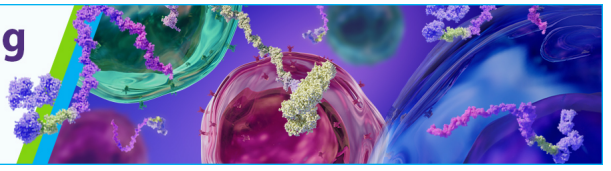

The Journal of Immunology

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J Immunol 2002; 168:2795-2802; ;
doi: 10.4049/jimmunol.168.6.2795
http://www.jimmunol.org/content/168/6/2795

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Regulation of Cytokine Expression by Ligands of Peroxisome Proliferator Activated Receptors[1]

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Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors with diverse actions including adipocyte differentiation and lipid metabolism. Recent studies have revealed anti-inflammatory activities, but the majority of these studies have been performed in monocyte/macrophages. In these studies, we investigate the effects of PPAR ligands in murine mitogen-activated splenocytes. Ciglitazone, a PPARγ ligand, consistently decreased IFN-γ and IL-2 production by mitogen-activated splenocytes and had modest effects on splenocyte proliferation. The effects of WY14,643, a representative of the fibrate class of PPARα ligands, on splenocyte proliferation and IL-2 levels are less marked than those observed with the PPARγ ligand. In addition, treatment with WY14,643 and other fibrates led to marked increases in supernatant concentrations of IL-4. However, treatment with a potent and specific PPARα ligand (GW7,647) did not augment IL-4. Also, WY14,643 induced IL-4 expression in splenocytes from PPARα knockout mice, suggesting that the fibrate effect on IL-4 was largely through a PPARα-independent mechanism. This increase in IL-4 was associated with and causatively related to augmented expression of CD23 by CD45R/B220$^+$ cells. We also demonstrate that PPARγ gene expression is up-regulated in T cells by mitogen activation, that it is positively regulated by IL-4 and WY14,643, and that it is blocked by anti-IL-4. Finally, we demonstrate that WY14,643 can modestly augment IL-4 promoter activity in a PPARα-independent manner. In concert, these findings support the roles of PPAR ligands in modulating inflammatory responses involving lymphocytes but also establish potent effects of the fibrate class of PPARα ligands on IL-4 expression that are receptor independent. *The Journal of Immunology,* **2002, 168: 2795–2802.**

Peroxisome proliferator activated receptors (PPARs),[3] members of the nuclear receptor superfamily, are ligand-activated transcription factors with diverse actions including the regulation of adipocyte differentiation and lipid metabolism (1). PPARs positively regulate gene expression by binding as heterodimers with retinoid X receptors to PPAR response elements (PPREs) in the promoters of target genes (2). PPARs can also negatively regulate gene expression in a ligand-dependent manner (3). PPARα regulates lipid homeostasis and is a target of fibrates, which are used clinically for the treatment of hypertriglyceridemia (4, 5). Fatty acids and eicosanoids are endogenous ligands for PPARα (6). In contrast, PPARγ is expressed in adipose tissue and is important in adipocyte differentiation. Ligands for PPARγ include the insulin-sensitizing thiazolidinediones and 15-deoxy-$\Delta^{(12,14)}$-prostaglandin J_2 (15d-PGJ_2) (7, 8).

Most recently, PPARs have been shown to mediate anti-inflammatory activities (3, 9, 10), and the majority of these studies have focused on effects in monocyte/macrophages and endothelial cells. PPARγ expression is augmented in activated peritoneal murine macrophages, and PPARγ ligands inhibit the expression of inducible nitric oxide synthase, gelatinase B, and scavenger receptor A genes, in part by antagonizing activities of the transcription factors AP-1, STAT, and NF-κB (3). Jiang et al. (10) have also demonstrated that PGJ_2 and troglitazone inhibited PMA-induced production of IL-1β, IL-6, and TNF-α by human peripheral blood monocytes. PPARα and PPARγ are expressed upon differentiation of human monocytes into macrophages, and treatment with both α and γ ligands induces apoptosis in macrophages (11). Studies performed with PPARα knockout mice have shown that they manifest a prolonged inflammatory response (9). Moreover, administration of thiazolidinediones, PPARγ ligands, is reported to attenuate inflammation in murine models of inflammatory bowel disease (12), non-obese diabetic mice (13), rodent models of atherosclerosis (14–16), and experimental allergic encephalitis (17). These studies support the concept that PPAR ligands exert anti-inflammatory actions.

More recently, several groups have demonstrated expression of PPARγ in T lymphocytes. PPARγ was identified in murine SJL-derived Th1 helper T cell clones specific for bovine myelin basic protein, and PPARγ ligands inhibited Ag-APC and anti-CD3, but not IL-2-stimulated, responses of the T cell clones. PPARγ ligands also inhibited IL-2 production (18). In human peripheral T lymphocytes, PGJ_2 and troglitazone, but not WY14,643, inhibited IL-2 production at 12 h, and PHA induced proliferation. The same investigators suggested that activated PPARγ physically associates with NFAT, blocking NFAT DNA binding and transcription of the IL-2 promoter (19).

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Received for publication July 25,2001. Accepted for publication January 17, 2002.

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[1] This work was supported by National Institutes of Health Grants DK45346 (to C.J.K.) and P42ES10337 (to C.K.G.), Department of Veterans Affairs Career Development Grants (to C.J.K. and R.C.), and a National Institutes of Health Training Grant in Hypertension (National Heart Lung and Blood Institute T32 HL0726). D.D. was supported by a National Kidney Foundation Fellowship Award. M.R. was supported by the American Heart Association, Western Affiliates Beginning Grant-In-Aid. D.A.K. takes part in the National Institutes of Health-supported Medical Scientist Training Program at the University of California, San Diego (Grant GM07198).

