FINAL REPORT

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Project Number: 13-005186

Project Title: Role of epithelial Toll Like Receptor dependent innate immune responses in the pathogenesis and treatment of PSC

A. SUMMARY OF THE PROJECT

A main pathogenetic mechanism able to explain PSC is still unidentified. The association of PSC with Inflammatory Bowel Disease (IBD) suggests the involvement of innate immune mechanisms. Unfortunately, the lack of experimental models has hampered this area of research. PSC has several common features with Cystic Fibrosis, a genetic disease caused by mutation in CFTR, a chloride channel involved in biliary secretory functions. We have recently shown that lack of CFTR is responsible for enhanced TLR4-dependent innate immune responses in the biliary epithelium of mice exposed to endotoxins, in the setting of experimental colitis. Moreover, cholangiocytes isolated from CFTR-KO mice exhibit a stronger TLR4/NF-κB-mediated inflammatory response to LPS. Similarly, we have preliminary evidence that cholangiocytes isolated from PSC patients and challenged with LPS, exhibit higher NF-κB transcriptional activity and IL-8 secretion, suggesting enhanced innate immune responsiveness to endotoxins. In addition, DCA-mediated bile acid toxicity causes more severe liver damage in CFTR-KO mice and induces the expression of High Mobility Group Box-1 (HMGB-1), a damage-associated molecule (DAMPs) released by dying cells and known to activate TLR4 and TLR2. Thus we hypothesize that a number of biliary damage conditions may be responsible for aberrant activation of the innate immunity in CF as well as in PSC. In this project, we plan to study the activation of innate immune pathways in response to endotoxins and bile acid toxicity in the CFTR-KO model and in human cholangiocytes PSC isolated from PSC patients. In addition, we will test if vitamin D nuclear receptors may represent a as novel therapeutic target in inflammatiory biliary diseases. These studies may lead to novel approaches for the treatment of sclerosing cholangitis.

B. STUDIES AND RESULTS FOR YEAR 2 OF THE PROJECT

During the second year of the project we have:

1. Completed the studies on the anti-inflammatory effect of PPAR-γ agonists. Our findings demonstrate that stimulation of PPARγ limits NF-κB-dependent inflammation in a CFTR-KO mouse model, an experimental model of sclerosing cholangitis. The following manuscript acknowledging PSC Partners Seeking a Cure was published and a copy is attached: *Scirpo R, Fiorotto R, Villani A, Amenduni M, Spirli C, Strazzabosco M. Stimulation of nuclear receptor peroxisome proliferator-activated receptor-γ limits NF-κB-dependent inflammation in mouse cystic fibrosis biliary epithelium. 2015. Hepatology 62: 1551-62.*

2. Generated a new in-vitro model of human biliary cells using the iPSC technology. Primary human cholangiocytes are difficult to isolate and in very short supply, as they need liver transplant samples. This model has several advantages compare to the primary human cholangiocytes isolated from patients. In fact, iPSCs can be derived from biopsy samples (i.e skin, blood) and they maintain a very high replicative potential that provides an unlimited source of patient-specific cells able to differentiate into the somatic cell of interest. As a proof of feasibility, we have successfully developed a method for the differentiation of human iPSC derived from healthy controls and from a CF patient carrying the ΔF508 mutation, into mature and functional cholangiocytes. Briefly, by recapitulating the different stages of liver development, we were able to differentiate our control and ΔF508 h-iPSCs into mature biliary epithelial cells (h-iPSC-BEC) and to culture them in polarized conditions. Therefore we performed an extensive characterization of these cells by gene expression analysis, immunocytochemistry, Western blot and functional assays (i.e TER measurement, CFL bile acid transport, cAMP production and GGT enzyme activity). Gene expression analysis showed the expression of biliary specification markers including CK19, CK7, HNF1b, SOX9, TGR5, CFTR (in both

control and ΔF508 - iPSC-BEC), aquaporin-1 and secretin receptor. By immunocytochemistry we confirmed the expression of CK19, CK7, HNF1b, SOX9, NOTCH2, ASBT and GGT. When cultured on transwell inserts coated with a thin layer of collagen, cholangiocytes formed an epithelial monolayer and developed epithelial apico/basal polarity as confirmed by the expression of primary cilia (α-tubulin) on the apical surface and the tight junction protein ZO-1 and the establishment of a TER (>1000 Ω/cm^2) (Millipore Co, MA). In addition, cholangiocytes on transwell inserts coated with a thicker layer of collagen self aggregated to form tubular structures with internal lumen and the ability to up-take the fluorescent bile acid CLF. h-iPSC-BEC cultured on transwell inserts and exposed to the hormone secretin on the basolateral side (where the secretin receptor is normally expressed) showed a raise of the cytoplasmic cAMP concentration thus confirming their functional maturity.

So far, we lack a valid experimental cellular model reproducing the human PSC disease and useable for investigating possible pathogenetic signaling pathways and/or testing novel therapeutic approaches. The method we have developed can be used for the generation of cholangiocytes from PSC patients therefore modeling the human PSC disease and useable for investigating possible pathogenetic signaling pathways and/or testing novel therapeutic approaches. These findings were presented as an oral communication at the EASL International Liver Congress 2016.

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Stimulation of Nuclear Receptor Peroxisome Proliferator–Activated Receptor-γ Limits NF-κB-Dependent Inflammation in Mouse Cystic Fibrosis Biliary Epithelium

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Cystic fibrosis–associated liver disease is a chronic cholangiopathy that negatively affects the quality of life of cystic fibrosis patients. In addition to reducing biliary chloride and bicarbonate secretion, up-regulation of toll-like receptor 4/nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB)–dependent immune mechanisms plays a major role in the pathogenesis of cystic fibrosis–associated liver disease and may represent a therapeutic target. Nuclear receptors are transcription factors that regulate several intracellular functions. Some nuclear receptors, including peroxisome proliferator–activated receptor-γ (PPAR-γ), may counterregulate inflammation in a tissue-specific manner. In this study, we explored the anti-inflammatory effect of PPAR-γ stimulation *in vivo* in cystic fibrosis transmembrane conductance regulator (Cftr) knockout mice exposed to dextran sodium sulfate and *in vitro* in primary cholangiocytes isolated from wild-type and from Cftr-knockout mice exposed to lipopolysaccharide. We found that in CFTR-defective biliary epithelium expression of PPAR-γ is increased but that this does not result in increased receptor activity because the availability of bioactive ligands is reduced. Exogenous administration of synthetic agonists of PPAR-γ (pioglitazone and rosiglitazone) up-regulates PPAR-γ-dependent genes, while inhibiting the activation of NF-κB and the secretion of proinflammatory cytokines (lipopolysaccharide-induced CXC chemokine, monocyte chemotactic protein-1, macrophage inflammatory protein-2, granulocyte colony-stimulating factor, keratinocyte chemoattractant) in response to lipopolysaccharide. PPAR-γ agonists modulate NF-κB-dependent inflammation by up-regulating nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha, a negative regulator of NF-κB. Stimulation of PPAR-γ *in vivo* (rosiglitazone) significantly attenuates biliary damage and inflammation in Cftr-knockout mice exposed to a dextran sodium sulfate–induced portal endotoxemia. *Conclusion*: These studies unravel a novel function of PPAR-γ in controlling biliary epithelium inflammation and suggest that impaired activation of PPAR-γ contributes to the chronic inflammatory state of CFTR-defective cholangiocytes. (HEPATOLOGY 2015;62:1551-1562)

