Published in final edited form as:

Gastroenterology. 2011 October ; 141(4): 1498–1508.e5. doi:10.1053/j.gastro.2011.06.052.

Loss of CFTR Affects Biliary Epithelium Innate Immunity and Causes TLR4–NF-κB–Mediated Inflammatory Response in Mice

Romina Fiorotto^{1,2}, Roberto Scirpo^{1,3}, Michael Trauner⁴, Luca Fabris^{2,5}, Rafaz Hoque¹, Carlo Spirlì^{1,2}, and Mario Strazzabosco^{1,2,3}

¹ Dept. of Internal Medicine, Section of Digestive Diseases, Yale University School of Medicine, New Haven CT, USA

² Center for liver Research (CelineR), and Division of Gastroenterology, Ospedali Riuniti Bergamo, Italy

³ Department of Clinical Medicine and Prevention, University of Milano-Bicocca, Milano, Italy

⁴ Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria

⁵ Department of Medical and Surgical Sciences “P.G.Cevese,” Università di Padova, Padova, Italy

Abstract

BACKGROUND & AIMS—Loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) in the biliary epithelium reduces bile flow and alkalinization in patients with CF. Liver damage is thought to result from ductal cholestasis, but only 30% of patients with CF develop liver defects, indicating that another factor is involved. We studied the effects of CFTR deficiency on Toll-like receptor 4 (TLR4)-mediated responses of the biliary epithelium to endotoxins.

METHODS—Dextran sodium sulfate (DSS) was used to induce colitis C57BL/6J-*Cftr*^{tm1Unc} (*Cftr*-KO) mice and their wild-type littermates. Ductular reaction and portal inflammation were quantified by keratin19 and CD-45 immunolabeling. Cholangiocytes isolated from wild-type and *Cftr*-KO mice were challenged with lipopolysaccharide (LPS); cytokine secretion was quantified. Activation of NF-κB, phosphorylation of TLR4, and activity of Src were determined. HEK-293 that expressed the secreted alkaline phosphatase (SEAP) reporter and human *TLR4* were transfected with CFTR cDNAs.

RESULTS—DSS-induced colitis caused biliary damage and portal inflammation only in *Cftr*-KO mice. Biliary damage and inflammation were not attenuated by restoring biliary secretion with *nor*-ursodeoxycholic acid, but were significantly reduced by oral neomycin and polymyxin B,

© 2011 The American Gastroenterological Association. Published by Elsevier Inc. All rights reserved.

Address correspondence: Mario Strazzabosco M.D., Ph.D., Dept. of Internal Medicine, Section of Digestive Diseases, Yale University School of Medicine, 333 Cedar Street, LMP 1080, 06520 New Haven, CT, USA; phone: (203)-785-7281, fax: (203)-785-7273, mario.strazzabosco@yale.edu.

Disclosure: None of the authors have any potential financial conflicts of interest related to this paper.

Author Contribution: R.F. was involved in the study design, acquisition, analysis, interpretation of data and drafting the manuscript; R.S. was involved in data acquisition and analysis; R.H. was involved in FACS analysis; M.T., L.F. and C.S. were involved in study design and critical revision of the manuscript; M.S. designed and supervised the study, including the interpretation of data and wrote the manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

indicating a pathogenetic role of gut-derived bacterial products. Cftr-KO cholangiocytes incubated with LPS secreted significantly higher levels of cytokines regulated by TLR4 and NF- κ B. LPS-mediated activation of NF- κ B was blocked by the TLR4 inhibitor TAK-242. TLR4 phosphorylation by Src was significantly increased in Cftr-KO cholangiocytes. Expression of wild-type CFTR in the HEK293 cells stimulated with LPS reduced activation of NF- κ B.

CONCLUSIONS—CFTR deficiency alters the innate immunity of the biliary epithelium and reduces its tolerance to endotoxin, resulting in a Src-dependent inflammatory response mediated by TLR4 and NF- κ B. These findings might be used to develop therapies for CF-associated cholangiopathy.

