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Peroxisome Proliferator-Activated Receptor γ Is Required for Regulatory CD4$^+$ T Cell-Mediated Protection against Colitis

Raquel Hontecillas[1] and Josep Bassaganya-Riera[1]

Peroxisome proliferator-activated receptor (PPAR) γ activation has been implicated in the prevention of immunoinflammatory disorders; however, the mechanisms of regulation of effector and regulatory CD4$^+$ T cell functions by endogenously activated PPAR-γ remain unclear. We have used PPAR-γ-deficient CD4$^+$ T cells obtained from tissue-specific PPAR-γ null mice (i.e., PPAR-γ fl/fl; MMTV-Cre$^+$) to investigate the role of endogenous PPAR-γ on regulatory T cell (Treg) and effector CD4$^+$ T cell function. Overall, we show that the loss of PPAR-γ results in enhanced Ag-specific proliferation and overproduction of IFN-γ in response to IL-12. These findings correlate in vivo with enhanced susceptibility of tissue-specific PPAR-γ null mice to trinitrobenzene sulfonic acid-induced colitis. Furthermore, the transfer of purified PPAR-γ null CD4$^+$ T cells into SCID recipients results in enteric disease. To test the assertion that the deficiency of PPAR-γ in Treg impairs their ability to prevent effector T cell-induced colitis, we performed cotransfer studies. These studies demonstrate that PPAR-γ-expressing, but not PPAR-γ null Treg, prevent colitis induced by transfer of naive CD4$^+$ T cells into SCID recipients. In line with these findings, the production of IFN-γ by spleen and mesenteric lymph node-derived CD4$^+$ T cells was down-regulated following transfer of PPAR-γ-expressing, but not PPAR-γ null, Treg. In conclusion, our data suggest that endogenous PPAR-γ activation represents a Treg intrinsic mechanism of down-regulation of effector CD4$^+$ T cell function and prevention of colitis. ***The Journal of Immunology,*** **2007, 178: 2940–2949.**

The inability of the immune system to down-regulate CD4$^+$ T cell responses leads to chronic autoimmune and inflammatory diseases such as multiple sclerosis, type 1 diabetes, rheumatoid arthritis, asthma or inflammatory bowel disease (IBD)[2] (1–4). Peroxisome proliferator-activated receptor γ (PPAR-γ) was originally identified as the molecular target for the thiazolidinedione (TZD) class of antidiabetic drugs and its potential for treating immunoinflammatory pathologies is currently under investigation (5). PPAR-γ activation by natural or synthetic agonists ameliorates experimental allergic encephalomyelitis, rheumatoid arthritis, experimental IBD, and eosinophilic airway inflammation (6–11). PPAR-γ is a nuclear hormone receptor activated by a diverse set of endogenous molecules, including lipids, their metabolites and derivatives, and drugs such as TZDs (12–14). Following ligand-induced activation, PPAR-γ heterodimerizes with the retinoid X receptor. This process is followed by dissociation from corepressors, association with coactivators, chromatin acetylation and remodeling, and binding to PPAR-γ-responsive elements in DNA promoter regions to enhance the transcription of target genes (15, 16). Through this pathway, PPAR-γ regulates lipid and glucose homeostasis (17). In contrast, PPAR-γ down-regulates the expression of proinflammatory cytokines by antagonizing the activities of transcription factors, such as AP-1, STAT, and NF-κB (18), and favoring the nucleocytoplasmic shuttling of the activated p65 subunit of NF-κB (19). For instance, TZDs have been proposed to elicit their anti-inflammatory effects by inducing the SUMOylation of PPAR-γ, which targets it to nuclear receptor corepressor histone deacetylase 3 complexes on inflammatory gene promoters such as AP-1 and NF-κB, resulting in a stable repressed state (20).

Although PPAR-γ involvement in the regulation of innate immune responses has been studied since the late 1990s (18, 21–23), only recently has the role of PPAR-γ in adaptive immunity been investigated. In vitro treatment of splenocytes or fractionated CD4$^+$ T cells with 15-deoxy-PGJ2 and TZD significantly reduces Ag and mitogen-induced proliferation (24, 25). PPAR-γ activation also regulates cytokine secretion by CD4$^+$ T cells (26). More specifically, PPAR-γ activation by rosiglitazone decreased IFN-γ production by splenocytes in vitro stimulated with PMA and ionomycin (27). Treatment of CD4$^+$ T cells with ciglitazone or 15-deoxy-PGJ2 triggered the physical association between PPAR-γ and NFATc1, resulting in IL-4 promoter inhibition and decreased IL-4 production (28), and 13-hydroxyoctadecadienoic acid (13-HODE) a putative endogenously generated PPAR-γ agonist, down-regulated IL-2 production by human peripheral blood T lymphocytes by reducing NFAT and NF-κB binding to the IL-2 promoter (29). IL-4 was shown to simultaneously increase the expression of PPAR-γ and 12,15-lipooxygenase, the enzyme involved in the generation of 13-HODE (30). Thus, it has been proposed that IL-4 indirectly down-regulates IL-2 production by T cells through PPAR-γ (29, 30). Overall, these results demonstrate that PPAR-γ is a negative regulator of adaptive immune responses. However, many of the effects attributed to PPAR-γ activation are only achieved at concentrations of synthetic agonists several orders of magnitude higher than those needed for receptor binding, suggesting that they occur through a PPAR-γ-independent mechanism (31). In addition, although initially considered an endogenous PPAR-γ ligand, many effects triggered by 15-dPGJ2 have later been shown to be PPAR-γ independent (31–33). Thus, additional studies are needed

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[2] Abbreviations used in this paper: IBD, inflammatory bowel disease; PPAR-γ, peroxisome proliferator-activated receptor γ; TZD, thiazolidinedione; Treg, regulatory T cell; TNBS, trinitrobenzene sulfonic acid; MLN, mesenteric lymph node; LPL, lamina propria leukocytes; DSS, dextran sodium sulfate; Teff, effector T cells.

to fully understand the intrinsic regulation of $CD4^+$ T cell function by PPAR-γ in a physiologically relevant setting in which the receptor is activated by naturally occurring endogenous ligands. Of note, *PPAR-*$\gamma^{+/-}$ mice have increased susceptibility to experimental allergic encephalomyelitis and dextran sodium sulfate (DSS) induced colitis (9, 34), which indicates that endogenous PPAR-γ agonists generated during inflammatory responses limit inflammation and disease severity.

Because PPAR-γ activation elicits anti-inflammatory actions, most efforts have been directed toward understanding how PPAR-γ suppresses effector $CD4^+$ T cell function. Although regulatory cells are crucial in maintaining peripheral tolerance through bystander suppression and infectious tolerance, and are actively involved in the contraction phase of the immune response, little is known about the role of Treg PPAR-γ in autoimmune disease prevention. Interestingly, many inflammatory and autoimmune conditions that could potentially be treated with PPAR-γ agonists arise from defects in the Treg compartment (4, 35). Four classes of $CD4^+$ Treg cells have been described (reviewed in Ref. 36). Natural Treg, which are thymus derived, constitute 90% of the peripheral $CD4^+CD25^+$ T cell subset, express in addition Foxp3, CTLA-4, and glucocorticoid-induced TNFR family-related protein, and suppress effector functions through a cell contact-dependent fashion. Induced Treg are more functionally and phenotypically heterogeneous in comparison to natural Treg and can be subdivided into: 1) induced $Foxp3^+$ Tregs, 2) Th3, and 3) Tr1. Induced $Foxp3^+$ Tregs can arise de novo from $CD4^+CD25^-Foxp3^-$ precursors following induction by TGF-β1 and are functionally characterized by producing TGF-β1. The Th3 regulatory cells also produce TGF-β1 and are involved in oral tolerance. Tr1 cells are IL-10-secreting Tregs, which are also induced by IL-10 in the course of chronic infections and are thought to be important in limiting tissue damage (36). To elucidate the role of PPAR-γ as a regulator of effector and regulatory $CD4^+$ T cell function, we used a loss-of-function approach whereby PPAR-γ was deleted in immune cells, including $CD4^+$ T cell subsets. We show that IFN-γ is markedly up-regulated in $CD4^+$ T cells lacking PPAR-γ and exposed to Th1-polarizing conditions. We also document that PPAR-γ down-regulates effector $CD4^+$ T cell Ag-specific proliferation. These in vitro findings correlated with increased susceptibility to trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice lacking PPAR-γ in immune and epithelial cells. To further dissect the role of PPAR-γ in effector $CD4^+$ T cell and Treg function, we cotransferred effector and regulatory $CD4^+$ T cells to SCID mice, and show for the fist time that the loss of PPAR-γ in Treg impairs their ability to control effector $CD4^+$ T cell responses.

