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Antagonizing Peroxisome Proliferator–Activated Receptor α Activity Selectively Enhances Th1 Immunity in Male Mice

Monan Angela Zhang,* Jeeyoon Jennifer Ahn,* Fei Linda Zhao,* Thirumahal Selvanantham,* Thierry Mallevaey,* Nick Stock,† Lucia Correa,† Ryan Clark,‡ David Spaner,*†,‡,§, and Shannon E. Dunn*,* †,‡,§

Females exhibit more robust Th1 responses than males. Our previous work suggested that this sex disparity is a consequence of higher expression of the androgen-induced gene peroxisome proliferator–activated receptor α (PPARα) in male CD4+ T cells. The objective of this study was to elucidate the cellular and molecular mechanism of how PPARα inhibits Th1 responses in male mice. In this study, we found that PPARα functions within CD4+ and CD8+ T lymphocytes and NKT cells to negatively regulate IFN-γ responses in male mice and identified Ifng as the gene target of PPARα repression. Treatment of male CD4+ T cells with the PPARα agonist fenofibrate induced the recruitment of PPARα and the nuclear receptor-interacting protein, nuclear receptor corepressor 1, to specific cis-regulatory elements in the Ifng locus. This recruitment associated with reduced histone acetylation at these sites. Knockdown of nuclear receptor corepressor 1 in primary male T cells abolished the effect of fenofibrate in reducing IFN-γ production. In contrast, treatment of male T cells with IS001, a novel antagonist of PPARα, increased Ifng gene expression and histone acetylation across the Ifng locus. Finally, we investigated the effects of IS001 on IFN-γ responses in mice during infection with the Th1-associated pathogen Listeria monocytogenes and observed that IS001 enhanced IFN-γ production by NKT, CD4+, and CD8+ T cells and improved the survival of male, but not female, mice. Our findings provide a novel mechanism of why IFN-γ responses are more robust in females and introduce a small-molecule IS001 that can be used to enhance Th1 immunity in males.

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T helper cells are classified into distinct subsets based on the expression of effector cytokines: IFN-γ for Th1, IL-4 for Th2, and IL-17A for Th17 cells (1). Naive CD4+ T cells acquire these distinct Th cell fates to orchestrate the appropriate immune response against invading pathogens (1). Th1 cells are specialized for the generation of cellular immunity against intracellular pathogens and are generated upon Ag receptor engagement in the presence of IL-12 (1). During Th1 differentiation, the IFN-γ receptor–STAT1 signaling pathway converges with the TCR signaling pathway to induce expression of T-bet, the master regulator of Th1 programming. T-bet promotes Th1 immunity by: 1) directly transactivating Ifng; 2) mediating epigenetic changes that make chromatin more accessible at the Ifng locus; and 3) increasing CD4+ T cell responsiveness to IL-12 (1–3). In addition to being the major Th1 effector cytokine, IFN-γ serves as an amplification signal in the Th1 pathway to further enhance T-bet expression (4).

One intriguing feature of Th1 immune responses is that they are more robust in females than in males (5–8). This sex difference is thought to underlie why women generate enhanced antiviral (9) and antitumor (10) immune responses, but also have a higher propensity to develop certain autoimmune diseases (8). Past studies have suggested that sex differences in Th1 responses arise because of suppressive effects of androgens at key nodes in the Th1 differentiation pathway including IL-12 production (11) and signaling (12, 13) and IFN-γ production downstream of Ag stimulation (6, 7). Until recently, the molecular players involved in this androgen-dependent regulation were not known.

We previously identified that the nuclear receptor peroxisome proliferator–activated receptor α (PPARα) is expressed at higher levels by male compared with female T cells both in humans and in mice and acts to limit Th1 cytokine production exclusively in the male sex (6, 7). We further showed that expression of PPARα is sensitive to androgen levels (6, 7) and that deficiency of this gene leads to heightened Th1 responses and more severe acute experimental autoimmune encephalomyelitis in male, but not female mice (6). Despite these advances, the precise mechanism of how PPARα limits Th1 immune responses is unknown.

Past studies have shown that PPAR family members (PPARγ, PPARα, and PPARδ) function to activate or repress target genes by regulating gene transcription (14). Positive regulation of target genes occurs through the binding of PPARs and retinoid X receptor (RXX) heterodimers to PPAR responsive elements in gene promoter regions (14). The binding of lipid ligands activates...
PPARs by promoting an allosteric change in the ligand-binding domain of the receptor that supports an increased association with coactivator proteins and a decreased association with corepressor proteins such as nuclear receptor corepressor 1 (NCOR) (14). In contrast, the repressive activities of PPARs feature more in inflammation control and instead occur through the indirect binding of PPARs to gene regulatory regions: a mechanism that has been termed “transrepression” (14). The molecular details of this transrepression mechanism are less understood, but have been resolved for certain gene targets of PPARγ including inducible NO synthase (iNOS) and retinoic acid–related orphan receptor (ROR) γ (Rore (15), 16). For example, it has been shown that treatment of macrophages with the ligand rosiglitazone induces the sumoylation and recruitment of PPARγ to the iNOS promoter, where it stabilizes the presence of the histone deacetylase (HDAC) 3/NCOR-containing corepressor complex, thereby inhibiting gene transcription (15). Whether PPARα works via a similar mechanism to repress Th1 responses in males is not known.

The aim of this study was to dissect the cellular and molecular mechanism of how PPARα inhibits Th1 immunity in male mice and to explore the utility of a novel small-molecule antagonist of PPARα to enhance Th1 immune responses.

Materials and Methods

**Mice**

SJL/J and C57BL/6 mice and breeder pairs of PPARα+/- (129S1/SvImJ) and PPARα-/- mice (129S4/SvJae-Ppargtm1Gonz/J) were from The Jackson Laboratory. Breeder pairs of PPARα+/- (model B6) and PPARα-/- (model ppara) on the B6 background were from Taconic Farms. The litters were also crossed onto the T-bet-/- background (The Jackson Laboratory). Work was conducted using 8–10-week-old mice in accordance with the guidelines of the Canadian Council on Animal Care under animal use protocols (AUP 1747 and AUP 3213) approved by the University Health Network.

**Experimental approach for in vitro studies**

For the majority of our in vitro studies (unless otherwise indicated), two or three independent experiments were conducted that each used immune cells that were pooled from mice of one cage per genotype. This was done to ensure that the dominant, high-testosterone males were included in our assays. Cage-housed males develop social hierarchies such that one or two males within a cage emerge to have high testosterone levels (17). We showed in our previous studies that the male mice with the highest testosterone levels also exhibit the highest levels of PPARα mRNA in T cells (6).

**Regulatory T cell suppression assay**

CD4+ T cells were enriched from spleens of mice using the mouse CD4+ isolation kit II (Miltenyi Biotec). Cells were stained with the following Abs: CD4-FTC (clone RM4-5), CD44-PE (clone L1M7), and CD25-allophycocyanin (clone PC61.5). CD4+CD25high regulatory T (Treg) cell and CD4+CD44hi/CD25- effector T (Teff) cell populations were sorted using the FACSaria II FACS sorter (BD Biosciences, Sickkids Flow Cytometry Facility). CD4+CD44hi/CD25- responding T cells (2.5 × 10^6/well) were cocultured together at the indicated ratio with CD4+CD25hi Treg cells in 96-well U-bottom plates with irradiated splenocytes (5 × 10^6/well) and anti-CD3 as described previously (18). The proliferation of responding T cells was measured between 72 and 90 h of culture using a [3H]thymidine incorporation assay.

