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Activation of PPAR-γ Signaling as a Novel Target to Limit NFκB-Dependent Inflammation in Cystic Fibrosis Biliary Epithelium

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1 SUMMARY

Cystic fibrosis-associated liver disease (CFLD) is a chronic cholangiopathy that negatively impacts the quality of life and survival of patients with Cystic Fibrosis (CF). CF is a disease of secretory epithelia caused by genetically-determined defective function of CFTR (cystic fibrosis conductance regulator), a cAMP-activated Cl^- channel that, in the liver, is uniquely expressed in the biliary epithelium. The pathogenesis of CFLD is thought to be related to the ductal cholestasis caused by the defective channel function of CFTR. However, a number of evidence indicate that, in association with the genetic defect, other pathogenetic factors concur to determine the liver phenotype. During the first year of my doctorate we have demonstrated that in CFTR-defective biliary epithelium, exposed to bacterial-derived endotoxins, TLR4/NF-κB-dependent innate immune responses are increased, indicating a loss of "endotoxin tolerance".

The current standard of care for CFLD is limited to the administration of choleretic agents. Based on our novel interpretation of the pathogenesis of CFLD, a rational therapeutic approach to the management of CFLD should be to target the exaggerated inflammatory response of the biliary epithelium.

Nuclear receptors (NRs), ligand-activated transcription factors that regulate several intracellular metabolic functions by controlling the expression of specific target genes, are now emerging as important regulators of inflammation in several conditions. In this work we sought to investigate the ability of NRs to modulate the TLR4/NF-κB-dependent innate immune responses in CF biliary epithelia.

Our data indicate that cholangiocytes express several subtypes of NRs, including Peroxisome Proliferator Activated Receptor (PPAR) subgroup α, γ and β/δ, Liver X Receptors (LXR) subgroup β, Farnesoid X Receptor (FXR) and Vitamin D Receptor (VDR).

Among these NRs, PPAR-γ was barely expressed in wild type (WT) cholangiocytes , but its expression, both at the gene and protein levels, was strongly and significantly more expressed in CF cells. In spite of its increased expression in the nucleus, PPAR-γ was not transcriptionally active in CF cholangiocytes; in fact the basal level of expression of PPAR-γ target genes (Acaa1b, Angptl4 and Hmgcs2) was similar to WT cholangiocytes. On the other hand, treatment of CF cholangiocytes with the PPAR-γ agonist pioglitazone significantly increased the expression of its target genes, indicating that the transcriptional activity of nuclear

PPAR-γ could be readily activated in the presence of proper agonists. Consistent with the higher expression level of the receptor, the increase in PPAR-γ target genes stimulated by pioglitazone was higher in CF than in WT cholangiocytes.

The observation that PPAR-γ, while inactive at baseline levels, was able to respond to exogenous stimulation, indicates that a defect in the availability of endogenous PPAR-γ activators might impair PPAR-γ function in CF and increase the receptor expression as counter-regulatory mechanism. Thus, to study the availability of endogenous PPAR-γ activators in CF, we performed lipidomic analysis of extracts from cultured cholangiocytes, and quantified the major ω-3 and ω-6 polyunsaturated fatty acids (PUFAs), i.e. the precursors of the active form of PPAR-γ ligands. We detected an increased ratio between arachidonic acid and docosahexaenoic acid (AA/DHA) and between arachidonic acid and linoleic acid (AA/LA) in CF cells compared to their controls. LA, the precursor of AA, is the main source of pro-inflammatory mediators, while DHA is an important precursor of anti-inflammatory eicosanoids that are able to activate PPAR-γ. This finding might explain, at least in part, the increased receptor expression in CF biliary cells.

Having shown that CF cells express higher amounts of PPAR-γ, that can be activated by exogenous ligands, but it is not transcriptionally active because of the lack of endogenous ligands, we next tested if exogenous activation of PPAR-γ by synthetic ligands, might decrease the TLR4/NF-κB-driven inflammation in CFTR-defective biliary epithelium. We found that in Cftr-KO cells, in the presence of pioglitazone, nuclear translocation of p65/NF-κB, as well as its transcriptional activity, were significantly inhibited, both before and after stimulation with bacterial LPS. Moreover, the gene expression and protein secretion of several NF-κB-dependent pro-inflammatory cytokines, such as LIX (LPS-induced CXC chemokine), MCP-1 (monocyte chemotactic protein-1), MIP-2 (macrophage inflammatory protein 2), G-CSF (granulocyte colony-stimulating factor) and KC (keratinocyte chemo-attractant) were significantly inhibited both at baseline and after LPS stimulation. The anti-inflammatory effect of pioglitazone was the result of a direct activation of PPAR-γ receptor, as shown by reversal of pioglitazone-induced protection in the presence of the PPAR-γ antagonist GW9662.

Finally, to understand the mechanism by which ligand-activated PPAR-γ blocks inflammation in CF cholangiocytes we analyzed the effect of pioglitazone on

NF-κB activation pathway steps, and found that pioglitazone up-regulated IκB-α, a negative regulator of NF-κB.

In conclusion, our results indicate that a decreased PPAR-γ function caused by a decreased availability of PPAR-γ endogenous agonists might be responsible for the chronic inflammatory state of CFTR-defective cholangiocytes. Stimulation of PPAR-γ signaling by the agonist pioglitazone is able to decrease NF-κB-dependent inflammatory responses by increasing IκB-α, thus may represent a novel strategy to limit inflammation in CFLD. Studies in mice models of liver damage in CFLD have been planned and may represent a proof of concept for anti-inflammatory treatment in CF.

2 INTRODUCTION AND BACKGROUND

2.1 Physiology of biliary epithelium and role of the Cystic Fibrosis Conductance Regulator in bile secretion.

The biliary epithelium is a complex three-dimensional network of conduits within the liver. It begins at level of canal of Hering, that represents the junction between hepatocytes and cholangiocytes (the epithelial cells lining the biliary tree), and then progressively merges into a system of interlobular, septal, major ducts and finally connects with the gallbladder and the intestine (figure 1) [1]. The main function of these epithelium is ductal bile formation (40% of total human bile) and its delivery to the gallbladder and the intestine.

Total bile formation is the result of the integrated function of hepatocytes and cholangiocytes. Hepatocytes produce the primary bile that generates the main osmotic driving force through the secretion of biliary acids, lipids, glutathione, organic cations and anions [2]. Solutes transport is mediated by several carriers located on both apical (canalicular) and basolateral (sinusoidal) membrane, and is associated with passive transport of water molecules through cellular junctions [3]. This primary bile is then modified during its transit toward the duodenum, by the biliary epithelium. This epithelium re-absorbs fluid, electrolytes, amino acids, glucose and bile acids and secrete water, Cl^- and HCO_3^-, and IgA immunoglobulins [2, 4].

Bile is a complex fluid containing 95% of water and 5% of several solutes, such as electrolytes, bile acids, bilirubin, lipids (cholesterol, lecitine), amino acids (glutamic acid, aspartic acid, glycine), vitamins (folic acid B9, piridossin B6, cobalamin B12), metals, inorganic molecules (Na^+, Cl^-, HCO_3^-). It also contains several enzymes and proteins including immunoglobulins (IgA). The bile performs multiple roles. For example, it is able to eliminate waste products of endogenous and exogenous compounds, such as bilirubin and drug metabolites, and to secrete bile salts, thereby allowing the intestinal absorption of lipids and vitamins. Moreover, it has the ability to dispose cholesterol from the blood [2, 3].

Besides bile formation, biliary epithelial cells are capable of performing a wide variety of functions. Firstly, they are involved in the reabsorption of biliary constituents (like glucose and glutathione), and in the enterohepatic circulation of

biliary salts; Moreover, they are able to interact with the immune system and different microorganisms, ensuring a defense against biliary infections [2, 5, 6]. On this regard, they present receptors for several cytokines, chemokines and also growth factors and angiogenetic factors, that also create a functional connection system between cholangiocytes and other hepatic cellular populations, like hepatocytes, stellate cells and endothelial cells [7] . In addition, several studies have described the ability of these cells to participate in regenerative and reparative liver processes upon endogenous and exogenous stimuli .

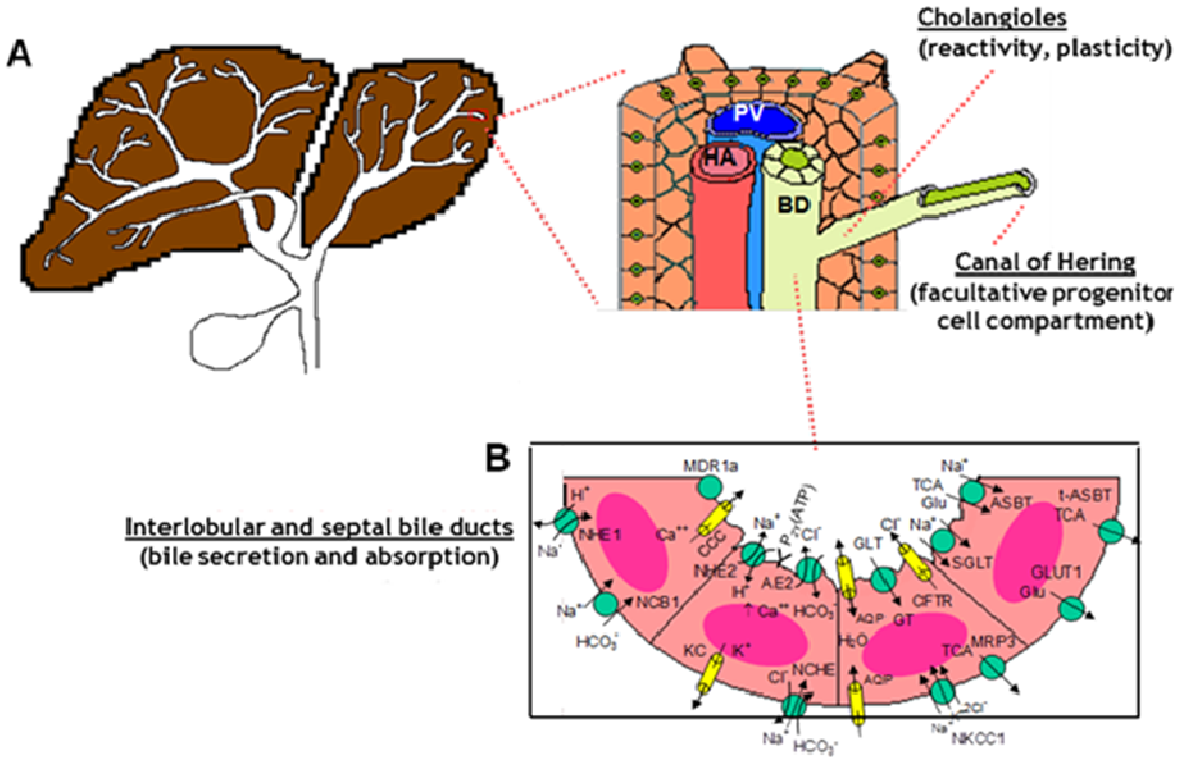


Figure 2. Morphologic and functional heterogeneity of biliary epithelium. (A) The intrahepatic bile duct epithelium is organized as a three-dimensional network of interconnecting conduits of different size inside the liver perfused by a periductular capillary plexus that originates from branches of the hepatic artery (HA). The biliary tree begins with the canals of Hering that constitutes a facultative progenitor cell compartment and connects hepatocytes with bile ducts. Cells lining the intrahepatic biliary tree have different functional and morphologic specializations: the cholangioles (size <15 μm) have some biologic properties such as plasticity, (ie, the ability to undergo limited phenotypic changes) and reactivity (ie, the ability to participate in the inflammatory reaction to liver damage); large size cholangiocytes (300-800 μm) modulate fluidity and alkalinity of the primary hapatocellular bile trough numerous different molecules located on their apical (luminal) and basolateral domain finally regulated by a complex of hormones, neuropeptides, and neurotransmitters. (B) PV, portal vein; HA, hepatic artery; BD, bile ducts; NHE1 or SLC9A, Na^+/H^+ exchanger; NCB1 or SLC4A4, $Na^+HCO_3^-$ cotransporter; KC, K^+ channel; NCHE, Na^+-dependent Cl^-/HCO_3^- exchanger; NHE2 or SLC9A2, Na^+/H^+ exchanger-2; AE2 or SLC4A2, Cl^-/HCO_3^- exchanger; P2Y, purinergic receptors; CCC, Ca^{++}-activated Cl^- channel; MDR1a or ABCB1, multidrug resistance protein 1a; AQP, aquaporins (ie, water channels); GT, glutamate transporter; CFTR

or ABCC7, cystic fibrosis transmembrane conductance regulator; SGLT1 or SLC5A1, Na^+-dependent glucose cotransporter-1; ASBT or SLC10A2, apical Na^+-dependent bile acid cotransporter; NKCC1 or SLC12A2, $Na^+/K^+/2Cl^-$ cotransporter; MRP3 or cMOAT2, or ABCC3, multidrug resistance protein 3; GLUT1 or SLC2A1, facilitated glucose transporter-1; t-ASBT, truncated ASBT; GLT, glutathione; Glu, glucose; TCA, taurocholate. (From Strazzabosco et al., 2005)

To perform all these different functions cholangiocytes are morphologically and functionally different along the biliary tree system [1]. Cholangiocytes that form the larger and interlobular ducts (diameter of 20-800 μm) are mainly involved in secretory functions, and express several ion carriers and hormone receptors in the plasma membrane [2, 5, 6]. The cholangiocytes that line the small ducts and the canals of Hering are mainly involved in other functions, such as plasticity (they can undergo phenotypic changes), reactivity (they can play a role in the inflammatory response after liver injury) and stemness (they may behave as progenitor cells).

In the last decade, the knowledge of the molecular basis of the secretory mechanisms has rapidly increased, and now it is clear that HCO_3^- and Cl^- secretion is the main driving force for biliary secretion. The net amount of fluid and bicarbonate secretion results from a fine balance between pro-secretory stimuli as secretin [2, 6], glucagon [8], VIP [9], acetylcholine [10], bombesin [11], and anti-secretory ones as somatostatin [12], insulin [13], gastrin [14] and endothelin-1 [15]. To coordinate all these different signals, the intrahepatic biliary epithelium expresses multiple adenyl cyclases (ACs) isoforms with an heterogenic expression among different cholangiocyte subpopulations [16]. For example the stimulation of adenylyl cyclases AC8, AC9 and sAC by secretin leads to a series of intracellular events: increase of cAMP levels, activation of protein kinase A (PKA), phosphorylation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), the stimulation of Cl^- and HCO_{3^-} efflux, and finally the inhibition of Na^+ influx that is normally performed by Na^+/H^+ exchanger (NHE3) [17].

CFTR is a low conductance chloride channel activated by cAMP and is expressed in the apical domains of many secretory epithelia, including biliary epithelium cholangiocytes. It is a membrane protein of 1480 amino acids belonging to *ATP-binding-cassette* (ABC) transporter family and is composed by two symmetrical motifs, each containing a membrane-spanning domain (MSD), usually composed by six transmembrane segments, and a nucleotide-binding domain (NBD) (figure 2) [18].