Materials and Methods

Mice

Tissue-specific PPAR-γ fl/fl; $MMTV\text{-}Cre^+$ (i.e., hemopoietic and epithelial cell-deficient) PPAR-γ null mice and PPAR-γ fl/fl; $MMTV\text{-}Cre^-$ littermates in a C57BL6/J background were generated by using the Cre-lox recombination system as previously described (37, 38). The mice were maintained in the animal facilities at Virginia Polytechnic Institute and State University and used at 6–8 wk. B6.CB17-*Prkdc*^{scid}/SzJ (SCID) were purchased from The Jackson Laboratory and housed under specific pathogen-free conditions in ventilated racks. All experimental protocols were approved by the institutional animal care and use committee at Virginia Polytechnic and met or exceeded guidelines of the National Institutes of Health Office of Laboratory Animal Welfare and Public Health Service policy.

Reagents

Media used for cell culture was RPMI 1640 supplemented with 10% FBS (HyClone), 25 mM HEPES buffer (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich), 0.1 mg/ml streptomycin (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 1 mM nonessential amino acids (Sigma-Aldrich), Con A (Sigma-Aldrich), 2 mM essential amino acids (Mediatech), and 2-ME. mAbs used for FACS analysis and cell culture assays were CD4 (clone L3T4), CD25 (clone PC61), CD45RB (clone 16A) IFN-γ (clone XMG1.2), CD3 (clone 17A2), and CD28 (clone 37.51), all from BD Pharmingen, and anti-mouse Foxp3 (clone FJK-16) from eBioscience. Anti-mouse IL-4-neutralizing Abs were obtained from R&D Systems. Recombinant mouse IL-2 and IL-12 were from PeproTech. OVA, IFA and TNBS, and type VIII collagenase were obtained from Sigma-Aldrich.

Immunization and evaluation of Ag-specific responses

PPAR-γ fl/fl; $MMTV\text{-}Cre^+$ and PPAR-γ fl/fl; $MMTV\text{-}Cre^-$ mice were immunized i.m. with 50 μg of OVA in IFA. Ag-specific responses to OVA were evaluated ex vivo 7 days postimmunization. Briefly, splenocytes were stimulated in 96-well round-bottom plates with increasing amounts of OVA and proliferation was measured on day 5. Cultures were pulsed for the last 20 h with 0.5 μCi of [^{3}H]thymidine.

$CD4^+$ T cell subset sorting

Splenocytes obtained from PPAR-γ fl/fl; $MMTV\text{-}Cre^+$ (i.e., PPAR-γ null) and PPAR-γ fl/fl; $MMTV\text{-}Cre^-$ (i.e., PPAR-γ floxed) littermate mice were enriched in $CD4^+$ T cells by magnetic negative sorting using the I-Mag cell separation system (BD Pharmingen). Briefly, cells were incubated with a mixture of biotinylated Abs followed by a second incubation with streptavidin particles and exposed to a magnet to remove unwanted cells. The purity of the $CD4^+$-enriched cell suspension was between 93 and 96%. CD4-enriched cells were used in in vitro assays, for adoptive transfer, or further purified by FACS. For FACS sorting, cells were labeled with CD45RB, CD4, and CD25 and separated into $CD4^+CD45RB^{high}CD25^-$ cells (i.e., effector T cells) and $CD4^+CD45RB^{low}CD25^+$ (i.e., Treg) in a FACSAria cell sorter (BD Biosciences). The purity of the FACS-sorted $CD4^+$ subsets was $\geq$98%.

$CD4^+$ T cell adoptive transfer

Six-week-old SCID mice were transferred with 5×10^5 unfractionated purified $CD4^+$ cells or with 4×10^5 $CD4^+CD45RB^{high}CD25^-$ from PPAR-γ fl/fl; $MMTV\text{-}Cre^+$ and PPAR-γ fl/fl; $MMTV\text{-}Cre^-$ mice. In cotransfer experiments, SCID mice received a mixture of 4×10^5 $CD4^+CD45RB^{high}CD25^-$ cells and 10^5 $CD4^+CD45RB^{low}CD25^+$ of all possible combinations of null and floxed naive and Treg (i.e., effector T cells (Teff))/Treg; null/null, null/floxed, floxed/floxed, and floxed/null). Mice were weighed on a weekly basis and clinical signs of disease were recorded daily for 8 wk. Mice that developed severe signs of wasting disease (weight loss $\geq$10% of their original weight) were sacrificed. Otherwise, mice were sacrificed 60 days after transfer.

Isolation of lymphocytes from colonic LP

Colons from SCID recipient mice were excised, placed in CMF (HBSS/10% FBS/0.251 EDTA)/HEPES (Mediatech), cleaned of contents, and sectioned into 5-mm pieces. To remove IEL, tissues were incubated for 45 min in 15 ml of CMF/EDTA. Medium was changed every 15 min. To release lamina propria lymphocytes (LPL), tissues were digested for 3 h with type VIII collagenase (Sigma-Aldrich) in HBSS. Medium was changed every hour and supernatants containing LPL were collected. After digestion, supernatants were pooled, spun at $400 \times g$. LPL were recovered from the interface of a 40–100% Percoll gradient.

$CD4^+$ T cell cultures and IFN-γ production

$CD4^+$ T cells purified by magnetic sorting were incubated for 4 days with plate-bound anti-mouse CD3 (2 μg/ml) and CD28 (1 μg/ml) and 50 U/ml rIL-2 (i.e., unskewed conditions) plus 10 ng/ml IL-12 and 2.5 mg/ml anti-mouse IL-4-neutralizing Ab (i.e., Th1-polarizing conditions). Fresh medium and cytokines were added on day 2. On day 4, cells were harvested, washed with complete RPMI 1640, enumerated, and stimulated in 96-well round-bottom plates coated with anti-mouse CD3 at 5 μg/ml for 16 h. Golgi Stop (BD Pharmingen) was added for the last 5 h. To measure IFN-γ production in the adoptive transfer experiments, splenocytes and mesenteric lymph node (MLN) cells obtained from SCID recipient mice were stimulated for 6 h with 5 μg/ml plate-bound anti-mouse CD3. Golgi Stop solution was added to cell cultures to prevent cytokine secretion.

For intracellular cytokine detection, cells were harvested and labeled with anti-mouse CD4-FITC, then simultaneously fixed and permeabilized with Cytofix-Cytoperm buffer (BD Pharmingen) and finally incubated with

PE-conjugated anti-mouse IFN-γ. Two-color FACS analysis was performed in a FACSCalibur cytometer (BD Biosciences), and data were analyzed with CellQuest Pro (BD Biosciences).