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[3] Abbreviations used in this paper: PPAR, peroxisome proliferator activated receptor; PPRE, PPAR response element; mPPAR, murine PPARα; RPL-19, ribosomal protein L19; TK, thymidine kinase; Luc, luciferase.

0022-1767/02/$02.00

Recent studies have demonstrated that PPAR ligands can also function in a receptor-independent manner. As an example, 15d-PGJ_2 has been shown to directly inhibit NF-κB gene expression by covalent modification of critical residues in IκB kinase and by modification of the DNA-binding domains of NF-κB (20–23).

In the current studies, we have examined the effects of both PPARα and PPARγ ligands on mitogen-activated splenocyte proliferation and cytokine expression. Our results demonstrate distinct effects of PPARα and PPARγ ligands on lymphocyte proliferation and cytokine secretion. We also delineate expression patterns of the PPARs. In addition, we find a marked effect of fibrates on IL-4 production that is independent of PPARα expression.

Materials and Methods

Experimental animals

Male and female C57BL/6 and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were used between 6 and 8 wk of age. Mice were housed and handled in accordance with Department of Veterans Affairs and National Institutes of Health guidelines under Institutional Animal Care and Use Committee-approved protocols. PPARα wild-type and homozygous knockout mice on a 129/Sv background were obtained from breeding pairs established by Dr. F. J. Gonzalez (Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD).

Reagents

Reagents used were Con A (Sigma-Aldrich, St. Louis, MO), WY14,643 (Chemsyn Science Laboratories, Lenexa, KS), ciglitazone (Alexis Biochemicals, San Diego, CA), ciprofibrate, gemfibrozil, PMA, and ionomycin (Sigma-Aldrich). GW7,647 was a kind gift from T. Willson (Glaxo Wellcome). Neutralizing Ab to IL-4 (anti-IL-4; 10 μg neutralizes 1,000 pg of IL-4) was provided by the National Cancer Institute (Frederick, MD). Mouse IL-4 and mouse IFN-γ were obtained from Roche (Indianapolis, IN).

Proliferation assays

After euthanasia, spleens were harvested from naive animals and prepared into single cell suspensions with a metal screen. RBCs were lysed by a room temperature incubation in a hypotonic solution (0.83% NH_4Cl, 20 mM Tris (pH = 7.6)). Proliferation assays were set up as previously described (24). Cells assayed for proliferation were pulsed with 1 μCi/well [^{3}H]thymidine (Amersham, Arlington Heights, IL) at 48 h and harvested 24 h later.

Cytokine ELISAs

Culture supernatant concentrations of IL-4, IFN-γ, and IL-2 were determined by sandwich ELISA with Abs purchased from BD PharMingen (San Diego, CA). IL-4 and IFN-γ concentrations were determined from culture supernatants after 96 h of activation with Con A. IL-2 was determined from culture supernatants after 24 h of activation. Ab dilutions that maximized signal to noise were determined for each Ab pair, and ELISAs were performed as previously described (24).

Flow cytometry

Spleens were harvested from naive BALB/c mice, prepared into single cell suspensions, stimulated with Con A, and treated with either 250 μM WY14,643 or ethanol (vehicle control) with or without anti-IL-4 (4,000 pg/ml of neutralizing activity). After 96 h, the cells were harvested and stained with 1 μg of both anti-CD23-FITC (IgE FcR; BD PharMingen) and anti-CD45R/B220-PE (B cell marker; BD PharMingen). Fluorescence was measured with an Elite Flow cytometer (Becton-Coulter, Mountain View, CA; Veterans Affairs Medical Center (VAMC) core FACS facility, San Diego, CA). For T and B cell separations, splenocytes were prepared, stimulated with Con A and isolated at 24 and 72 h, stained with anti-CD3ε-FITC (T cell marker) and anti-CD45R/B220-PE, and separated by MOFLO (Cytomation, Fort Collins, CO; VAMC core FACS facility) for further studies.

Northern blots

Single cell splenocyte suspensions isolated from BALB/c mice were stimulated with Con A and treated with or without murine IL-4 (50 U/ml) or WY14,643 (250 μM) or ethanol (control). The cells were then harvested at 24, 48, and 72 h, and poly(A)$^+$ RNA was isolated with the Oligotex mRNA mini kit (Qiagen, Valencia, CA). Five micrograms of poly(A)$^+$ RNA per lane was run on a 1% agarose/formaldehyde gel, transferred onto a nylon filter, and UV cross-linked. The PPARγ probe was derived from pCMX-murine PPARγ (3). Autoradiography was performed and a probe for glyceraldehyde-3-phosphate dehydrogenase was used to control for differences in loading and transfer of RNA.

RT-PCR

Total RNA was prepared from splenocyte populations with the RNeasy Mini kit (Qiagen) and stored at −70°C with 40 U of RNase-OUT inhibitor (Life Technologies, Gaithersburg, MD). Two micrograms of each sample was treated with 1 μl of DNase I, amplification grade (Life Technologies). The DNase was inactivated and cDNA was prepared with the Superscript II Preamplification System (Life Technologies) according to the instructions. Semiquantitative PCR was performed with *Taq* DNA polymerase (Qiagen) with primers for PPARα and PPARγ, which amplified 433 bp and 253 bp, respectively: PPARα, 5′-GAACATCGAGTGTCGAATATGTGG-3′, 5′-ATGTATGACAAAAGGCGGGTTG-3′; PPARγ, 5′-CCCTTTACCACAGTTGATTTCTCCAG-3′, 5′-CTGATGCTTTATCCCCACAGACTCGG-3′. Primers for ribosomal protein L19 (RPL-19) cDNA were included to control for reaction efficiency and variations in mRNA concentration in the original reverse transcriptase reaction, RPL-19: 5′-CGCTGTGGTAAAAAGAAGGTGTG-3′, 5′-TGCTCCATGAGAATCCGCTTG-3′. The amplification cycle for PPARα is 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min for 34 cycles; PPARγ is 94°C, 55°C, 72°C for 30–31 cycles; and RPL-19 is 94°C, 57°C, 72°C for 32 cycles. Fifteen microliters of the samples was analyzed on a 5% polyacrylamide gel, stained with ethidium bromide, and read on a ChemiImager 4400 (Innotech, San Leandro, CA).