**In vitro T cell stimulations, cytokine measurements, and flow cytometry**

CD4+ and CD8+ T cells were isolated from spleens and lymph nodes of mice using magnetic beads (Miltenyi Biotec or Stemcell Technologies). The purity of isolated cells was routinely monitored and ranged between 94 and 98%. Purified cells were resuspended in complete RPMI 1640 media or X-VIVO-20 media (Lonza) supplemented with 2 mmol/l t-glutamine and were cultured in 96-well flat-bottom plates (2 × 10^5/well) that were precoated with anti-CD3 (clone 145-2C11) and anti-CD28 (clone 37.51) (each at 0.5 μg/ml). Th polarizations were conducted as described previously (7) using the following cytokines and Abs from eBioscience: 10 ng/ml IL-12 + 10 μg/ml anti-IL-4 (clone 11B11) for Th1-, 10 ng/ml IL-4 + 10 μg/ml anti-IFN-γ (clone XMG1.2) for Th2-, or 3 μg/ml TGF-β + 50 ng/ml anti-IL-6 + 10 μg/ml anti-IFN-γ + 10 μg/ml anti-IL-10 (clone clone 1B11) for Th17-polarizing conditions. Cytokine levels in culture supernatants were measured using Ready-SET-Go! ELISA kits (eBioscience). PMA/ionomycin stimulations and cell surface and intracellular cytokine stainings were performed as described previously (19, 20). Intracellular staining for transcription factors was performed using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience). The following Abs and tetraters were used in this study and were purchased from eBioscience unless otherwise noted: CD4-PE–Cy5 (GK1.5), CD8-FITC (53-6.7), TCR-β-PE–Cy7 (H56-597), IFN-γ–PE (XMG1.2), NKp46–allophycocyanin–eFluor780 (29A1.4), NKp46–Alexa Fluor 700 (29A1.4; BD Biosciences), GATA-3–PE (TWA1), T-bet–PE–Cy7 (4B10), RORγt–PE (B2D), and unloaded and PBS57-loaded mCD1d tetramer–allophycocyanin (National Institutes of Health Tetramer Core Facility). Data were acquired using an LSRII (BD Biosciences) (Sickkids Flow Cytometry Facility) and analyzed using FlowJo software (V9+) (Tree Star).

**Measurement of mRNA levels**

Levels of specific mRNAs were measured using real-time RT-PCR (Roche LightCycler 480; Roche) as described previously (7) using FastStart Universal SYBR Green Master mix (Roche) and specific primer pairs (Supplemental Table I). The following amplification parameters were used: 95°C for 15 min, followed by 50 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 30 s. The relative abundance of each PCR product was determined using the standard curve method and normalized to β-actin mRNA levels.

**Determination of IC50 of IS001 for PPARα and other nuclear hormone receptors**

The IC50 values of IS001 for PPARα and other nuclear hormone receptors were determined using the following commercial reporter assay kits from Indigo Biosciences: human PPARα (catalog number BI00111), murine PPARα (catalog number MO0111), human PPARδ (catalog number IB0121), human PPARγ (catalog number IB00101), human estrogen receptor β (catalog number IB00411), human glucocorticoid receptor (catalog number IB00201), human thyroid hormone receptor β (catalog number IB01101), and human RXRα (catalog number IB00801). In brief, reporter cells provided with the kit were resuspended in the provided media and dispensed into 96-well assay plates with a positive control agonist (i.e., 20 nmol/l GW7647 for PPARα assays) and different quantities of IS001. Following an overnight incubation, media was discarded from wells and replaced with luciferase detection reagent provided with the kit. Luminescence was measured using a Molecular Devices FlexStation (Molecular Devices). The luminescence intensity of light emission from the luciferase reaction was directly proportional to the relative level of nuclear receptor activation in the reporter cells.

**Small interfering RNA knockdown of gene expression**

Transfection of mouse CD4+ T cells was performed using the Mouse T Cell Nucleofector Kit and the Nucleofector device (X-001 program) according to kit directions (Amaxa). Cells were transfected with 2 μg ON-TARGETplus SMARTpool small interfering RNAs (siRNAs) (Dharmacon) designed against mouse PPARα (L-040740-01), mouse NCOR (L-058556-00-0005), or nontargeting siRNAs (ON-TARGETplus control pool D-001810-10-05; Thermo Scientific). A pmxGFP expression vector provided with the kit was also transfected at the same time as the siRNAs. After electroporation, mouse CD4+ T cells were recovered from the electroporation cuvette and then were allowed to rest overnight in supplemented Mouse T Cell Nucleofector Medium (Amaxa). The following day, a portion of cells was used for flow cytometric analysis of GFP expression and real-time RT-PCR measurement of PPARα mRNA or NCOR mRNAs, whereas remaining cells were stimulated with anti-CD3 and...
anti-CD28 for measurement of cytokine levels in culture supernatants or acetylated histone 4 (H4-Ac) by chromatin immunoprecipitation (ChIP) (see details below).

Phosphosignaling analysis

T cells were washed with ice-cold serum-free X-VIVO 20 (Lonza) and resuspended at a concentration of 140 × 10⁶ cells/ml. Cells were stimulated with anti-CD3 and anti-CD28, and cell lysates were prepared as described previously (21). Protein concentrations were determined using the Quanti Kit (Molecular Probes), and proteins were separate by SDS-PAGE using NuPAGE 4–12% Bis-Tris Gel (Life Technologies) and NuPAGE MOPS 2× running buffer (Life Technologies). Proteins were transferred to polyvinylidene difluoride membranes overnight at 4°C using the Mini Trans-Blot Cell (Bio-Rad). Western blotting was conducted as described previously (22) by first probing blots overnight at 4°C using the SimpleChIP Enzymatic detection reagent (GE Healthcare).

ChIP

CD4⁺ and CD8⁺ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (5 μg/ml each) for 2 and 4 h, respectively, and ChIP was performed as previously described (7) using the SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology). DNA was immunoprecipitated using specific Abs (acetyl-histone H4K8, 2594S, 1:50 [Cell Signaling]; di(trimethylated histone 3 lysine 27 [H3K27Me3], 9733S, 1:50 [Cell Signaling]; PPARx, 101710, 1:50 [Cayman Chemical]; NCO1, PAI-844A, 1:50 [Thermo Scientific]; and isotype control IgG from native rabbit sera, 2729P, 1:500 [Cell Signaling]) and amplified using real-time PCR. Primer sequences are listed in Supplemental Table I.

Pharmacokinetic studies for IS001

Two CD-1 mice were gavaged orally with IS001 (30 mg/kg) in 0.5% methylcellulose in PBS. Blood samples were taken from mice at various time points, and plasma samples were prepared and frozen at –80°C prior to analysis. Standards were prepared by adding known amounts of IS001 to thawed mouse plasma to yield a concentration range from 0.8 to 4000 ng/ml. Plasma samples were precipitated using acetonitrile (1:4, v/v) containing the internal standard buspirone and 1.5% acetic acid. The levels of IS001 and buspirone were analyzed using a quadrupole mass spectrometer system that consisted of the mass spectrometer (Sciex API-3200; Sciex) that was interfaced to an HPLC system containing two LC10Avp pumps, a static bed mixer (Shimadzu), and a LEAP PAL autoinjector (Leap Technologies, Carrboro, NC). The analyze mixture (10 μl) was then injected on the column, and the samples were analyzed in positive ion mode by multiple reaction monitoring liquid chromatography-tandem mass spectrometry high-performance liquid chromatography method. The mobile phase contained 10 mmol/l ammonium acetate in water with 0.05% formic acid (solvent A) and 10 mmol/l ammonium acetate in 50% acetonitril/50% methanol with 0.05% formic acid (solvent B). The flow rate was maintained at 1 ml/min, and the total run time was 2.5 min. Analytes were separated using a linear gradient as follows: 1) the mobile phase was held for 30 s at 5% solvent; 2) solvent B was increased from 5 to 95% over the next 0.2 min; 3) solvent B was then held constant for 1.3 min at 95%; and 4) solvent B was then returned to the initial gradient conditions. The pharmacokinetic test parameters of the compound were calculated by a noncompartmental analysis using WinNonlin (Pharsight). Maximal plasma concentrations and their time of occurrence were both obtained directly from the measured data. The calibration curves were constructed by plotting the peak-area ratio of the analyzed peaks against the corresponding nominal concentrations of test compound in the plasma samples.