Cystic fibrosis (CF) is an autosomal recessive disease that severely affects the secretory epithelia of several organs, including pancreas, lungs, liver, and gut. It is caused by mutations of the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic adenosine monophosphate–regulated chloride channel expressed at the apical membrane of most epithelial cells.[1,2] About 30% of CF

Abbreviations: AA, arachidonic acid; CF, cystic fibrosis; CFLD, cystic fibrosis–associated liver disease; CFTR, cystic fibrosis transmembrane conductance regulator; CK19, cytokeratin 19; DHA, docosahexaenoic acid; DSS, dextran sodium sulfate; FXR, farnesoid X receptor; G-CSF, granulocyte colony-stimulating factor; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha; KC, keratinocyte chemoattractant; KO, knockout; LA, linoleic acid; LIX, LPS-induced CXC chemokine; LPS, lipopolysaccharide; LXR, liver X receptor; MCP-1, monocyte chemotactic protein-1; MIP-2, macrophage inflammatory protein-2; NF-κB, nuclear factor kappa light chain enhancer of activated B cells; NR, nuclear receptor; PCR, polymerase chain reaction; PIO, pioglitazone; PPAR, peroxisome proliferator–activated receptor; ROSI, rosiglitazone; TLR, toll-like receptor; VDR, vitamin D receptor; WT, wild type.

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patients present liver abnormalities that may evolve into a clinically significant chronic liver disease (CF-associated liver disease [CFLD]). Consistent with the expression of CFTR in the biliary epithelium, CFLD manifests as a chronic and progressive cholangiopathy that can eventually progress into sclerosing cholangitis and focal biliary cirrhosis.[3,4]

The pathogenesis of CFLD was thought to be uniquely associated with the ductal cholestasis caused by the impaired bile flow and biliary alkalinization consequent to the defective channel function of CFTR at the cholangiocyte level. The resulting hyperviscous secretions would accumulate into the bile ducts, causing retention of hydrophobic bile acids and toxins that would damage the biliary epithelium.[3,5,6] However, derangement of epithelial immunity has recently emerged as an important pathogenic component of CFLD and other manifestations of CF.[7-10] Recent data have demonstrated that when defective in CFTR the biliary epithelium up-regulates toll-like receptor 4 (TLR4)/nuclear factor kappa light chain enhancer of activated B cells (NF-κB)–dependent innate immune responses when exposed to bacteria-derived endotoxins, leading to peribiliary inflammation.[11]

This novel interpretation of the pathogenesis of CFLD paves the way for new approaches to management. The current standard of care for CFLD is limited to the administration of ursodeoxycholate, a bile acid which is able to stimulate the choleretic function of hepatocytes and cholangiocytes and to modify the composition of the bile acid pool toward a reduced toxicity.[12,13] However a pathogenesis-based therapeutic approach should also target the TLR4/NF-κB-dependent inflammatory pathway in the biliary epithelium.

Nuclear receptors (NRs) constitute a superfamily of ligand-dependent transcription factors that regulate a variety of cellular functions, including metabolic homeostasis, apoptosis, cell proliferation, and differentiation.[14-16] Moreover, selected classes of NRs, such as glucocorticoid receptors, liver X receptors (LXR) and peroxisome proliferator–activated receptors (PPARs), are also able to modulate inflammation by controlling TLR-dependent signaling pathways. However, the biological functions regulated by a given NR, as well as the underlying mechanisms, vary in a tissue-specific manner.[17-23] In the biliary epithelium, NRs participate in the regulation of many cholangiocytic functions, including protection from bile-circulating toxics (vitamin D receptor [VDR], farnesoid X receptor [FXR]), secretion (glucocorticoid receptor, FXR), and cell proliferation (hormone receptors).[24] However, it is not known if PPAR-γ possesses anti-inflammatory properties in CF biliary epithelium or its potential mechanism of action.

In this study, we analyzed the pattern of NRs expressed in wild-type (WT) and CFTR-defective cholangiocytes and investigated the ability of the NR PPAR-γ to modulate innate immune responses initiated by activation of TLRs. We show that PPAR-γ expression is up-regulated in CFTR-defective cholangiocytes and provide evidence that stimulation of PPAR-γ is able to negatively regulate NF-κB-dependent innate immune responses in CF biliary epithelium, by stimulating the NF-κB negative regulator nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha (IκBα). We also show that administration of the PPARγ agonist rosiglitazone (ROSI) ameliorates liver damage in CF mice *in vivo*. Our results suggest that PPAR-γ signaling may represent a novel target to control biliary inflammation in CF.

Materials and Methods

Additional methods are detailed in the Supporting Information.

Animals and Experimental Protocols. All procedures were performed according to protocols approved by the Yale University Institutional Animal Care and Use Committee. Congenic C57BL/6J-Cftr^{tm1Unc} mice (Cftr-knockout [KO]), an accepted model for human CF disease, and their WT littermates were used for *in vivo* experiments and for the isolation of primary cholangiocyte cell lines. Animals were bred in our facility or provided by the CF Core Center Animal Core

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(Case Western Reserve University, Cleveland, OH) and maintained as described.[11,13,25] Cftr-KO mice and WT littermates were exposed to dextran sodium sulfate (DSS)[11] alone or in combination with the PPAR-γ agonists pioglitazone (PIO) and ROSI (10 mg/kg, intraperitoneally daily). At the end of the treatment, mice were sacrificed and their liver tissues harvested, fixed in formalin, and then embedded in paraffin for histochemical analysis.

Cell Culture and Treatments. Mouse cholangiocytes were isolated from WT and Cftr-KO mice as described.[11,13] After the first passage, cells were plated into 25-cm^2 tissue culture flasks coated with rat-tail collagen as described.[11,13] Before selected experiments, cells were cultured in Transwell inserts with a 0.4-μm-pore semipermeable membrane (Becton, Dickinson, and Co., Franklin Lakes, NJ). Under this condition, cells grow as a polarized monolayer that can be accessed through both the apical and basolateral domains separately. Establishment of a confluent monolayer was routinely checked, measuring transepithelial resistance and membrane potential difference (Millicell ERS System; Millipore, Billerica, MA). One week after confluence, transepithelial resistance was $>1000\ \Omega \cdot cm^2$ and cells were ready for analysis. Wild-type and Cftr-KO cholangiocytes were exposed to lipopolysaccharide (LPS, 100 ng/mL), PIO (10-50 μM), ROSI (10-50 μM), and GW9662 (10 μM). PIO and ROSI were administered 2 hours before and during LPS stimulation. GW9662 was administered 3 hours before and during LPS treatment.

Western Blotting. PPAR-γ protein expression was assessed by western blot in cytosolic and nuclear protein fractions of WT and Cftr-KO cholangiocytes, using an antibody against PPAR-γ (Cell Signaling Technology; rabbit, 1:1000, 5% milk in trishydroxymethylaminomethane-buffered saline Tween 20). IκBα was investigated on total lysates of WT and Cftr-KO cholangiocytes, treated as described above, using an antibody against total IκBα (Cell Signaling Technology; rabbit, 1:1000, 5% bovine serum albumin in trishydroxymethylaminomethane-buffered saline Tween 20). For additional details, refer to the Supporting Information.

