites were used as competitors to test for specificity of DNA complex. Antibodies against NFAT supershifted the protein-DNA complex. C, Jurkat cells were co-transfected with a reporter construct directed by the NFAT distal site of the IL-2 promoter and either a PPARγ-wild type (+PPARγ) or control DNA plasmid (−PPARγ). Cells were treated with different ligands, stimulated by PHA/PMA as shown, and collected for analysis of reporter gene activity 24 h later.

![Fig. 4. PPARγ associates with NFAT in T cells. PPARγ-NFAT complexes were detected after immunoprecipitation and protein immunoblot analysis of T cells. Cells lysates from T cells induced by PHA/PMA or ligands were prepared. Immunoprecipitation was carried out using anti-PPARγ antibodies. Immune complexes were washed and analyzed by immunoblotting with monoclonal anti-NFATc1 antibodies.](http://www.jbc.org/)

![Fig. 5. Troglitazone and 15d-PGJ2 inhibit DNA binding and transcriptional activation of Sp-1. A and B, DNA binding of transcription factors NFAT (A) and Sp-1 (B) induced by PHA/PMA in human peripheral blood T cells as demonstrated by EMSA. Cells were treated with dimethyl sulfoxide-control or troglitazone or 15d-PGJ2 and incubated with medium (−) or PHA/PMA (+) for 2 h at 37 °C. Nuclear extracts (5 μg) were incubated with a 32P-labeled probe. Arrow indicates migrational location of each DNA complex. Excess amounts of unlabeled oligonucleotides containing wild type (wt) or mutated (mt) NFAT sites were used as competitors to test for specificity of DNA complex. Antibodies against NFAT supershifted the protein-DNA complex. C, Jurkat cells were co-transfected with a reporter construct directed by the NFAT distal site of the IL-2 promoter and either a PPARγ-wild type (+PPARγ) or control DNA plasmid (−PPARγ). Cells were treated with different ligands, stimulated by PHA/PMA as shown, and collected for analysis of reporter gene activity 24 h later.
introduced a PPARγ2 expression vector (pSG5hPPARγ2) together with a PPRE-driven luciferase reporter construct (AOx) into Jurkat T cells. Fig. 2B (upper panel) shows that activation of a PPAR-dependent promoter required co-expression of PPARγ in Jurkat T cells. Upon addition of the PPARγ ligand troglitazone or 15d-PGJ2, luciferase activity was significantly increased compared to overexpression of PPARγ. To determine whether changes in IL-2 mRNA levels can be ascribed, at least in part, to differences in promoter activity, Jurkat T cells were co-transfected with IL-2 luciferase reporter constructs and a PPARγ expression plasmid. PMA/PHA treatment resulted in a marked increase in IL-2 promoter activity. Lack of PPARγ expression rendered the Jurkat cells unresponsive to the inhibitory effects of troglitazone and 15d-PGJ2 on PHA/PMA-induced IL-2 promoter activation (Fig. 2B, lower panel). However, this effect was restored by transfection of PPARγ. These data suggest that the inhibitory effects of troglitazone and 15d-PGJ2 on IL-2 promoter activity are directly dependent on the expression and activation of PPARγ.

The transcription factor NFAT plays an essential role in IL-2 gene expression. NF-ATc1 is also involved in the proliferation of peripheral T lymphocytes (16). Therefore, we evaluated transcriptional activity and DNA binding of NFAT to determine whether NFAT might be a target for negative regulation of T-cell activation by PPARγ ligands. As shown in Fig. 3A, the specific binding of NFAT probe corresponding to the human IL-2 promoter is strongly induced by PHA/PPMA, whereas equivalent nuclear extracts from troglitazone- or 15d-PGJ2-treated cells displayed diminished binding capacity to the 32P-radiolabeled probes. Moreover, the inhibitory effect of PPARγ ligands on NF-κB and AP-1 DNA-binding activities was also observed in T cells activated by PHA/PHA (data not shown). In contrast, the DNA binding of Sp-1 (17) was unaffected by troglitazone and 15d-PGJ2 (Fig. 3B). The EMSA data obtained correlated with the effect of PPARγ ligands on the transcriptional activity analyzed by a reporter construct directed by the NFAT distal site of the IL-2 promoter. The transcriptional activation of the above reporter construct was abrogated by troglitazone or 15d-PGJ2 in the presence of PPARγ overexpression (Fig. 3C). Thus, one conceivable explanation for the repression of IL-2 transcription in troglitazone- or 15d-PGJ2-treated cells could be due to selective disruption of the transcriptional regulation of the IL-2 promoter.