Experimental infection with L. monocytogenes

L. monocytogenes (EGD strain) were grown in brain–heart infusion media as previously described (23). For survival studies, C57BL/6J mice were infected i.p. with the LD₅₀ dose of the pathogen. For immune studies, a dose of 100,000 CFU was used to elicited optimal NK and NKT IFN-γ responses (19), whereas a sublethal dose of 20,000 CFU was used for experiments that assessed CD4⁺ and CD8⁺ T lymphocyte responses to avoid mouse lethality prior to the time of the peak T cell response. Endpoints for survival studies consisted of a 20% loss in body weight. For IS001 treatment studies, mice were administered the drug (30 mg/kg in 150 μl 0.5% methylcellulose in PBS) or vehicle by gavage, twice daily (9 to 10 h apart), starting 1 d prior to infection.

Serum was also collected from mice at 2 d postinfection with 100,000 CFU pathogen by cardiac puncture. Bacterial load was determined in the spleen at 48 h postinfection. For this, spleens were weighed and dissociated through a 70-micron nylon cell strainer into a petri dish that contained 1 × PBS. A 10 fold-dilution series of homogenates was prepared and streaked onto plates containing the brain–heart infusion agar. After 48 h, CFUs were counted, and the CFU/ml was calculated as the CFU on the plate/dilution factor/0.1 ml plated/mg tissue.

Statistical approach

Data are presented as means ± SEM. When data were parametric (kurtosis and skewness <2) and group variances were homogenous (Bartlett homogeneity test), a two-tailed t test (n = 2 groups) or a one-way ANOVA (n > 2 groups) was used to detect differences among groups. When data were nonparametric, ranks were compared among groups using a Mann–Whitney U test (n = 2 groups) or Kruskal–Wallis test (n > 2 groups). A difference in mouse survival between groups was determined using a log rank test. A p value ≤ 0.05 was considered significant.

Results

PPARx acts within effector CD4⁺ T cells to limit Th1 cytokine production

Previously, we reported that T cells from male, but not female, PPARα−/− mice proliferate more robustly and produce higher levels of Th1 cytokines than PPARα+/+ (wild-type [WT]) counterparts in vivo during experimental autoimmune encephalomyelitis and in vitro after activation with anti-CD3 and anti-CD28 (6). This hyper-Th1 response occurred exclusively in males, suggesting a role for this nuclear receptor in the sex-dependent regulation of Th1 immunity (6). To further explore the cellular basis of the enhanced Th1 responses in PPARα−/− males, we first conducted conventional in vitro Treg suppression assays. Consistent with our previous observations, CD4⁺CD25⁺CD44low Teff cells isolated from spleens of PPARα−/− males proliferated more robustly than WT counterparts (two-tailed t test, p = 0.022) and produced higher levels of IFN-γ (two-tailed t test, p = 0.042) (Teff only, open circles, left versus right panels, Fig. 1A). In contrast, WT and PPARα−/− CD4⁺CD25⁺ Treg cells did not exhibit any differences in their capacity to suppress the expansion or IFN-γ production by either WT (Fig. 1A, left panel) or PPARα−/− (Fig. 1A, right panel) Teff cells. Further supporting a role for PPARα in male CD4⁺ T effector cells, PPARα mRNA levels were also more highly expressed in male versus female T cells, particularly within the Teff cell compartment (Fig. 1B).

We also explored a role for PPARα in the APC compartment by culturing irradiated PPARα−/− and WT splenocytes (as a source of APC) with purified naive MOG p35–55 TCR-transgenic CD4⁺ T cells and peptide Ag. However, varying the genotype of APC in these assays did not influence the proliferation or IFN-γ production by either WT (Fig. 1A, left panel) or PPARα−/− (Fig. 1A, right panel) Teff cells. Further supporting a role for PPARα in male CD4⁺ Teff cells, PPARα mRNA levels were more highly expressed in male versus female T cells, particularly within the Teff cell compartment (Fig. 1B).

PPARα represses Th1 responses

We observed that PPARα deficiency was associated with enhanced Th1 cytokine production by male T cells; however, it has also been reported that certain ligand activators of PPARα enhance IL-4 production (24). To distinguish which Th pathway is the target of PPARα-deficiency, we compared cytokine production by male PPARα−/− and WT CD4⁺ T cells that were stimulated under Th0-, Th1-, Th17-, or Th2-polarizing conditions. Regardless of the type of skewing condition, male PPARα−/− CD4⁺ T cells produced IFN-γ levels of Th1 cytokines than PPARα+/+ (wild-type [WT]) counterparts in vivo during experimental autoimmune encephalomyelitis, and in vitro after activation with anti-CD3 and anti-CD28 (6). This hyper-Th1 response occurred exclusively in males, suggesting a role for this nuclear receptor in the sex-dependent regulation of Th1 immunity (6). To further explore the cellular basis of the enhanced Th1 responses in PPARα−/− males, we first conducted conventional in vitro Treg suppression assays. Consistent with our previous observations, CD4⁺CD25⁺CD44low Teff cells isolated from spleens of PPARα−/− males proliferated more robustly than WT counterparts (two-tailed t test, p = 0.022) and produced higher levels of IFN-γ (two-tailed t test, p = 0.042) (Teff only, open circles, left versus right panels, Fig. 1A). In contrast, WT and PPARα−/− CD4⁺CD25⁺ Treg cells did not exhibit any differences in their capacity to suppress the expansion or IFN-γ production by either WT (Fig. 1A, left panel) or PPARα−/− (Fig. 1A, right panel) Teff cells. Further supporting a role for PPARα in male CD4⁺ T effector cells, PPARα mRNA levels were also more highly expressed in male versus female T cells, particularly within the Teff cell compartment (Fig. 1B).

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This increased IFN-γ production by male PPARα−/− CD4⁺ T cells was acompañed by decreases in the production of
IL-4 under Th17-skewing conditions and decreases in the production of IL-17 under most conditions examined (Fig. 2A). These changes in Th cytokine production were accompanied by increases in the intranuclear staining of T-bet and decreases in the intranuclear staining of GATA-3 and RORγT (Fig. 2B).

We also stimulated naive WT and PPARα−/− CD4+ T cells with anti-CD3 and anti-CD28 under Th0 conditions and examined the time course of expression of various Th-associated genes. We observed that PPARα−/− CD4+ T cells displayed a striking enhancement in Ifng gene expression relative to WT CD4+ T cells at 4 and 16 h poststimulation (Fig. 2C). This Th1 skewing in the cytokine gene signature in PPARα−/− CD4+ T cells was evident at the earliest time point examined (4 h) and was accompanied by increases in the expressions of a number of Th1-associated genes including Stat1 (at 4 h), Tbx21 (at 16 h), and eomesdermin (Eomes) (at 16 h) and decreases in the peak expressions of Gata3 (at 4 h) and Rorc (at 8 h) mRNAs (Fig. 2C).