Keywords

UDCA; mouse model; inflammatory response; signaling

INTRODUCTION

Cystic Fibrosis (CF) is a common autosomic recessive disease that involves multiple organs, caused by mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR)¹. In the liver, CFTR is selectively expressed by the bile duct epithelium, where it regulates Cl^- and HCO_3^- secretion in response to hormonal and paracrine stimuli^{1, 2}. About 30% of CF patients present liver abnormalities and up to 10% develop clinically significant liver disease and hepatobiliary complications characterized by a chronic cholangiopathy, eventually evolving into sclerosing cholangitis and focal biliary cirrhosis^{3, 4}. CF cholangiopathy negatively impacts the quality of life and survival of CF patients, and may require liver transplantation in cases that progress to organ failure¹.

In response to cAMP/PKA, CFTR promotes Cl^- efflux into the bile, acting as a Cl^- channel and also facilitating the activation of Ca^{2+} -dependent Cl^- channels via apical release of ATP. The increased luminal Cl^- gradient generates the driving force for HCO_3^- secretion by the apical AE-2 exchanger, resulting in a bicarbonate-rich choleresis⁵. In CF, because of defective CFTR function, this mechanism is impaired; as a consequence, bile flow is reduced, and less alkaline biliary secretions accumulate into the bile ducts, causing retention of hydrophobic bile acids and toxins⁴.

CF liver disease is not associated with specific CFTR mutations, and even if the secretory defect is common to all CF patients, it remains still unclear why liver complications affect only a percentage of them. To date, the search for modifier genes has yet to provide definitive results⁶. Thus, beside the genetic defects, other intercurrent pathogenic co-factors may play a role in determining the clinical phenotype.

CFTR-defective mice do not spontaneously develop liver disease, unless under specific inbreeding conditions. Blanco et al. showed that induction of colitis via oral administration of dextrans resulted in severe bile duct injury in homozygous Cftr-KO mice, but not in heterozygous or wild-type littermate⁷. Thus, we reasoned that, the development of CF cholangiopathy requires “two hits”: i.e. those intercurring events are responsible for the development of the liver phenotype in CF because the biliary epithelium is more easily damaged by potential toxic insults such as endotoxemia.

The response of the biliary epithelium to endotoxins (LPS) is regulated by the innate immune system⁸. Among the pattern recognition receptors, toll like receptors 4 (TLR4) is the major component of the endotoxin receptor complex, together with CD14 and the LPS-binding protein. TLR4 is expressed by cholangiocytes, together with TLR2, TLR3 and TLR⁹. 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”. In fact, when the biliary epithelium is exposed to low level of endotoxins, no significant biliary or portal inflammation is generated^{10, 11}.

Recently, the role of innate immunity in liver diseases has generated much interest. The innate immune system plays a pathogenic role in acute and chronic inflammatory diseases of the liver, of diverse etiology, from alcoholic, to drug-induced or viral hepatitis^{10, 11}. For example, in chronic HCV patients, a single nucleotide polymorphism in TLR4 that reduces TLR4 activity is associated with a decreased risk of developing liver fibrosis¹².

In this paper, we have used the DSS colitis model to induce an inflammatory cholangiopathy in CFTR-KO mice (C57BL/6J-*Cftr*^{tm1Unc}) and studied the role of innate immunity, and in particular of TLR4 regulation in the pathogenesis of CF-related liver disease.

MATERIALS AND METHODS

Material and reagents are listed in the supplementary material

Animals and experimental protocol—Experiments were performed according to protocols approved by the Yale University IACUC. Congenic B6.129P2-*Cftr*^{tm1Unc} mice, (harbouring a mutation, S489X, blocking CFTR transcription), and their wild type littermates were used as an accepted model for the human CF disease¹³. Genotype for each mouse and breeding were performed as described^{14, 15} (see supplementary material).