Immunohistochemistry. Liver slides 4 μm thick from paraffin-embedded tissues were processed and stained as described[11] with the cholangiocyte-specific marker cytokeratin 19 (CK19) to visualize the ductular reaction and with the leukocyte-specific marker CD45 to analyze the inflammatory cell infiltrate. Quantification of the CK19-positive and CD45-positive areas by morphometric analysis was performed as described.[11]

Statistical Analysis. Results are shown as mean ± standard deviation. Statistical comparisons were made using one-way analysis of variance or the Wilcoxon-Mann-Whitney two-sample rank sum test, where appropriate. The statistical analysis was performed using SAS software (SAS Institute Inc., Cary, NC). $P < 0.05$ was considered significant.

Results

Expression of the NR PPAR-γ Is Increased in CFTR-Defective Cholangiocytes. In the liver, NRs are expressed by different cellular subtypes, such as hepatocytes, stellate cells, macrophages, and cholangiocytes, with each displaying a specific expression pattern.[24,26] By real-time polymerase chain reaction (PCR) analysis, we compared the expression of different NRs in primary cholangiocytes isolated from WT and Cftr-KO mice, cultured on Transwell inserts and grown as polarized monolayers. Gene expression of multiple NRs was detected (Fig. 1A) including PPAR subgroups α, γ, and δ; LXR subgroup β; FXR; and VDR. Among these transcripts, PPAR-δ, LXR-β, and FXR were expressed at higher levels, compared to PPAR-α and VDR, in both groups of cells. On the contrary, the expression of PPAR-γ was significantly increased in Cftr-KO cholangiocytes compared to WT cells. Western blot analysis on cytosolic and nuclear protein fractions showed that in both groups of cells the expression of PPAR-γ was mostly nuclear and significantly increased in CF cells, which confirmed our gene expression results (Fig. 1B).

Expression of PPAR-γ Target Genes Is Not Increased in Cftr-KO Cholangiocytes But Is Up-Regulated After Stimulation With PPAR-γ Agonists. PPARs are constitutively bound to the DNA, repressing the transcription of specific genes. When bound to the proper ligands (endogenous or synthetic), these receptors activate the transcription of specific target genes that regulate several cellular functions.[27] The expression level of these signature genes is considered indicative of the receptor activation rate. To investigate whether the increased expression of PPAR-γ in CF compared to WT cells is associated with a higher transcriptional activity of the receptor, we studied by real-time PCR the gene expression of metabolic target genes known to be transcriptionally modulated by PPAR-γ: acetyl-coenzyme A synthase 1B (Acaa1b), angiopoietin-like 4 (Angptl4), and 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (Hmgcs2).[28] Expression of these genes in Cftr-KO cells was similar to that in WT cells, indicating a comparable basal activity of PPAR-γ (Fig. 2A). Treatment with specific PPAR-γ agonists PIO and ROSI (10-50 μM) significantly increased the expression of PPAR-γ target genes (Fig. 2B), indicating that PPAR-γ receptors in both WT and Cftr-KO cells can be activated by

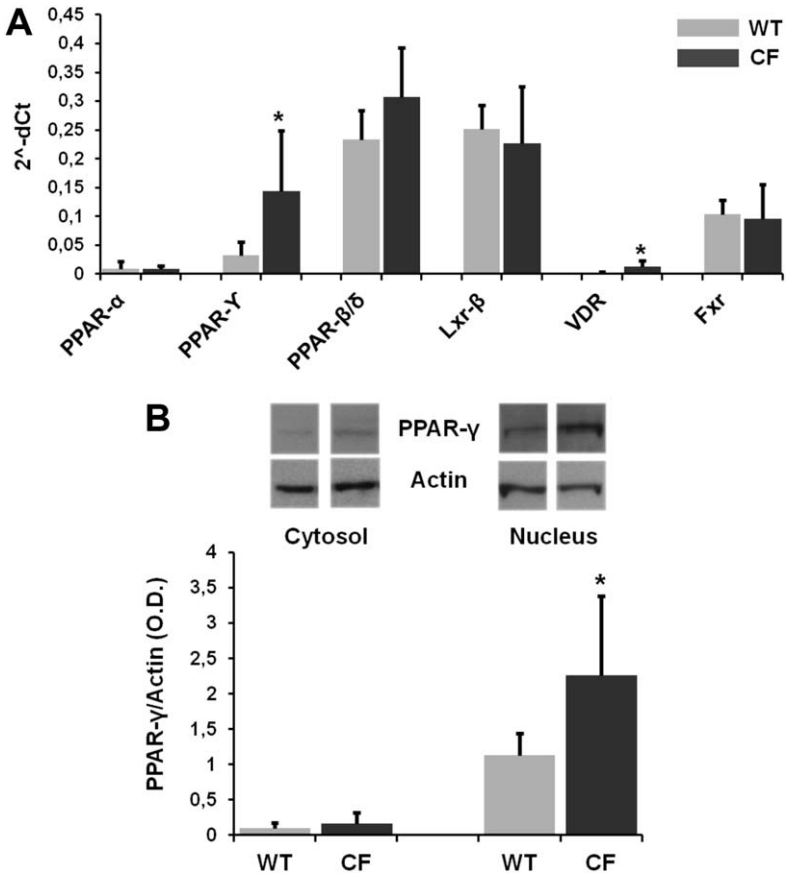

Fig. 1. (A) Gene expression of the NR PPAR-γ is increased in CFTR-defective cholangiocytes. Real-time PCR analysis of NRs in WT and Cftr-KO (CF) cholangiocytes. Data were performed in duplicate in n = 5 (WT) and n = 6 (CF) different cell lines, normalized to the HPRT housekeeping gene. *$P < 0.05$ versus WT. (B) Protein expression of PPAR-γ is increased in CFTR-defective cholangiocytes. Western blot of PPAR-γ in cytosolic and nuclear protein fractions from WT and Cftr-KO (CF) cholangiocytes. Data were performed in n = 3 experiments. *$P < 0.05$ versus WT.

exogenous ligands. These findings show that PPAR-γ in CF-defective cholangiocytes can be readily activated by exogenous agonists and suggest that an imbalance in the level of endogenous activators might explain why the increased expression of PPAR-γ does not correlate with an increased activity of the receptor.

Consistent with this hypothesis, we documented an imbalance between ω-3 and ω-6 polyunsaturated fatty acids in Cftr-KO cholangiocytes. We extracted total lipids from WT and Cftr-KO cholangiocytes and performed a lipidomic analysis by gas chromatography of the major ω-3 and ω-6 polyunsaturated fatty acids involved in the metabolic cascade that leads to the production of natural ligands of PPAR-γ.[21,29-32] We detected a significant increase in the ratio of arachidonic acid/linoleic acid (AA/LA) and a trend toward an increased ratio of AA/docosahexaenoic acid (DHA) (Fig. 3).