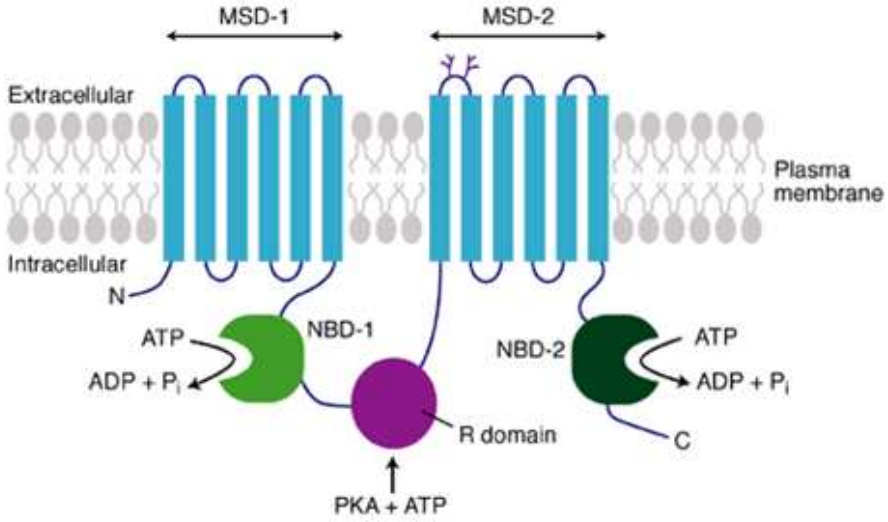


Figure 2. Model showing domain structure of cystic fibrosis transmembrane conductance regulator (CFTR). MSD, membrane-spanning domain; NBD, nucleotide-binding domain; R, regulatory domain; PKA, cAMP-dependent protein. kinase. (From Davidson and Dorin, 2001)

These two motifs are linked by a third common domain (regulatory R domain) that presents consensus phosphorylation sites that are targeted by PKA and PKC [18-21]. The efflux of chloride through this channel creates an electrochemical gradient that allows the activation of an anion exchanger (AE2) that transports bicarbonate into the biliary lumen, in exchange with Cl^- (figure 3).

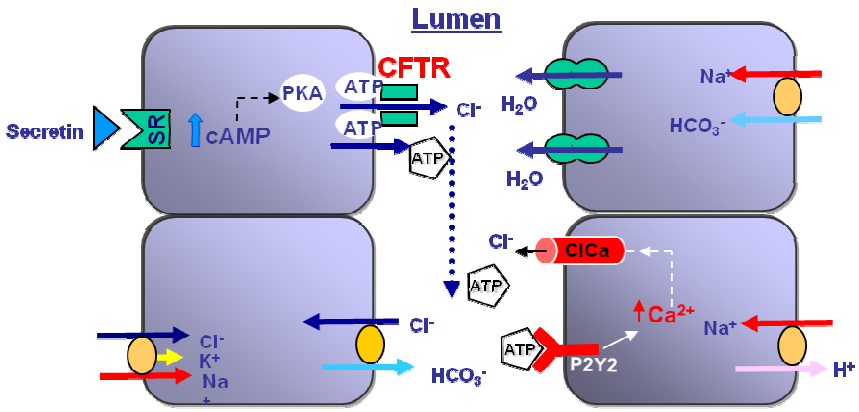


Figure 3. Role of CFTR in the biliary epithelium. In the liver CFTR is localized in the apical membrane of biliary epithelium where in response to secretin secretes chloride into the lumen and regulates the alkalinity and fluidity of the bile in cooperation with AE2 and aquaporins. It is also involved in ATP secretion and activation of purinergic signaling.

In addition to CFTR, there are other important chloride channels involved in production of bile. For example, the apical Ca^{2+}-dependent chloride channel, that is stimulated by the purinergic signaling at level of the biliary lumen. On the other hand, the efflux of Cl^- from cholangiocytes into the lumen is counterbalanced

through other Cl^- uptake systems, including the basolateral Na^+-K^+-$2Cl^-$ cotransporter NKCC1/SLC12A2, responsible also for maintaining cell volume homeostasis [22]. All the ionic effluxes into the lumen lead to the influx of water to maintain the osmotic balance inside/outside the cell; this process occurs both paracellulary and through specific water channels, called aquaporins 1 and 2 (AQP1 and AQP2) [23, 24].

All the secretory functions of biliary epithelium are also subject to a paracrine regulation. The hepatocytes secrete molecules (i.e. bile salts, glutathione and purinergic nucleotides) into the bile canaliculi that are delivered to receptors and carriers located in the apical domain of cholangiocytes [6]. Among these, ATP is released from hepatocytes and cholangiocytes themselves (autocrine loop) into the bile and it binds the apical purinergic receptor P2Y2 of cholangiocytes stimulating the efflux of Cl^- (through Ca^{2+}-dependent chloride channels) and the influx of HCO_3^- (through exchanger channels Na^+/H^+ on the basolateral side, NHE-1) [25].

2.2 Cholestatic diseases of biliary epithelium.

The term “cholangiopathy” refers to several genetic or acquired diseases affecting biliary epithelial cells. Cholangiopathies that target the intrahepatic biliary epithelium can cause high morbidity and mortality and are the main indication for liver transplant in the pediatric population [7, 26].

Cholangiopathies include several diseases with different pathogenesis: immune-mediated (primary biliary cirrhosis, graft versus host disease, primary sclerosing cholangitis), infective (cytomegalovirus or cryptosporidium), toxicological (several common drugs) or ischemic (arterial stenosis, post-transplant and chronic rejection). In addiction genetic or developmental diseases, that lead to an impair bile duct biology, are known to cause progressive cholangiopathies [7] (i.e Cystic Fibrosis, Alagille Syndrome, ADPKD, Caroli Disease, Biliary Atresia, etc.) (see table at the end of the paragraph).

The pathophysiology of cholangiopathy is characterized by several mechanisms, that include cell loss (lysis or apoptosis), proliferation of cholangiocytes, portal inflammation, fibrosis and impairment of bile production (cholestasis) [7]. Cholestasis is the biochemical, functional and clinical consequence of impairment in bile flow and in the secretion of organic and inorganic compounds

into the bile. As a consequence bile solutes accumulate in the hepatocytes and also the blood, determining classical clinical symptoms, such as jaundice and itch [2, 7].

The specific mechanisms that lead to ductal cholestasis in chronic cholangiopathies are not completely understood. In general cholestatic cholangiopathies are characterized by a decrease in the amount of bile flow, caused by immune-mediated, infective, metabolic, genetic or ischemic process. Independently of the specific cause, the resulting changes in bile components may further exacerbate the biliary epithelium damage [7]. Once the epithelium is damaged, cholestasis can be worsened by fibro-inflammatory obliteration of bile ducts, or by an altered response of secretory function (inflammation-mediated) in the biliary epithelium [27, 28].

	Incidence	Genetic Defect	Pathology	Clinical Features and Outcome	Current Therapy
AGS	1:70,000	*JAGGED1* (a ligand of Notch receptors)	Bile duct paucity, extensive ductular metaplasia of hepatocytes	Severe pruritus, jaundice, rare progression to cirrhosis; hypercholesterolemia; extrahepatic manifestations may be important (stroke, renal failure)	OLT if persistent cholestasis, intractable pruritus, or, rarely ESLD
CF	3–20% of patients with CF	*CFTR* (a CAMP-dependent Cl-channel)	Ductal cholestasis and focal biliary cirrhosis	Cholestasis, splenomegaly and hypersplenism, variceal bleeding; pancreatic and pulmonary infections	UDCA, supportive therapy, OLT
MDR3 deficiency	Rare	*MDR3* (a canalicular phosphatidylcholine translocator)	Marked ductular reaction and early fibrosis	Cholestasis, rapid progression to cirrhosis; portal hypertension may be an early complication	OLT if ESLD; UDCA in selected cases
ADPKD	1:1,000	*PKD1* and *PKD2* (polycystin 1 and 2)	DPM, biliary microhamartomas and cysts	Renal failure, cyst complications (mass effect, hemorrage, infection, rupture), preserved liver function; severe hepatic cystic disease with pregnancy and estrogen therapy	Cyst aspiration and sclerosis; cyst fenestration; liver resection; OLT if complications; combined liver-kidney Tx if renal failure
ARPKD	1:20–40,000	*PKHD1* (fibrocystin)	DPM, biliary microhamartomas and cysts	Renal failure, recurrent cholangitis, portal hypertension	OLT if complications; combined liver-kidney Tx if renal failure
ADPLD	Nearly 50% of PLD cases	*PRKCSH* (hepatocystin) and *SEC63* (a endoplasmic reticulum translocator)	DPM, biliary microhamartomas and cysts	Similar to ADPKD except for renal involvement	Similar to ADPKD
Caroli	Rare	*PKHD1* in some cases	DPM and cystic dilatation of the intrahepatic bile ducts	Recurrent cholangitis, cyst complications, cholangiocarcinoma (7%)	Partial hepatectomy in segmental forms; OLT if complications
CHF	Rare	*MPI* (phosphomannose isomerase) or *PKHD1* in some cases	DPM and billiary microhamartomas, surrounded by a dense fibrous stroma	Portal hypertension, recurrent cholangitis, cholangiocarcinoma (rare)	OLT in complicated cases

Table. Genetic or developmental diseases, that lead to an impair bile duct biology, causing progressive cholangiopathies. ADPKD, autosomal dominant polycystic kidney disease; ADPLD, autosomal dominant polycystic liver disease; AGS, Alagille syndrome; ARPKD, autosomal recessive polycystic kidney disease; CF, cystic fibrosis; CHF, congenital hepatic fibrosis; DPM, ductal plate malformation; ESLD, end-stage liver disease; MDR3, multidrug resistance 3; OLT, orthotopic liver transplant; UDCA, ursodeoxycholic acid; Tx, transplant. (From Strazzabosco et al. 2005)

2.3 Pathophysiology of cholangiopathy in Cystic Fibrosis.

Cystic Fibrosis (CF) is the most common lethal autosomal recessive disease in Caucasians, affecting 1 in every 3000 live births. CF is a systemic disorder that affects secretory epithelia in pancreas, lungs, sweat glands, Wolffian ducts, liver and gut. It is caused by mutations that occur in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [20, 29]. This gene, located on the long arm of chromosome 7, encodes a 1480 amino-acid residue polypeptide. All the mutations that disrupt CFTR are classified on 5 different classes. Class I mutations determine a premature transcription termination resulting in a non-functional truncated protein, or no expression. Class II are missense mutations (ΔF508-Cftr) that determine misfolded proteins and their consequent retention in the endoplasmic reticulum. Class III cause an impaired secretion of Cl^- because of an altered activation by ATP. Class IV determine a defective chloride transport across the membrane, and class V cause an abnormal splicing process that decreases the total amount of functional CFTR [30]. To date, more than 1500 mutations have been identified. The most common mutation is a deletion in exon 10 that causes the deletion of a phenylalanine, at position 508. This mutation, called ΔF 508 is present in the majority of patients with Cystic Fibrosis.

The primary cause of mortality and morbidity in CF is lung disease; as a consequence of chronic infections and inflammation the lung tissues are progressively damaged, eventually leading to respiratory failure [31].

However, about 30% of CF patients present, also, biochemical liver abnormalities and about 10% of these develop clinically significant liver disease and hepatobiliary complications characterized by a chronic cholangiopathy that can eventually evolve into sclerosing cholangitis and focal biliary cirrhosis. Few is known about the pathogenesis of this phenotype. However, the development of mouse models in the last decade allowed researchers to better address mechanisms involved in the pathogenesis of cystic fibrosis-associated liver disease (CFLD) [32].

In the liver, CFTR is expressed specifically in the apical membrane of cholangiocytes where, in response to stimulation by gastrointestinal hormones, is responsible for the fluid and electrolyte content of bile (paragraph 2.1). Thus, the liver disorder in CF impacts the secretory function of the biliary epithelium and leads to a thickened and inspissated bile [31, 33]. Ultrastructural investigation of cholangiocytes in CF patients revealed pathological changes (such as abnormal cell

shapes, necrosis and increased periductal collagen deposition) also in asymptomatic patients, clearly suggesting that the primary event that leads to liver disease is the injury of bile duct cells as a result of an increased susceptibility of biliary epithelium to damages [31, 34, 35].

Liver disease in patients with CF has been classically considered a consequence of the impaired bile flow and biliary alkalinization caused by the defective channel function of CFTR. This would cause a chronic cholestasis that predisposes the epithelium to damages caused by retention of cytotoxic compounds (bile acids) and xenobiotics. The resulting chronic sclerosis cholangiopathy, may then progress to focal biliary cirrhosis and eventually organ failure [1, 7, 27, 36]. The histological finding of bile plugs in the interlobular bile ducts of patients with cystic fibrosis was often used as proof of the pathogenetic relevance of reduced bile secretion. However, this finding is actually rare and jaundice is a very late and uncommon sign in patient with CF [35]. Furthermore, CFTR-defective mice do not spontaneously develop liver disease, unless under specific inbreeding conditions [37].

Blanco et al. showed that induction of colitis and portal endotoxemia via oral administration of dextran sodium sulfate (DSS), a protocol known to cause portal release of bacterial products, resulted in severe bile duct injury in homozygous Cftr-KO mice, characterized by inflammation and cholangiocyte proliferation, but not in heterozygous or wild-type littermate [38]. In this model, the biliary defect acquired with the CF background may influence the cell response to damage, so that injurious agents that would not result in progressive liver disease in wild type mice would cause organ damage in CF suggesting that, beside the genetic defects, other pathogenic co-factors must be playing a role in determining the clinical phenotype.

2.4 Biliary epithelium and innate immunity.

Because of its anatomical location, the liver is constantly exposed to gut-derived bacterial products, but in healthy conditions it is able to rapidly clear most of the portal endotoxins. To protect itself against enteric pathogens the biliary epithelium expresses different Toll-like receptors (TLRs) [39, 40]. TLRs belong to a family of receptors that can recognize and discriminate between specific patterns of microbial components (i.e. LPS, flagellin, DNA, RNA) [39-41]. Specifically, gene

expression of all nine isoforms of TLRs were detected in biliary cells and protein expression was confirmed for TLR 2,3,4 and 5 [40]. TLRs are all transmembrane molecules, and their signaling is mediated by association of their internal domains (TIR domains) with different combinations of adaptor molecules (MyD88, MAL, TRIF, TRAM) [39, 40, 42]. For example, signal transduction in response to LPS is mediated by TLR4 [39, 40]. Following stimulation by LPS, TLR4 activates an intracellular signaling cascade that ultimately stimulates the NF-κB-dependent expression of several cytokines and chemokines, as Tumor necrosis factor (TNFα), IL-1, IL-6, IL-12, IL-8, G-CSF, LIX, that trigger the inflammatory process (figure 4) [39-41, 43, 44]. However, in the healthy liver, the presence of microbial products coming from the intestine does not elicit an inflammatory reaction, because a fine regulation of TLR signals maintains a safe level of "tolerance" [39, 40, 45].