Cftr-KO mice and WT littermates were exposed to Dextran sodium sulfate (DSS), to induce an acute colitis and portal endotoxemia⁷. Mice received DSS by oral administration (solved in Peptamen), using 100 mg/day for 9 days, followed by 85 mg/day for 5 days. Biliary secretion was restored in *Cftr*-KO mice treated with DSS by administering 24-Norursodeoxycholic Acid (nor-UDCA)¹⁶. Nor-UDCA was administered to wild-type and *Cftr*-KO mice at doses of 15 mg/mouse/day, for one week before the administration of DSS (at doses reported above) and continued for the following two weeks of DSS treatment. Bowel decontamination was obtained by treatment with 100mg of Polymyxin B (to bind endotoxins) and 220 mg of neomycin/Kg body weight/day added to the drinking water for 2 weeks before and during the DSS treatment¹⁷. After treatment mice were sacrificed, blood was taken by direct cardiac puncture or from the portal vein and serum was stored for analysis. Liver and colonic tissues were harvested and fixed in formalin then embedded in paraffin (two main lobes). Alanine aminotransferase (ALT) activity was measured by the MMPC facility at Yale University.

Immunohistochemistry—Details of the immunohistochemistry protocol, quantification of K19 and CD45 positive structures are reported in the supplementary material.

Isolation and characterization of cholangiocytes—Mouse cholangiocytes were isolated from wild-type and *Cftr*-KO mice as described^{15, 18, 19}. cholangiocytes were maintained in 25-cm² tissue culture flasks, plated on rat-tail collagen, as described^{15, 18, 19}. The biliary phenotype was confirmed by staining for K19, and acetylated α -tubulin.

Endotoxins treatment of WT and *Cftr*-KO cholangiocytes—WT and *Cftr*-KO cholangiocytes were cultured in transwell inserts with a 0.4 μ m pore semipermeable membrane (Becton Dickinson Lab, Franklin Lakes, NJ, USA); these cells behave as a polarized monolayer that can be accessed through both the apical and basolateral domain separately. Establishment of a confluent monolayer with competent tight junction was routinely checked measuring transepithelial resistance and membrane potential difference (Millicell ERS system; Millipore, Billerica, MA). One week after confluence, transepithelial

resistance was $>1000 \Omega\text{-cm}^2$ ^{15, 20}. WT and Cftr-KO cholangiocytes were exposed to LPS (100 ng/ml), for 6, 12 and 24 hours from the basolateral or the apical side; both basolateral and apical overnatant were collected for cytokine analysis and proteins were extracted for western blot experiments.

Determination of cytokine secretion—Culture supernatant was used for the analysis of 32 mouse chemokines and cytokines by using the Milipore's MILLIPLEXTM mouse Cytokines/Chemokines kit coupled with BioPlex Luminex platform following manufacture's instructions.

Western blotting—Nuclear and cytosolic fractions were obtained by using the NE-PER[®] extraction kit (Pierce, Rockford, IL) following manufacture's instructions. Total cell lysate was extracted by using a lysate buffer (50 mM Tris-HCl, 1% NP40, 0,1% SDS, 0,1% Deoxycholic acid, 0.1 mM EDTA, 0,1 mM EGTA) containing fresh protease and phosphatase inhibitor cocktail. For details and antibodies used see supplementary material.

HEK-BlueTM-hTLR4 cells and NF- κ B activation assay—HEK-BlueTM-hTLR4 cells (InvivoGen, San Diego, CA) are engineered HEK293 cells that stably co-express the human TLR4 and an NF- κ B inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. NF- κ B activation is measured using the QUANTI-BlueTM detection colorimetric assay that detects SEAP production following TLR4 activation. Cells were seeded in 96 well plates at a density of 15×10^3 cells/well and transfected 6h later with 30 ng of WT-CFTR cDNA²¹ (a generous gift from Dr. Bompadre) or equivalent cDNA containing an empty vector using FuGENE[®] Transfection Reagent (Roche Diagnostic GmbH, Germany) following the manufacture's instruction; 24h later cells were stimulated with LPS (100ng/ml) in the growth medium. NF- κ B-induced SEAP activity was determined 24h later using SynergyTM 2 plate reader (Biotek[®] Instruments, Winooski, VT).

Statistical Analysis—Results are shown as mean $\pm$ SD. Statistical comparisons were made using one-way analysis of variance or the Wilcoxon–Mann–Whitney 2-sample rank sum test, where appropriate. In the latter, the *P* value was obtained from the exact permutation null distribution. The statistical analysis was performed using SAS software (SAS Institute Inc, Cary, NC). *P* values <0.05 were considered significant.