PPAR-γ Stimulation Inhibits LPS-Induced NF-κB Activation in Cftr-KO Cholangiocytes. We have shown that CFTR-defective cholangiocytes respond to LPS with increased NF-κB activation compared to WT cells.[11] To understand whether activation of PPAR-γ may regulate the LPS-dependent inflammatory response in the biliary epithelium, we treated WT and Cftr-KO cholangiocytes with PIO or ROSI (50 μM) alone or in combination with LPS (100 ng/mL) for 6 hours, and NF-κB transcriptional activity was assessed by a luciferase-based gene reporter. As reported,[11] Cftr-KO cholangiocytes responded to LPS with a higher NF-κB transcriptional activity with respect to control cells. Moreover, in Cftr-KO cells, treatment with PIO or ROSI significantly inhibited NF-κB activation in both groups of cells at the basal level and after LPS challenge (Fig. 4). Toxic cellular effects of PIO and ROSI at the experimental concentration used were excluded by performing a lactate dehydrogenase assay (Supporting Fig. S1).

The Inhibitory Effect of PPAR-γ Agonists on NF-κB Activity in CF Cells Is Dependent on a Direct Activation of PPAR-γ. Thiazolidinediones also may have receptor-independent effects.[33] To understand whether the inhibitory effect of PIO and ROSI on NF-κB activity in CF cells was dependent on a direct activation of PPAR-γ, we used two different approaches. First, polarized Cftr-KO cholangiocytes were treated with LPS (100 ng/mL) or the combination of LPS with PIO or ROSI (50 μM), in the presence or absence of the chemical antagonist GW9662 (10 μM).[34] We found that pretreatment with GW9662 significantly prevented the inhibitory effect of both agonists on the

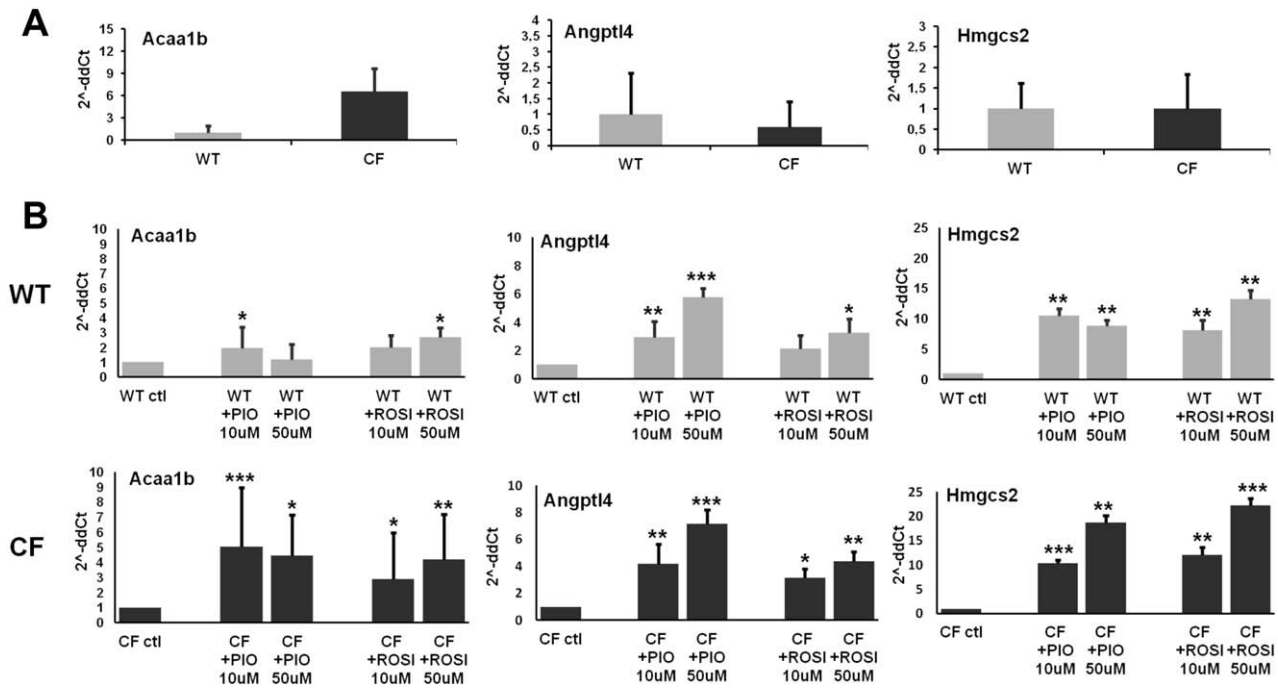

Fig. 2. Expression of PPAR-γ target genes is not increased in Cftr-KO cholangiocytes but is up-regulated after stimulation with PPAR-γ agonists. Gene expression of PPAR-γ target genes in WT and Cftr-KO (CF) cholangiocytes before (A) and after 12 hours of treatment with 10-50 μM of the agonists PIO and ROSI (B). Data were generated in duplicate in n = 4-6 different experiments, normalized to the HPRT housekeeping gene, and expressed as 2^{-ddCt} to the respective internal control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Abbreviations: Acaa1b, acetyl-coenzyme A acyltransferase 1B; Angptl4, angiopoietin-like 4; Hmgcs2, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2.

transcriptional activation of NF-κB (Fig. 5A). Secondly, experiments were repeated after PPAR-γ gene silencing. Gene expression analysis in silencing conditions confirmed a significant decrease of PPAR-γ gene (Fig. 5B). PPAR-γ gene silencing significantly abolished the anti-inflammatory effect of PIO and ROSI (Fig. 5C). Collectively, these findings indicate that the anti-inflammatory property of PIO and ROSI is receptor-dependent.

PPAR-γ Stimulation Inhibits LPS-Induced Secretion of NF-κB-Dependent Proinflammatory Cytokines in Cftr-KO Cholangiocytes. Sustained activation of NF-κB in endotoxin-stimulated Cftr-KO biliary cells is responsible for a robust production of specific proinflammatory cytokines/chemokines as described.[11] Based on the inhibitory effect on NF-κB activation, we investigated whether PPAR-γ activation also decreases the secretion of NF-κB-dependent inflammatory cytokines.

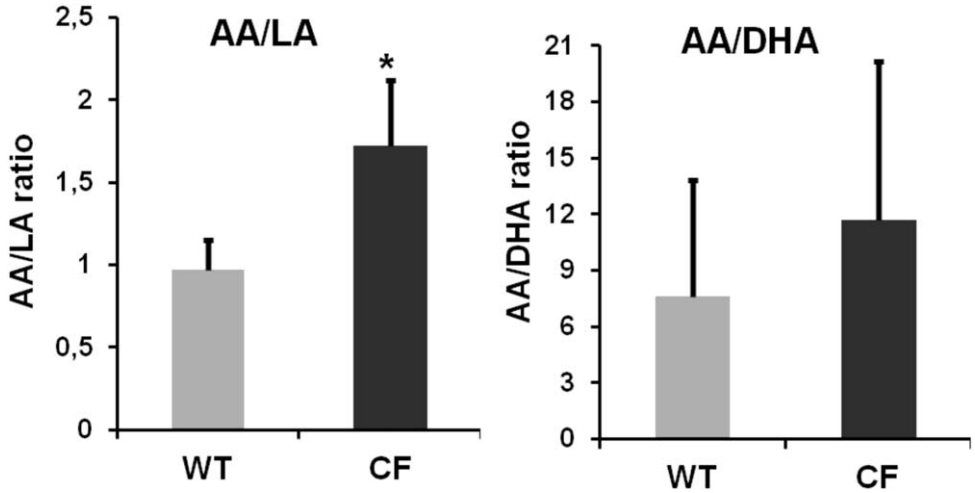

Fig. 3. Cftr-KO cholangiocytes show an imbalance between ω-3 and ω-6 polyunsaturated fatty acids. Lipidomic analysis of total lipids extracted from WT and Cftr-KO (CF) cholangiocytes. Data were performed in n = 4 experiments. $*P < 0.01$.