Plasmids and transient transfections

The (acyl CoA oxidase)$_3$-thymidine kinase-luciferase ((AOX)$_3$-TK-Luc) construct has been described previously (3). The pSG5-PPARα was cloned from the pCMX-PPARα expression vector. The IL-4 promoter construct (pGL3-IL-4-Luc) contains an 828-bp fragment (−767/+61) isolated from the C57BL/6 mouse and was a kind gift from J. Trama (University of California, San Diego, CA). The mutated pGL3-IL-4mut-Luc construct was generated by converting cytidines, within the putative PPRE, to thymidines with the Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Jurkat T lymphocytes were cultured in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS (Gemini Bio-Products, Calabasas, CA), 130 U/ml penicillin, 130 μg/ml streptomycin, and 2.5 mM L-glutamine (Omega Scientific, Tarzana, CA). Jurkat cells were transfected by electroporation at 280 V/960 μF using a Bio-Rad (Hercules, CA) electroporator with a capacitance extender. Three hours after transfection, the cells were treated with PPAR ligands in the presence or absence of PMA and ionomycin. Luc activity was assessed as described (25).

Statistics

Differences were statistically analyzed using unpaired Student's *t* test. Analysis was accomplished with STATVIEW v4.5 (Abacus Concepts, Berkeley, CA).

Results

PPARγ ligands and fibrates exert distinct effects on mitogen-induced splenocyte proliferation and cytokine expression

We first examined the effects of the PPARγ ligand ciglitazone on mitogen-induced splenocyte proliferation and cytokine expression. Fig. 1*A* depicts the results of those studies when splenocytes from C57BL/6 female mice were used. Consistent with recent reports, we observed that the PPARγ ligand ciglitazone impairs Con A-induced splenocyte proliferation and IL-2 production (18, 19). The most profound effect of ciglitazone, however, was on the levels of IFN-γ in supernatants of activated splenocytes. In the presence of 50 μM ciglitazone, IFN-γ concentrations were <10% of the levels seen with vehicle alone. Ciglitazone had no effect on IL-4 expression. The same experiments were additionally conducted using age- and gender-matched BALB/c splenocytes. These results are depicted in Fig. 1*B* and demonstrate similar inhibitory effects on lymphocyte proliferation and the elaboration of IFN-γ and IL-2.

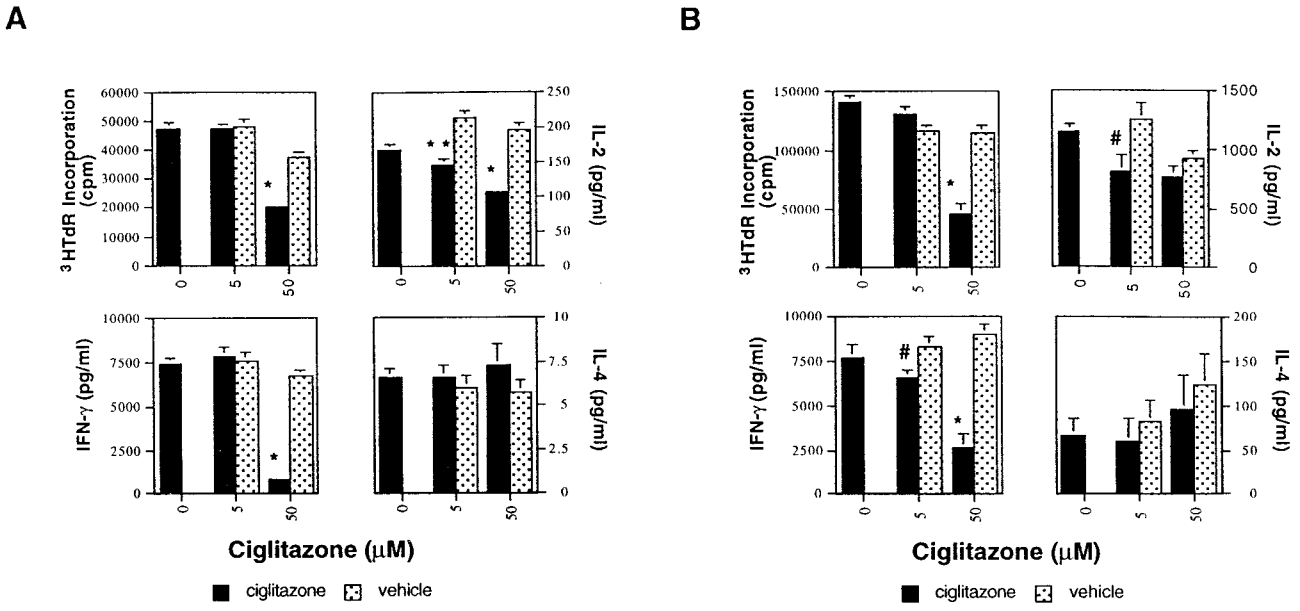

FIGURE 1. Ciglitazone alters proliferation and cytokine elaboration in mitogen-activated splenocytes. Ciglitazone or vehicle control (ethanol) was added to mitogen-activated splenocytes harvested from C57BL/6 mice (*A*) and BALB/c mice (*B*), and supernatant cytokines were measured by ELISA. In both mouse strains there is a moderate reduction in proliferation and IL-2 and a profound decrement in measured IFN-γ levels. Values represent the results of two separate experiments. #, $p < 0.05$ vs control; **, $p < 0.01$ vs control; *, $p < 0.0001$ vs control.