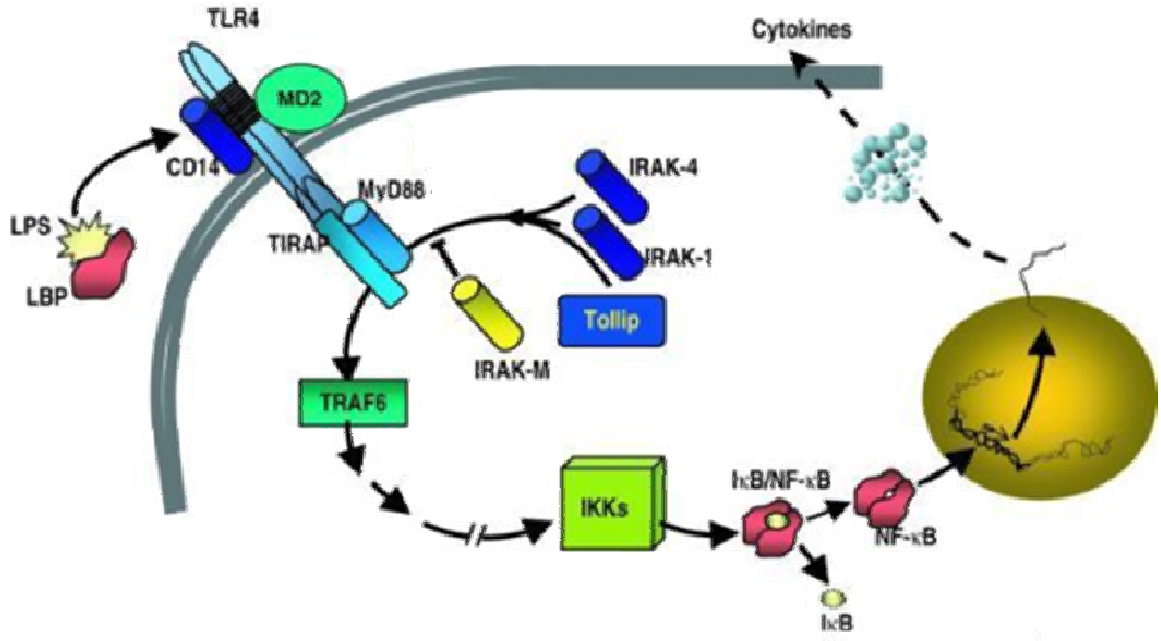


Figure 4. Cell recognition of lipopolysaccharide (LPS). **LBP, lipopolysaccharide binding protein; TLR4, Toll-like receptor 4; IRAK, IL-1 receptor-associated kinase; Tollip, Toll interacting protein; MD2, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; TIRAP, TIR domain-containing adapter protein (also known as MYD88 adaptor-like protein); TRAF6, TNF receptor-associated factor-6; IKKs, IκB kinases; NF-κB, nuclear factor kappa B; IκB, kappa B inhibitor. (From Villar et al, 2004)**

Tolerance to endotoxins is achieved through several mechanisms, including down-regulation of TLRs expression, down-regulation of the co-receptor MD-2 [46], and up-regulation of the expression of negative regulators of the TLR4 pathway [39, 40].

In the last decade, the innate immunity system in cystic fibrosis has generated much interest since it plays a pathogenic role in acute and chronic inflammatory state in CF patients and animal models [25, 47]. In fact, growing body of evidence suggest that defective CFTR affects several component of innate immunity. Studies of CF lung disease indicate that altered airway epithelial innate immune functions are responsible for exaggerated and ineffective inflammatory responses that fail to clear pulmonary pathogens [48]. For example, studies on cystic fibrosis fetuses have detected an endogenous activation of NF-κB and recruitment of leukocytes in tracheal explants [49]. In line with this, studies *in vitro* using epithelial CF cell lines from patients or CF mouse models confirmed an increased NF-κB signaling and the involvement of several pro-inflammatory pathways [50-58].

Derangement of epithelial immunity is becoming an important issue for many CF-associated disorders, as CFLD. Our recent studies demonstrated that liver disease in Cystic Fibrosis is the result of an altered TLR4-dependend innate immunity in CFTR-defective biliary epithelium that generates an exaggerated inflammatory response to intercurring events. Using the dextran sodium sulfate (DSS)-induced colitis, that allow bacterial components to be delivered from the gut to the liver, we showed that Cftr-KO mice developed a strong inflammatory cholangiopathy, characterized by the expansion of the ductular reactive components and portal inflammatory reaction with extensive infiltration of mononuclear cells, on the contrary of WT mice that showed no liver toxicity under the same experimental conditions (Figure 5) [59].

Moreover, restoration of biliary secretion with nor-UDCA*,* an analog of the therapeutic bile acid UDCA, previously shown by us to stimulate bile flow and biliary HCO3- output in Cftr-KO mice, did not reduce the biliary damage or the inflammatory infiltrate in DSS-treated Cftr-KO mice, indicating that the main pathogenic factors involved in determining liver damage under our experimental conditions was not the impaired ion secretion and reduced bile flow, but rather an increased susceptibility of CFTR defective biliary epithelium to endotoxin-mediated injury. Additional *in vitro* studies indicated that this phenomenon was due to an altered innate immune response of CFTR-defective biliary epithelial cells, characterized by an exaggerated TLR4-dependent activation of NF-κB, accompanied by an elevated LPS-induced secretory profile of inflammatory cytokines, compared to controls [59].

This novel hypothesis paves the way for new approaches to the treatment of CF liver disease and suggests that rational therapeutic approach to CFLD should be to target the inflammatory process and the TLR signaling pathway.

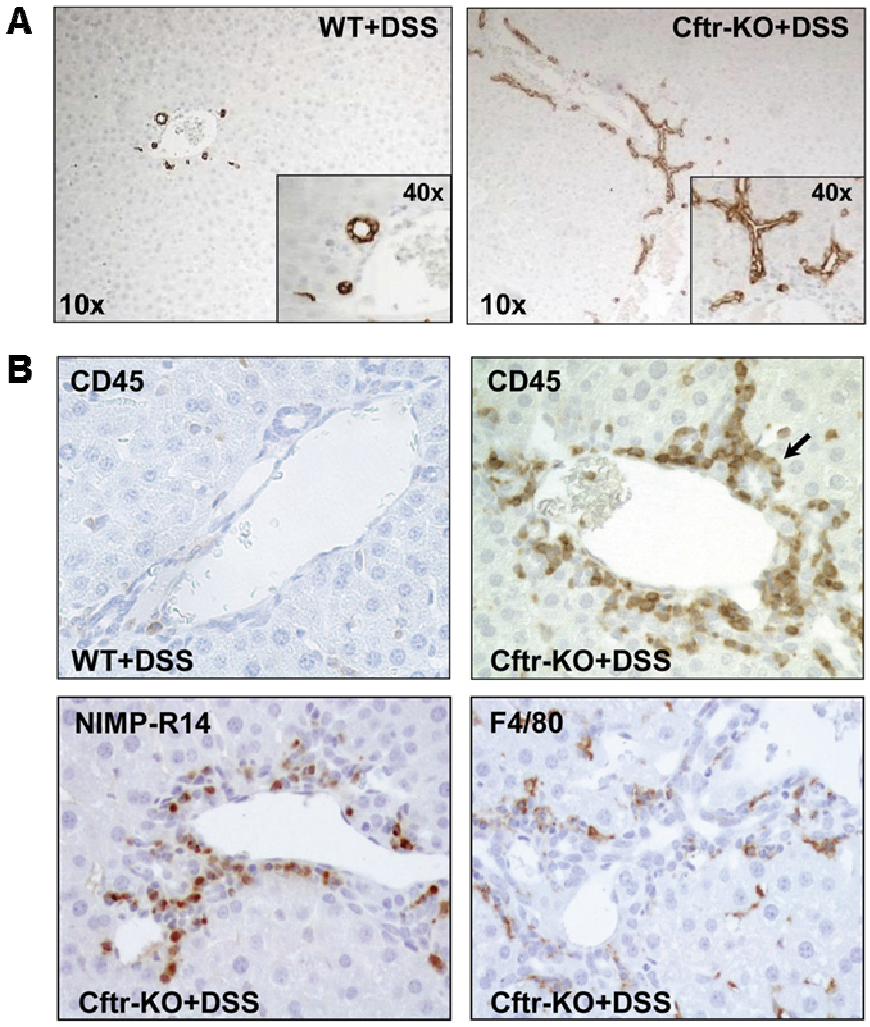


Figure 5. Characterization of DSS-colitis model. (A) CK-19 immunolabelling in liver tissue from wild type and Cftr-KO treated with DSS. Cftr-KO but not wild type mice show liver damage characterized by bile duct proliferation and inflammation. (B) Characterization of portal inflammation in CFTR-KO mice. (CD45, leukocytes marker; NIMP-R14, neutrophil marker; F4/80, macrophage marker).

2.5 Nuclear receptors: integration of metabolism and inflammation.

Nuclear receptors (NRs) constitute a superfamily of ligand-dependent transcription factors that, by controlling the transcriptional activity of several genes, exert a variety of cellular functions, including cellular homeostasis, lipid and carbohydrates metabolism, apoptosis, tissue repair, cell proliferation and

differentiation [60-62]. All members of this family share two conserved structures that are important for their functions: a central DNA-binding domain and a carboxy-terminal ligand-binding domain. The latter domain is essential to confer specificity to each receptor and to mediate ligand-regulated transcriptional activation and repression functions, contributing also, in most cases, to receptor homodimerization or heterodimerization [63].

Three broad classes of NRs have been described. The first class contains the prototypical ligand-driven receptors activated by a wide range of steroid compunds and hormones, mediating most of the biological effects of glucocorticoids, mineralcorticoids, oestrogens, progestins and androgens. These receptors, such as glucocorticoid receptor (GR), retinoic acid receptor (RAR) and vitamin D receptor (VDR) are usually expressed in the cytoplasm and, after binding to a specific ligand, they activate and translocate into the nucleus to control the transcription of specific target genes. The second subset comprises the so called 'orphan receptors', for which regulatory ligands have not been identified. The third class is represented by the 'adopted' orphan receptors, whose naturally ligands were identified long after their discovery. This subset of receptors comprises a broad range of metabolite-activated transcription factors, mainly recognized by retinoic acid, fatty acid metabolites and oxysterols [64, 65].

An important feature of most nuclear receptors is their ability to form heterodimers with the retinoic X receptor (RXR), an obligate step to perform their transcriptional functions. In the absence of specific activating ligands, most of these RXR heterodimers are bound to DNA, complexed with co-repressors, able to repress target gene expression. Upon ligand binding, conformational changes of the receptor allow co-repressors to be rapidly exchanged with co-activators and the activation of target gene transcription [64, 66]. Examples of receptors included in this class are the farnesoid X receptor (FXR), the liver X receptors (LXRs) and the peroxisome proliferator-activated receptors (PPARs). This positive/negative control at the transcription level is usually referred to as the *transactivation* mechanism [66-68].

Different NRs, in particular VDR, LXRs and PPARs, appears to be able to control inflammatory responses by regulating the expression of pattern recognition receptors, signal transduction proteins, effector molecules, and negative regulators. The mechanism by which LXRs and PPARs activates/inhibits the expression of inflammation-related genes is different from the classic transactivation pathway

illustrated above, and is called *transrepression* (figure 6 summarize all the mechanisms discussed).

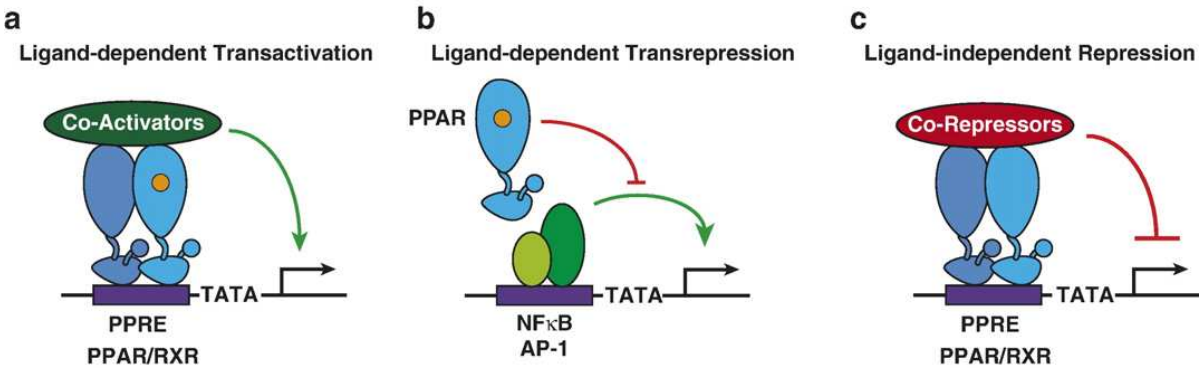


Figure 6. Transcriptional activities of the peroxisome proliferator-activated receptors. PPARs can both activate and inhibit gene expression. (a) Ligand-dependent transactivation. PPARs activate transcription in a ligand-dependent manner by binding directly to specific PPAR-response elements (PPRE) in target genes as heterodimers with RXR. Binding of agonists ligand leads to the recruitment of coactivator complexes that modify chromatin structure and facilitate assembly of the general transcriptional machinery to the promoter. (b) Ligand-dependent transrepression. PPARs repress transcription in a ligand-dependent manner by antagonizing the actions of other transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). (c) Ligand-independent repression. PPARs bind to response elements in the absence of ligand and recruit corepressor complexes that mediate active repression. This complex antagonizes the actions of coactivators and maintains genes in a repressed state in the absence of ligand. (From Ricote and Glass, 2007).

Transrepression activity is based on the direct association ('tethering') of NRs with master regulators of inflammation, such as NF-κB, activator protein 1(AP-1), nuclear factor of activated T cells (NFAT), or a direct sequence-specific DNA binding that regulate the transcription of inhibitors of the inflammatory cascade, as for the NF-κB inhibitor IκB-α (figure 7) [64, 69-72].

The ability of these receptors to integrate metabolic and inflammatory pathways makes them important targets for the study of human metabolic diseases, such as atherosclerosis and type 2 diabetes, and for the management of several inflammatory conditions [64, 73, 74].