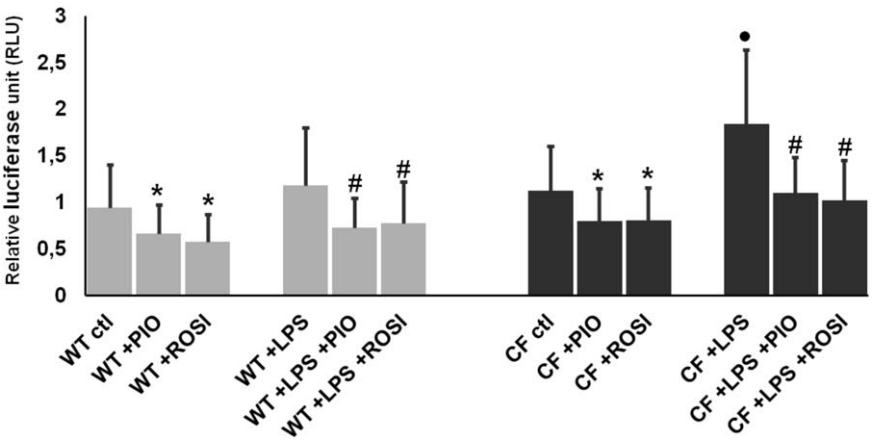

Fig. 4. PPAR-γ stimulation inhibits LPS-induced NF-κB activation in Cftr-KO cholangiocytes. Luciferase-based NF-κB gene reporter of WT and Cftr-KO (CF) cells before and after 6 hours of treatment with LPS (100 ng/mL) alone or in combination with PIO (50 μM) or ROSI (50 μM). Bar graph represents the ratio between the NF-κB-dependent expression of luciferase and the constitutive expression of *Renilla*. Data were performed in n = 4 experiments. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LPS, $^{\bullet}P < 0.05$ versus WT+LPS.

The effect of PIO and ROSI was analyzed for the following panel of proinflammatory cytokines: interleukin-1 isoforms α and β, interleukin-6, granulocyte colony-stimulating factor (G-CSF), keratinocyte chemoattractant (KC), LPS-induced CXC chemokine (LIX), monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein 2 (MIP-2). After treatment with PIO or ROSI (50 μM), LPS (100 ng/mL), or their combination for 12 hours, the medium was collected and proteins were harvested to normalize for the number of cells. Cytokine concentrations were assessed using a microsphere-based multiplex immunoassay (Luminex; MILLIPLEX MAP). In line with our previous studies, CF cells secreted a higher amount of cytokines with respect to WT cells. Moreover, treatment with both agonists significantly reduced the secretion of MCP-1, G-CSF, MIP-2, KC, and LIX (Fig. 6).

PPAR-γ Stimulation Inhibits the Activation of NF-κB Through an IκBα-Dependent Mechanism. To study the mechanism by which ligand-activated PPAR-γ blocks inflammation in CF cholangiocytes, we analyzed the effect of PIO and ROSI on crucial steps that lead to activation of the NF-κB pathway. In resting cells, IκBα retains the NF-κB subunits p50/p65 in a cytoplasmic inactive complex. In the presence of inducers of NF-κB, IκBα is phosphorylated by the kinase complex inhibitor of NF-κB kinase and degraded so that the activated p65 subunit can translocate to the nucleus.[35] We measured the protein expression of total IκBα and found that it was significantly reduced in CF compared to WT cells, which is consistent with a higher activation of NF-κB (Fig. 7A).

However, treatment with PIO and ROSI significantly increased IκBα protein expression after LPS challenge (Fig. 7C), indicating that PPAR-γ stimulation controls NF-κB activation by inducing its negative regulator, IκBα. In addition, we found that both PIO and ROSI significantly up-regulated IκB-α gene expression (Fig. 7B), suggesting that PPAR-γ agonists modulate IκB-α protein at the transcriptional level.

PPAR-γ Stimulation* In Vivo *Reduces Biliary Damage and Inflammation in Cftr-KO Mice Treated With DSS. To confirm *in vivo* the anti-inflammatory properties of PPAR-γ activation in CF cholangiopathy, we treated Cftr-KO mice with DSS to induce a portal endotoxemia, as described,[11] in the presence or absence of PPAR-γ agonists. At the end of the treatment, liver tissues were harvested and stained with CK19, to quantify the biliary and progenitor cell compartment expansion as a marker of biliary damage, and with CD45, to quantify the amount of leukocyte infiltration in the portal space as a marker of inflammation. DSS-treated Cftr-KO mice show bile duct proliferation and a significant infiltration of inflammatory cells in the portal space.[11,36] Unfortunately, treatment with PIO showed significant liver toxicity in both CF and WT mice treated with DSS (data not shown) and was not considered for further investigation. Conversely, in Cftr-KO mice treated with DSS and ROSI (10 mg/kg, intraperitoneally daily), bile duct proliferation (Fig. 8A) and inflammatory cell infiltration (Fig. 8B) were significantly reduced, as determined by computer-assisted morphometric analysis of CK19-positive and CD45-positive areas, consistent with a protective effect of PPAR-γ activation *in vivo*.