Next we focused our attention on how functional co-association of PPARγ with transcription factors NFAT might mediate T-cell suppression. We tested for complex formation between PPARγ and NFAT in a co-immunoprecipitation experiment. Fresh human peripheral T lymphoid cells were induced by PMA/PHA and treated with troglitazone or 15d-PGJ2. Cell extracts were prepared and immunoprecipitated with a PPARγ-specific antibody; immunoprecipitates were developed on Western blots with an NFATc1-specific antibody. As shown in Fig. 4, The NFATc1 complex can be co-precipitated with PPARγ in cells induced by PMA/PHA and troglitazone or 15d-PGJ2. Furthermore, the addition of anti-PPARγ antibody induced high affinity binding of extracts to the NFAT probes as determined by EMSA (data not shown), demonstrating that removal of PPARγ with this antiserum increases the target specificity of NFAT. These data indicate that a direct physical protein-protein interaction occurs between nuclear receptor PPARγ and transcription factors NFAT.

Recent studies have provided insights into the mechanisms by which steroid nuclear receptors regulate cytokine genes (18). Corticosteroids may bind directly as a dimer to regulatory sequences (GRE) in target gene promoters and subsequently affect activation of transcription. Dexamethasone has also been shown to inhibit the IL-2 promoter by interference with AP-1. The current model of dexamethasone inhibition has demonstrated a physical interaction between corticosteroid receptor and NF-κB. In the case of PPARγ, a non-steroid nuclear receptor, it has been reported that PPARγ agonists specifically inhibit expression of tumor necrosis factor α through inhibition of tumor necrosis factor receptor α promoter activity in monocytes (9). However, no physical co-precipitation of PPARγ with other transcription factors was shown. The data presented here demonstrate that PPARγ ligands suppress transactivation of IL-2 via down-regulation of the IL-2 promoter in a PPAR-dependent manner. The transcriptional regulation of the IL-2 gene has been analyzed extensively at the level of the IL-2 promoter. cis-Acting elements for several transcription factors have been identified within this regulatory region. The factors that bind to these motifs include NFAT, AP-1, and NF-κB proteins. Also, a binding site for Sp-1 has been identified immediately upstream of the distal NFAT site. Our data show that PPARγ ligands selectively block DNA binding and transcriptional activation of transcription factors such as NFAT regulating the IL-2 promoter in T cells.

From our data, PPARγ down-regulates the IL-2 promoter by a novel mechanism that involves protein-protein interactions. As shown in Fig. 4, formation of a molecular complex between PPARγ and NFAT may occur in T cells, which could block PHA/PMA-induced DNA binding and transactivation. Although we have focused on the interaction between NFAT and PPARγ, we cannot exclude the possibility that PPARγ interaction with AP-1 or NF-κB may also play an important role in blocking IL-2 gene transcription. However, activation and function of NFAT is an absolute requirement for IL-2 transcription.

Taken together, these data provided the evidence that the inhibitory role of PPARγ in the immunomodulation of T lymphocytes is based on functional interaction between PPARγ and T-cell-specific transcription factors. Such a model could highlight the importance of transcriptional cross-talk in T-cell biology and may lead to a new molecular class of immunomodulators. PPARγ ligands may be of therapeutic value in human T-cell-mediated diseases by targeting IL-2 gene expression.

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