In a recent work examining the effects of PPARγ ligands on IL-2 production by activated T cells, no effects of PPARα ligands were observed (19). To further explore potential effects of PPARα ligands on lymphocyte proliferation and cytokine expression, we additionally tested a range of in vitro concentrations of WY14,643 in these assays. As shown in Fig. 2*A*, WY14,643 markedly inhibits IFN-γ expression in splenocytes isolated from C57BL/6 mice, with only a modest inhibitory effect on lymphocyte proliferation and IL-2 expression. In contrast to the effects of ciglitazone, coculture with WY14,643 additionally markedly augments IL-4 expression. Activated splenocytes from C57BL/6 mice produce relatively low concentrations of IL-4; therefore, we tested the effects of WY14,643 on activated splenocytes from BALB/c mice. These results are depicted in Fig. 2*B* and demonstrate a profound augmentation of IL-4 levels in the supernatants from splenocytes activated in the presence of WY14,643. Further studies revealed marked up-regulation of IL-4 mRNA in splenocytes treated with WY14,643 for 96 h (data not shown), which correlates with these observed increases in IL-4 protein.

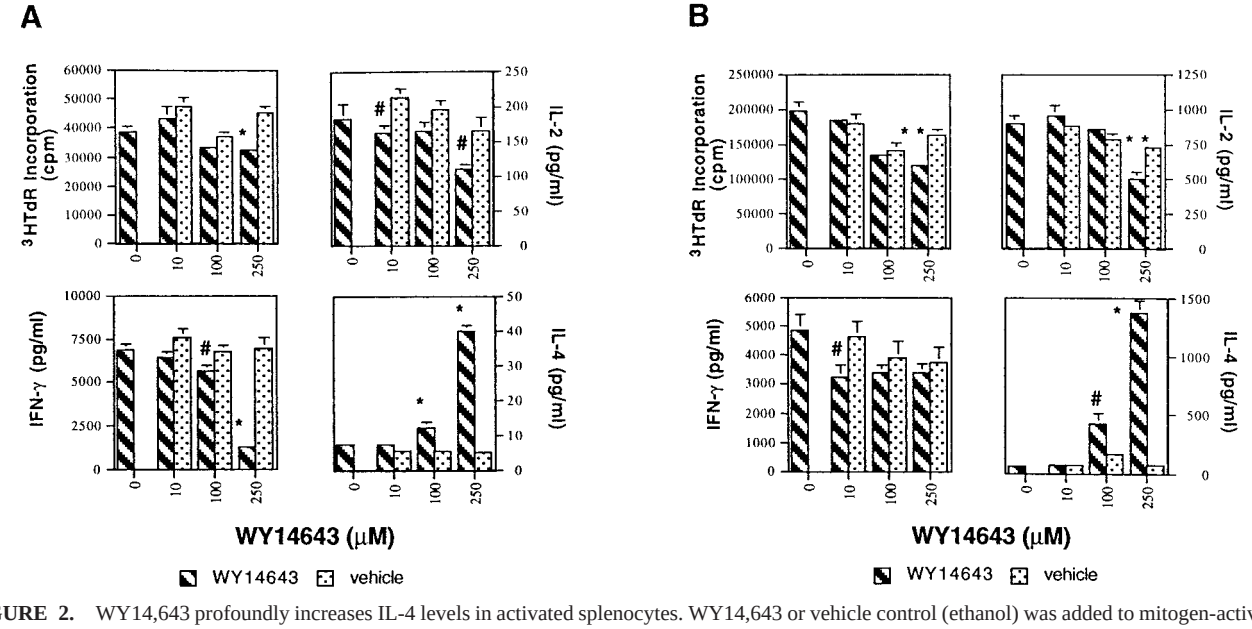

FIGURE 2. WY14,643 profoundly increases IL-4 levels in activated splenocytes. WY14,643 or vehicle control (ethanol) was added to mitogen-activated splenocytes harvested from C57BL/6 mice (*A*) and BALB/c mice (*B*), and supernatant cytokines were measured by ELISA. In both mouse strains there is an increase in IL-4 levels, but this increment is >15-fold in the BALB/c splenocyte culture supernatants. Values represent the results of two separate experiments. #, $p < 0.05$ vs control; **, $p < 0.01$ vs control; *, $p < 0.0001$ vs control.

This augmentation of IL-4 levels is similarly seen if BALB/c splenocytes are activated by Con A in the presence of graded concentrations of ciprofibrate or gemfibrozil, low-affinity members of the fibrate class of PPARα agonists (Fig. 3). Recently, PPAR ligands have been shown to have both PPAR-dependent and -independent actions (20–23, 26–28); therefore, we evaluated the effect of a very specific and potent PPARα ligand, GW7,647 (EC_{50} = 6 nM) (29), on activated BALB/c splenocytes. When GW7,647 was added to mitogen-activated mixed splenocyte cultures, there was no appreciable increase in IL-4, even at a concentration of 10 μM (>1000-fold above its EC_{50}) (Fig. 4). This suggests that the fibrate-induced increment in IL-4 may occur through a PPARα-independent mechanism. To determine whether WY14,643 exerts its effects on IL-4 expression through a PPARα-dependent or -independent mechanism, splenocytes from PPARα knockout mice were used. Fig. 5 shows that WY14,643 augmented supernatant IL-4 levels in PPARα knockout mice nearly to the extent observed in the wild-type mice.