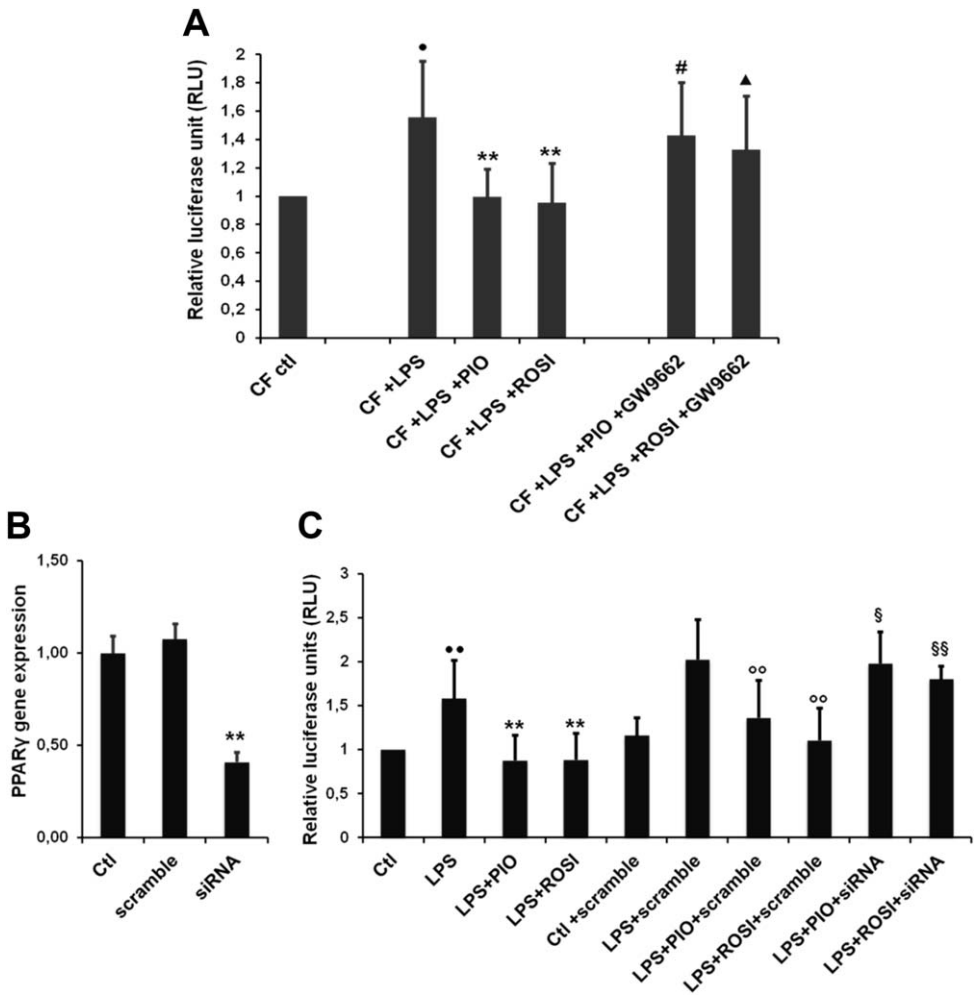

Fig. 5. The inhibitory effect of PPAR-γ agonists on NF-κB activity in CF cells is receptor-dependent. (A) Luciferase-based NF-κB gene reporter of Cftr-KO (CF) cells before and after 6 hours of LPS (100 ng/mL) alone or in combination with PIO and ROSI (50 μM) with or without the PPAR-γ antagonist GW9662 (10 μM). Data were generated from n = 4 experiments. Bar graph represents the ratio between the NF-κB-dependent expression of luciferase and the constitutive expression of *Renilla*. (B) Gene expression analysis confirmed a significant reduction of PPAR-γ messenger RNA in silencing condition. (C) Luciferase-based NF-κB gene reporter of Cftr-KO (CF) cells before and after 6 hours of LPS (100 ng/mL) alone or in combination with PIO and ROSI (50 μM) with or without small interfering RNA specific for PPAR-γ (30nM) or with scrambled oligonucleotides (30 nM). Data were generated from n = 5 experiments. Bar graph represents the ratio between the NF-κB-dependent expression of luciferase and the constitutive expression of *Renilla*. (A) $^{**}P < 0.01$ versus LPS, $^{\bullet}P < 0.05$ versus control, $^{\#}P < 0.05$ versus LPS+PIO, $^{\blacktriangle}P < 0.05$ versus LPS+ROSI; (B) $^{**}P < 0.01$ versus scramble; (C) $^{\bullet\bullet}P < 0.01$ versus control, $^{**}P < 0.01$ versus LPS, $^{\S}P < 0.05$ versus LPS+PIO+scramble, $^{\S\S}P < 0.01$ versus LPS+ROSI+scramble, $^{\circ\circ}P < 0.01$ versus LPS+scramble. Abbreviation: siRNA, small interfering RNA.

Discussion

CFLD is the third most common cause of death in patients with CF. Little is known about the pathogenesis and etiology of CFLD, and current clinical management is limited to supportive care and the administration of choleretic bile acids, such as ursodeoxycholate.[3,4,12,13] Treatment with ursodeoxycholate has shown some benefit; however, recent data on the pathophysiology of the disease suggest that treatment of CFLD should also aim at controlling innate immune responses and NF-κB-dependent inflammation.[7,8,10,37] Studies from our group demonstrated that TLR4/NF-κB-dependent innate immune responses are deregulated in cholangiocytes that lack CFTR and are exposed to bacteria-derived endotoxins.[11]

Among drugable pathways that can be targeted to decrease innate immune responses, NRs are attracting attention. Several specific drugs are in development or already on the market. In this study, we investigated the ability of PPAR-γ to modulate the innate inflammatory responses initiated by activation of TLRs. In particular, our results provide evidence that activation of PPAR-γ signaling is able to repress NF-κB-dependent inflammation in CF biliary epithelium by stimulating the gene and protein expression of the NF-κB-negative regulator

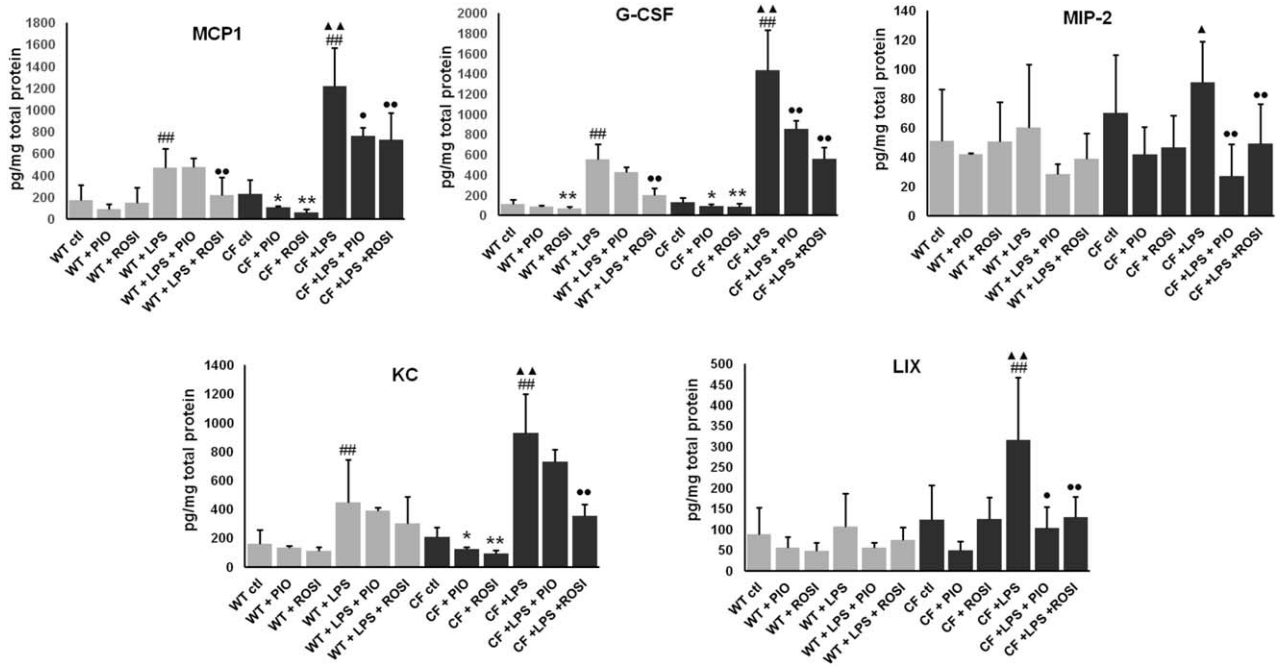

Fig. 6. PPAR-γ stimulation inhibits LPS-induced secretion of NF-κB-dependent proinflammatory cytokines in Cftr-KO cholangiocytes. Luminex assay of proinflammatory cytokines MCP1, MIP-2, KC, G-CSF, and LIX secreted in culture medium of WT and Cftr-KO cholangiocytes, before and after 12 hours of treatment with LPS (100 ng/mL) alone or in combination with PIO or ROSI (50 μM). Data were generated from n = 4 experiments and are normalized for the total cellular protein content. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control, $^{\#}P < 0.05$ versus control, $^{\#\#}P < 0.01$ versus control, $^{\bullet}P < 0.05$ versus LPS, $^{\bullet\bullet}P < 0.01$ versus LPS; $^{\blacktriangle}P < 0.05$ versus WT+LPS, $^{\blacktriangle\blacktriangle}P < 0.01$ versus WT+LPS.