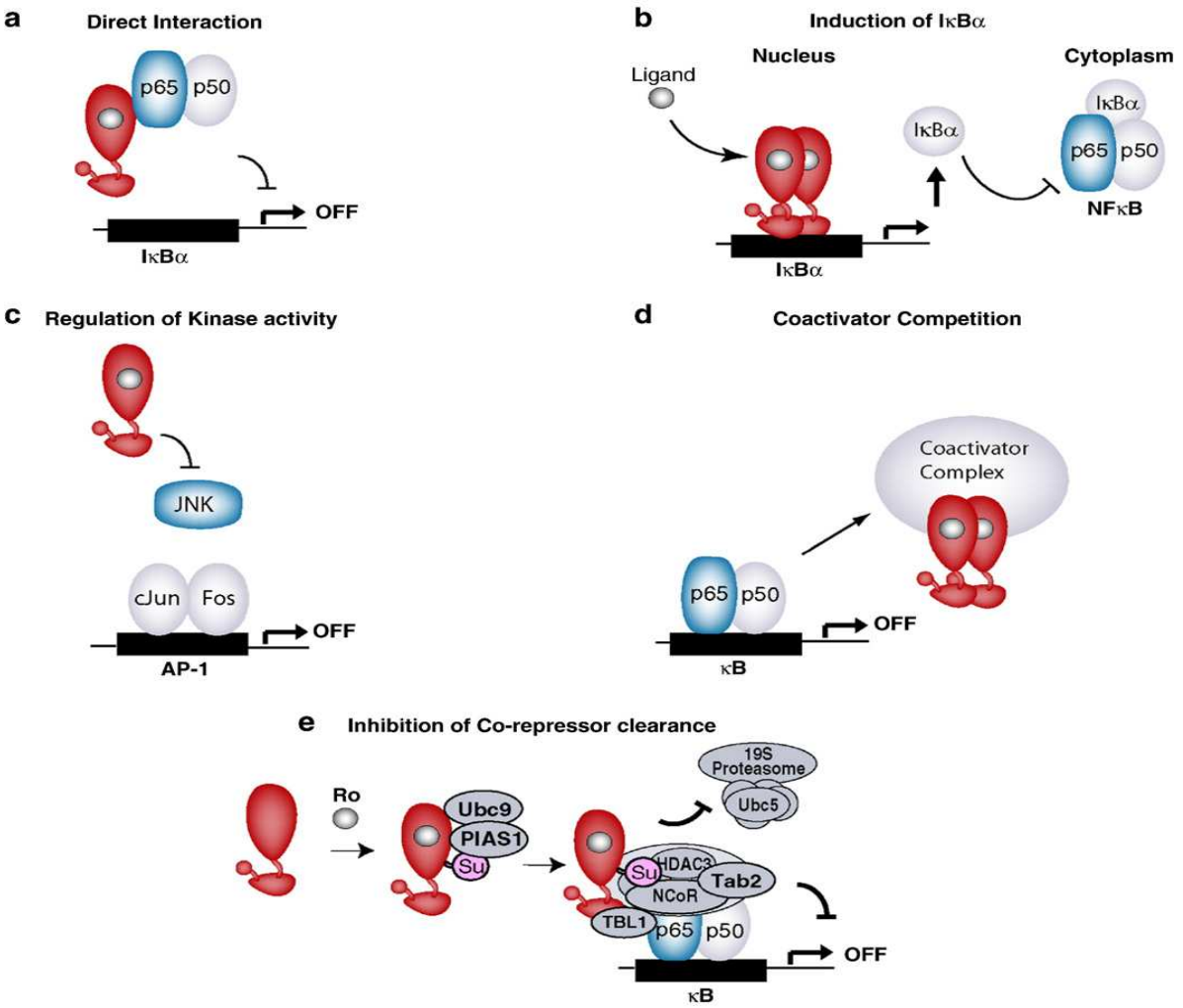


Figure 7. Mechanisms of PPAR-mediated transrepression. (a) Direct interaction between PPAR and p65 subunit. (b) Induction of IκBα expression. (c) Activation of PPAR inhibits c-Jun N-terminal kinase (JNK) MAPK activity. (d) Competition for a limiting pool of coactivators, such as CREB-binding protein. (e) Corepressor-dependent model of transrepression. PPARγ can inhibit inflammatory responses by blocking the signal-dependent clearance of NCoR corepressor complexes. LPS stimulation promotes the ubiquitin-dependent proteosomal degradation of NCoR corepressor complexes. In the presence of ligand, PPARγ is sumoylated and targeted to the NCoR corepressor complexes on gene promoters, preventing the clearance of these complexes. AP-1, activator protein-1; NCoR, nuclear-receptor co-repressor complexes; HDAC3, histone deacetylase 3; TBL1, transducin-β-like 1, TBL1; TBL1-related protein, TBLR1; PIAS1, protein inhibitor of activated STAT1; Tab2, TAK1-binding proteins; SUMO conjugate on target lysine instead of cysteine (From Ricote et al, 2007).

2.5.1 Peroxisome-proliferator-activator receptors.

The PPAR subfamily is composed by three different isoforms: PPAR-α, PPAR-β/δ and PPAR-γ. Several studies identified the natural occurring ligands of these receptors to be mono- and poly-unsaturated fatty acids (MUFAs and PUFAs), eicosanoids components of lipoproteins, and derivatives of linoleic acids. The

relative tissue quantity of endogenous ligands for PPARs is expected to modulate the activation state of a given PPAR subtype in a given cell. Their expression has been found in vascular and immunological cell types, such as monocytes and macrophages, endothelial cells, lymphocytes, dendritic cells, epithelial cells, and also different types of liver cells [75, 76].

All the three PPAR isoforms may negatively regulate inflammation and immune responses initiated by activation of TLRs in immune cells, such as monocytes/macrophages, endothelial cells, adipose tissue and epithelial cells. This negative control occurs through different mechanisms, including direct interactions with NF-κB and AP-1 subunits, modulation of kinase activities, competitive binding for co-activators and stabilization of co-repressors (as described in 2.5 in figure 7) [72, 75-79]. Among all the PPARs, the γ isoform (PPAR-γ) has been described to modulate inflammatory responses in several systems, by controlling the production of pro-inflammatory cytokines induced by LPS and interferon gamma (IFN-γ) through the inhibition of NF-κB, STAT1 and AP-1 (figure 8) [80-82].

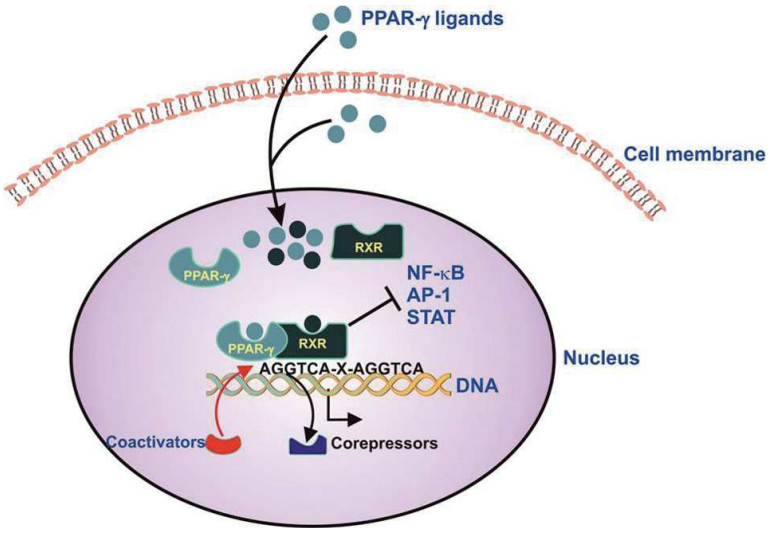


Figure 8. PPAR-γ-mediated gene regulation. The PPAR-γ/RXR heterodimer binds to PPAR response elements (PPRE). Ligand-activated heterodimer recruits coactivator to and/or derecruits corepressor molecules from the transcriptional start site, leading to increased transcription of selected genes. Activation of PPAR-γ can also inhibit the expression of genes regulated by specific proinflammatory transcriptin factors, such as NF-κB, AP-1 and STAT. (From Green et al, 2011).

The identification of endogenous ligands able to activate PPAR-γ is still ongoing, but several studies have already described different ω-6-and ω-3 polyunsaturated fatty acids (PUFAs) as potent PPAR-γ activators [83].

Most of these ligands belong to the eicosanoids family. Eicosanoids are lipid mediators derived from precursors present in the cell membrane, such as arachidonic acid (AA) and linoleic acid (LA), or from the intermediate metabolite docosahexaenoic acid (DHA), through a complex metabolic cascade, as illustrated in figure 9.

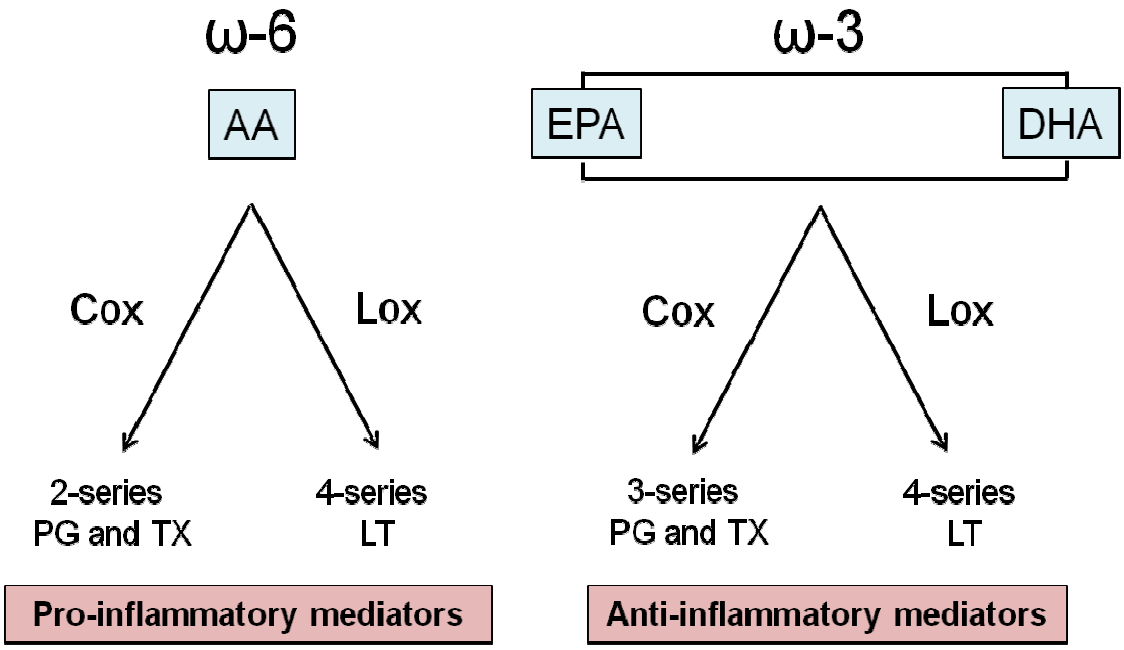


Figure 9. Formation of bioactive lipid mediators. The precursor ω-6 fatty acid arachidonic acid (AA) and the ω-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can generate a range of pro-inflammatory and anti-inflammatory lipid mediators via actions of several enzymes, mainly lipoxygenase (LOX) and cyclooxygenase (COX). AA forms a range of proinflammatory mediators, such as the 2-series prostaglandins (PG) and thromboxane (TX), via COX, and the 4-series leukotrienes (LT), via multiple LOX action. An array of anti-inflammatory mediators, including the 3-series PG and TX, via COX, and the 4-series LT, via LOX, is generated from EPA and DHA.

This metabolic pathway is performed through the action of two important enzymes, the lipoxygenase and cyclooxygenase, whose expression levels are modulated in a cell-specific and stimulus specific manners.

All the bioactive lipids generated in this cascade can have pro-inflammatory properties, as well as anti-inflammatory effects, depending on the lipid derivative and the cell type. More in depth, the ω-6 AA is a precursor of several pro-inflammatory eicosanoids and leukotrienes and few pro- and anti-inflammatory prostaglandins, while the ω-3 PUFAs, mainly DHA, are thought to be the main source of several anti-inflammatory eicosanoids [84-87].

In addition to these naturally occurring activators, several synthetic ligands have been developed, some of them already in use for clinic management of

dyslipidemia, such as fibrates, or type-2 diabetes, such as the thiazolidine-2,4-diones (TZDs) rosiglitazone and pioglitazone, that function as strong PPAR-γ activators [88].

3 AIM OF THE STUDY

Cystic Fibrosis (CF) is a common genetic disease, caused by mutations in CFTR, a protein that regulates fluid secretion in a number of organs. About the 30% of CF patients are affected by liver disease (CFLD), a condition that can compromise survival and quality of life of these patients. Unfortunately a cure is not yet available.

Defective CFTR function alters bile flow and biliary alkalinization at the cholangiocyte level, and this defect was initially thought to cause liver damage. However, we have recently demonstrated that in addition to cholestasis CFLD may result from an altered TLR4/NF-κB-dependent signalling immune leading to loss of endotoxin tolerance and exaggerated inflammatory responses of CFTR-defective biliary epithelium to intercurring events.

Currently, the therapy of CFLD is limited to the administration of bile acids that stimulate the choleretic function of the biliary epithelium. However, we have shown that the recovery of bile secretion is not sufficient to ameliorate liver damage in an experimental model in which CFTR-defective liver cells are exposed to endotoxins. Thus, we suggest that the correct therapeutic strategy to treat CFLD should target the biliary epithelium inflammatory response by targeting the TLR4/NF-κB pathway.

Recent evidences indicate that Nuclear Receptors (NRs) play an important role in the modulation of innate inflammatory responses initiated by activation of TLRs. Thus, NRs represent interesting therapeutic targets in cystic fibrosis. In this study we tested the hypothesis that *activation of nuclear receptors might represent a novel therapeutic strategy to control the TLR4/NF-κB-driven inflammatory response in CFTR-defective biliary epithelium.* This thesis will address the following specific aims:

1) study the specific expression pattern of NRs expressed in WT and CFTR-defective cholangiocytes and their mechanism of regulation;

2) study the role of NRs in the regulation of innate immune responses in Cftr-KO cholangiocytes.

4 MATERIALS AND METHODS

4.1 Isolation and culture of primary cholangiocytes from WT and Cftr-KO mice.

Mouse cholangiocytes were isolated from wild-type and C57BL/6J-Cftr^{tm1Unc} mice [47, 89, 90], a widely used rodent model of Cystic Fibrosis. In this mouse model the gene encoding for Cystic Fibrosis Transmembrane Regulator is knocked-out leading to a complete “loss of function”, model in which any CFTR mRNA is not detectable.

Briefly, mice were anaesthetized by isoflurane, the portal vein was cannulated and the liver perfused *in situ* with 250 ml of cold-ringer-HCO_3 buffer (KRB) for 5 minutes. After liver perfusion, 2-3 ml of liquid trypan blue agar was injected into the portal vein. Interlobular and primary branches of portal vein, hepatic artery and bile ducts were exposed by removing surface hepatocytes by forceps. Under a dissecting microscope, bile ducts were microdissected by using the blue agar-filled portal vein and hepatic artery for reference. Further microdissection is performed under higher magnification to remove residual hepatocytes, component of the portal veins and hepatic arteries, and excess of connective tissues. The isolated bile ducts were microdissected again, and then plated into college gel. In one-two weeks biliary cells usually start to outgrow. When subconfluent, they are passaged and plated again on the top of rat-tail collagen, cultured on an enriched medium (La Russo et al.) slightly modified by increasing fetal bovine serum (FBS) to 10%. The biliary phenotype and maintenance of the normal polarity was confirmed by staining for cytokeratin 7, for acetylated α-tubulin and by measuring transepithelial resistance.

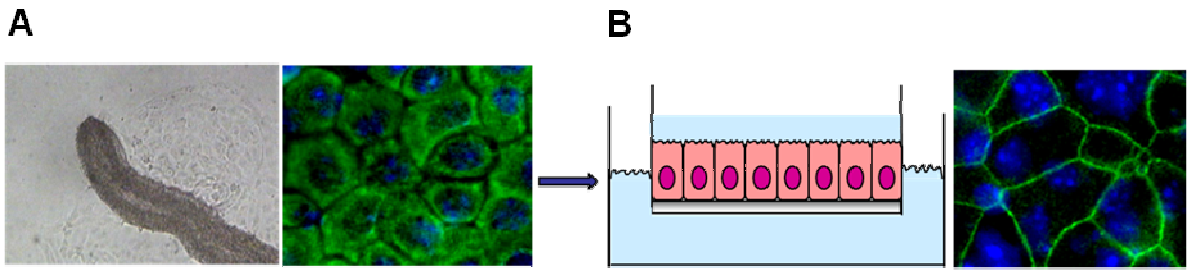


Figure 10. Isolation of primary cholangiocytes from wild type and Cftr-KO mice. (A) The bile duct was isolated and microdissected. Cells were plated and stained for cholangiocytes marker (CK19/DAPI). (B)

These cells were then cultured on transwell inserts with a semi-permeable membrane and stained for ZO-1/DAPI.