It has been previously reported that male PPARα−/− mice develop a fatty liver with age due to defects in lipid metabolism (25). Though we used young (8-wk-old) adult mice in our experiments to avoid this possible confounder, it remained possible that the Th1 bias we observed in PPARα−/− T cells related to other metabolic abnormalities (25) or developmental defects (26) in the PPARα−/− mouse or to the compensatory upregulation of other nuclear receptors such as PPARγ and PPARδ (6). Thus, to further address the involvement of PPARα in the regulation of the Th fate, we investigated the effects of the well-known PPARα agonist fenofibrate and a novel antagonist of PPARα IS001 on Th cytokine production by WT CD4+ T cells. IS001 was designed to be a competitive antagonist of PPARα and would act to displace endogenous fatty acid ligands (i.e., unsaturated fatty acids such as linoleic acid) that are constitutively bound to PPARα in vivo or in vitro when cells are cultured in media that contains serum or fatty acid-bound albumin. Preliminary experiments using nuclear hormone reporter assays determined that the IC50 of IS001 for mouse and human PPARα is in the nanomolar range and further showed that this drug exhibits a 50–200-fold selectivity for mouse and human PPARα compared with other nuclear receptors (Fig. 3A).

Upon treatment of CD4+ T cells with IS001, we observed that the drug had the effect of enhancing IFN-γ production by activated CD4+ T cells relative to vehicle control treatment (Fig. 3B), but did not decrease IL-4 or IL-17 production (Fig. 3B). In fact, IS001 had the effect of slightly enhancing the production of IL-4 (Fig. 3B), which appeared to relate to a stimulatory effect of this drug on T cell proliferation (Supplemental Fig. 1A). In contrast, treatment with fenofibrate selectively reduced the production of IFN-γ by CD4+ T cells (Fig. 3C). These effects of IS001 and fenofibrate on IFN-γ production were not observed in cultures of PPARα−/− CD4+ T cells (Supplemental Fig. 1B for IS001) (see Ref. 7 for fenofibrate), indicating that they were PPARα dependent.
To further verify that PPARα is uniquely regulating the Th1 pathway, we also conducted siRNA knockdown studies in primary male WT CD4+ T cells. For these studies, we used male T cells from SJL mice, for which we can achieve reasonable transfection efficiencies and PPARα mRNA knockdown (Fig. 3D, Fig. 3 legend). Similar to findings using the antagonist, transfection with PPARα siRNAs resulted in a heightened production of IFN-γ by CD4+ T cells upon stimulation with anti-CD3 and anti-CD28 stimulation, but did not influence the productions of IL-17 or IL-4 (Fig. 3E). Together with our pharmacologic data, these results support a mechanism by which PPARα inhibits the Th1 pathway directly as opposed to an indirect mechanism involving promotion of Th2 or Th17 pathways.

To determine which gene in the Th1 pathway is the target of PPARα-dependent regulation, we focused on the three Th1-associated genes (Tbx21, Eomes, and Ifng) that are known to act early in the Th1 pathway downstream of early TCR and CD28 stimulation (1) and were expressed at higher levels in PPARα−/− compared with WT CD4+ T cells (Fig. 2C). IFN-γ and T-bet act in a feed-forward loop to enhance each other’s expression during

**FIGURE 2.** PPARα−/− CD4+ T cells are skewed toward Th1. (A and B) Lymph nodes were isolated from WT or PPARα−/− male SV.129 mice (n = 5 mice/group), pooled within groups, and dissociated into a single-cell suspension. Cells were then stimulated in complete RPMI 1640 with anti-CD3 and anti-CD28 under Th0- (no added cytokines), Th1- (IL-12 + anti-IL-4), Th2- (IL-4 + anti–IFN-γ), or Th17-polarizing (TGF-β + IL-6 + anti–IFN-γ + anti-IL-4) conditions. (A) Cytokine levels in supernatants at 60 h in one experiment of three. Values are means + SEM readings in triplicate cultures. (B) Intranuclear staining of T-bet, GATA-3, and RORγt in Th0 cells in the CD4+ T cell live gate after 60 h of stimulation. These plots are representative of two independent experiments. (C) Naive CD4+ T cells were isolated from spleen and lymph nodes of PPARα+/+ or PPARα−/− male mice (n = 5 mice/group), pooled within groups, and stimulated with anti-CD3 and anti-CD28 for various times (0, 4, 8, 16, or 24 h). After the indicated times, cells were harvested for isolation of total RNA. Transcript levels for the indicated genes were measured by real-time PCR and are expressed relative to β-actin mRNAs. Values are means + SEM of triplicate PCR reactions in one representative experiment of two that were performed. *Significantly different (p < 0.05) from PPARα+/+ by t test (two-tailed). FMO, fluorescence-minus-one.

Ifng is the gene repressed by PPARα
PPARα REGULATES SEX DIFFERENCES IN IFN-γ PRODUCTION

We had observed that the effect of PPARα in limiting Ifng gene expression in male CD4+ T cells occurred even under Th0 conditions, suggesting that this transcription factor may be acting as a rheostat, modulating the strength of TCR or CD28 signals that lead to Ifng transcription. Although previous reports have suggested a role for PPARα in the negative regulation of the phosphorylation of p38 (28) or the abundance of IκBα (29), we did not observe any aberrations in the expression or phosphorylation of key signaling intermediates downstream of the TCR and CD28 in male PPARα−/− CD4+ T cells (Supplemental Fig. 2). Thus, PPARα must operate more distally in this pathway, possibly at the Ifng locus itself to control Ifng expression.

Transcription of Ifng is regulated by both TCR and cytokine-dependent signals and downstream transcription factors that bind to cis elements at the promoter, but also at a number of highly conserved noncoding sequences (CNS) located far upstream and downstream of Ifng (Fig. 5A) (1–3). The binding of transcription factors to CNS sites is supported by epigenetic modifications such as histone acetylation that make chromatin at the Ifng locus more accessible (2, 3). H4-Ac is one epigenetic mark that accumulates at CNS sites at the Ifng locus during Th1 differentiation (30). This acetylation event occurs as a result of the T-bet–dependent removal of the NCOR/Sin3A/HDAC-containing corepressor complex at the Ifng locus (30).

To investigate whether PPARα may be regulating histone acetylation, we started by comparing the abundance of H4-Ac at CNS sites at the Ifng locus in male WT and PPARα−/− CD4+ T cells using a ChIP assay. Consistent with previous reports (3), we observed a more notable induction in H4-Ac in WT CD4+ T cells under Th1 compared with Th0 polarization.

Th1 differentiation, whereas Eomes is important for optimal expression of IFN-γ in CD4+ T cells and the major inducer of IFN-γ expression in the absence of T-bet (1).

To investigate whether it was the higher T-bet expression in PPARα−/− CD4+ T cells that was driving the enhanced IFN-γ mRNAs, we generated PPARα−/+ and PPARα−/− mice on the T-bet−/− background, isolated naive CD4+ T cells from these mice and stimulated these cells with anti-CD3 and anti-CD28. IL-12 was also added to media to help amplify the low-level IFN-γ mRNA expression in T-bet−/− T cells. We observed that PPARα−/− T-bet−/− CD4+ T cells still exhibited higher IFN-γ mRNA levels and cytokine production than PPARα+/− T-bet−/− CD4+ T cells (Fig. 4A), suggesting that T-bet is not required for the PPARα-dependent regulation of IFN-γ production. Interestingly, in the absence of T-bet, there was an early compensatory increase in Eomes mRNA expression in the PPARα−/− CD4+ T cells (Fig. 3A) that corresponded with the timing of the early peak in T-bet expression that is observed in WT T cells postactivation (Fig. 2C).