FACS analysis of IL-12Rβ2 molecule expression

IL-12Rβ2 expression on sorted $CD4^+$ T cells was detected by incubating cells with unlabeled hamster anti-mouse IL-2Rβ2 mAb or hamster IgG, followed by biotin-labeled mouse anti-hamster IgG, and streptavidin-PE-Cy5. Data acquisition was done on a FACSCalibur (BD Biosciences) and analyzed on CellQuest Pro.

FACS analysis of $Foxp3^+$ Treg

Splenocytes, MLN cells, or LPL from recipient mice were incubated with anti-mouse CD4 and anti-mouse CD25. Cells were then fixed and permeabilized for 20 min at 4°C and then incubated with anti-mouse Foxp3 (eBioscience) diluted in permeabilization buffer for 30 min. Ten thousand $CD4^+$ cells were analyzed by FACS.

TNBS-induced colitis

TNBS was administered per rectum to PPAR-γ fl/fl; MMTV-Cre^+ and PPAR-γ fl/fl; MMTV-Cre^- mice to induce a transmural, infiltrative colitis driven by a Th1-polarized immune response. This model facilitates the evaluation of early or initiating events associated with the development of IBD. The unchallenged (control) mice were treated with vehicle alone (50% ethanol in PBS). More specifically, to induce TNBS colitis, 2.0 mg of TNBS (pH 1.5–2.0; Sigma-Aldrich) in 50% ethanol was administered per rectum to lightly anesthetized mice (with isoflurane) with a 3.5-French polyethylene catheter inserted into the rectum. The catheter tip was 4 cm proximal to the anal verge, 100 μl of fluid (TNBS/ethanol or 50% ethanol alone) was slowly instilled into the colon, and the mouse was held in a vertical position for 1 min. All mice used in TNBS challenge studies were euthanized 72 h following TNBS administration. Mice were monitored daily for the development of clinical signs of disease and weighed on days 0 and 3. Colons were excised and fixed in 10% buffered formalin and later sectioned and stained with H&E. Microscopic lesions were graded from 1 to 4 depending on the severity of lymphocytic infiltration, erosion of the epithelium, and enlargement of the mucosa.

IFN-γ ELISA

IFN-γ concentrations were quantitated in $CD4^+$ T cell culture supernatants from cells cultured in unskewed and Th1-polarizing conditions using the mouse IFN-γ ELISA kit (BD Biosciences Pharmingen) according to the manufacturer's instructions.

Statistical analyses

Parametric data were analyzed using the ANOVA followed by Scheffe's multiple comparison method. Nonparametric data were analyzed by using the Mann-Whitney's *U* test followed by a Dunn's multiple comparisons test. ANOVA was performed by using the general linear model procedure of SAS, release 6.0.3 (SAS Institute). Statistical significance was assessed at a $p \leq 0.05$.

Results

The loss of PPAR-γ results in enhanced lymphocyte proliferation

PPAR-γ fl/fl; MMTV-Cre^+ mice lack PPAR-γ protein in epithelial and hemopoietic cells. The loss of PPAR-γ in hemopoietic cells does not affect the numbers of $CD4^+$ and $CD8^+$ T cells, B cells, or macrophages in spleen, MLN, or thymus of healthy mice (Ref. 38 and our unpublished data). To determine whether the loss of PPAR-γ affected the induction of Ag-specific immune responses, PPAR-γ fl/fl; MMTV-Cre^+ mice (i.e., tissue-specific PPAR-γ null) and PPAR-γ fl/fl; MMTV-Cre^- littermates were immunized with OVA, and Ag-specific proliferation was evaluated ex vivo 7 days postimmunization. As shown in Fig. 1, proliferation of PPAR-γ null splenocytes was significantly higher than that of PPAR-γ-floxed littermates for all concentrations of OVA tested ex vivo. In addition, proliferation to Con A was significantly higher ($p \leq 0.05$) in splenocytes from PPAR-γ fl/fl; MMTV-Cre^+

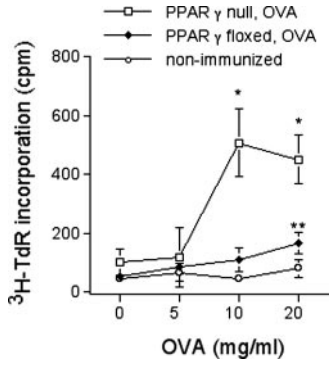

FIGURE 1. The loss of PPAR γ enhances Ag-specific proliferation. Tissue-specific PPAR-γ null mice (PPAR-γ fl/fl; MMTV-Cre^+) lacking PPAR-γ in epithelial and hemopoietic cells, and PPAR-γ-floxed (PPAR-γ fl/fl; MMTV-Cre^-) littermates were immunized i.m. with 50 μg/ml OVA in IFA. Splenocytes were isolated 7 days postimmunization and stimulated ex vivo with increasing concentrations of OVA for 5 days. [^{3}H]Thymidine was added for the last 16 h. Data are presented as means ± SE. (* or **, $p \leq 0.05$) of an experiment with five mice per group.

($65.7 \times 10^3 \pm 19.8 \times 10^3$ cpm vs $18.8 \times 10^3 \pm 2.4 \times 10^3$ cpm). These data suggest that, although not required for lymphocyte development or maintenance in the periphery, endogenous PPAR-γ activation limits lymphocyte proliferation.

PPAR-γ null $CD4^+$ T cells overexpress IFN-γ in response to IL-12

Previous experiments have shown that transfection of Jurkat T cells with a PPAR-γ expression plasmid suppressed the IFN-γ promoter (27). We tested whether the loss of PPAR-γ would enhance IFN-γ production by $CD4^+$ T cells exposed to Th1-polarizing conditions. Purified $CD4^+$ T cells isolated from spleens of PPAR-γ fl/fl; MMTV-Cre^+ or PPAR-γ fl/fl; MMTV-Cre^- littermate mice were cultured under unskewed (Fig. 2, *A* and *D*) or Th1-polarizing conditions (i.e., IL-12 and anti-IL-4-neutralizing Abs) (Fig. 2, *B* and *E*) for 4 days. Cells were then harvested and restimulated with anti-CD3 for 16 h to evaluate IFN-γ production.

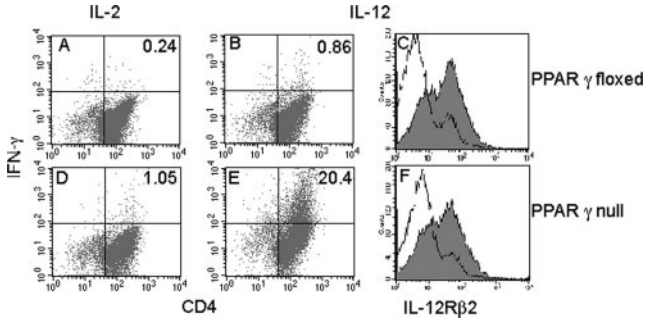

FIGURE 2. PPAR-γ regulates the expression of IFN-γ without changing the levels of IL-12Rβ2 in $CD4^+$ T cells under Th1-polarizing conditions. $CD4^+$ T cells purified from spleens of PPAR-γ fl/fl; MMTV-Crei (PPAR-γ floxed, *top panel*) and PPAR-γ fl/fl/; MMTV-Cre^+ mice (PPAR-γ null, *bottom panel*) were cultured in RPMI 1640 supplemented with 50 U/ml IL-2 alone (*A* and *D*) or with 10 ng/ml IL-12 and 2.5 μg/ml neutralizing IL-4 Abs (*B* and *E*) in plates coated with anti-mouse CD3 (2 μg/ml) and CD28 (1 μg/ml). On day 4, cells were harvested, washed, and restimulated with plate-bound anti-CD3 (5 μg/ml) overnight. The expression of IFN-γ was severe colitis (*D*). Transference of unfractionated floxed measured by intracellular flow cytometry. IL-12Rβ2 was measured on day 4 on cells stimulated in the conditions described above (*C* and *F*). Plots are representative of three separate experiments with similar results.