Enriched culture medium: epidermal growth factor, dexamethasone, triiodothyronine, EDTA, collagenase IV, forskolin, insulin, were purchased by Sigma-Aldrich (St Louis, MO). Minimum essential medium, Dulbecco's minimal essential medium, Ham's F12 medium, fetal bovine serum, minimum essential medium nonessential amino acids solution, minimum essential medium vitamin solutions, glyceryl monostearate, chemically defined lipid concentrate, soybean trypsin inhibitor, penicillin/streptomycin, gentamycin, trypsin/EDTA, and glutamine were purchased from Invitrogen (Carlsbad, CA).

4.2 Endotoxin, pioglitazone and GW9662 treatments of WT and Cftr-KO cholangiocytes

WT and Cftr-KO cholangiocytes were cultured in transwell inserts with a semi permeable membrane (BD Falcon™); under these conditions they behave as a polarized monolayer that can be accessed through both the apical and basolateral domain separately. Once confluent and with a transepithelial resistance >1000 Ω/cm^2 (Millicell-ERS System; Millipore Co, Bedford, MA), WT and Cftr-KO cholangiocytes were exposed to LPS (100 ng/ml) (Sigma-Aldrich), pioglitazone (10-50 µM) (Sigma-Aldrich) and GW9662 (10 µM) (Sigma-Aldrich). Pioglitazone, potent and selective PPAR-γ agonist, was administered two hours before and during LPS stimulation. GW9662, irreversible PPAR-γ antagonist, was administered only one hour before pioglitazone treatment.

Basolateral and apical overnatants were collected and then RNA and cell proteins were purified from the cultured cells.

4.3 Real-Time PCR Array

Total RNA was isolated using Trizol reagent (Invitrogen) and quantified with 'Nanodrop 1000' spectrometer (Thermo Scientific) with an absorbance of 270 nm. 1 µg RNA was converted into PCR template with the High capacity cDNA reverse Transcription kit (Applied Biosystem). 50 ng of total PCR template was then

combined with the TaqMan® Universal PCR Master Mix (Applied Biosystem), and real-time PCR was performed using an ABI 7500 termalcycler (Applied Biosystem). TaqMan probes (dual-labeled with 5'-FAM and 3'-TAMRA) and primers for all genes analyzed in this study are designed to cover the exons terminals in order to exclude genomic DNA co-amplification (Applied Biosystem).

Data were analyzed with the ΔΔCt method with normalization of the raw data to Hypoxanthinephophoribosyltransferase (HPRT) housekeeping gene.

4.4 Determination of cytokine secretion

Culture supernatant was used for the analysis of 32 mouse chemokines and cytokines by using the Milipore's MILLIPLEX™ mouse Cytokines/Chemokines kit coupled with BioPlex Luminex platform following manufacture instructions. Luminex® uses proprietary techniques to internally colorcode microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites the fluorescent dye PE. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals.

4.5 Western blotting

Nuclear and cytosolic fractions were obtained by using the NE-PER® extraction kit (Pierce, Rockford, IL) following manufacture instructions. Total cell lysate was extracted by using the CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich), with fresh protease and phosphatase inhibitor cocktails.

For all of the experiments protein lysates were loaded in 4-12% SDS-polyacrylammide gel and run at constant voltage of 200mV with 30 mA. Then

proteins were transferred on a nitrocellulose membrane using constant voltage of 30 mV with 170 mA. To verify the efficiency of transfer all the membranes was stained with Ponceau-Red (Sigma-Aldrich). To block all the non-specific link sites all the membranes were incubated with 0.1% Tween 20 in PBS containing 5% dry milk (Euroclone).

PPAR-γ protein expression was assessed by Western Blotting 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 TBS-tween)

NF-κB nuclear translocation was assessed by Western Blotting in nuclear protein fractions of WT and Cftr-KO cholangiocytes, lysed before and 6 hours after LPS stimulation, using an antibody against p65 (Santa Cruz Biotechnology, Rabbit, 1:1000, 5% milk in TBS-tween), the component of heterodimeric complex p50/p65 that translocates to the nucleus to regulate the expression of cytokines. Antibody against Histone H3 (Sigma, Rabbit, 1:2000, 5% milk in TBS-tween) was used to normalized for nuclear protein content.

IκB-α was investigates on total lysates of WT an Cftr-KO cholangiocytes, treated as described above, using antibodies against total IkB-α (Cell Signaling, Rabbit, 1:1000, 5% Bovine serum Albumin in TBS-tween) and phospho-IkB-α (Cell Signaling, Mouse, 1:1000, 5% Milk in TBS-tween)

4.6 PPAR-γ DNA-binding assay

The capacity of PPAR-γ to bind DNA was measured by the TransAM™ PPAR-γ Transcription Factor Assay Kit (Active Motif). The TransAM format is a high-throughput assay that measure transcription factor binding to a consensus-binding site. TransAM Kit contain a 96-stripwell plate to which the consensus-binding site oligonucleotide that contains a PPRE (5’ -AACTAGGTCAAAGGTCA -3’) has been immobilized. Nuclear extract containing PPAR-γ is added to each well and the transcription factor binds to this oligonucleotide.

4.7 NF-κB transcriptional activity

Transcriptional activation of NF-κB was assessed using the Cignal NF-κB Reporter luciferase Kit (SaBiosciences-Qiagen) which is designed to monitor the activity of NF-κB-regulated signal transduction pathways in cultured cells. The kit contains transfection-ready NF-κB reporter construct (a mixture of inducible NF-κB-responsive firefly luciferase construct and constitutively expressing renilla luciferase construct), as well as positive and negative controls. The NF-κB-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the NF-κB transcriptional response elements (TRE).

Cells were plated in 24 well plates and 24 hours later (50%–70% confluent) were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer instructions. Each transfection contained 200 ng of NF-κB reporter or positive or negative control. After 24 hours cells were treated as described above.

The activity of firefly and renilla luciferases were measured with the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) following manufacture instructions.

4.8 Lipidomic analysis by Gas chromatography

Extraction of Total Lipids. About $10x10^6$ cells were pelleted (dry weight was measured and used for normalization) and 3 ml ice-cold chloroform/methanol (2:1, v/v) containing 0.01% BHT (Butylated hydroxytoluene, Sigma) was added to the cell pellet. 50 µl of TG, DAG, and FFA internal standards were then added. To separate organic and aqueous phases were added 1 ml of iced-cold chloroform and 1 ml of iced cold H_2O. After vortexing for thirty seconds, samples were centrifuged for 15 min at 4^oC, 4000 rpm, and the bottom (organic phase chloroform) was collected. The extracted lipid samples were dried with a gentle flow of nitrogen.

TLC Separation of NEFA, TG, DAG, and phospholipids. Samples were reconstituted with chloroform and loaded onto TLC plates and TG, DAG, and FFA were loaded as reference compounds. TLC plates were, then, developed with

hexane: diethyl ether: acetic acid (60:13:0.65), or (80:20:1). Total FFA spots were scraped and directly esterified with sulfuric acid in MeOH.

Derivitization to Fatty Acid Methyl Esters (FAME) for GC-MS. 2ml of 2% H2SO4:MeOH were added to screw cap glass tubes containing FA standards or scaped silica gel. The tubes were purged with N2, sealed with vortex, and transferred to a heating block for 70 min at 80 ℃. The reaction is terminated by the addition of 500 ul of 1 N sodium hydroxide. Total FAME were extracted with heptanes (0.5ml x 3). Heptane layers were combined for GC-MS analysis.

4.9 Statistical analysis.

Results are shown as mean ± SD. Statistical comparisons were made using Student's t test, with analysis of variance where appropriate. All statistical analysis were analyzed with the SPSS 13.0 (statistical package for social science 2013). P values <0,05 were considered significant.

5 RESULTS

5.1 Biliary epithelial cells express several subsets of NRs.

In the liver NRs are expressed by different cellular subtypes, such as hepatocytes, stellate cells and macrophages, each displaying a specific expression pattern. To profile the expression of NRs in the biliary epithelium we performed a real time PCR analysis of NRs gene expression in primary cholangiocytes isolated from wild-type and CFTR-KO mice, cultured on transwell inserts and grown as a polarized monolayers. Gene expression of multiple NRs was detected (figure 11A) including peroxisome proliferator activated receptor (PPAR) subgroup α, γ and δ, liver X receptors (LXR) subgroup β, farnesoid X receptor (FXR) and vitamin D receptor (VDR). Among these transcripts, PPAR-δ, LXR-β and FXR where expressed at higher levels, as compared to PPAR-α and VDR; Their levels were comparable between WT and Cftr-KO cells.

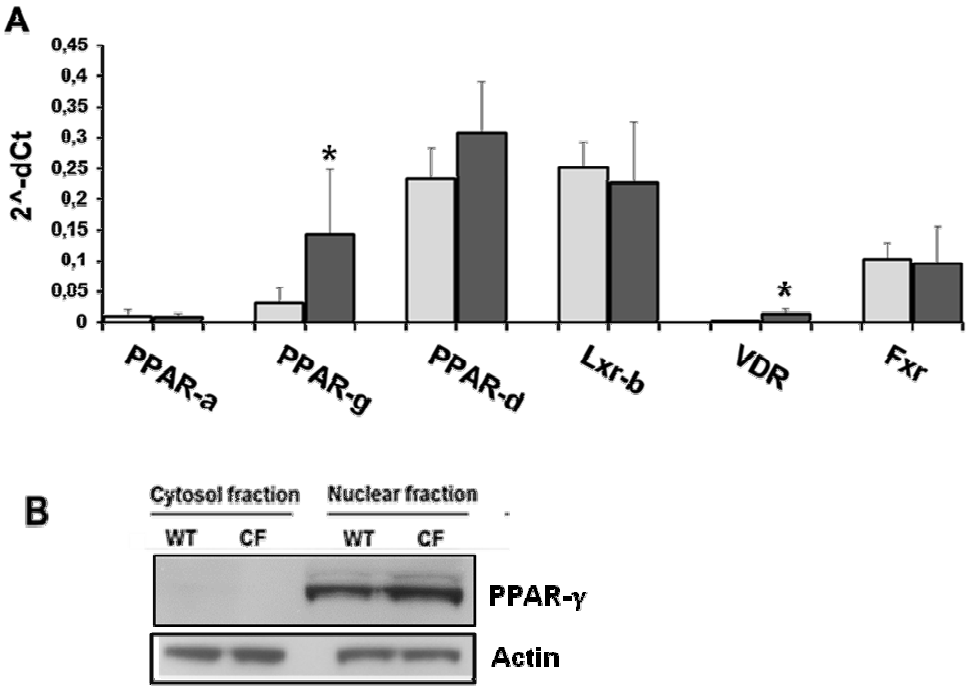


Figure 11. A) Real-time PCR analysis of nuclear receptors in WT and Cftr-KO cholangiocytes. Data were performed in duplicate in n=5 (WT) and n=6 (CF) different cell lines, normalized to HPRT housekeeping gene, and are expressed as 2^-ddCt. B) Western Blot in cytosolic and nuclear fractions using an antibody against PPAR-γ.

On the contrary, the expression of PPAR-γ was significantly increased in Cftr-KO cholangiocytes as compared to wild type cells. Increased expression of PPAR-γ in CF cells was confirmed also at the protein level, as shown by the western blot

analysis on cytosolic and nuclear protein fractions of wild type and Cftr-KO cells (figure 11B). Western blot analysis showed that in both WT and CF cells the localization of the receptor was mostly nuclear.

In the absence of activated ligands, PPAR receptors reside into the nucleus bound to the peroxisome proliferator response elements (PPREs), that consist of repeated DNA sequences, repressing the expression of target genes [64, 66, 71]. This receptor/DNA interaction occurs independently from ligands and is prerequisite of the transcriptional control. We tested the ability of the PPAR-γ to bind the PPAR response elements (PPRE) on the DNA, through an ELISA-based kit and, in proportion to the amount of the receptor present in the nuclear proteins pool, more binding activity was detected in CF cells respect to WT (figure 12), and was not influenced by the agonist pioglitazone as expected.

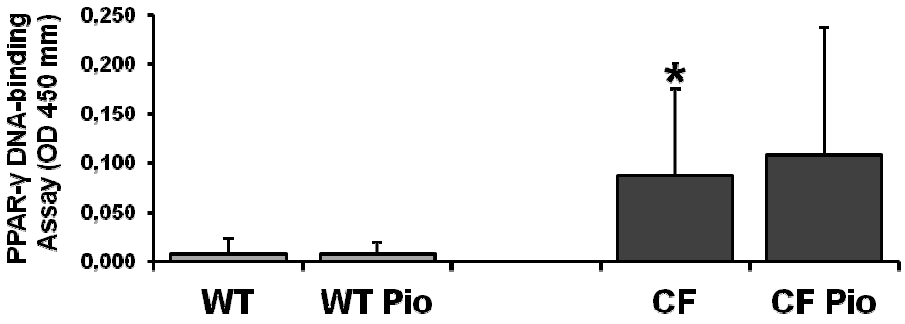


Figure 12. PPAR-γ DNA-binding assay, measuring the ability of the receptor to bind to specific peroxisome proliferator response elements on DNA (PPRE). Data were performed in n=5 experiments. *p<.05 vs WT.

This additional evidence indicated that nuclear PPAR-γ in CF cells was properly positioned on the DNA response sequences.

5.2 PPAR-γ target genes expression (transactivation pathway) in Cftr-KO cholangiocytes, is activated after stimulation with PPAR-γ ligand pioglitazone.

PPARs are constitutively bound to the DNA, repressing the transcription of specific genes. Upon arrival of activator ligands, these receptors are able to switch on the transcription of specific target genes that regulate several cellular functions [66]. The expression level of these signature genes is considered as indicative of the receptor activation rate.

To investigate whether the increased expression of PPAR-γ in CF as respect to WT-cells is associated to an higher PPAR-γ transcriptional activity, we studied by RT-PCR the gene expression of specific PPAR-γ target genes: acetyl-Coenzyme A synthase 1B (Acaa1b), angiopoietin-like 4 (Angptl4), monoglyceride lipase (Mgll), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2) [91]. Polarized wild type and Cftr-KO cholangiocytes were used for these experiments. Expression of PPAR-γ target genes in Cftr-KO cells was comparable to that of wild type cells (figure 13 A), indicating a comparable basal activity of PPAR-γ.