RESULTS

DSS treatment causes an inflammatory cholangiopathy in Cftr-KO mice but not in wild type mice

Following the protocol of Blanco et al.⁷, we exposed Cftr-KO mice and WT littermates to dextran sodium sulfate (DSS), an experimental condition known to induce acute colitis and portal endotoxemia^{7, 22}. The histological grade of colitis induced by DSS treatment and the concentration of endotoxin in the portal blood were similar in WT and Cftr-KO mice (supplementary fig.1 and supplementary fig.2). However, treatment with DSS generated an inflammatory cholangiopathy only in homozygous Cftr-KO mice. As shown in figure 1A, on the contrary to WT mice, Cftr-KO mice exposed to DSS, developed significant biliary damage, characterized by the expansion of the ductular reactive component and portal inflammatory reaction, with extensive infiltration of mononuclear cells in the portal space. Significant increased ALT levels in the serum of Cftr-KO mice exposed to DSS confirmed the liver injury (supplementary fig.3). K19 immunolabeling demonstrated biliary epithelial damage, proliferation of dysmorphic reactive biliary structures and activation of progenitor cells. As shown in figure 1B and in supplementary table 1 the percentage of K19-positive staining area quantified by computer-assisted morphometric analysis, was significantly

higher in Cftr-KO mice treated with DSS (Cftr-KO+DSS (n=7): $1.65\% \pm 0.32$ vs Cftr-KO (n=4): $0.7\% \pm 0.13$, $p < 0.01$) while it was not increased in WT mice (WT+DSS (n=7): $0.56\% \pm 0.22$ vs WT (n=5): $0.58\% \pm 0.18$, $p = 0.45$). Characterization of the inflammatory infiltrate (fig.2, supplementary table 1) showed an increased recruitment of CD45⁺ cells around the bile ducts in livers of DSS-treated Cftr-KO mice, as compared to livers of similarly treated WT mice. Most leukocytes were positive for the neutrophil-specific marker NIMP-R14 (figure 2A). FACS analysis of the non-parenchymal CD45⁺ cell fraction from whole livers of DSS-treated Cftr-KO mice (figure 2B), showed a large percentage of CD45⁺/F4/80⁺ and low percentages of NK, T and B cells.

Restoration of biliary secretion does not attenuate DSS induced liver damage in Cftr-KO mice

If CF cholangiopathy was the consequence of impaired fluid and HCO₃⁻ secretion by cholangiocytes, restoration of biliary and HCO₃⁻ secretion would improve biliary damage. *NorUDCA* (*nor*-ursodeoxycholic acid) stimulates bile flow and biliary HCO₃⁻ output in Cftr-KO mice, independently from CFTR, due to its ability to undergo cholehepatic shunting¹⁶. However, treatment with *norUDCA* did not reduce the K19 positive area in DSS-treated Cftr-KO mice (Cftr-KO+DSS+*norUDCA* (n=5): $2.09\% \pm 0.63$ vs Cftr-KO+DSS (n=7): $1.65\% \pm 0.32$, $p = 0.07$) (fig.1C and supplementary table 1). Immunolabeling for CD45 in the portal area showed no difference in *norUDCA* treated mice (supplementary table 1). Because of the lack of effect of *norUDCA* we reasoned that, rather than to impaired bile flow, liver damage in Cftr-KO mice could be explained by an increased susceptibility of the Cftr-defective biliary epithelium to bacterial products.

Administration of neomycin/polymyxin-B reduces liver damage in Cftr-KO mice treated with DSS

To study if the translocation of bacterial products from the intestine into the circulation constitutes the main pathogenetic factor determining the liver damage, we treated Cftr-KO mice with polymyxin-B and neomycin before and during DSS treatment. Administration of 100mg of polymyxin-B and 220 mg of neomycin/Kg body-weight/day into the drinking water (a concentration known to prevent the growth of intestinal bacteria in mice¹⁷) significantly reduced the K19 and CD45 positive areas in Cftr-KO mice, as well as the amount of circulating LPS in both WT and Cftr-KO mice (fig.1C, supplementary table 1 and supplementary fig.2).