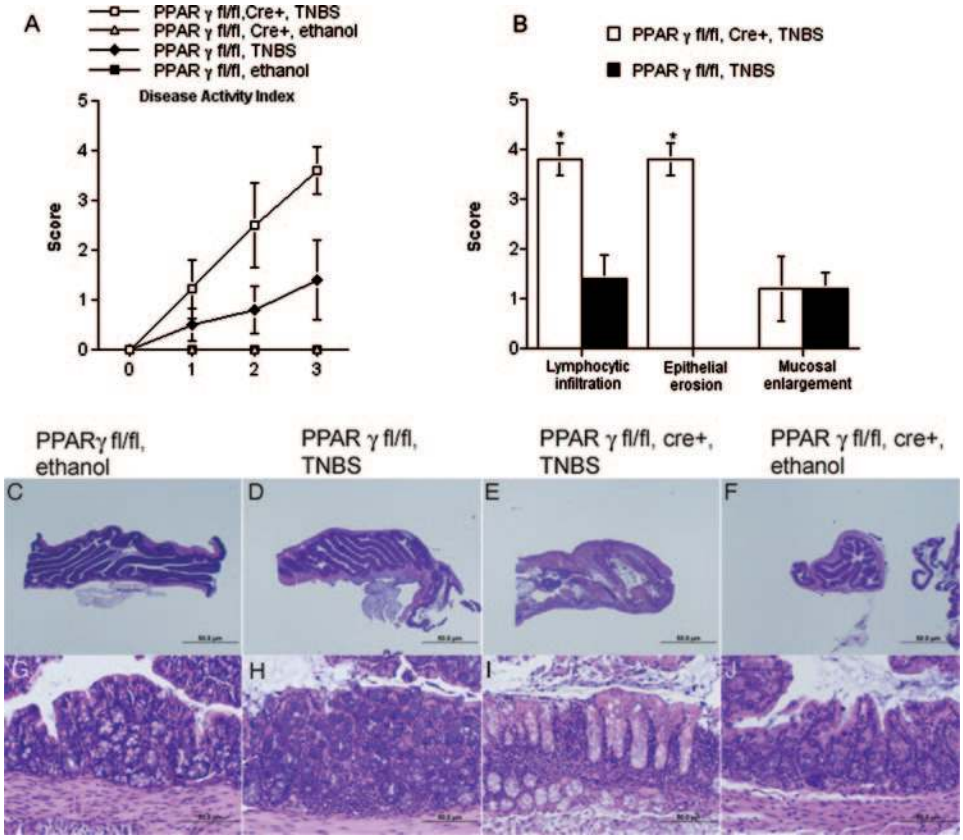

FIGURE 3. Mice lacking PPAR-γ in colonic immune and epithelial cells develop more severe colitis. PPAR-γ fl/fl; MMTV-Cre$^+$ and PPAR-γ fl/fl; MMTV-Cre$^-$ mice were challenged intrarectally with TNBS in ethanol or vehicle alone. A disease activity index was calculated based on daily clinical scores (*A*). Mice were sacrificed 3 days after TNBS challenge. Lymphocytic infiltration, epithelial erosion, and mucosal enlargement were scored in H&E-stained colonic sections. Histological scores shown are means ± SE from an experiment with eight mice per group (*B*). Representative micrographs of colonic longitudinal (*C–F*) and cross-sections (*G–J*) on day 3 after TNBS challenge.

The results of intracellular cytokine staining show an increase in IFN-γ production resulting from the loss of PPAR-γ in CD4$^+$ T cells (Fig. 2*E*). These results were confirmed when the same experiment was repeated and IFN-γ protein secretion was measured by ELISA in cell supernatants (data not shown). The overexpression of IFN-γ in response to IL-12 stimulation was not due to differences in IL-12Rβ2 levels in PPAR-γ null vs floxed CD4$^+$ T cells (Fig. 2, *C* and *F*).

Loss of colonic PPAR-γ expression exacerbates TNBS-induced colitis

Excessive CD4$^+$ T cell activation can lead to chronic inflammatory diseases such as IBD. Previously, we have shown that conjugated linoleic acid, a polyunsaturated fatty acid, protects mice from DSS colitis through a mechanism dependent on the expression and activation of PPAR-γ in epithelial and immune cells. In addition, PPAR-γ mRNA expression was completely shut down in colons with severe inflammation (37). The intrarectal administration of TNBS represents a well-established model of CD4$^+$ T cell-induced colitis characterized by a predominant Th1 response induced by the excessive production of IL-12 by APC (39). PPAR-γ fl/fl; MMTV-Cre$^+$ and PPAR-γ fl/fl; MMTV-Cre$^-$ mice were challenged intrarectally with TNBS or vehicle (i.e., ethanol/PBS) alone, and clinical signs of disease were monitored for 3 days. We found that TNBS-treated tissue-specific PPAR-γ null mice developed more severe disease characterized by lethargy, signs of abdominal pain, and,

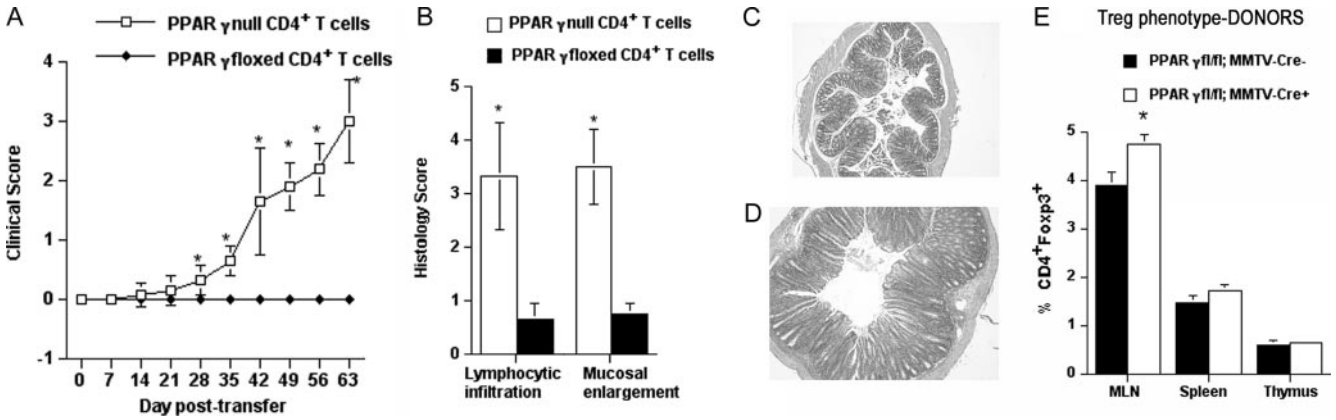

FIGURE 4. SCID mice transferred with PPAR-γ null CD4$^+$ T cells develop colitis. SCID mice received 5 × 10^5 unfractionated CD4$^+$ T cells obtained from spleens of PPAR-γ fl/fl; MMTV-Cre$^+$ or PPAR-γ fl/fl; MMTV-Cre$^-$ mice. Recipients of PPAR-γ null cells developed clinical signs (*A*) and had higher histological scores (*B*) consistent with the development of CD4$^+$ T cells did not induce colitis (*C*), whereas the recipients of PPAR-γ null CD4$^+$ T cells developed colitis characterized by thickening of the mucosa with lymphocytic infiltration and crypt hyperplasia (*D*). Average scores are presented as means ± SE (●, $p \leq 0.05$) of an experiment with 10 mice per group. *E*, Percentage of CD4$^+$ Foxp3$^+$ T cells in MLN, spleen, and thymus of donor mice.