Because the increase in IL-4 was associated consistently with a decrease in IFN-γ in the C57BL/6 cultures and occasionally in the BALB/c cultures, we next explored whether the augmentation of IL-4 was dependent on a decrement, even a transient one, in IFN-γ. BALB/c splenocytes were stimulated with Con A and treated with 250 μM WY14,643 with or without anti-IL-4 (10 μg/well, which neutralizes 5,000 pg/ml) or IFN-γ (10,000 pg/well, which represents 50,000 pg/ml) from the initial setup of the cultures. As shown in Fig. 6, even in the presence of extraordinary concentrations of IFN-γ from the time of splenocyte activation, WY14,643 treatment still resulted in a marked augmentation of IL-4 levels in culture supernatants. Also, when anti-IL-4 was added to the WY14,643-treated splenocyte cultures, there was a modest augmentation in the supernatant concentrations of IFN-γ. These results suggest that the WY14,643 effect on IL-4 is direct rather than dependent on a decline in IFN-γ.

WY14,643 augments expression of CD23 in an IL-4-dependent manner

The expression of CD23 on B cells is up-regulated by IL-4. To ascribe functional significance to the augmentation of IL-4 by WY14,643, we examined the expression of CD23 and CD45R/B220 on BALB/c splenocytes activated with Con A in the presence or absence of WY14,643 and/or neutralizing Abs to IL-4 (neutralizes 4000 pg/ml). As depicted in Fig. 7, WY14,643 treatment elicits an increase in the percentage of CD45R/B220$^+$ splenocytes in the Con A-activated cultures. In addition, WY14,643 increases the percentage of CD45R/B220$^+$ CD23$^+$ cells within these cultures. This augmentation of double-positive cells is reversed by the presence of neutralizing Abs to IL-4, supporting the contention that WY14,643 augments expression of CD23 through its effects on IL-4.

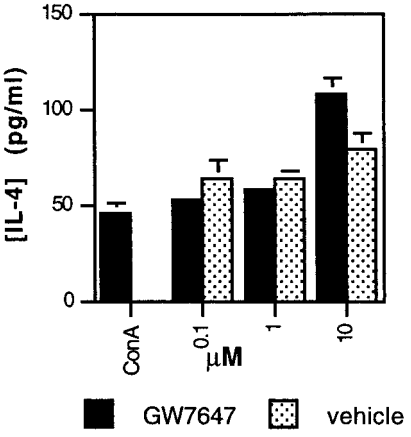

FIGURE 4. GW7647, a highly specific PPARα ligand, does not increase IL-4 levels. Splenocytes from BALB/c mice were activated with Con A and treated with GW7647. IL-4 in culture supernatants was measured by ELISA. Despite levels >1000-fold above its EC_{50} for activation of PPARα, there was no appreciable increase in IL-4.

PPARγ mRNA expression in activated T cells is dependent on IL-4

The mRNA encoding PPARγ is expressed at a low level in cultures of Con A-activated splenocytes. We have been able to detect it on Northern blots only by using poly(A)$^+$ RNA. Fig. 8*A* demonstrates the up-regulation of PPARγ mRNA with activation and marked induction with either rIL-4 or WY14,643. To further delineate which cells in Con A-activated spleen were expressing PPARγ, we activated BALB/c splenocytes and separated them by flow cytometry into CD3ϵ^+ cells and CD45R/B220$^+$ cells. As seen in Fig. 8*B*, the PPARγ mRNA can be detected by semiquantitative RT-PCR at 72 h after activation in the CD3ϵ^+ cells (T cells), but not in the CD45R/B220 population (B cells). The clear up-regulation of this mRNA at 96 h after activation is completely inhibited by neutralizing Ab to IL-4 (Fig. 8*C*). PPARα mRNA is expressed early in Con A-activated splenocytes and virtually disappears by 72 h (Fig. 8*D*). Thus, we have shown that fibrates increase IL-4 levels, which secondarily up-regulate PPARγ expression.

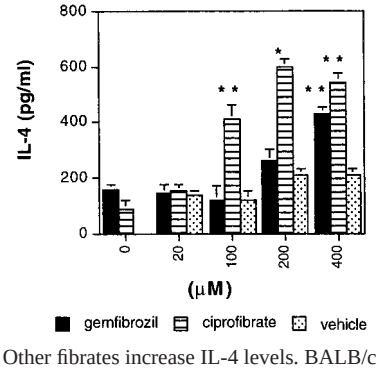

FIGURE 3. Other fibrates increase IL-4 levels. BALB/c splenocyte cultures were activated with Con A and treated with gemfibrozil, ciprofibrate, and vehicle control (ethanol). IL-4 levels in supernatants were measured by ELISA after 96 h of culture. Both gemfibrozil and ciprofibrate cause increases in measured IL-4 levels. *, $p < 0.0001$ vs control; **, $p < 0.01$ vs control.

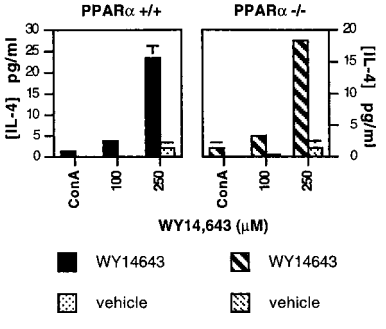

FIGURE 5. WY14,643 increases supernatant IL-4 levels in mixed splenocyte cultures from PPARα knockout mice. Splenocytes were isolated from wild-type and PPARα knockout mice, stimulated with Con A, and treated with increasing concentrations of WY14,643. WY14,643 treatment augmented supernatant IL-4 levels in PPARα knockout mice nearly to the extent observed in the wild-type mice. Values represent the results of two separate experiments.