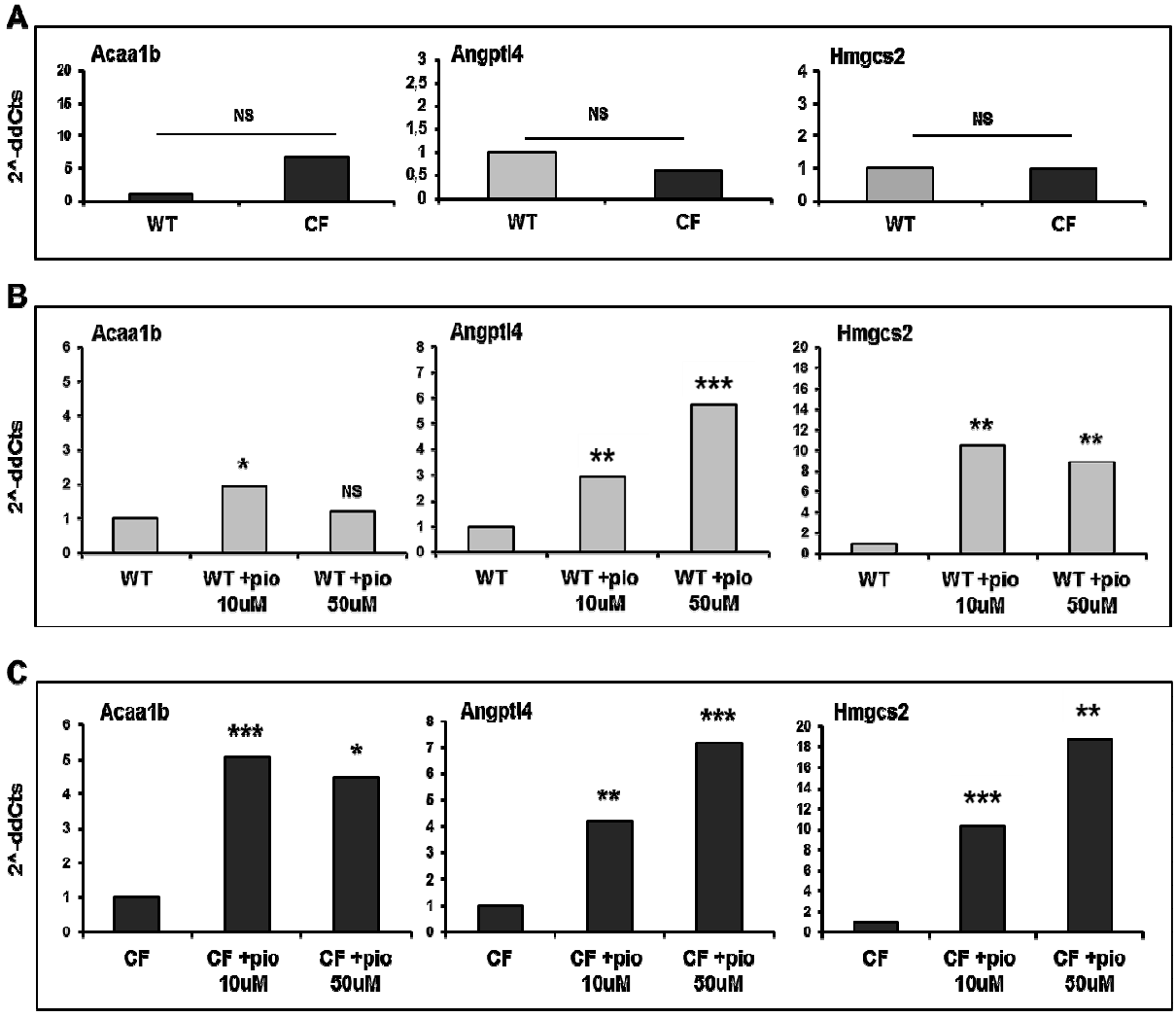


Figure 13. Real-time PCR analysis of PPAR-γ target genes in WT and Cftr-KO cholangiocytes before (panel A) and after treatment with 10-50 μM of the agonist Pioglitazone (panel B-C). Data were performed in duplicate in n=4-6 different experiments, normalized to HPRT housekeeping gene, and are expressed as 2^-ddCt to the respective internal control (WT in panel A, WT unstimulated in panel B, CF unstimulated in panel B). Acaa1b: acetyl-Coenzyme A acyltransferase 1B; Angptl4: angiopoietin-like 4; Hmgcs2: 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2. NS= not significant; *p<.05; **p<.01; ***p<.001. Internal variability of each group was calculated based on standard deviation of dCts and is reported in the following table:

	ctl	pio 10 µM	pio 50 µM	ctl	pio 10 µM	pio 50 µM
Acaa1b	0,98	1,43	1,03	3,07	3,9	2,71
Angptl4	1,32	1,11	0,63	0,8	1,45	1,03
Hmgcs2	0,61	1,08	0,94	0,83	0,72	1,38
	Wild type			**Cftr-KO**		

Treatment with the specific PPAR-γ agonist Pioglitazone (10 and 50 µM) significantly increased the expression of PPAR-γ target genes (figure 13 B-C), indicating that PPAR-γ receptor in both wild type and Cftr-KO cells can be activated by exogenous ligands. Consistent with the higher expression level of the receptor, the increase in PPAR-γ target genes stimulated by pioglitazone was likely higher in CF than in WT cholangiocytes.

5.3 CFTR-KO cholangiocytes show an imbalance between ω-3 and ω-6 poly-unsaturated fatty acids (PUFAs).

ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) are the precursors of bioactive lipids produced at the cell membrane level, some of which constitute the active form of PPAR-γ endogenous ligands. These fatty acids are able to modulate inflammation and immune processes, by producing pro- or anti-inflammatory mediators. The amount and type of these FA are finely regulated to guarantee a proper balance of the active lipids produced [84-87]. To understand if a decrease in endogenous activators limits PPAR-γ function in CF cells, we extracted total lipids from WT and Cftr-KO cholangiocytes and performed, by gas chromatography, a lipidomic analysis of the major ω-3 and ω-6 polyunsaturated fatty acids (PUFAs), involved in the production of PPAR-γ natural ligands. We detected an increase trend of arachidonic acid (AA) and a decrease trend of linoleic acid (LA) and docosahexaenoic acid (DHA) (figure 14 A) that resulted in an increased final ratio between arachidonic acid and docosahexaenoic acid (AA/DHA) and between arachidonic acid and linoleic acid (AA/LA) (figure 14 B) in CF cells compared to their controls.

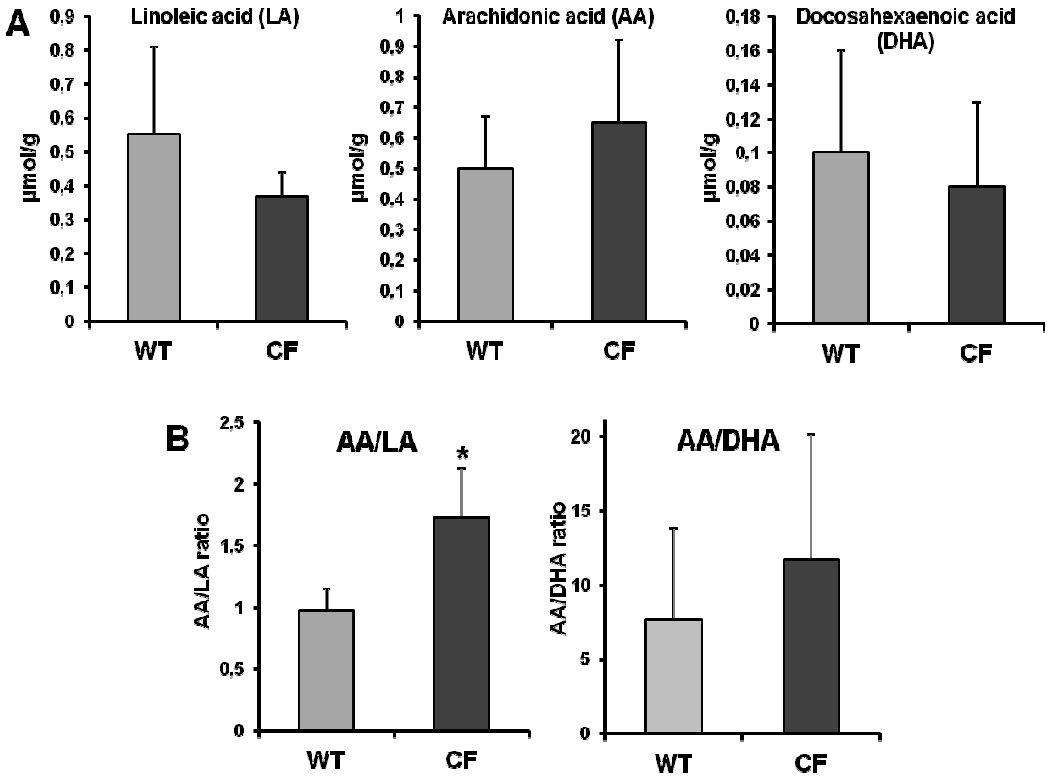


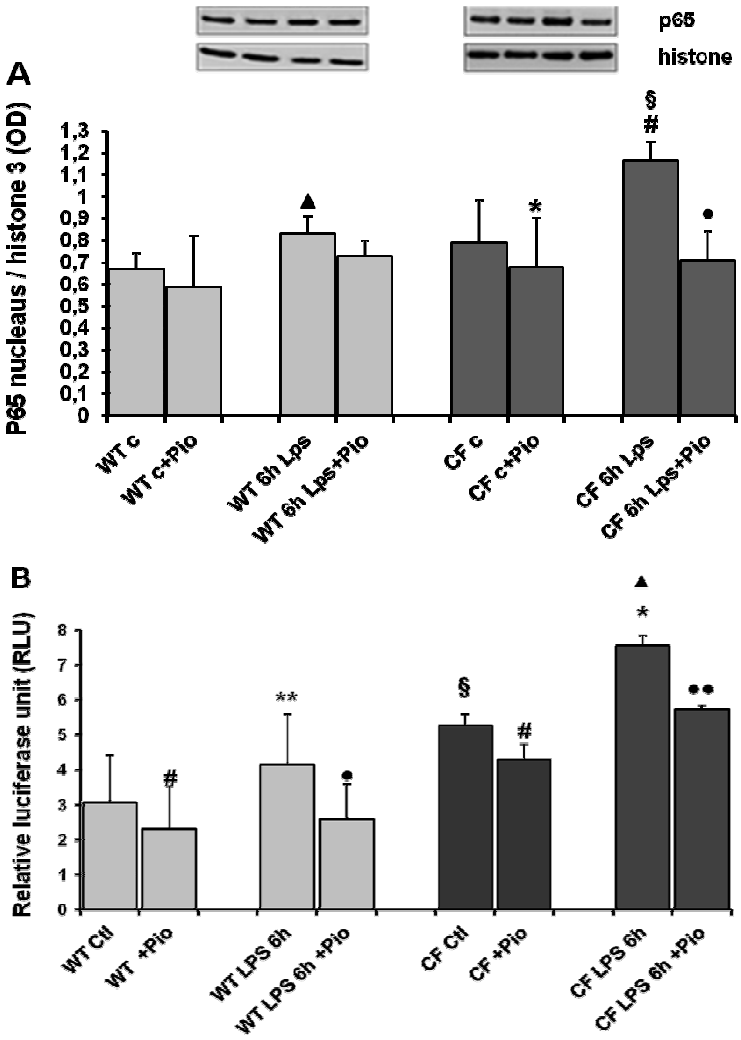
Figure 14 A-B. Lipidomic analysis of total lipids extraction from WT and CFTR-KO cholangiocytes. AA: arachidonic acid; DHA: docosahexaenoic acid; LA: linoleic acid. Data were performed in n=4 experiments, and expressed as micromoles/grams of total protein. *p<.01.

LA, the precursor of AA, is the main source of pro-inflammatory mediators, and was likely reduced in CF cells, while DHA, important precursor of anti-inflammatory eicosanoids that are able to activate PPAR-γ, was rather decreased. This findings indicate that there is a lack of endogenous activators of PPAR-γ in CF that may be responsible for the increased expression of PPAR-γ in CF biliary cells.

5.4 Pioglitazone inhibits LPS-induced NF-κB activation in Cftr-KO cholangiocytes.

We have shown that CFTR-defective cholangiocytes respond to LPS with an increased NF-κB activation, as compared wild type cells [59]. To understand whether activation of PPAR-γ may regulate the LPS-dependent inflammatory response in the biliary epithelium, we treated wild type and Cftr-KO cholangiocytes with pioglitazone (50 µM), LPS (100ng/ml) or their combination for 6 hours. NF-κB activity was assessed from the expression of p65/NF-κB in the nuclear protein fraction (using western blot), and by a luciferase-based NF-κB gene reporter, to

directly measure the transcriptional activity of NF-κB. Cftr-KO cholangiocytes responded to LPS with a significantly higher NF-κB nuclear translocation and transcriptional activity as respect to control cells. Moreover, in Cftr-KO cells, treatment with pioglitazone significantly inhibited NF-κB activation at both basal level and after LPS challenge, (Figure 15 A-B).



<u>Figure 15. (A) Western Blot in nuclear fractions</u> of WT and Cftr-KO cells before and after 6h of LPS (100 ng/ml) and Pioglitazone (50 μM) stimulation, using an antibody against p65. Data were performed in n=4 experiments. Bar graph represents optical density quantification of p65 in the nucleus normalized for histone 3. *p<.01 vs CF c; **p<.01 vs CF 6h Lps; ▲p<.05 vs WT c; #p<.01 vs CF c; §p<.01 vs WT 6h Lps. <u>(B) Luciferase-based NF-κB gene reporter</u> of WT and Cftr-KO cells before and after 6h of LPS (100 ng/ml) and Pioglitazone (50 μM) stimulation. Data were performed in n=4 experiments. Bar graph represent the ratio between the NF-κB-dependent expression of luciferase and the constitutive expression of renilla. *p<.01 vs CF c; **p<.001 vs WT ctl; #p<.01 vs ctl; §p<.05 vs WT ctl; •p<.05 vs Lps 6h; ••p<.01 vs Lps 6h; ▲p<.01 vs WT Lps 6h;

The weaker effect of pioglitazone in WT is again consistent with the lower expression of PPAR-γ in these cells.

5.5 The inhibitory effect of pioglitazone on NF-κB activity in CF cells is dependent on a direct activation of PPAR-γ.

Pioglitazone may have also receptor-independent effects [92]. To understand whether the inhibitory effect of pioglitazone on NF-κB activity in CF cells was dependent on a direct activation of PPAR-γ, we repeated the experiments in the presence of a specific PPAR-γ antagonist GW9662 [93]. Polarized CFTR-KO cholangiocytes were treated with LPS (100ng/ml) or with the combination of LPS and pioglitazone (50 µM), with and without a pretreatment of 1 hour of GW9662 (10µM). We found that pretreatment with the antagonist GW9662 significantly prevented the inhibitory effect of pioglitazone on the activation of NF-κB, as shown in figure 16, indicating that the anti-inflammatory property of pioglitazone depends in part on the receptor activation.

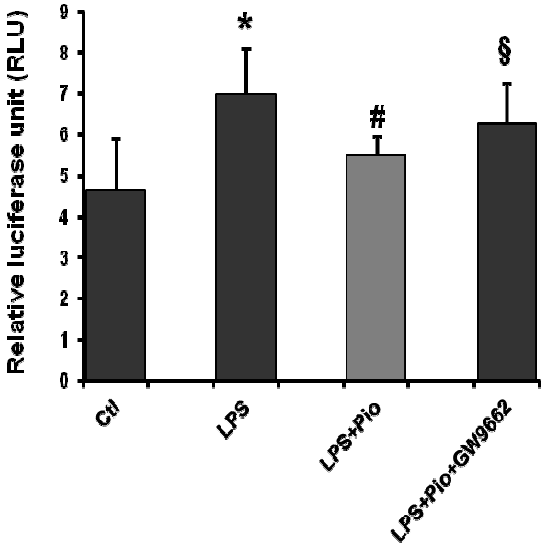


Figure 16. Luciferase-based NF-κB gene reporter of Cftr-KO cells before and after 6h of LPS (100 ng/ml) and Pioglitazone (50 µM) stimulation with and without a pretreatment of 1 hour with the PPAR-γ antagonist GW9662. Data were performed in n=4 experiments. Bar graph represent the ratio between the NF-κB-dependent expression of luciferase and the constitutive expression of renilla. *p<.001 vs CF ctl; # p<.05 vs LPS; § p<.05 vs LPS +Pio.