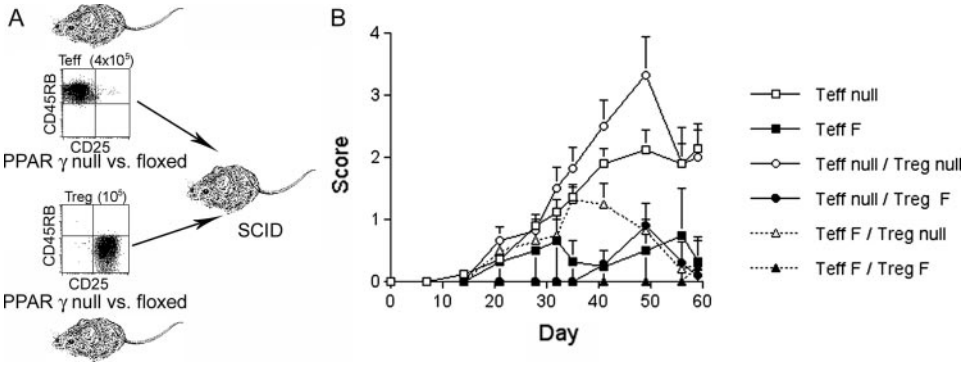

FIGURE 5. $CD4^+CD45RB^{high}CD25^-$ (effector) and $CD4^+CD45RB^{low}CD25^+$ (Treg) cells were obtained from spleens of PPAR-γ fl/fl; MMTV-Cre^- (floxed) and PPAR-γ fl/fl; MMTV-Cre^+ (null) mice and used in cotransfer experiments. Schematic representation of the experiment in which SCID mice received 4×10^5 effector cells from either genotype alone or in combination with null or floxed 10^5 Treg (*A*). A disease activity index score of clinical signs of colitis and wasting disease was recorded on a weekly basis (*B*). Mice that received null effector cells alone or with null Treg had the highest scores, whereas the transfer of floxed Treg cells prevented clinical disease. Results are presented as mean ± SE of groups of at least seven mice.

in some cases, rectal bleeding than PPAR-γ-floxed mice (Fig. 3*A*). Histological examination of H&E-stained colonic specimens showed more severe lymphocytic infiltration of the lamina propria, epithelial erosion, and necrosis of the epithelium in PPAR-γ null mice (Fig. 3, *B* and *I*). These data are in line with our in vitro findings and indicate that the loss of PPAR-γ in immune cells results in accelerated onset and exacerbation of immunoinflammatory pathologies.

$CD4^+$ T cells lacking PPAR-γ exhibit a colitogenic phenotype

Because PPAR-γ fl/fl; MMTV-Cre^+ mice lack PPAR-γ protein expression in hemopoietic and epithelial cells, our previous findings on the increased severity of TNBS-induced colitis in PPAR-γ fl/fl; MMTV-Cre^+ mice were inconclusive regarding the contribution of PPAR-γ expressed in $CD4^+$ T cells, a cell type which abundantly infiltrates the intestine of IBD patients,

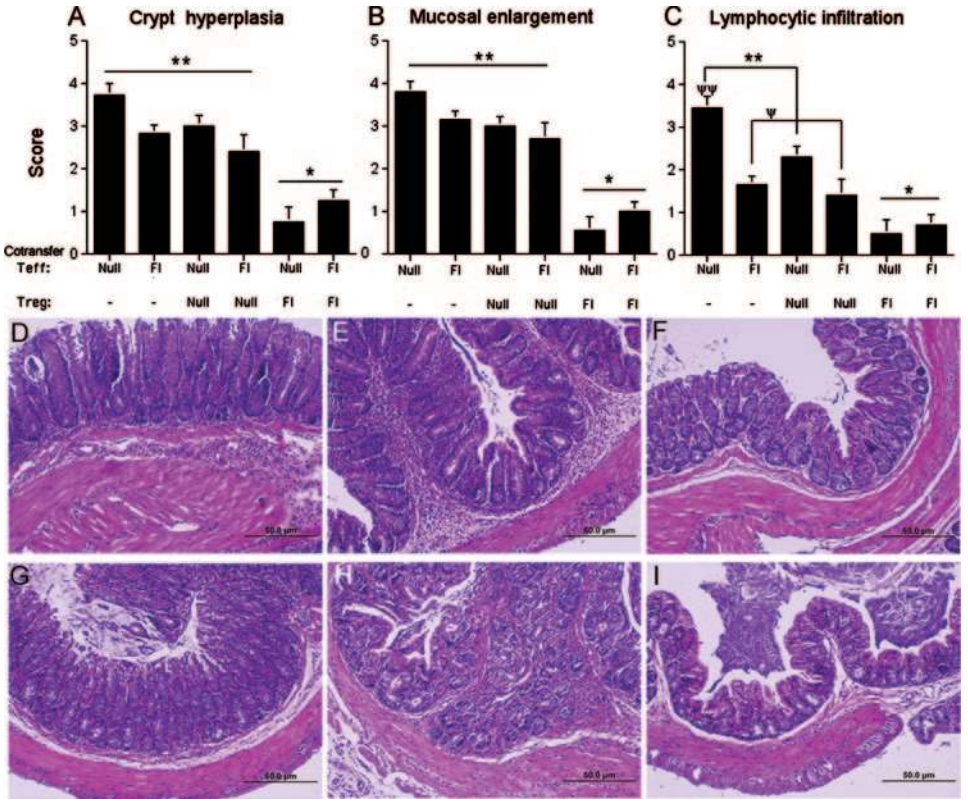

FIGURE 6. Histopathological assessment of H&E-stained colonic sections collected from SCID mice 8 wk after transfer of 4×10^5 PPAR-γ null or floxed $CD4^+CD45RB^{high}CD25^-$ effector cells alone or in combination with either 10^5 PPAR-γ-floxed or null Treg. Recipients of PPAR-γ null (*D*) or floxed (*G*) effector cells alone developed severe colitis characterized by mucosal enlargement, hyperplasia of the epithelium and lymphocytic infiltration of the mucosa and submucosa. Lesions were not prevented by the cotransfer of null Treg along with effector T cells of ether genotype (*E* and *H*), whereas floxed Treg were able to prevent colitis (*F* and *G*). Average scores for crypt hyperplasia (*A*), mucosal enlargement (*B*), and lymphocytic infiltration (*C*) for each recipient group. Cotransfer of floxed Treg cells prevented crypt hyperplasia and mucosal enlargement induced by effector cells of either genotype (∗ and ∗∗ were significantly different ($p \leq 0.05$); ∗ vs ∗∗, $p \leq 0.05$). Mice that received PPAR-γ-floxed effector cells alone or with Treg of either genotype had lower scores of lymphocytic infiltration than mice that received PPAR-γ null effector cells (∗ vs ∗∗, $p \leq 0.05$ and ψ vs ψψ, $p \leq 0.05$).