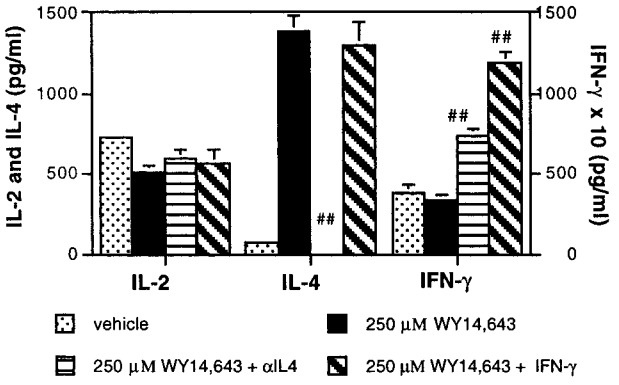

FIGURE 6. The WY14,643-induced increment in IL-4 is independent of IFN-γ. Splenocytes activated with Con A were treated with 250 μM WY14,643 or vehicle and anti-IL-4 (10 μg/well) or murine IFN-γ (10,000 pg/well) when the splenocyte cultures were set up. Cytokines were measured by ELISA. Despite the high levels of IFN-γ achieved in the supernatants (>10,000 pg/ml), there was no significant decrement in IL-4 production in WY14,643-treated splenocytes. ##, $p < 0.0001$ vs 250 μM WY14,643 treatment.

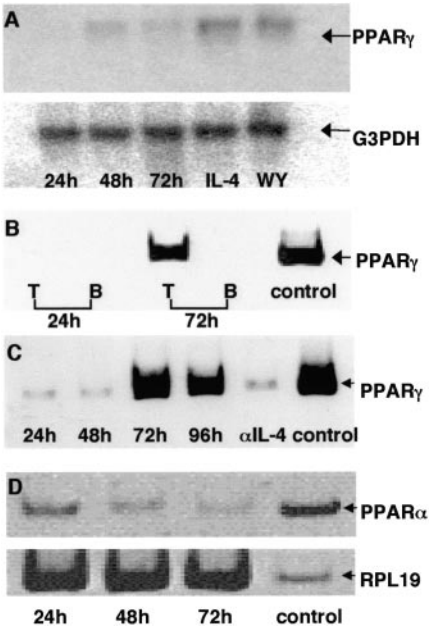

FIGURE 8. PPARγ mRNA expression in activated T cells is dependent on IL-4. *A*, Northern blot of PPARγ expression. BALB/c splenocytes were stimulated with Con A, and 5 μg of poly(A)$^+$ RNA was isolated at different time points: 24, 48, 72, 72 h + IL-4 (50 U/ml), and 72 h + WY14,643 (250 μM). *B*, PPARγ is expressed in T cells at 72 h. RT-PCR was performed in the linear phase of amplification (31 cycles). BALB/c splenocytes were stimulated with Con A and harvested at 24 h and 72 h. Cells were separated by MOFLO into T cells (FITC-CD3ϵ) and B cells (PE-CD45RB220); the control represents a PPARγ plasmid control. *C*, RT-PCR of PPARγ in the linear phase of amplification (30 cycles). Mixed splenocyte cultures from BALB/c mice stimulated with Con A were harvested at the indicated time points: 24, 48, 72, and 96 h. αIL-4 corresponds to 96-h cultures treated with anti-IL-4 (4,000 pg/ml of neutralizing activity); the control is a PPARγ plasmid. *D*, RT-PCR for PPARα in the linear phase of amplification (34 cycles). Stimulated splenocyte cultures from BALB/c mice were harvested at 24, 48, and 72 h. Expression of PPARα is most robust at 24 h, with virtually no evident expression by 72 h. The control represents a PPARα plasmid. Another reaction was performed to detect expression of RPL-19, a rRNA, to control for equivalent PCR efficiency and variations in mRNA concentrations.

WY14,643 modestly up-regulates the IL-4 promoter and this effect appears to be PPARα independent

Intriguingly, the murine (and human) IL-4 promoter contains a nearly perfect PPRE located 317 bp from the transcription start site. This raised the possibility that regulation of IL-4 expression by fibrates may be through both PPARα-dependent and PPARα-independent mechanisms. To establish a positive control for PPAR-dependent transactivation, Jurkat cells were transfected with a PPRE-driven Luc reporter ($(AOX)_3$-TK-Luc). Fig. 9*A* shows that in the absence of both PPARα and stimulation, WY14,643 cannot activate the PPRE reporter. The Jurkat cells express low levels of PPARα (data not shown). However, if the cells are activated with PMA and ionomycin or are cotransfected with the PPARα expression vector (pSG5-murine PPARα (mPPARα)), there is a 2-fold increase in promoter activity stimulated by 5 μM WY14,643. A total of 50 μM WY14,643 could stimulate the $(AOX)_3$-TK-Luc construct to nearly the same extent in the presence or absence of PPARα. Jurkat cells were transfected with the IL-4 promoter construct (pGL3-IL-4Luc) with or without pSG5-mPPARα (Fig. 9*B*). WY14,643 was able to activate transcription of the IL-4 promoter when the cells were stimulated, regardless of whether pSG5-PPARα was cotransfected. Higher concentrations of WY14,643 that exerted stronger effects on IL-4 expression in cultured primary splenocytes could not be evaluated in Jurkat cells due to toxicity (data not shown). To assess whether WY14,643 activated gene transcription through the putative PPRE (at −317 bp relative to the start site), we mutated this site. As predicted, mutation of the PPRE within the IL-4 promoter did not alter the ability WY14,643 to activate transcription in activated Jurkat cells (Fig. 9*C*). We also used GW7,647 in the IL-4 promoter studies and there was no appreciable effect of this potent PPARα ligand on the IL-4 promoter (data not shown). These studies further confirm that WY14,643 acts in a PPARα-independent manner. Furthermore, the ability of WY14,643 to activate IL-4 promoter transcription is weak, suggesting that additional mechanisms may prove important in mediating the WY14,643-induced increase in IL-4. Alternatively, higher concentrations of WY14,643 could have exerted stronger effects on the IL-4 promoter construct; however, doses of WY14,643 greater than 50 μM greatly decreased cell viability in the Jurkat cell cultures.