IκBα. We also provide evidence that administration of the PPAR-γ agonist ROSI ameliorates liver biliary damage and inflammation *in vivo* in CFTR-defective mice, providing direct evidence that this mechanism is of pathogenetic relevance.

NRs include a wide range of ligand-activated transcription factors that influence cellular responses by altering gene expression.[14-16] Although initially recognized for their role in metabolism and cellular homeostasis, some NRs, including PPARs, are now emerging as important negative regulators of inflammation, even though the knowledge of their mechanisms of action is still limited.[17-23]

NRs are differentially expressed in distinct liver cell types.[24,26] We found that primary cholangiocytes isolated from WT and CFTR-defective mice express multiple receptors, including VDR, LXR-β, FXR, and all three isoforms of PPAR, consistent with previously published data.[26] Conflicting reports have described PPAR-γ expression in CF.[38] PPAR-γ was decreased in CF airway epithelial cells[39] and unchanged in CF whole liver.[40] Interestingly, we observed that Cftr-KO cholangiocytes had a significantly higher gene and protein expression of PPAR-γ compared to control cells, in which PPAR-γ is expressed at low levels. This finding led us to further investigate whether the increased PPAR-γ expression was correlated with an increase in the receptor activity in CF cells. PPARs are constitutively bound to the DNA, where they repress the transcription of specific target genes. Upon binding of activator ligands, the transactivation pathway is switched on and the transcription of these genes is activated.[27] We analyzed the expression of classical genes activated by PPAR-γ and found that this was comparable between CF and control cells, indicating that, in spite of an increased expression of PPAR-γ in CF cholangiocytes, the transcriptional activity of the receptor was not different from WT cells.

Similar to other PPAR members, in a given tissue, the activation state of the receptor is modulated by the relative amount of endogenous PPAR-γ ligands.[29,32] We reasoned that proper activation of PPAR-γ in CF might be impaired by a limited availability of endogenous activators. Eicosanoids are signaling molecules derived from key fatty acid precursors present at the cell membrane level, such as LA, or from the intermediate metabolite AA and DHA. Selected classes of anti-inflammatory polyunsaturated fatty acids can act as PPAR-γ endogenous ligands.[21,29-32] Altered ratios of AA/LA and AA/DHA have been reported in the blood and mucosal samples of CF patients and Cftr-KO mice.[41-43] Consistent with the above findings, our lipidomic analysis showed an increase in AA/LA and AA/DHA ratios in Cftr-defective cells compared to their controls. Recent data showed

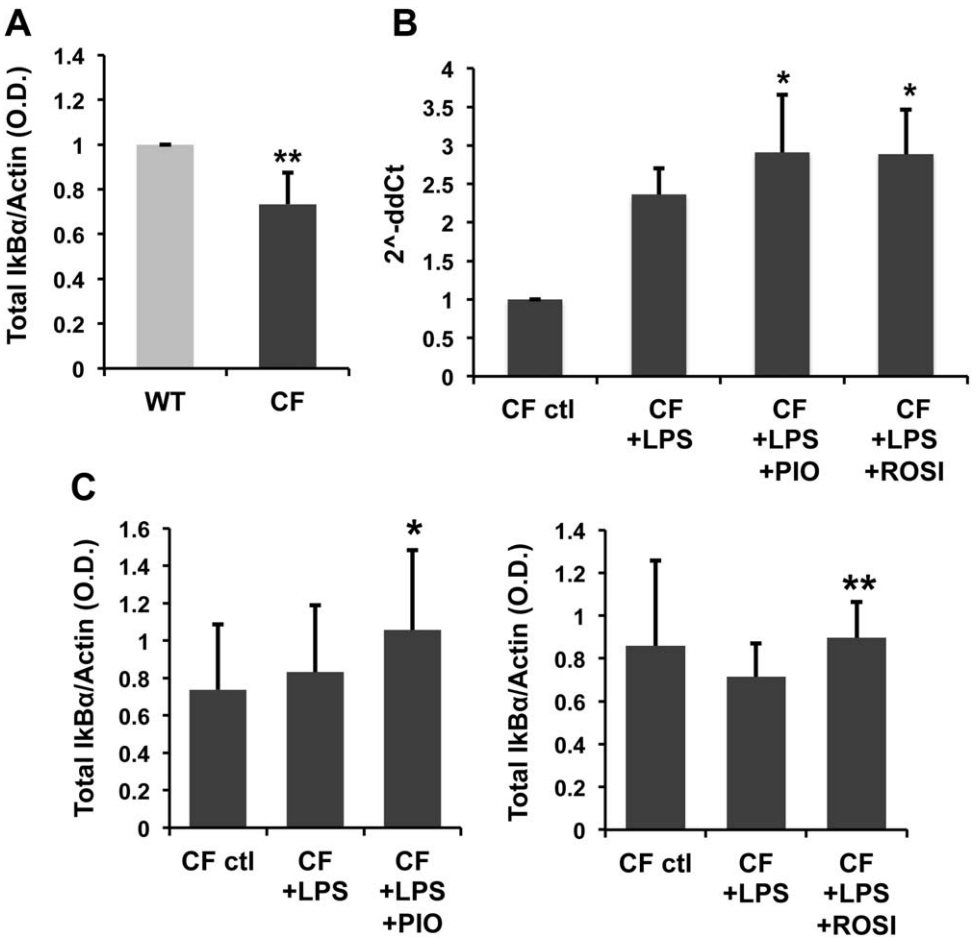

Fig. 7. PPAR-γ stimulation inhibits the activation of NF-κB through an IκBα-dependent mechanism. Protein expression of total IκBα was analyzed by western blot. (A) Data represent the average of densitometric analysis of n = 7 experiments in WT and CF cells, normalized for actin. $^{**}P < 0.01$ versus WT. (B) Real-time PCR analysis of IκBα gene in CF cells treated for 6 hours with LPS (100 ng/mL) alone or in combination with PIO or ROSI (50 μM). Bar graph represents the average of n = 4 independent experiments in duplicate, normalized to glyceraldehyde 3-phosphate dehydrogenase. $^{*}P < 0.05$ versus LPS. (C) CF cells were treated as in (B). Bar graph represents the average of densitometric analysis of n = 3 experiments, normalized for actin. $^{*}P < 0.05$ versus CF+LPS, $^{**}P < 0.01$ versus CF+LPS. Abbreviation: O.D., optical density.

that CFTR may alter AA/LA metabolism through an adenosine monophosphate–activated protein kinase–mediated increase in $\Delta 5$ and $\Delta 6$ desaturases.[44] Altered AA/LA ratio and a lower production of DHA-mediated endogenous activators of PPAR-γ in CF biliary cells may account for the increased expression of the receptor as a compensatory mechanism to counteract a lower activation state.