Because T-bet and Eomes are both induced downstream of IFN-γ receptor–STAT1 signaling (4, 27), it remained possible that the higher expressions of these transcription factors in PPARα−/− CD4+ T cells was related to the higher IFN-γ production by PPARα−/− CD4+ T cells during the early stages of activation. Thus, we repeated T cell stimulations in the presence of an IFN-γ neutralizing Ab, which functioned to bind any secreted IFN-γ. We found that under these conditions, the differences in the early expressions of T-bet and Eomes mRNAs between WT and PPARα−/− CD4+ T cells disappeared, whereas the levels of IFN-γ mRNAs in PPARα−/− CD4+ T cells remained elevated above WT (Fig. 3B). Together, these data strongly suggest that Ifng is the gene repressed by PPARα in male CD4+ T cells.

Deficiency in PPARα is associated with enhanced histone acetylation at the Ifng locus

PPARα specifically inhibits the Th1 pathway. (A) The IC50 of the IS001 antagonist for human and mouse PPARα as well as for thyroid hormone receptor β (TRβ), RXRα, glucocorticoid receptor (GR), and estrogen receptor β (ERβ) as determined using nuclear hormone-based luciferase reporter assays. WT CD4+ T cells isolated from SJL mice (n = 5 mice/group) were pooled, and preincubated in X-VIVO-20 media with 100 mmol/l IS001 (B) or 2.5 μmol/l fenofibrate (C) or DMSO (0.1%) vehicle for 4 h prior to stimulation with anti-CD3 and anti-CD28. Shown are the cytokine levels detected in culture supernatants after 48 h. Values are means ± SEM of triplicate cultures, and data presented are representative of three independent experiments. *Significantly different (p < 0.05) from WT by t test (two-tailed). (D and E) Total mouse CD4+ T cells were isolated from male SJL mice (n = 5 mice/group), pooled, and electroporated with a vector encoding GFP (to monitor for transfection efficiency) plus mouse mRNA expression and remaining cells were transferred to anti-CD3–

T-bet specifically inhibits the Th1 pathway. (A) The IC50 of the IS001 antagonist for human and mouse PPARα as well as for thyroid hormone receptor β (TRβ), RXRα, glucocorticoid receptor (GR), and estrogen receptor β (ERβ) as determined using nuclear hormone-based luciferase reporter assays. WT CD4+ T cells isolated from SJL mice (n = 5 mice/group) were pooled, and electroporated with a vector encoding GFP (to monitor for transfection efficiency) plus mouse mRNA expression and remaining cells were transferred to anti-CD3–

To investigate whether PPARα may be regulating histone acetylation, we started by comparing the abundance of H4-Ac at CNS sites at the Ifng locus in male WT and PPARα−/− CD4+ T cells using a ChIP assay. Consistent with previous reports (3), we observed a more notable induction in H4-Ac in WT CD4+ T cells under Th1 compared with Th0 polarization.
conditions (Fig. 5B). However, when comparing the levels of H4-Ac between WT and PPARα−/− CD4+ T cells, it was apparent that PPARα−/− CD4+ T cells showed a higher abundance of H4-Ac at the Ifng promoter and at most CNS sites examined under both Th0 and Th1 conditions (Fig. 5C, 5D). However, no differences in the abundance of H4-Ac were observed between quiescent WT and PPARα−/− CD4+ T cells (Fig. 5E), indicating that PPARα only influences H4-Ac at the Ifng locus in activated T cells.

We also compared the abundance of H3K27-Me, a repressive mark that accumulates at CNS sites in the Ifng locus upon Th2 differentiation (31). Though naive CD4+ T cells show only limited H3K27-Me at certain CNS sites in the Ifng locus, we noted that this abundance was lowered in PPARα−/− compared with WT cells after stimulation (Fig. 5F), further consistent with the notion that male PPARα−/− CD4+ T cells are not only more poised to become Th1 but may be less poised to become Th2 cells.

Next, we investigated whether the small-molecule PPARα antagonist IS001 could evoke a similar increase in H4-Ac across the Ifng locus as seen in PPARα−/− CD4+ T cells. For these experiments, we stimulated both male and female CD4+ T cells with anti-CD3 and anti-CD28 for 4 h in the presence of either IS001 or vehicle control. Similar to findings of male PPARα−/− CD4+ T cells, treatment of male WT CD4+ T cells with the antagonist increased H4-Ac at the Ifng promoter and at most CNS sites (Fig. 5G, left panel) as well as increased the levels of IFN-γ mRNAs (Fig. 5H). Consistent with the broad-scale nature of these changes, IS001 treatment also enhanced T-bet mRNA levels in male T cells (Fig. 5H). This effect of the antagonist in increasing H4-Ac was not observed in assays conducted using female CD4+ T cells (Fig. 5G, right panel), confirming a sex-specific modulation of the Ifng gene by PPARα.

**Ligand-dependent transrepression of Ifng by PPARα**

How PPARα represses the expression of Ifng is not presently known. Previously, Pascual et al. (15) resolved the molecular mechanism of how the related nuclear receptor PPARγ represses the transcription of Inos in macrophages. It was found that the ligand agonist rosiglitazone promotes the sumoylation and recruitment of PPARγ to the Inos promoter, where it stabilizes the presence of an NCOR-containing corepressor complex, rendering the gene refractory to LPS stimulation (15). NCOR mediates the repressive effects of PPARs and other nuclear receptors by recruiting HDACs to gene-regulatory regions (32). Given that PPARα is also reported to be sumoylated upon ligand treatment (33), we hypothesized that PPARα may operate by a similar mechanism to repress Ifng.

We therefore tested whether treatment of male CD4+ T cells with the PPARα ligand fenofibrate would enhance the recruitment of PPARα to regulatory elements at the Ifng gene and whether this recruitment would be associated with reduced H4-Ac and enhanced NCOR at these sites. For these studies, we used Th1-polarized CD4+ T cells that exhibited a higher basal level of H4-Ac (Fig. 5B). We observed that treatment with fenofibrate reduced IFN-γ mRNAs and the abundance of H4-Ac at two CNS sites (CNS-22 and CNS-34) and at the Ifng promoter relative to levels in the vehicle control group (Fig. 6A). These changes were independent of T-bet, because fenofibrate treatment did not lower T-bet expression within the 4-h time frame of the experiment (Fig. 6B). In addition, the three cis-regulatory elements that showed reduced H4-Ac also displayed an increased abundance of NCOR and PPARα as detected by ChIP (Fig. 6C, 6D).