5.6 Pioglitazone inhibits LPS-induced expression and 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 pro-inflammatory

cytokines/chemokines as described in our previous studies [59]. Based on the inhibitory effect of pioglitazone on NF-κB activation, we sought to investigate whether PPAR-γ activation decreases also the expression of NF-κB-dependent inflammatory cytokines. First, we analyzed by RT-PCR the effect of pioglitazone on a panel of cytokine gene expression at baseline and after administration of LPS for 6 and 12 hours. The following panel of pro-inflammatory cytokines was analyzed: interleukin (IL)-1 isoform α and β, interleukin (IL)-6, granulocyte colony-stimulating factor (G-CSF), keratinocyte chemo-attractant (KC), LPS-induced CXC chemokine (LIX), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2). Our data showed that in CF cells, pioglitazone (50 μM) significantly inhibits the gene expression of MCP-1, LIX and MIP-2, before and after administration of LPS (Figure 17).

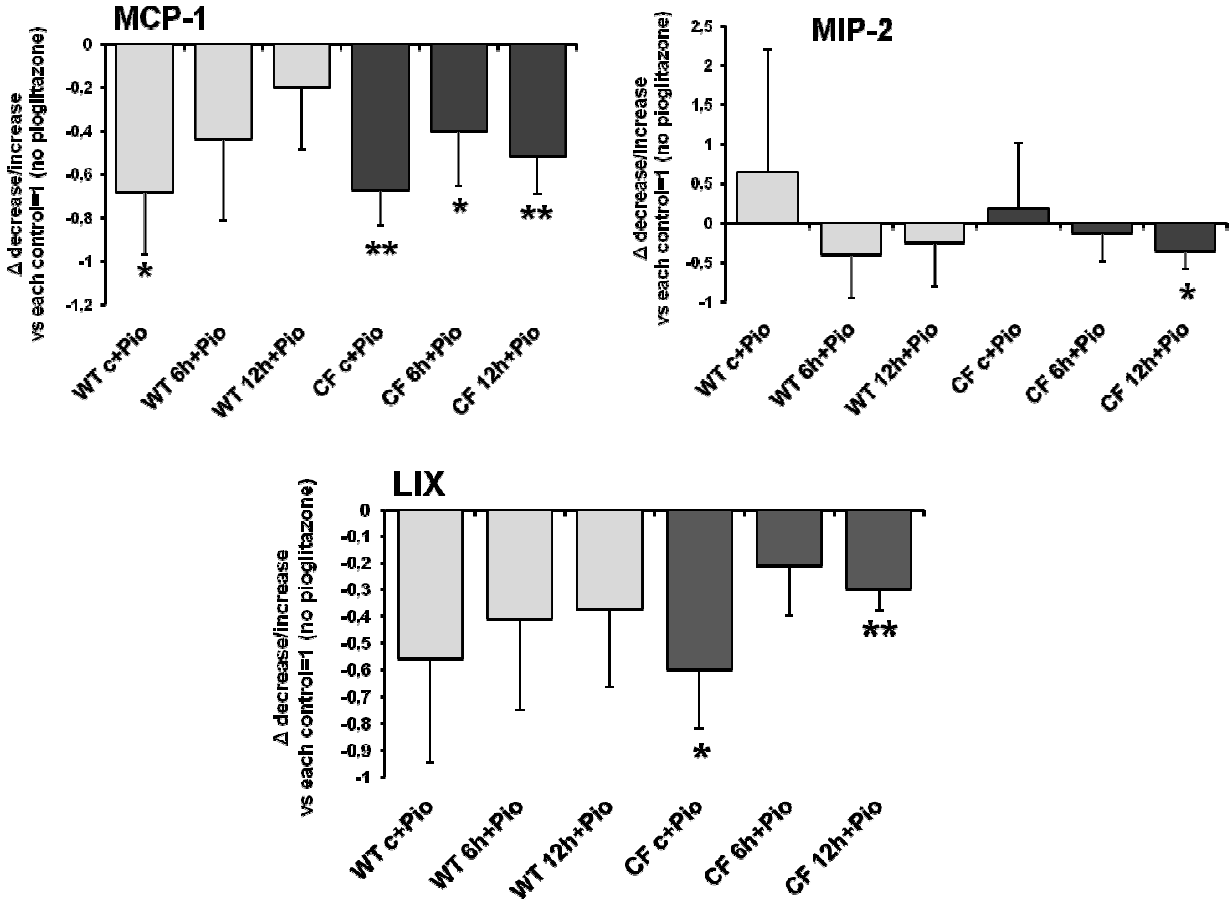


Figure 17. Real-time PCR analysis of proinflammatory cytokines genes monocyte chemotactic protein-1 (MCP1), LPS–induced chemokine (LIX) and macrophage inflammatory protein 2 (MIP-2), in WT and CFTR-KO cholangiocytes before and after treatment with LPS in combination with the agonist Pioglitazone. Data presented were performed in n=3-4 experiments and are expressed as Δ decrease/increase induced by pioglitazone treatment versus each control (no pioglitazone). (*p<.05; **p<.01).

To understand if transcriptional modulation of cytokines by pioglitazone reflects also a reduced production/secretion of cytokines, we measured the same panel of cytokines, in the apical and basolateral media of cholangiocyte cultures.

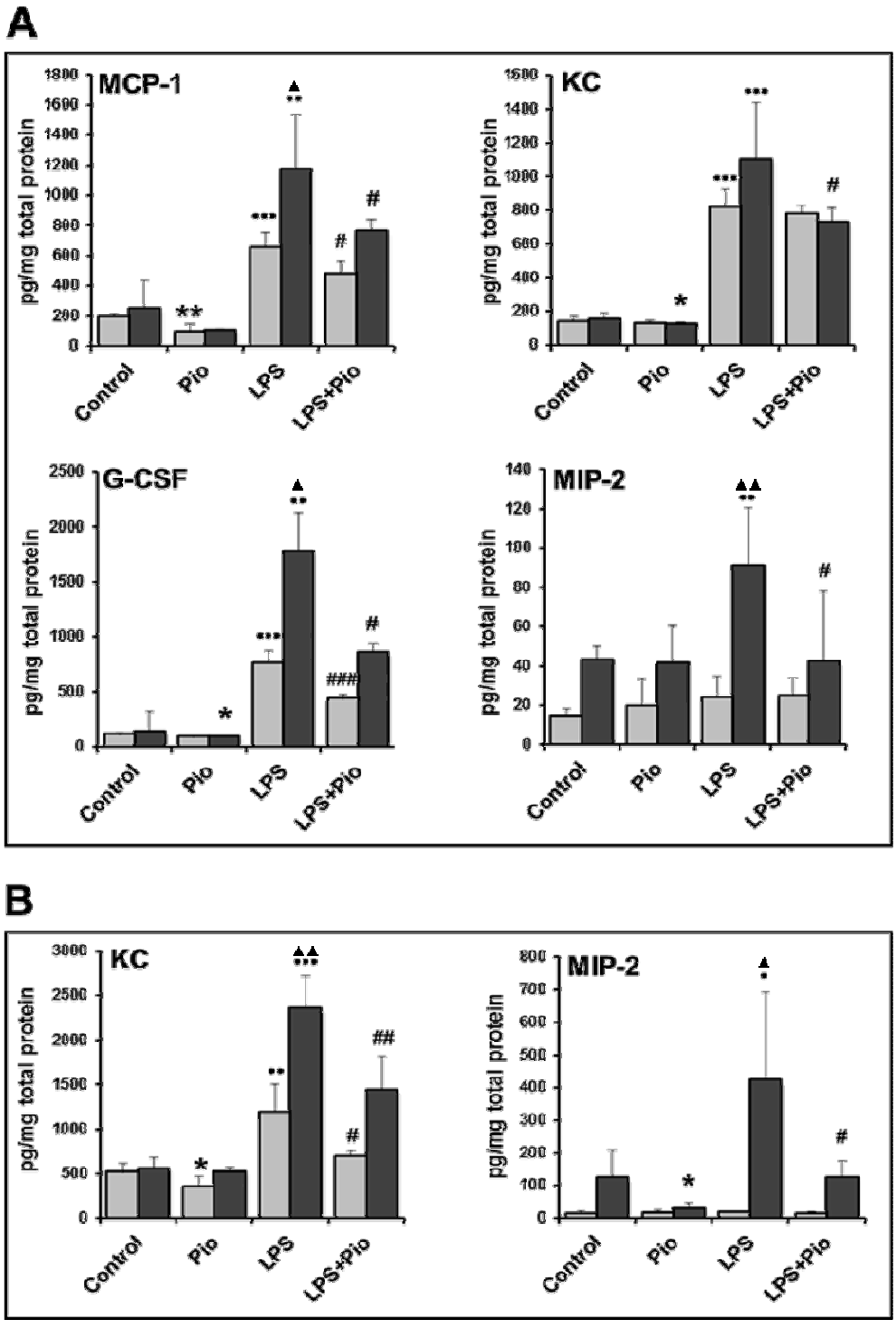


Figure 18. Microsphere-based multiplex immunoassay (Luminex, MILLIPLEX™ MAP) of proinflammatory cytokines monocyte chemotactic protein-1 (MCP1), macrophage inflammatory protein 2 (MIP-2), keratinocyte chemo-attractant (KC) and granulocyte colony-stimulating factor (G-CSF) secreted in the apical (A) or basolateral (B) medium of WT and CFTR-KO cholangiocytes, before and after treatment with LPS and in combination with the agonist pioglitazone. Data presented were performed in n=4

experiments and are normalized for the total cellular protein content. *p<.05, **p<.01 vs control; $^{\#}$p<.05, $^{\#\#}$p<.01 $^{\#\#\#}$p<.001 vs LPS; $^{\bullet}$p<.05, $^{\bullet\bullet}$p<.01, $^{\bullet\bullet\bullet}$p<.001 vs control, $^{\blacktriangle}$p<.05 vs WT LPS, $^{\blacktriangle\blacktriangle}$p<.01 vs WT Lps.

WT and Cftr-KO cells were polarized in transwell inserts and, once confluent and with a transepithelial resistance >1000 Ω/cm^2, treated with pioglitazone, LPS or their combination for 12 hours as described above. After treatment, both apical and basolateral medium were collected, and proteins 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, respect to WT cells. Moreover, treatment with pioglitazone significantly reduced the apical secretion of MCP-1 and G-CSF and MIP-2 as well as the basolateral and apical secretion of KC and MIP-2 (figure 18 A-B).

5.7 Pioglitazone inhibits the activation of NF-κB via an IκB-α-dependent mechanism.

To study the mechanism by which ligand-activated PPAR-γ blocks inflammation in CF cholangiocytes, we analyzed the effect of pioglitazone on crucial steps that lead to activation of the NF-κB pathway. Activation of the transcription factor NF-κB is finely controlled. In resting cells, the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκB-α) retains the NF-κB subunits p50/p65 in a cytoplasmic inactive complex.

In the presence of inducers of NF-κB, IκB-α is phosphorylated by the kinase complex IKK and degraded, so that the activated p65 subunit can translocate to the nucleus [94]. PPARs can block the activation of NF-κB by controlling the phosphorylation or the expression of IκB-α [69, 70, 95]. We detected no changes in phosphorylated IκB-α measured by western blot in the total protein fraction of WT and Cftr-KO cholangiocytes, treated with pioglitazone (50 μM), LPS or their combination (data not shown). Thus, we measured the protein expression of IκB-α and found that the basal expression of total IκB-α was significantly reduced in CF, as compared to control cells. Furthermore, treatment with pioglitazone significantly increased the IκB-α protein expression both at basal level and after LPS challenge, while showing no significant effects in wild type cells, at any time point (Figure 19). Levels of IκB-α were thus specular to the activation of NF-κB.

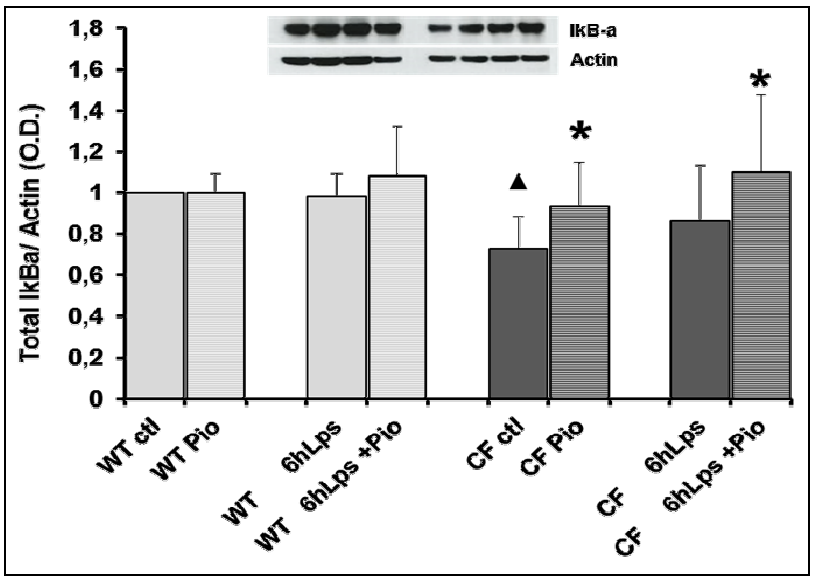


Figure 19. Western Blot in total protein fraction of WT and Cftr-KO cells before and after 6h of LPS (100 ng/ml) and Pioglitazone (50 μM) stimulation, against total IKB-α. Data were performed in n=4 experiments. Bar graph represents optical density quantification of total IKB-α normalized for Actin. *p<.05 vs CF c and vs CF 6h Lps; ▲p<.05 vs CF 6h Lps;

6 DISCUSSION

Cystic fibrosis represents one of the most frequent autosomal recessive genetic disorder among Caucasians, affecting approximately 1 in 3000 newborns. The gene affected encodes for the Cystic fibrosis transmembrane conductance regulator, a cAMP-activated chloride channel expressed on the apical surface of most secretory epithelia. Clinical manifestations are highly heterogeneous, the most frequently affected organs being lung, pancreas, intestine and liver [20, 29].

Liver complications affects about 30% of CF patients; beside the pulmonary phenotype, liver disease (CFLD) is one of leading causes of death in CF. Consistent with the expression of CFTR in the biliary epithelium, CFLD manifests as a chronic and progressive cholangiopathy that may evolves into sclerosing cholangitis and eventually biliary cirrhosis.

The pathogenesis of the disease has been initially interpreted as the result of impaired bile flow and biliary alkalinization caused by 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 then damage the biliary epithelium [33-35].

Emerging evidence suggests that several other factors may be responsible for the pathogenesis of this disease. Recent data generated by our group have demonstrated that, in association with the genetic defect, other pathogenetic factors are necessary to determine the liver phenotype. More in depth, during the first year of this doctoral project we have published a study showing that lack of CFTR predisposes the biliary epithelium to an increased innate immune response when exposed to bacterial-derived endotoxins. CFTR-defective mice exposed to DSS-induced colitis develop a strong inflammatory cholangiopathy characterized by ductular reaction and recruitment of inflammatory cells in the portal space. Additional *in vitro* studies confirmed that CFTR-defective biliary epithelial cells, when exposed to bacterial LPS, trigger a hyperactivation of TLR4/NF-κB signaling [59].