Cftr-defective cholangiocytes respond to LPS with an increased cytokine secretion with respect to WT

To investigate the response of CFTR-defective cholangiocytes to an endotoxin stimulation, we isolated and cultured primary cholangiocytes from WT and Cftr-KO mice, as previously described^{15, 18, 19}. We exposed CFTR-defective and WT cholangiocytes to LPS (100 ng/ml) from the apical or the basolateral side, for 0, 6, 12 and 24 hours and, measured the secretion of multiple cytokines in the apical and basolateral media using a microsphere-based multiplex immunoassay (Luminex, MILLIPLEXTM MAP). Figure 3 shows the changes in the apical and basolateral medium of the cytokines which secretion, after apical (A) or basolateral (B) LPS treatment, was significantly different between Cftr-KO and WT cholangiocytes. Granulocyte colony stimulating factor (G-CSF), keratinocyte chemoattractant (KC), IL-1 α , IL-6, monocyte chemotactic protein-1 (MCP-1), lipopolysaccharide-induced CXC chemokine (LIX) after basolateral LPS stimulation and macrophage inflammatory protein 2 (MIP-2) after apical LPS stimulation, were significantly increased in CF, as compared to WT cholangiocytes; their concentrations peaked at 12 hrs after stimulation. Interestingly, several of these cytokines were mildly, but significantly increased in CF cells, even before the challenge with LPS (see supplementary table 2 and

fig.3). Altered secretion of these cytokines in response to LPS, suggests that CFTR-defective cholangiocytes have an exaggerated innate immune response to TLR ligands.

Innate immunity of the biliary epithelium is finely tuned to prevent excessive inflammatory reactions in response to its continuous exposure to low concentration of endotoxins coming from the intestine through the portal circulation^{10, 11}. We measured by western-blot the expression of IRAK-M, known to confer endotoxin tolerance in human cholangiocytes⁹ and of A20, a protein involved in LPS-tolerance in enterocytes²³; both were unchanged in Cftr-KO mice (see supplementary fig.4).

Cftr-defective cholangiocytes respond to LPS with increased TLR4-dependent NF- κ B activation

The biliary epithelium expresses TLR4^{10, 11}. TLR4 specifically mediates the response to LPS through the activation and nuclear translocation of NF- κ B^{24, 25}. We have quantified by western blot the expression of p65 in the nuclear fraction of WT and Cftr-KO cholangiocytes, before and after exposure to LPS for 6 and 12 hrs (figure 4A,B) and found that p65 expression, already significantly increased in Cftr-KO at baseline, was further and significantly increased after LPS stimulation, as compared to WT. After LPS, nuclear immunofluorescence for p65 was higher in Cftr-KO cells with respect to WT cells (not shown). These data strongly indicate that CFTR-defective cholangiocytes have an excessive activation of TLR/NF- κ B signaling after exposure to LPS. To prove the direct involvement of TLR4 signaling, we treated WT and Cftr-KO cholangiocytes with TAK-242, (a selective inhibitor of TLR4 that blocks the interactions between TLR4 and its adaptor molecules^{26, 27}) and measured NF- κ B transcriptional activity, after LPS administration. As shown in fig.4C, LPS-dependent- NF- κ B activation in Cftr-KO cells was significantly inhibited by TAK-242.

Src-dependent phosphorylation of TLR4 is significantly increased in Cftr-KO cholangiocytes

TLR4 can be activated through the phosphorylation of its TIR domains by Src, a non receptor tyrosine kinase²⁸. Using an antibody that recognizes a phosphorylated tyrosine residue at position 674, we studied TLR4 phosphorylation (expressed as ratio between phosphorylated and total TLR4) in WT and CF cholangiocytes, before and after treatment with LPS for 12 h. As shown in fig. 5A,B, as compared to WT cholangiocytes, the level of TLR4 phosphorylation in CF-cholangiocytes is significantly higher both at basal condition and upon LPS stimulation. Total TLR4 expression was comparable in WT versus Cftr-KO cells before and after LPS stimulation (not shown). Pretreatment with PP2 (10 μ M), a specific inhibitor of Src kinase family²⁹, completely blunted TLR4 phosphorylation in Cftr-KO cells.