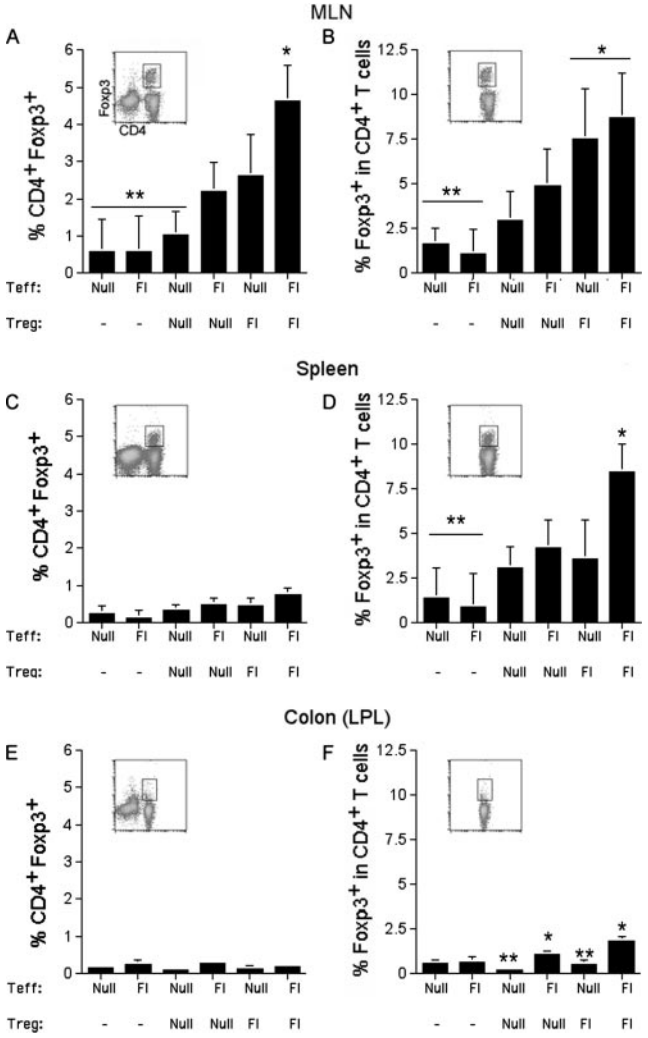

FIGURE 7. SCID mice received 4×10^5 PPAR-γ null or floxed $CD4^+CD45RB^{high}CD25^-$ effector cells alone or in combination with either 10^5 PPAR-γ-floxed or null Treg. Lymphocytes were isolated from MLN, spleen, or lamina propria 8 wk after transfer and the presence of $Foxp3^+CD4^+$ Treg cells was assessed by flow cytometry. The percentage of Treg was higher in mice cotransferred with floxed effector and Treg cells in MLN, spleen, and lamina propria. The percentage of $Foxp3^+CD4^+$ cells was significantly higher (∗ vs ∗∗, $p \leq 0.05$) in MLN and spleen of mice that received floxed cells compared with mice receiving effector cells alone. In the lamina propria, the percentage of $Foxp3^+$ within $CD4^+$ T cells was significantly higher (∗ vs ∗∗, $p \leq 0.05$) in mice that were injected with PPAR-γ-floxed effector cells. Gating used in the flow cytometric analysis is show in the dot plot inserts for each tissue. Results are presented as means ± SE of groups of 6–10 mice.

to the down-regulation of experimental IBD. To investigate whether $CD4^+$ T cell PPAR-γ was important for IBD protection in vivo, we conducted an adoptive transfer experiment in which immunodeficient SCID mice were reconstituted with unfractionated PPAR-γ null or PPAR-γ-floxed $CD4^+$ T cells. Although the transfer of naive $CD4^+CD45RB^{high}$ T cells into immunologically naive recipients results in intestinal immunopathology, it is well established that the transfer of unfractionated $CD4^+$ T cells containing Treg ($CD4^+CD25^+$ T cells) does not cause disease (40). Hence, when both naive T cells and Treg are transferred together, the immunoregulatory actions of Treg protect against excessive Th1 polarization, IFN-γ overproduction, and effector T cell-induced colitis. However, we found that the transfer of PPAR-γ null $CD4^+$ T cells induced detectable clinical disease in SCID recipients by 4 wk following adoptive transfer, which became significantly more severe throughout the course of the study (Fig. 4*A*). Histological examination demonstrated that colons recovered from recipients of PPAR-γ null $CD4^+$ T cells had a significantly greater lymphocytic infiltration and crypt hyperplasia than those recovered from recipients of PPAR-γ-floxed $CD4^+$ T cells (Fig. 4, *B–D*). Our finding represents the first in vivo evidence suggesting a defective regulation of the $CD4^+$ T cell compartment in the absence of PPAR-γ. Although PPAR-γ fl/fl; MMTV-Cre^+ mice have similar percentages of $CD4^+$ T cells, a possible explanation for these results is the presence of lower $CD4^+Foxp3^+$ Treg cells within the $CD4^+$ subset. We next measured the percentage of Treg cells in spleen, thymus, and MLN of PPAR-γ fl/fl; MMTV-Cre^+ and PPAR-γ fl/fl; MMTV-Cre^- littermate mice. No differences were found in spleen and thymus, although the percentage of Treg was slightly but significantly higher in the MLN of PPAR-γ fl/fl; MMTV-Cre^+ mice (Fig. 4*E*).

To determine whether the colitogenic phenotype of PPAR-γ null $CD4^+$ T cells was due to alterations of Treg functions or to intrinsic defects in effector $CD4^+$ T cells, we next conducted a series of experiments in which SCID mice received PPAR-γ null or floxed $CD4^+CD45RB^{high}CD25^-$ T cells (i.e., effector cells) alone or in combination with $CD4^+CD45RB^{low}CD25^+$ Treg cells of either genotype (Fig. 5*A*). Following adoptive transfer, we monitored the appearance of clinical signs associated with colitis or wasting disease. Mice began to show signs of disease as soon as 3 wk posttransfer (Fig. 5*B*). The recipients of PPAR-γ null effector $CD4^+$ T cells alone presented more severe disease activity indices than the recipients of PPAR-γ-floxed effector cells alone. Moreover, although cotransfer of PPAR-γ-floxed Treg in combination with PPAR-γ null effector cells significantly delayed the development of clinical signs, no improvement on overall disease activity index scores was observed in the group of mice that simultaneously received PPAR-γ null Treg and PPAR-γ null effector cells. As anticipated, PPAR-γ-floxed Treg cells completely prevented disease in mice receiving PPAR-γ-floxed effector cells. Although the cotransfer of PPAR-γ null effector and PPAR-γ-floxed Treg had no effect on the initial phases of the disease, from day 30 onward the presence of PPAR-γ-expressing cells was associated with recovery as determined by the decrease in average clinical scores. These data indicated that the loss of PPAR-γ suppresses Treg and enhances effector $CD4^+$ T cell functions.