We next investigated whether the stimulation of PPAR-γ with synthetic ligands controls TLR4/NF-κB-driven inflammation in CFTR-defective biliary epithelium. We have shown that CFTR-defective biliary epithelial cells, in response to endotoxins, activate a strong TLR4-dependent inflammatory response, characterized by a higher NF-κB activity, which in turn is responsible for a sustained and increased production of several proinflammatory cytokines.[11] To study the effect of PPAR-γ stimulation on NF-κB activation, we tested the effect of two different thiazolidinedione compounds. Thiazolidinediones are a family of potent synthetic agonists that activate PPAR-γ and include troglitazone, ciglitazone, ROSI, and PIO. Of these, troglitazone has been removed from the market due to significant hepatotoxicity. However, ROSI and PIO are currently Food and Drug Administration–approved in the United States for type 2 diabetes treatment and act as strong insulin sensitizers.[45,46] We found that treatment of WT and CF cells with PIO and ROSI alone or in the presence of LPS significantly reduced the activation of NF-κB in CF cells, as shown by the significant reduction in the transcriptional activity of the NF-κB promoter. Moreover, the effect of thiazolidinediones on the activation of NF-κB was significantly reversed by pretreatment with GW9662, a selective PPAR-γ antagonist that irreversibly blocks the ligand-binding pocket of the receptor, and by experiments using gene silencing, indicating the

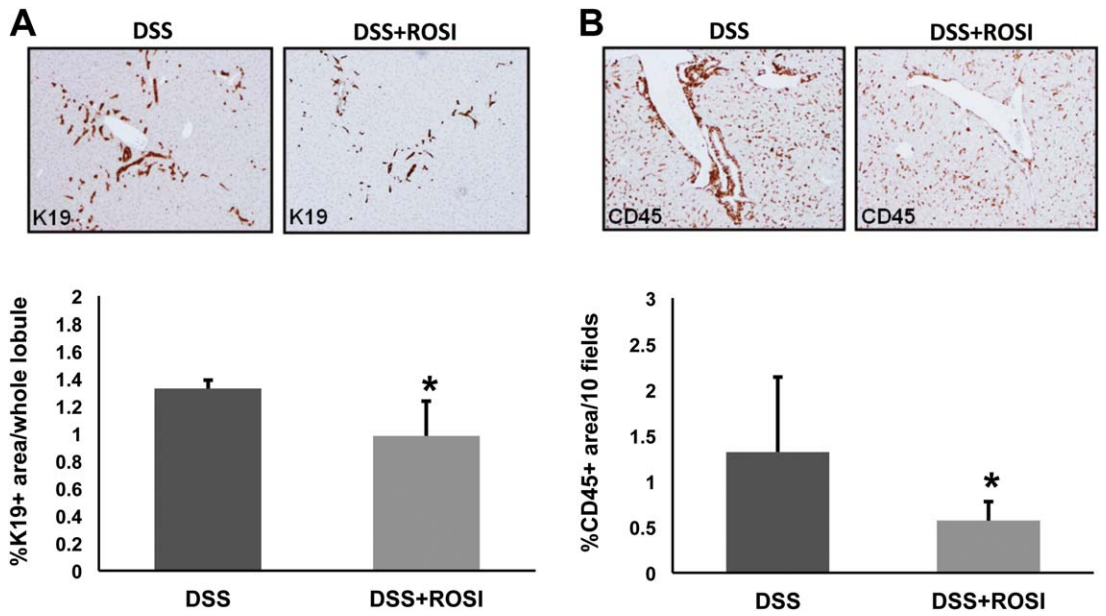

Fig. 8. PPAR-γ stimulation reduces biliary damage and inflammation in Cftr-KO mice treated with DSS. Cftr-KO mice were treated with DSS (n = 5) or with DSS and ROSI (n = 5) (10 mg/kg, intraperitoneally daily). At the end of the treatment, liver tissues were harvested and stained with the cholangiocyte-specific marker CK19 (A) or the leukocyte-specific marker CD45 (B). Computer-assisted morphometric analysis of CK19-positive and CD45-positive areas shows that rosiglitazone treatment significantly reduced bile duct proliferation and inflammatory cell infiltration in Cftr-KO mice treated with DSS. *$P < 0.05$ versus DSS only. Abbreviation: K19, cytokeratin 19.

specificity of the effects and the activation of a receptor-dependent pathway.

Furthermore, PPAR-γ activation significantly reduced the secretion of proinflammatory cytokines downstream of the NF-κB pathway. As previously described, CF cells secreted a higher amount of cytokines, with respect to WT cells, after stimulation with LPS. After treatment with PIO and ROSI, the secretion of MCP-1, G-CSF, MIP-2, KC, and LIX was significantly reduced in both groups of cells, with a more prominent effect in CF cells. The weaker effect in control cells, where PPAR-γ is less expressed, suggests that the anti-inflammatory effect was dependent on the amount of receptor available. Interestingly, G-CSF and especially KC (the murine homolog of human interleukin-8) are strongly involved in proliferation, survival, and chemotaxis of neutrophils, which we have previously described as the major constituents of the inflammatory infiltrate in Cftr-KO livers, in the setting of endotoxin-induced damage.[11]

PPARs may control inflammation through multiple mechanisms unique to a given cell type. Some of these mechanisms occur in the cytoplasm by interfering with the NF-κB activating machinery.[47] The activation of NF-κB requires the inactivation by phosphorylation of its cytoplasmic inhibitor, IκBα, by the inhibitor of NF-κB kinase complex.[35] To dissect the mechanism by which ligand-activated PPAR-γ blocked inflammation in CF cholangiocytes, we analyzed the effect of PIO and ROSI on different steps of the NF-κB pathway. The gene and protein expression of IκBα in CF cells increased significantly after treatment with both agonists in combination with LPS. These findings indicate that the anti-inflammatory effect of PPAR-γ activation occurs, at least in part, through a transcriptional control of the NF-κB negative regulator.

We next investigated the effect of PPAR-γ activation *in vivo* in Cftr-KO mice treated with DSS, a model previously used by others and ourselves to induce an inflammatory cholangiopathy, caused by the translocation of intestinal bacteria into the portal circulation.[11,36] PIO treatment in WT and CF mice showed a significant hepatotoxic effect and, therefore, was not used for further experiments. Conversely, Cftr-KO mice treated with DSS in combination with ROSI showed a significant decrease in biliary damage and portal inflammation compared to the control group, where only DSS was administered. Liver toxicity by PIO was clearly unrelated to the stimulation of PPAR-γ because no liver toxicity was seen with ROSI, a more PPAR-γ-specific agonist.[48] PIO is commonly used in humans for the treatment of diabetes, and therefore, our hypothesis is that liver toxicity in our experimental conditions is due to a species-related or strain-related off-target effect of PIO. However, further toxicological studies will eventually be needed to clarify this aspect.

In conclusion, these studies show a novel function of the NR PPAR-γ in controlling biliary epithelium inflammation. PPAR-γ is expressed more in CF biliary epithelium, but defective production of bioactive ligands impairs the proper activation of the receptor, contributing to the chronic inflammatory state of CFTR-defective cholangiocytes. On the other hand, exogenous stimulation of PPAR-γ has anti-inflammatory properties in the CF biliary epithelium and limits the TLR4/NF-κB-dependent innate immune responses to endotoxins. Finally, our *in vivo* results strongly indicate the pathophysiological relevance of the described mechanism and the potential value of PPAR-γ as a therapeutic target in CF and possibly in other inflammatory cholangiopathies.

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