Though the increased presence of PPARα at the Ifng promoter and CNS-22 and CNS-34 enhancer regions correlated with the accumulation of NCOR, it remained unclear whether NCOR was required for the repressive activities of PPARα on IFN-γ production. Therefore, we investigated whether siRNA-mediated knockdown of NCOR expression in primary T cells would abrogate the effects of fenofibrate in decreasing IFN-γ production. For these studies, we used SJL male T cells, which are

**FIGURE 4.** IFN-γ is the gene target of PPARα-dependent suppression. WT or PPARα−/− naive CD4+ T cells were isolated from mice on the T-bet+/+ or T-bet−/− background (cells pooled from n = 5 mice/group). These cells were stimulated in complete RPMI 1640 with anti-CD3 and anti-CD28 for 24 h in the absence (A) or presence (B) of anti-IFN-γ (10 μg/ml). For the experiment using T-bet−/− CD4+ T cells (in A), media was also supplemented with mouse rIL-12. Transcript levels were measured using real-time PCR and normalized to β-actin mRNAs. IFN-γ levels in culture supernatants were measured at 24 h of culture using ELISA. Values are means ± SEM of triplicate reactions (left three panels) or triplicate cultures (right panel) and representative of two independent experiments. *Significantly different (p < 0.05) from PPARα−/− group by t test (two-tailed).
PPARα REGULATES SEX DIFFERENCES IN IFN-γ PRODUCTION

A

CNS-54  CNS-34  CNS-22  CNS-5  CNS-1  **Ifng** Promoter  Coding Region

**Ifng** Locus

B

![Graph showing fold increase in H4-Ac vs Th0 and Th1](image)

C

![Graph showing H4-Ac fold increase in Th0 and Th1](image)

D

![Graph showing H4-Ac fold increase in Th0 and Th1](image)

E

![Graph showing H4-Ac fold increase in Th0 and Th1](image)

F

![Graph showing H4-Ac fold increase in Th0 and Th1](image)

G

![Graph showing H4-Ac fold increase in Th0 and Th1](image)

H

![Graph showing mRNA levels of T-bet and IFN-γ](image)

**FIGURE 5.** Reducing PPARα activity enhances H4-Ac at the Ifng locus. (A) Schematic of the **Ifng**. (B and C) Naive CD4+ T cells or Th1-polarized cells grown from spleens of male WT or PPARα−/− mice (n = 5/group) were stimulated in complete RPMI 1640 with anti-CD3 and anti-CD28 for 4 h. ChIP was performed using anti–H4-Ac and primers that spanned CNS sites and the **Ifng** promoter. (B) Mean ± SEM fold increase of H4-Ac in stimulated versus nonstimulated CD4+ T cells in one experiment. The relative abundance of H4-Ac in the WT and PPARα−/− CD4+ T cells is shown under Th0 (C) and Th1 conditions (D). (F) Mean ± SEM relative abundance of H4-Ac at the various CNS sites and the **Ifng** promoter in naive quiescent WT and PPARα−/− CD4+ T cells. (F) Naive PPARα−/− and PPARα−/− CD4+ T cells were activated for 4 h with anti-CD3 and anti-CD28 (8 μg/ml each), and ChIP was performed using a di/triethylated histone H3K27 Ab. Shown is the relative abundance of H3K27-Me at the various CNS sites and the **Ifng** promoter. *Significantly different (p < 0.05) by two-tailed t test. (G and H) Naive CD4+ T cells were isolated from male or female WT mice and were stimulated in X-VIVO-20 media with anti-CD3 and anti-CD28 in the presence of 100 nmol/l ISO001 or DMSO vehicle. ChIP was performed as described above for H4-Ac (G), and T-bet and IFN-γ mRNAs were measured using real-time PCR (male cells only) (in H). All of these studies were conducted using samples from mice (n = 5 mice pooled/group). The values are means ± SEM of triplicate PCR measurements from one experiment, and data shown are representative of two to three independent experiments. All ChIP data except for that in (B) reflects the abundance of immunoprecipitated DNA normalized to input DNA less the minimal signal generated from the IgG control. *Significantly different (p < 0.05) from PPARα+/+ (C–F) or vehicle (G and H) by t test (two-tailed).

Amenable to transfection and gene knockdown (Fig. 6E, 6F). Similar to our previous findings in SV.129 CD4+ T cells (Fig. 3C), fenofibrate treatment reduced IFN-γ production by CD4+ T cells transfected with nontargeting siRNAs (Fig. 6G) and reduced H4-Ac at CNS-34, CNS-22, and the **Ifng** promoter sites (Fig. 6H). Knockdown of NCOX reversed these effects of fenofibrate (Fig. 6G, 6H), confirming that PPARα requires NCOX for the repression of **Ifng**. Together, these data suggest that PPARα represses **Ifng** gene expression by recruiting NCOX and associated HDACs to important regulatory elements in the **Ifng** locus.

**PPARα also limits IFN-γ production by male NKT and CD8+ T cells**

IFN-γ is not only produced by CD4+ T cells, but also in large amounts by cytotoxic CD8+ T lymphocytes, NK, and NKT cells (34). It has also been reported that these cell types exhibit a higher production of IFN-γ in females than in males (35–37). To gain
FIGURE 6. Fenofibrate induces PPARα recruitment to specific regulatory elements in the $\text{ifng}$ locus to repress H4-Ac. (A–D) Th1-polarized CD4$^+$ T cells grown from male WT mice ($n = 5$ group) were stimulated for 4 h with anti-CD3 and anti-CD28 in X-VIVO-20 media in the presence of the PPARα ligand (fenofibrate) or DMSO vehicle. (B) Total RNA was isolated from T cells, and T-bet and IFN-γ mRNAs were measured using real-time RT-PCR measurement and expressed relative to $\beta$-actin mRNAs. ChIP was performed as described in Fig. 5 legend using anti–H4-Ac (A), anti-NCOR (C), and anti-PPARα (D) Abs. For ChIP studies in (A) and (C), the data are the relative abundance of immunoprecipitated DNA normalized to total input DNA less the minimal signal generated from the IgG control, whereas (D) shows the fold change of the relative abundance of PPARα in the fenofibrate- versus DMSO-treated samples. Values in (A)–(C) are means + SEM of triplicate PCR reactions and representative of two independent experiments, whereas values in (D) are means + SEM of fold-change values obtained in two independent experiments. In (A)–(D), an asterisk denotes a significant difference ($p < 0.05$) from vehicle counterpart by two-tailed $t$ test. (E–H) CD4$^+$ T cells were isolated from male SJL/J mice and transfected with either nontargeting or NCOR-specific siRNAs along with a GFP reporter plasmid. After a 12-h overnight rest, cells were treated 4 h with fenofibrate and then stimulated with anti-CD3 and anti-CD28 for either 4 h (ChIP and mRNA measurement) or 48 h (cytokine measurement). (E) Efficiency of transfection as determined by flow cytometry measurement of GFP expression. (F) Abundance of NCOR mRNAs (relative to $\beta$-actin mRNAs) in CD4$^+$ T cells stimulated with NCOR or nontargeting siRNAs. (G) IFN-γ production by CD4$^+$ T cells, and (H) H4-Ac at CNS-34, CNS-22, and $\text{ifng}$ promoter sites using ChIP. Values in (F)–(H) are means + SEM of triplicate PCR reactions or cultures in one experiment done of two that were performed. In (G) and (H), an asterisk indicates a significant difference ($p < 0.05$) from nontargeting siRNA vehicle as determined using a one-way ANOVA and Tukey post hoc test.
insights into the breadth of regulation of IFN-γ production by PPARα, we stimulated spleenocytes from male and female WT and PPARα<sup>−/−</sup> mice with PMA/ionomycin and examined IFN-γ responses by NK and NKT cells by intracellular cytokine staining. We observed that the frequencies of IFN-γ-producing NK cells did not differ between WT and PPARα<sup>−/−</sup> groups in either sex, though there was a tendency for a higher frequency of IFN-γ<sup>+</sup> cells in the male PPARα<sup>−/−</sup> versus the male WT group (Fig. 7A). In contrast, IFN-γ responses to PMA/ionomycin were higher in the NKT cell compartment in male PPARα<sup>−/−</sup> as compared with WT mice (Fig. 7B).