Because in some instances clinical disease associated with $CD4^+$ T cell adoptive transfer does not correlate with colitis severity (41), we also performed a histological examination of colonic specimens of recipient mice on day 60 after transfer. Overall, microscopic lesions corresponded with $CD4^+$ T cell-induced colitis. Mononuclear cell infiltrates in the mucosa and submucosa, mucosal enlargement, excessive crypt epithelial cell proliferation, and globlet cell depletion were found in the most severely affected mice. On average, the group of mice that received PPAR-γ null effector T cells had the highest scores for crypt hyperplasia and mucosal enlargement (Fig. 6, *A* and *B*). The cotransfer of PPAR-γ null Treg in combination with either PPAR-γ null or floxed effector T cells failed to prevent the development of crypt hyperplasia and enlargement of the colonic mucosa (Fig. 6, *A* and *B*). In contrast, the cotransfer of PPAR-γ-floxed Treg significantly improved microscopic inflammatory lesions when compared with the other groups (Fig. 6, *A–C*), suggesting that expression of PPAR-γ by Treg is required for optimal anti-inflammatory efficacy. The analysis of the amount of lymphocytes present in the lamina propria showed a significantly higher degree of infiltration by PPAR-γ null effector T cells when compared with PPAR-γ-floxed cells. PPAR-γ null Treg did not change the extension of lymphocytic

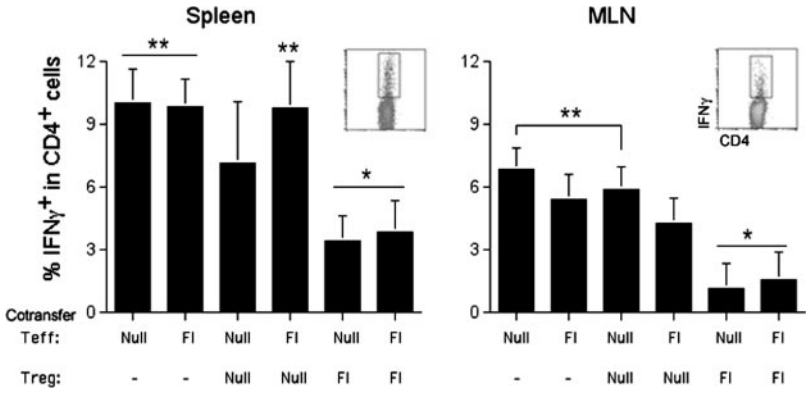

FIGURE 8. Lymphocytes were isolated from spleen and MLN of SCID mice transferred with 4×10^5 PPAR-γ null or floxed $CD4^+CD45RB^{high}CD25^-$ effector cells alone or in combination with either 10^5 PPAR-γ-floxed or null Treg. Cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and intracellular IFN-γ in $CD4^+$ T cells was measured by flow cytometry. Recipient mice of PPAR-γ-floxed Treg had the lowest percentage of IFN-γ^+CD4^+ T cells in MLN and spleen (* vs **, $p \leq 0.05$). Gating used in the flow cytometric analysis is show in the dot plot inserts for each tissue. Results are presented as means ± SE of groups of 6–10 mice.

infiltration when administered in combination with either PPAR-γ null or floxed effector cells (Fig. 6*C*). Compared with mice that received effector T cells on their own, the cotransfer of PPAR-γ-floxed Treg was associated with a significantly lower degree of lymphocytic infiltration in the colonic lamina propria (Fig. 6*C*).

The loss of PPAR-γ limits the expansion of Foxp3$^+$ Treg cells systemically and at the colonic mucosal level

Because the inability of PPAR-γ null Treg to prevent colonic lesion development could have been due to alterations in their expansion and/or tissue-homing properties, we examined the presence of Treg cells in spleen, MLN, and colonic lamina propria of SCID recipient mice. Whereas CD25 is a good marker for the Treg phenotype in healthy naive mice, in the course of an inflammatory response, some $CD4^+CD25^+$ will be activated effector T cells (36). Thus, we used intracellular Foxp3 expression by $CD4^+$ T cells as a marker of Treg. At week 8 posttransfer, $Foxp3^+$ cells were found in all three compartments. Overall, the MLN had the highest ratio of $Foxp3^+$ cells followed by spleen and lamina propria (Fig. 7, *A*, *C*, and *E*), although similar ratios between spleen and MLN were found when the analysis was done gating on $CD4^+$ T cells only (Fig. 7, *B*, *D*, and *F*). The level of Foxp3 expression, as assessed by mean florescence intensity, was higher in Treg cells present in the MLN irrespective of the genotype (data not shown). Small but detectable numbers of $Foxp3^+$ cells were found in some of the mice that did not receive $CD4^+CD45RB^{low}CD25^+$ Treg cells, consistent with de novo induction of Treg cells from naive $CD4^+$ T cell precursors. The highest percentage of $Foxp3^+$ cells was found in the MLN, spleen, and lamina propria of mice that received PPAR-γ-floxed Treg and effector cells (Fig. 7, *A–D*), suggesting that indeed the lack of PPAR-γ negatively impaired the expansion of Treg. However, the cotransfer of PPAR-γ-floxed Treg and PPAR-γ null Teff slightly limited the expansion of $Foxp3^+$ cells in the spleen and lamina propria, which suggests the existence of a possible interaction between effector and regulatory cells.

PPAR-γ-expressing Treg effectively reduce IFN-γ-producing CD4$^+$ T cells

Treg cells contribute to immune homeostasis and maintenance of peripheral tolerance by preventing the initiation or down-regulating of ongoing $CD4^+$ T cell effector responses. In IBD and autoimmune diseases, the disruption of the regulatory-effector axis results in excessive induction of IFN-γ-secreting $CD4^+$ T cells. To test whether the loss of PPAR-γ in Treg cells had any impact on IFN-γ production in vivo, cytokine production was assessed by intracellular flow cytometry in spleen and MLN cells isolated from SCID recipients following a 6-h stimulation period with anti-CD3 (Fig. 8). We found a significant reduction of IFN-γ production by $CD4^+$ T cells in both MLN and spleen of mice that received PPAR-γ-floxed Treg, irrespective of the genotype of effector T cells. In contrast, no differences in IFN-γ production were found between mice that received PPAR-γ null Treg and recipients of effector T cells of either genotype alone.

Discussion

The high levels of expression of PPAR-γ in immune cells and secondary lymphoid organs suggest an important role for PPAR-γ in regulating the induction and execution of immune responses. Consistent with this theory, whole-body PPAR-$\gamma^{+/-}$ mice and immune cell-specific PPAR-γ null mice are more susceptible to autoimmune and intestinal inflammatory diseases (34, 37, 42). Furthermore, PPAR-γ activation by synthetic or natural agonists in an inflammatory setting limits the extent of tissue injury (10, 11). Epithelial cells, macrophages, effector $CD4^+$ T cells, and Treg play an important role in the immunopathogenesis of IBD (39). Expression of PPAR-γ by macrophages (43) and epithelial cells (44) has already been shown to be required for protection against DSS colitis. However, the preponderant theory is that dysregulated $CD4^+$ T cell responses are central determinants of IBD pathogenesis. In support of this theory, Th1 polarization is enhanced in the colonic mucosa of patients with CD (45, 46). Furthermore, $CD4^+$ T cells make up the main lymphocyte population that infiltrate mucosal tissues in all models of IBD so far studied, and in instances in which effector T cells are deleted in vivo, inflammation can be ameliorated (39). Our findings demonstrate that PPAR-γ is required for down-modulating $CD4^+$ effector and enhancing Treg function in a mouse model of intestinal inflammation.