Currently, the treatment for CFLD is limited to the administration of bile acids, such as UDCA, able to stimulate the choleretic function of hepatocytes and cholangiocytes and to modify the composition of the bile acid pool. However, a previous study has shown that the ability of UDCA to stimulate choleresis at the cholangiocyte level depends on the presence of a functional CFTR [47], suggesting that if UDCA is of benefit in cystic fibrosis cholangiopathy, its mechanisms of action

must be different from stimulation of ion secretion in cholangiocytes. Thus, we suggest that, in addition to the use of choleretic drugs, the correct therapeutic strategy should be to target the TLR4/NF-κB pathway the biliary epithelium.

In this work we sought to investigate the capability of nuclear receptors (NRs) to modulate the innate inflammatory responses initiated by activation of TLRs. Our results suggest that activation of NRs may constitute a novel strategy to control biliary inflammation. In particular, we provide evidence that activation of PPAR-γ signaling is able to inhibit NF-κB-dependent innate immune responses in CF biliary epithelium, by stimulating its negative regulator IκB-a. .

Nuclear receptors comprise a wide range of ligand-activated transcription factors that influence cellular responses by altering gene expression. Although NRs were initially described for their role in metabolism and cellular homeostasis, these transcription factors are now emerging as regulators of inflammation. In the last few years several studies have described the role of NRs in the resolution of inflammation [64, 73, 74, 88]. However, knowledge of their mechanisms of action and specific targeted tissues is still partial.

NRs have been shown to coordinate several key aspects of hepatic physiology and pathophysiology. Agonists of LXRs, FXRs and PPARs have been tested in experimental models of fibrosis and inflammation for their ability to cross-regulate the production of inflammatory mediators with metabolic processes. For example, activation of hepatic stellate cells (HSC) in course of fibrogenesis is associated with a decreased expression and activity of PPAR-γ. Stimulation of this receptor with specific ligands is able to suppress several marker of HSC activation, such as expression of collagen and alpha smooth muscle actin (alpha-SMA) [96]. Moreover, several studies indicated that PPARs, mainly the β/δ and γ isoforms, exert protective effect in the liver against inflammation and fibrosis, in mouse and rat model of NASH. On this regard, administration of PPARs agonists to mice and rats fed a choline/methionine-deficient diet prevents fibrosis development, stimulates antioxidant gene expression, β-oxidation of fatty acids and suppresses inflammation [97].

Expression of NRs show different patterns between the different liver cell types. Therefore, as a first approach, we analyzed the gene expression of the specific subtypes of nuclear receptors in primary cholangiocytes isolated from wild type and CFTR-defective mice. We found that cholangiocytes express multiple

receptors, with LXR-β, FXR and PPAR-δ expression being highly expressed in both wild-type and CF cells. Multiple studies have already examined the role of these latter receptors in cholangiocytes physiology, describing an important control function on cholesterol metabolism and flux. By regulating the expression of Niemann Pick C1 Like L1 (NPC1L1), for example, PPAR-δ promote apical cholesterol import from bile into cholangiocyte. In parallel, LXR-β/PPAR-β/δ-dependent increase of ABCA1 transporter, promote cholesterol efflux from the basolateral membrane [98]. This capacity to modulate cholesterol flux though the cell is already considered an important target in the study of cholestatic liver diseases.

Interestingly, the γ isoform of PPARs resulted up-regulated in CFTR-KO cholangiocytes compared to their controls. This increased expression was further confirmed at the protein level. Controversial data about altered expression or function of this receptor have been reported in intestine and lung tissues from CF mice. In ileum and colonic tissues from the same mice this altered expression was associated with an altered nuclear localization/activity of the receptor. These initial studies were not confirmed in a subsequent study that showed comparable amounts of PPAR-γ mRNAs in WT and CFTR-defective colonic epithelial cells. These latter findings, and parallel studies on CF airway/bronchial epithelia, rather suggested that defective PPAR-γ function was secondary to the altered recruitment of co-activator molecules at the DNA level, impacting the transcriptional control of PPAR-γ [99].

Based on the above findings, we further investigated whether the upregulation of PPAR-γ expression in CF cholangiocytes reflected into an increase in receptor activity. PPARs normally reside into the nucleus bound to the DNA, repressing the transcription of specific target genes. Upon arrival of activator ligands, NRs witch on the transcription of specific target genes. This is the classical “transactivation pathway” that has been mainly described in metabolic processes [66]. To evaluate PPAR-γ activation in WT and Cftr-KO cholangiocytes we measured the expression of the specific target genes acetyl-Coenzyme A synthase 1B (Acaa1b), angiopoietin-like 4 (Angptl4), monoglyceride lipase (Mgll) and 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2) [91]. We found that the expression of Acaa1b, Angptl4 and Hmgcs2 was comparable between CF and control cells (Mgll was almost undetectable in cholangiocytes), indicating that, in spite of an increased nuclear expression of PPAR-γ in CF cholangiocytes, its transcriptional activity was not different from WT cells. However, PPAR-γ in CF cells

was responsive to exogenous activators, as shown by the upregulation of target genes expression, after treatment with the PPAR-γ synthetic agonist pioglitazone.

A reduced function of PPAR-γ have been previously reported in CF and explained with the retention of the receptor into the cytoplasm, a condition that would inhibit its binding to the DNA [100] . In our condition, this does not appear to be the case; in fact, when we measured the amount of receptor associated with DNA in the nuclear compartment we found a higher DNA-binding in CF cells, as expected by the higher nuclear amount of receptor, as compared to WT cells.

Given that no alterations in the DNA-binding process were detected and that exogenous administration of PPAR-γ ligands was able to activate the receptor in CF cholangiocytes, we reasoned that the increased expression of an inactive receptor in CF cells could result from a decrease in the PPAR-γ endogenous activators. Consistent with this hypothesis, a recent study showed that in CF colonic epithelia a defect in PPAR-γ function was the result of a reduction in endogenous activators, instead of the receptor itself. This was associated with a deficiency in essential fatty acids with decreased levels of eicosanoid precursors [91]. Eicosanoids are signaling molecules derived from fatty acids present in the cell membrane, such as arachidonic acid (AA) and linoleic acid (LA), or from the intermediate metabolite docosahexaenoic acid (DHA). Consistent with these previous observation, our lipidomic analysis of the major ω-3 and ω-6 polyunsaturated fatty acids (PUFAs), precursors of the active form of PPAR-γ endogenous ligands, showed an imbalance in most of the lipids analyzed. in particular AA and DHA and LA as indicated by the increased ratio AA/DHA and AA/LA in CF cells compared to their controls. LA represent the precursor of AA, the main source of pro-inflammatory mediators. On the contrary, DHA has been recognized as an important precursor of anti-inflammatory eicosanoids that are well known activators of PPAR-γ. Reduced level of LA, in parallel with elevated AA levels, may be explained by an increased LA metabolism, whereas DHA decrease may be the result of altered modifications of its desaturation and elongation cascade. This is the first report showing an imbalance of bioactive lipids in the biliary epithelium, and, at least in part, explain why we detect increased inactive receptor in CF biliary cells. Collectively, all these initial evidence, led us to investigate whether the exogenous activation of PPAR-γ, by synthetic ligands, might hamper the TLR4/NF-κB-driven inflammation in CFTR-defective biliary epithelium.

Thiazolidinediones is a family of potent synthetic agonists that activate PPAR-γ, and include troglitazone, ciglitazone, rosiglitazone and pioglitazone. Of these, troglitazone was removed from the market due to significant hepatotoxicity, while rosiglitazone and pioglitazone are currently FDA approved for type-2 diabetes treatment, as strong insulin sensitizers. In addition to their metabolic effects, these activators have shown anti-inflammatory properties in several systems. Pioglitazone, in particular, was shown to limit inflammatory signatures of airway epithelia in cystic fibrosis mice [101-103]. Additional studies indicated that inhibition of inflammation from pioglitazone occurs in a cell and tissue-specific manner, following PPAR-γ-dependent and PPAR-γ-independent mechanisms .

We have shown that CFTR-defective biliary epithelial cells, in response to endotoxin injury, activate a strong TLR4-dependent inflammatory response, characterized by a higher activation of NF-κB transcription factor in CF cholangiocytes, as compared to WT cells. This higher activation is responsible for a sustained and robust production of several pro-inflammatory cytokines [59]. To test the effect of PPAR-γ stimulation on NF-κB activation, we treated WT and CF cells with the agonist pioglitazone in the presence of LPS. Pioglitazone significantly reduced the increased nuclear activation of NF-κB in CF cells, both before and after LPS administration, as shown by the significant reduction of p65 nuclear translocation and a decrease in the transcriptional activity of the NF-κB promoter. This effect was weaker in WT controls, where PPAR-γ is less expressed, reaching a significant effect only in the transcriptional activation assay, and suggest that the anti-inflammatory effect could depend on the amount of receptor available.

To better elucidate if the inhibitory effect of pioglitazone on NF-κB activity in CF cells was dependent on a direct activation of its receptor, we repeated the experiments in the presence of a receptor antagonist. GW9662 is a potent and selective PPAR-γ antagonist that blocks, irreversibly, the ligand-binding pocket of the receptor [93]. We found that pretreatment with GW9662 partially prevented the effect of pioglitazone on the activation of NF-κB, indicating that the anti-inflammatory property of pioglitazone depends in part on the receptor activation.

Since Pioglitazone was able to prevent NF-κB activation in CF cholangiocytes, we investigated also the effect of PPAR-γ activation on the gene expression and protein secretion of pro-inflammatory cytokines downstream to the NF-κB pathway. Consistent with its effects on NF-κB, in CF cells, pioglitazone

treatment was able to reduce the gene expression of monocyte chemotactic protein-1 (MCP-1), LPS-induced CXC chemokine (LIX) and macrophage inflammatory protein 2 (MIP-2), at basal level and after 6 and 12 hours of LPS stimulation. This inhibitory effect of pioglitazone on cytokines production was even more prominent when the amount of cytokines secreted on cultured medium was measured, by Luminex immunoassay. As previously shown by us, CF cells secreted an higher amount of cytokines, respect to WT cells, after stimulation with LPS. After treatment with pioglitazone the apical and/or basolateral secretion of MCP-1, granulocyte colony-stimulating factor (G-CSF) and keratinocyte chemo-attractant (KC) was significantly reduced in both group of cells, while the apical and basolateral secretion of MIP-2 was inhibited only in CF cells. Interestingly, LIX, G-CSF and especially KC (the murine homologue of human IL-8) are strongly involved in proliferation, survival and chemotaxis of neutrophils, that we have previously described as the major constituents of the inflammatory infiltrate in CFTR-KO livers, in the setting of experimental colitis [59].

PPARs have the ability to interfere with a number of transcriptional pathways involved in inflammatory responses. Some of these mechanisms occur in the cytoplasm by interfering with the NF-κB activating machinery. NF-κB is usually kept sequestered in the cytoplasm by its inhibitor IκB-α. The release/activation of NF-κB requires the phosphorylation of this inhibitor, performed by the IKK kinase complex. Once phosphorylated, IκB-α is rapidly polyubiquitinated and degraded by the proteasome [94]. To better understand the mechanism by which ligand-activated PPAR-γ blocked inflammation in CF cholangiocytes, we analyzed the effect of pioglitazone on different steps of activation of the NF-κB pathway. While the phosphorylation state of IκB-α, before and after LPS stimulation, was similar in WT and CF cells, we found that the expression of total IκB-α in CF cells increased significantly after treatment with pioglitazone, both at basal level and after LPS stimulation. Similar mechanism of inhibition of NF-κB by the α and γ isoform of PPARs has been previously demonstrated in other system (ie.lung, myotubes, smooth muscle cells) [69, 70]. Interestingly, the basal expression of total IκB-α was significantly reduced in CF, compared to control cells, likely consistent with a higher activation of NF-κB , typical feature of CFTR-defective cells.

In conclusion, this study shows, for the first time, that stimulation of NRs may represent a novel strategy to control inflammation in cystic fibrosis biliary epithelium.

In fact, our data demonstrate that PPAR-γ is more expressed in CF biliary epithelium, but a defective production of bioactive ligands may impair the correct activation of the receptor. Exogenous stimulation of PPAR-γ by the agonist pioglitazone has anti-inflammatory properties in CF cholangiocytes by limiting the TLR4/NF-κB-dependent innate immune responses to LPS. This protective role depends on the induction of the NF-κB negative regulator IκB-α. In figure 20 are summarized our findings.

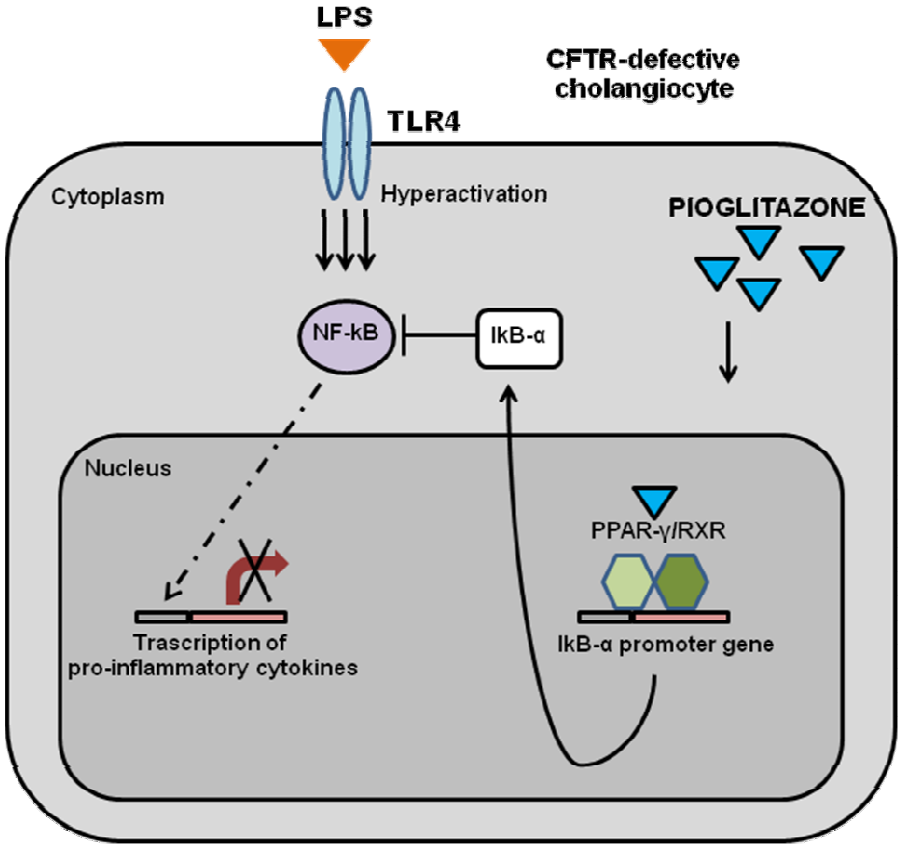


Figure 20. Suggested model of inhibition of inflammation by PPAR-γ agonist pioglitazone in CFTR-defective biliary epithelium.

The results described in this study represent an important step forward in understanding the physiology and pathophysiology of the biliary epithelium and describe a novel function of the nuclear receptor PPAR-γ in controlling biliary epithelium inflammation. Furthermore, the relevance of the mechanisms described in CF liver disease may potentially be extended to other chronic inflammatory liver diseases.

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