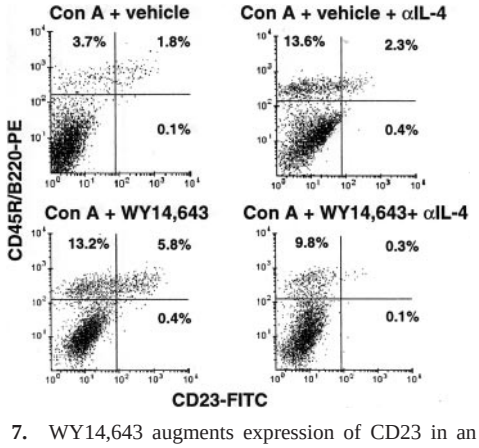

FIGURE 7. WY14,643 augments expression of CD23 in an IL-4-dependent manner. BALB/c splenocytes were activated with Con A and stained after 96 h with CD45R/B220-PE and CD23-FITC. WY14,643 treatment augments the number of double-positive cells, an effect that is negated with concomitant treatment with neutralizing Ab to IL-4. Percentages of positive cells in each gate are represented. αIL-4, Anti-IL-4.

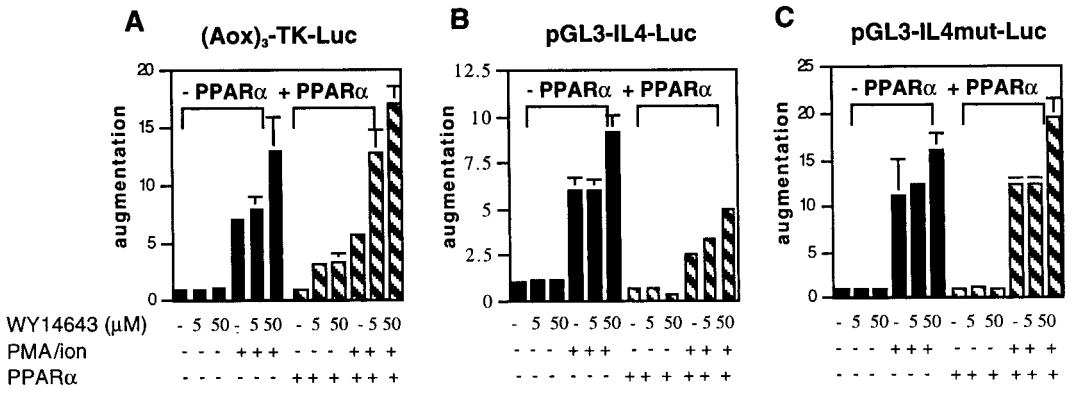

FIGURE 9. WY14,643 modestly induces IL-4 promoter activity in activated Jurkat T lymphocytes. *A*, Jurkat cells were transfected with a PPRE-driven Luc reporter, $(AOX)_3$-TK-Luc, and a PPARα expression vector, pSG5-mPPARα. *B*, Jurkat cells were transfected with a luciferase reporter gene under transcriptional control of the IL-4 promoter (−767/+61, pGL3-IL-4-Luc). *C*, Jurkat cells were transfected with a Luc reporter gene under transcriptional control of the IL-4 promoter that had been mutated in the putative PPRE (pGL3-IL-4mut-Luc). Cells were cotransfected with 5 μg of reporter plasmid and 750 ng of the mPPARα expression vector (pSG5-mPPARα) as indicated. Cells were also treated with combinations of WY14,643, (PMA, 3 ng/ml), and ionomycin (3 μg/ml). Bars represent SD; values are representative of experiments performed at least three times.

Discussion

In these studies, we have demonstrated that both synthetic PPARα and PPARγ ligands exert significant yet distinct effects on mitogen-activated lymphocyte proliferation and cytokine expression. Ciglitazone, the PPARγ ligand, consistently decreases IFN-γ and IL-2 production by mitogen-activated splenocytes and has modest effects on splenocyte proliferation. The effects of WY14,643 on lymphocyte proliferation and IL-2 levels are less pronounced than those observed with the PPARγ ligand; however, treatment with three different low-affinity fibrates led to marked increases in supernatant concentrations of IL-4. In contrast, a highly specific and potent PPARα ligand did not significantly augment IL-4 levels, suggesting that fibrates increase IL-4 through a PPARα-independent mechanism. This was confirmed in PPARα knockout mice. We have also shown that the increase in IL-4 was associated with and causatively related to augmented expression of CD23 by $CD45R/B220^+$ cells. In addition, the temporal expression of the PPARγ and PPARα nuclear receptors in resting and activated splenocytes differs, and treatment of splenocytes with WY14,643 or rIL-4 results in augmented PPARγ gene expression. In contrast, neutralizing Abs to IL-4 block up-regulation of PPARγ in activated T cells.

Several recent studies have documented an effect of PPARγ ligands on T cell proliferation and IL-2 production (18, 19). Yang et al. (19) found that PPARγ ligands, but not WY14,643, inhibited IL-2 production and mitogen-induced proliferation in human peripheral blood T cells. They proposed that PPARγ ligands exert this effect by regulating the IL-2 promoter, blocking NFAT DNA binding and transcriptional activity. Clark et al. (18) described PPARγ1 expression in murine helper T cell clones and freshly isolated splenocytes and documented inhibition of proliferative responses and of IL-2 secretion by PPARγ ligands. This inhibition of proliferative responses may be attributable in part to apoptosis of activated T cells in the presence of PPARγ ligands (30). Our findings confirm these earlier results and additionally demonstrate inhibition of IFN-γ secretion by the PPARγ ligand ciglitazone. Our preliminary studies suggest that this effect on IFN-γ occurs through a PPARγ-dependent mechanism (data not shown). Recently, Chtanova and Mackay (31) have shown with microarray techniques that polarized Th2 cells express greater levels of PPARγ2 mRNA than Th1 cells. The higher levels of PPARγ expression in the setting of endogenous ligands could result in lower IFN-γ expression observed in the Th2 population. Furthermore, the IL-4 produced by the Th2 cells could be responsible for up-regulating PPARγ expression in these cells.