We show that the loss of PPAR-γ enhances Ag-specific lymphocyte proliferation. PPAR-γ protein expression increases as T cells transit from resting to activated state. Initial studies demonstrated that PPAR-γ activation by synthetic ligands induces lymphocyte apoptosis (25). However, apoptosis occurred at high doses of synthetic agonist, which are generally recognized to be cytotoxic and exert PPAR-γ-independent effects in immune cells (47). Thus, apoptosis could result from drug toxicity, rather than from an active process triggered by PPAR-γ itself. Our results are in line with previous observations by Clark et al. (24) demonstrating that the TZD ciglitazone inhibits the ability of T cell clones and freshly isolated splenocytes to proliferate. Similar findings were reported by Yang et al. (29), who demonstrated that PPAR-γ activation by the linoleic acid metabolite 13-HODE limited lymphocyte proliferation by blocking NFATc binding to the IL-2 promoter. Although these studies were conducted with unfractionated spleen cells, none of them considered a possible role for PPAR-γ in Treg

function. Our results, in addition, show that the loss of PPAR-γ in cells exposed to a Th1-inducing cytokine environment significantly increases the frequency of IFN-γ-producing $CD4^+$ T cells. The overproduction of IFN-γ by PPAR-γ null $CD4^+$ T cells could result from defects in intrinsic effector $CD4^+$ T cell down-modulatory mechanisms and/or defects in extrinsic regulatory functions elicited by Treg. Cunard et al. (27) reported that the reduction of IFN-γ secretion by T cells treated with TZD was due to the inhibition of the IFN-γ proximal promoter in Jurkat T cells transfected with a PPAR-γ plasmid. These findings support the concept of intrinsic regulation of IFN-γ production through PPAR-γ. In contrast, naturally occurring Treg suppressed IFN-γ production by responder cells in a model of autoimmune gastritis, which correlated with decreased expression of the Th1 differentiation transcription factor T-bet (48). However, the role of PPAR-γ as an internal enhancer of Treg function has not been investigated. Because we did not use any exogenous agonist, our results also indicate that endogenous ligands produced by $CD4^+$ T cells or PPAR-γ activators provided through tissue culture serum maintain this nuclear transcription factor in a constitutive activation state sufficient to suppress IFN-γ. This is in line with a report showing enhanced IFN-γ levels in splenocyte cultures treated with the PPAR-γ antagonist GW6992 (27).

We next used TNBS, a Th1-driven colitis model, to investigate whether our in vitro findings correlated in vivo with enhanced disease susceptibility. Previously, it had been reported that TZD ameliorate DSS and TNBS colitis and that *PPAR-*$\gamma^{+/-}$ mice are more susceptible to ischemia and reperfusion injury (11, 42, 49). We used PPAR-γ fl/fl; $MMTV\text{-}Cre^+$ mice, which lack PPAR-γ in epithelial and hemopoietic cells, and confirmed that the disease was exacerbated in mice lacking PPAR-γ compared with their PPAR-γ-expressing littermates. It has been shown that PPAR-γ ligands decrease IL-12 production (10), and the loss of PPAR-γ in bone marrow-derived dendritic cells enhances IL-12p40 mRNA expression in response to LPS (our unpublished data). Thus, the TNBS model did not allow us to rule out the possibility that increased levels of IL-12 or IL-23 production by PPAR-γ null APC could have also contributed to the disease outcome. We then used $CD4^+$ T cell adoptive transfer and Teff/Treg cotransfer models to better understand the role of PPAR-γ in regulating both Treg and effector $CD4^+$ T cell function. Naive $CD4^+$ T cells transferred to immunodeficient mice expand in MLN and spleen. Once activated, they acquire a Th1 phenotype and home into the colonic lamina propria where they undergo further divisions. However, when Treg are simultaneously transferred with naive $CD4^+$ T cells, the Th1 response is dampened and effector cells infiltrate the gut mucosa without causing disease (36). Moreover, others have established that Treg migration into the colonic lamina propria is not necessary for colitis prevention. Instead, Treg colonize MLN and spleen where they prevent Th1 differentiation (50). Our results are in consistency with Treg preferential migration and localization in the MLN and spleen, characteristic of preventive models of adoptive transfer colitis. Our data demonstrate that Treg from PPAR-γ-expressing mice expanded to a greater extent in vivo than PPAR-γ-deficient Treg, therefore, limiting the induction of IFN-γ-producing effector T cells.

There have been contradictory reports regarding the localization of PPAR-γ expression in the colon. Saez et al. (51) showed that PPAR-γ is expressed mainly in crypt epithelial cells, whereas Dubuquoy et al. (52) found that PPAR-γ protein was present in epithelial cells at the villous tip. The latter also described PPAR-γ presence in mononuclear cells of the lamina propria of healthy mice. Interestingly, PPAR-γ was not detected in the epithelium of germfree mice, whereas the expression of PPAR-γ in the lamina propria of these mice was intact, suggesting that bacterial colonization, without inflammation, does not down-regulate PPAR-γ in the lamina propria. In contrast, Katayama et al. (53) found that DSS colitis abrogated PPAR-γ expression in lamina propria mononuclear cells. PPAR-γ agonists are effective, preventive interventions against colitis although, due to inflammation-induced PPAR-γ gene suppression, they fail to treat established inflammation (54). In this regard, gene therapy using a replication-deficient adenovirus vector expressing PPAR-γ restored the PPAR-γ expression in the gut and the ability of PPAR-γ agonists to down-regulate colitis (53). We demonstrate for the first time that adoptive transfer of PPAR-γ-expressing, but not PPAR-γ null, Treg prevents disease development, which is indicative of the relevance of endogenous PPAR-γ activation in maintaining intestinal homeostasis. Powrie and colleagues (41) have shown that Treg were able to cure colitis induced by previously transferred naive $CD4^+$ T cells. It would be of particular interest to elucidate whether, in addition to preventing colitis, Treg PPAR-γ is also required for curing established IBD.

Of note, only PPAR-γ-expressing Treg were able to completely prevent inflammation induced by effector cells of either genotype. This suggests that PPAR-γ expression and/or activation by endogenous agonists is required for optimal Treg function and IBD protection. Wohlfert and Clark (55) demonstrated that PPAR-γ activation by synthetic ligands enhances the TGF-β1-dependent conversion of naive T cells into $Foxp3^+$-induced Treg in vitro. However, the mechanism by which PPAR-γ enhances Treg activity remains unknown. The transcription factor Foxp3 is required for natural and induced Treg development (56). TGF-β1 regulates Foxp3 expression in peripheral $CD4^+CD25^+$ natural Treg but it is not required for Treg generation in the thymus. For instance, *TGF-*$\beta1^{-/-}$ mice have reduced numbers of $CD4^+CD25^+$ splenocytes, which express lower levels of Foxp3 than wild-type littermates. Furthermore, adoptive transfer of *TGF-*$\beta1^{-/-}CD4^+CD25^+$ into $TGF^{+/+}$ recipient mice restores Foxp3 expression (57). Foxp3 is expressed at normal levels in PPAR-γ null $CD4^+$ T cells recovered from recipient mice. In addition, natural $CD4^+CD25^+$ Treg cells from PPAR-γ fl/fl; $MMTV\text{-}Cre^+$ mice express similar levels of Foxp3 than floxed littermates (our unpublished results). Moreover, a small subset of $Foxp3^+$ cells was detected in SCID mice that received effector T cells of either genotype alone, indicating that induction of Treg de novo from naive $CD4^+$ T cells was not affected by the loss of PPAR-γ. Collectively, these data indirectly suggest that TGF-β1 signaling is not altered in PPAR-γ-deficient Treg cells.

In summary, our findings are the first to demonstrate in vivo alterations in Treg function due to the deficiency of PPAR-γ. The relevance of these findings is 2-fold. First, the expression of PPAR-γ in the colonic mucosa is shut down during severe inflammatory responses in experimental models of colitis and in patients with ulcerative colitis (52). In addition, rare alleles of PPAR-γ have been found in patients with Crohn's disease (58). Thus, decreased levels of PPAR-γ could result in impaired Treg activity and consequently in the prolongation of the inflammatory response. Second, the use of Treg-based clinical therapeutic approaches to treat autoimmune and inflammatory conditions by ex vivo expansion and activation of Treg has been proposed. Specific ex vivo targeting of PPAR-γ in Treg could be used to enhance their function for effective treatment of autoimmune diseases while avoiding the negative side effects, i.e., congestive heart failure, hepatic steatosis, and weight gain associated with systemic administration of synthetic agonists.

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Disclosures

The authors have no financial conflict of interest.

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