Next, we investigated whether PPARα is involved in regulating the sex difference in IFN-γ production by CD8<sup>+</sup> T cells. Consistent with our findings for CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells isolated from spleens of male, but not female, PPARα<sup>−/−</sup> mice produced higher levels of IFN-γ than WT counterparts in response to anti-CD3 and anti-CD28 stimulation (Fig. 8A). Similarly, treatment with IS001 increased IFN-γ production by male, but not female, CD8<sup>+</sup> T cells relative to levels in the vehicle control group (Fig. 8B). The increased IFN-γ production by male CD8<sup>+</sup> T cells in response to IS001 treatment correlated with a higher abundance of H4-Ac at most CNS sites examined (Fig. 8C, <i>top panel</i>). In contrast, the abundance of H4-Ac was not changed or was lowered at CNS sites in female CD8<sup>+</sup> T cells treated with IS001 (Fig. 8C, <i>bottom panel</i>). These results indicate that PPARα controls sex differences in IFN-γ production by conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as NKT cells.

**IS001 treatment enhances IFN-γ responses in male mice during L. monocytogenes infection**

IFN-γ signaling has been shown to be crucial for mounting cellular immune responses against intracellular pathogens including the Gram-positive bacterium <i>L. monocytogenes</i> (38). Experimental infection of mice with <i>L. monocytogenes</i> has been an instrumental model for deciphering the importance of IFN-γ in innate and cellular immunity (19, 38–41). We therefore were interested in whether treatment with the PPARα antagonist IS001 would enhance IFN-γ production by innate and adaptive immune cells in male mice and improve resistance against experimental infection with this pathogen. For these studies, mice were infected with <i>L. monocytogenes</i> (i.p.) and treated twice daily via oral gavage with either vehicle (0.5% methylcellulose) or IS001 (30 mg/kg), starting 1 d prior to infection. Preliminary, pharmacokinetic studies established that a 30-mg/kg oral dose of IS001 resulted in plasma concentrations of IS001 (Supplemental Fig. 3) that would specifically antagonize PPARα based on the determined IC<sub>50</sub> values (Fig. 3A).

During experimental infection with <i>L. monocytogenes</i>, early IFN-γ production by NK and NKT cells in the spleen and liver is important for the initial control of the pathogen (39, 40). To investigate whether there was a sex-dependent effect of IS001 on NK and NKT cell IFN-γ production, we infected male and female mice i.p. with 100,000 CFU of <i>L. monocytogenes</i> and treated mice twice daily with IS001 or vehicle starting 1 d prior to infection. We then examined IFN-γ production by innate cells in the spleen at 24 h postinfection, the time of the peak IFN-γ response. We found that significant differences in IFN-γ responses were apparent between the sexes and between treatment groups within the NKT cell compartment, but not in the NK cell compartment (Fig. 9A, 9B). Specifically, we observed that a higher frequency of female NKT cells produced IFN-γ compared with male NKT cells upon infection (comparing vehicle-treated male and female groups in Fig. 9B; <i>p = 0.048</i>, <i>t</i> test), and this was associated with a higher level of IFN-γ in the serum of female mice (Fig. 9C). Oral dosing of IS001 had the effect of enhancing IFN-γ responses by NKT cells and increasing serum IFN-γ in male, but not female mice.
FIGURE 8. PPARα negatively regulates IFN-γ production by male CD8+ T cells. Total CD8+ T cells isolated from spleens of male or female WT and PPARα−/− mice (n = 5/group) were stimulated with anti-CD3 and anti-CD28 (A) or treated with 100 nmol/l IS001 or vehicle prior to stimulation (B and C). (A) Shown are the mean ± SEM cytokine levels in culture supernatants of male and female cells after 24 h in one of two independent experiments that were done. Male and female data were collected from independent experiments and were thus not displayed in the same panel. *Significantly different from the WT group by two-tailed t test. (B) Shown are the mean ± SEM cytokine levels in culture supernatants of male and female CD8+ T cell cultures in one of two representative experiments that were done. *Significantly different (p < 0.05) from the vehicle counterpart. †Significantly different (p < 0.05) from sex-matched counterpart. Data were compared using a one-way ANOVA and Tukey post hoc test. (C) ChIP for H4-Ac was performed as described in Fig. 3 legend. Values are means ± SEM of triplicate cultures or PCR reactions of one representative experiment of two that were done. Data are expressed as the relative abundance of immunoprecipitated DNA normalized to total input DNA less the minimal signal generated from the IgG control. *Significant difference (p < 0.05) from vehicle by two-tailed t test.

Discussion

It has been suggested that sex differences in Th1 responses arise because of the higher IL-12 production by APC and enhanced T cell responsiveness to this cytokine in females (11, 12). We previously observed that even in the absence of IL-12, male CD4+ T cells have a lower intrinsic ability than female CD4+ T cells to produce IFN-γ, which associated with higher expression of PPARα (6, 7). The present study identifies Ifng as the gene repressed by PPARα and provides evidence that PPARα inhibits Ifng by recruiting NCOR to key regulatory elements of the Ifng locus. Furthermore, we observed that treatment of mice with a small-molecule PPARα antagonist IS001 enhanced IFN-γ production by NKT, CD4+, and CD8+ T cells selectively in male mice, further implicating PPARα as a major factor controlling sex differences in IFN-γ responses and support the application of IS001 as an adjuvant to boost innate and cellular immune responses in males.
mice (24), suggesting that the effect occurred through a PPARα-independent mechanism. In contrast, the study by Gocke et al. (42) reported that the induction of IL-4 in T cells by gemfibrozil was PPARα-dependent and associated with the direct recruitment of PPARα to the IL-4 promoter (42). Taken together, the current evidence suggests that PPARα functions primarily to inhibit IFN-γ production by T cells; however, at high doses, certain fibrate drugs may have the added effect of inducing Th2 cytokine production, which may or may not be PPARα dependent.

In addition to elucidating the Th1 pathway as the prime target of PPARα-dependent regulation, our studies identified the Ifng to be the gene target of suppression. There were three key pieces of evidence that supported this conclusion. First, in examining the profile of Th-related genes in activated CD4+ T cells, it was apparent that Ifng was the gene that showed a striking and sustained difference in expression between WT and PPARα−/− CD4+ T cells. Secondly, when we neutralized extracellular IFN-γ, the differences in T-bet and Eomes mRNA expression between WT and PPARα−/− CD4+ T cells were further accentuated. Finally, the ability of fibrates to enhance IFN-γ responses selectively in males during L. monocytogenes infection (Fig. 9).
and PPARα−/− CD4+ T cells disappeared, whereas the expression of Ifng remained persistently elevated in PPARα−/− CD4+ T cells. Finally, in our ChIP studies that investigated the effect of fenofibrate on H4-Ac, we observed the drug to inhibit IFN-γ but not T-bet mRNAs. Notably, our finding that PPARα targets IFN-γ rather than T-bet, does contrast with the mechanism elucidated for the PPARγ-dependent control of the Th17 lineage, in which it was found that PPARγ directly represses ROR-γt expression (16). Why evolution favored Ifng rather than Tbx21 as a target of PPARα in the control of Th1 immunity is not known, but may relate to the key importance of early production of IFN-γ in the amplification of T-bet expression during early Th1 programming (4).