PPARα ligands have been previously implicated in the control of inflammatory responses. PPARα-deficient mice have a prolonged response to inflammatory stimuli, such as arachidonic acid and leukotriene B_4 (9). PPARα ligands inhibit the cytokine-activated expression of IL-6, VCAM, and cyclooxygenase-2 (32–34). In vivo administration of PPARα ligands to aged mice diminishes the augmented NF-κB expression typically seen in such animals, as well as the elevated splenocyte levels of IL-6 and IL-12 (35). PPARα ligands may inhibit NF-κB functional expression and DNA binding activity in part by augmenting the expression of IκBα (36). In a similar manner, in vivo administration of WY14,643 to aged mice corrects the dysregulation of IFN-γ and splenic inducible NO synthase seen in aged mice (37).

A smaller literature exists regarding the augmentation of cytokine production or immune responses by PPARα activators. WY14,643 stimulates the synthesis of IL-8 and monocyte chemotactic protein-1 by human aortic endothelial cells (38). Enioutina et al. (39) have demonstrated that the blunted mucosal and systemic humoral immune responses seen in aged mice can be restored with dietary supplementation of PPARα activators, such as WY14,643. Our own findings of augmented IL-4 expression in the presence of WY14,643 may provide a partial explanation as to why humoral immune responses are augmented in the aged mice fed WY14,643.

Two lines of evidence, dose response and genetic, have consistently shown that the fibrate-induced augmentation of IL-4 in mixed splenocyte cultures may be mediated in a PPARα-independent manner. High doses of WY14,643 (100–250 μM; EC_{50} = 5 μM), ciprofibrate (100−400 μM), and gemfibrozil (200−400 μM) are required to increase IL-4 levels. At high concentrations, PPAR ligands are known to exert PPAR-independent effects. It is for this reason that we used a highly specific PPARα ligand, GW7,647. At concentrations greater than 1000-fold above its EC_{50}, there was no appreciable increase in IL-4. This agent is related to the fibrate class of ligands, and it is possible that at much higher concentrations it could increase IL-4 levels; however, the lack of effect at concentrations far above its EC_{50} suggests that the fibrates are not functioning in a PPARα-dependent manner. Furthermore, treatment of splenocytes isolated from PPARα knockout mice with WY14,643 resulted in a similar augmentation of supernatant IL-4 levels in both wild-type and knockout animals.

Increasingly, PPAR-independent effects of PPAR ligands are being discovered (20, 21, 23, 26–28, 40). 15d-PGJ_2 has been shown to covalently modify critical residues in IκB kinase and the DNA-binding domains of NF-κB. Thiazolidinediones have also been demonstrated to inhibit translation initiation by depleting intracellular calcium stores in a PPARγ-independent manner (26). Many of the previous studies using PPAR ligands may need to be reevaluated to confirm whether the reported effects occur though PPAR receptors.

Paradoxically, the IL-4 promoter contains a highly conserved PPRE ~317 bp upstream of the transcription initiation site. Preliminary studies with WY14,643 have shown modest induction of the promoter, though we could never use the same concentrations of WY14,643 that were used in the splenocyte cultures, due to toxicity in the Jurkat cells. However, as predicted by our earlier studies, mutation of the PPRE did not alter promoter activity. Also, treatment with GW7,647 in PPARα-activating concentrations induced transcription of the PPRE-driven reporter, but did not activate the IL-4 promoter construct (data not shown). Further studies will need to be performed to assess whether WY14,643 has a direct effect on the promoter or whether it modifies other coactivators or repressors.

PPARγ expression is induced in macrophages (41) and microglial cells (42) by IL-4. Our current studies extend this paradigm to activated T cells. Although we do not see expression of PPARγ in resting T cells exposed to IL-4 (R. Cunard and C. J. Kelly, unpublished observations), we clearly demonstrate that PPARγ mRNA is up-regulated in activated T cells and superinduced by WY14,643 and that up-regulation of PPARγ is inhibited in the presence of blocking Abs to IL-4.

Our studies use high concentrations of PPAR ligands, which raises the concern of the physiologic relevance of these findings. Fibrates have a high degree of plasma protein binding (95–99%) (43), and we use 10% FCS for splenocyte cultures. Our review of the literature confirms that high doses of fibrates are required, and peak levels of the therapeutic agent gemfibrozil are in the 100 μM range (33, 44). Furthermore, endogenous PPAR ligands, including prostaglandins, are found in high concentrations in splenocytes (45), and the metabolism of arachidonic acid is greatly increased in inflammatory states (20).

Fibrates are currently in clinical use as hypolipidemic agents. In a similar manner, PPARγ ligands of the thiazolidinedione family are in broad use in patients with type 2 diabetes mellitus. Recent studies have demonstrated that PPARγ ligands may well have beneficial effects in rodent models of atherosclerosis (14–16), colitis (12), arthritis (46), and experimental allergic encephalitis (17). The full extent of immunomodulatory effects of these compounds in humans has not yet been delineated and remains an important focus for continued investigation.

Acknowledgments

We thank Judy Nordberg and the rest of the staff in the San Diego VAMC Core Flow Cytometry facility. We thank Tim Willson and Glaxo Wellcome for supplying us with GW7647. We also thank F. J. Gonzalez (Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD) for providing PPARα knockout mice.

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