Our experiments also resolved a potential mechanism for how PPARα represses Ifng. We observed that treatment with fenofibrate induced the corecruitment of PPARα and NCOR to the Ifng promoter as well as CNS-22 and CNS-34 sites, which are distal elements in the Ifng locus that are required for Th1 lineage-specific expression (2). The enhanced abundance of NCOR was also associated with reduced histone acetylation at these regulatory elements, which is consistent with the role of NCOR in tethering HDACs to gene control regions (32). Furthermore, our finding that knockdown of NCOR abrogated the effects of fenofibrate in reducing H4-Ac and IFN-γ production, proved that NCOR is required for the effect of PPARα on IFN-γ expression. These findings thus mirror the mechanism elucidated for the PPARγ-dependent inhibition of Inos, where it was found that PPARγ inhibited expression of this gene by recruiting and stabilizing the presence of NCOR and HDAC3 at the promoter region of this gene. In addition to the effects of PPARα on histone acetylation, our studies provided limited evidence that PPARα may also regulate the accumulation of the repressive histone mark H3K27-Me, because the levels of H3K27-Me were higher at various CNS sites in PPARα−/− versus WT CD4+ cells. Future studies will investigate whether PPARα is directly responsible for the altered methylation status at the Ifng promoter, by examining whether H3K27-Me or Enhancer of Zeste Homolog 2, the enzyme that catalyzes this histone methylation event, accumulate differently at the Ifng locus in fenofibrate- or IS001-treated T cells.

Why PPARα was recruited to CNS-22 and CNS-34 and not other CNS sites is not clear, but could relate to the fact that these two regulatory elements are considered to be pioneering elements within the Ifng locus (2, 43). In particular, CNS-22 is thought to serve as a primary node for the recruitment of trans factors that initiate the remodeling of the locus during Th1, Tc1, and NK cell differentiation (2, 43). CNS-22 and CNS-34 along with the Ifng promoter also contain binding sites for NF-kB, a transcription factor that acts downstream of TCR and CD28 signals to induce Ifng gene expression (2, 44). Though our study did not reveal a role for PPARα in regulating the kinetics of IkB degradation, in past work, we showed that nuclear extracts from male PPARα−/− CD4+ T cells exhibit higher NF-kB p65 DNA binding activity than WT nuclear extracts using an oligo-based DNA-binding assay (6). This result indicated that in addition to regulating chromatin remodeling, PPARα may regulate the expression of Ifng by impacting distal events in NF-kB signaling that influence the DNA binding of this transcription factor (such as phosphorylation of NF-kB).

One inconsistency that we observed between the effects of fenofibrate and IS001 on H4-Ac and was that IS001 influenced H4-Ac at all CNS sites, whereas fenofibrate influenced H4-Ac only at CNS-22 and CNS-34. This inconsistency most likely relates to differences in the types of Th cells that were used in ChIP studies and the effects of these short-term drug treatments on T-bet expression. In the ChIP experiments using IS001, naïve CD4+ T cells were used that exhibited low baseline T-bet mRNA expression and H4-Ac. Stimulation of naïve CD4+ T cells over 4 h in the presence of the antagonist had the effect of enhancing T-bet expression, a necessary prerequisite for inducing broad-scale changes in H4-Ac at the Ifng locus (30). In contrast, ChIP experiments using fenofibrate were conducted using Th1-polarized cells that already exhibited high T-bet mRNA expression, which was not impacted in the short-term by ligand treatment; hence no broad-scale changes in H4-Ac were apparent and T-bet-independent effects of PPARα could be revealed.

The finding that PPARα regulates IFN-γ expression through the CNS-34 site may also explain why NK cell IFN-γ production was not significantly impacted by PPARα deficiency. Although CNS-22 is involved in the establishment of H4-Ac marks and for induction of Ifng transcription in both NK cells and adaptive lymphocytes, the human homolog of CNS-34 has been shown to be important in the regulation of IFN-γ in CD4+ T cells and NKT cells, but not in NK cells (2, 45). Thus, it is possible that the PPARα-dependent control of Ifng may be diluted in NK cells due to the decreased importance of CNS-34 in this regulation. Consistent with this notion, we did observe a trend for a higher IFN-γ response by PPARα−/− NK cells and by WT NK cells treated with the PPARα antagonist.

Our in vivo studies using IS001 also provided the proof of concept that antagonizing PPARα activity with a small molecule can be used as an approach to boost IFN-γ production in males to aid in the clearance of intracellular pathogens. In addition to its role in combating bacterial infection, IFN-γ production is crucial for antitumor immune responses, and rIFN-γ or IL-12 have been used with some success in clinical trials to treat various malignancies (46–48). Because systemic administration of these cytokines is associated with side effects and dose-related toxicities (47, 48), alternative approaches are being pursued to enhance IFN-γ signaling including injection of tumors with adenovirus encoding IFN-γ (47) and administration of small-molecule activators of STAT1 signaling (49). We propose that treatment with IS001 may serve as yet another approach to boost IFN-γ responses in males to treat chronic infection or malignancy.

In conclusion, our studies in PPARα provide a molecular mechanism for why IFN-γ responses are more robust in females and introduce a novel small molecule that can be used to augment the production of this cytokine in males.

Acknowledgments
We thank Dr. Peppi Prasit and the scientific team from Inception Sciences for providing IS001, Dr. Dana Philpott from the University of Toronto for providing L. monocytogenes, and the National Institutes of Health Tetramer Core Facility for the CD1d tetramers.

Disclosures
N.S., L.C., and R.C. are employees of Inception Sciences who developed the IS001 antagonist. N.S. is also the director of Inception Sciences and holds equity in the company. The other authors have no financial conflicts of interest.

References


Supplemental Fig. 1. Titration of IS001 and the effect of this compound on CD4\(^+\) T cell proliferation and IFN-\(\gamma\) production. Total CD4\(^+\) T cells were isolated from male PPAR\(\alpha^{++}\) and PPAR\(\alpha^{-/-}\) mice (N=5/group) and were pre-incubated with various concentrations of IS001 or DMSO vehicle (0.1% volume) prior to stimulation with anti-CD3 and anti-CD28. (A) The proliferation of T cells was measured between 72-90 h by \[^{3}H\]-thymidine incorporation assay and is expressed in counts per minute (CPM). (B) IFN\(\gamma\) production was measured after 48 hours using an ELISA kit. Values represented are means +/- SEM of triplicate cultures and data are representative of 2-3 independent experiments. * indicates a significant difference from the vehicle-treated counterpart by two-tailed T-test (level of significance, \(P<0.05\)).
Supplemental Fig. 2. PPARα−/− male CD4+ T cells do not exhibit a differential phosphorylation or abundance of key signaling intermediates downstream of TCR/CD28. Total CD4+ T cells isolated from male WT and PPARα−/− mice (N=6-8 mice/group) and were stimulated with anti-CD3 and anti-CD28. At the indicated times after stimulation, cells were lysed with 2 x lysis buffer and were boiled. Proteins were separated by SDS-PAGE and western blotting was performed using the indicated antibodies. β-actin was used as a loading control. These blots are representative of 2 independent experiments.
Supplemental Fig. 3. Blood concentrations of IS001 after a single oral dose of IS001. IS001 was prepared as a 30 mg/kg suspension in 0.5% methylcellulose and was provided orally to two CD-1 mice. Blood was collected after 1, 2, 4, 6 and 24 h after gavage and the levels of IS001 were measured via mass spectrometry. The levels in the blood were below the level of detection (0.01 µM) by 24 h. Values are means + SEM of levels observed in the two mice.
Supplemental Table I: Primer sequences used for real-time RT-PCR and ChIP studies

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<th>Primer Names</th>
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<tr>
<td>IFNγ REV</td>
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The primer sequences used to amplify the *Ifng* promoter and CNS sites were provided by Dr. Robin Hatton and Dr. Casey Weaver (University of Alabama Birmingham).