The Src family of tyrosine kinases, are non-receptor tyrosine kinases that play important roles in cell proliferation, survival, adhesion, and motility, as well as in inflammation³⁰. A role for protein tyrosine kinases in intracellular signaling initiated by LPS has been documented in several studies^{29, 31, 32}. To compare the activity of Src in WT and CF cholangiocytes, we determined, by Western blot, the ratio between the active Src (i.e. phosphorylated at tyrosine 418) and the total amount of Src. As shown in fig. 5C,D, Src activity is significantly increased in CF as compared to WT cells. Total Src expression level was comparable in WT and CF cells (not shown). Consistent with the inhibition of TLR4 receptor phosphorylation by PP2, increased activity of Src may represent a key event leading to altered TLR4-mediated innate immunity response in Cftr-KO cholangiocytes.

Expression of wild type CFTR reduces LPS-mediated NF- κ B activation in HEK-BlueTM–hTLR4 cells

To test the hypothesis that CFTR expression reduces TLR4 activation in response to LPS, we induced the expression of CFTR in HEK-BlueTM–hTLR4 cells by transfection with a WT-CFTR cDNA construct (see fig.6A). These cells are designed for studying the stimulation of h-TLR4 by monitoring the activation of a NF- κ B reporter gene and do not express endogenous CFTR. As expected, stimulation with LPS induces a significant NF- κ B activation (not shown). As shown in fig.6B, cells transfected with CFTR show a significant decrease in the activation of NF- κ B, after stimulation with LPS (24h) as compared to cells transfected with an empty vector. These data are consistent with the hypothesized role of CFTR in the regulation of TLR4 signaling responses in epithelial cells.

DISCUSSION

In this study we used the DSS colitis model to induce biliary damage in *Cftr*-KO mice (C57BL/6J-*Cftr*^{tm1Unc}) and found that correction of bile secretion with the hypercholeretic bile acid *nor*UDCA, did not improve liver damage. On the contrary, liver damage was decreased by bowel decontamination. CFTR-defective cholangiocytes exposed to LPS showed an increased TLR4-mediated activation of NF- κ B and secreted more TLR4-dependent cytokines than WT cholangiocytes. Furthermore we showed that expression of CFTR in epithelial cells reduces the LPS-induced activation of TLR4 signaling and provided evidence for a Src-mediated upregulation of TLR4 signaling in CFTR-deficiency. These results indicate that defective function of CFTR impacts on the innate immunity of the biliary epithelium, predisposing cholangiocytes to generate a strong TLR4 mediated inflammatory response.

Consistent with the “two hits” hypothesis, exposure of CFTR-defective mice to DSS, in spite of similar degree of colitis and levels of circulating endotoxins, caused significant biliary damage in homozygous *Cftr*-KO mice, but not in wild type mice. Epithelial damage was characterized by proliferation of dysmorphic reactive biliary structures and portal inflammation, with extensive infiltration of mononuclear cells, mostly positive for neutrophil-specific marker NIMP-R14 and for the macrophage marker F4/80.

The classical interpretation of the pathogenesis of CF-cholangiopathy placed the emphasis on the consequences of the defective channel function of CFTR on bile flow and bicarbonate secretion¹. The resulting thickened and less alkaline secretions would accumulate into the bile ducts causing retention of hydrophobic bile acids and toxins that would then damage the biliary epithelium. The histological finding of mucus bile plugs in the interlobular bile ducts of patients with cystic fibrosis was used as proof of the pathogenetic relevance of reduced bile secretion. However, this finding is actually rare and jaundice is a very late sign in patient with CF¹.

To challenge this hypothesis we treated CFTR-KO mice with *nor*UDCA. Due to its ability to undergo cholehepatic shunting, *nor*UDCA is known to stimulate bile flow and biliary HCO₃ output in *Cftr*-KO mice, through a CFTR-independent mechanism¹⁶. However, administration of *nor*UDCA to *Cftr*-KO mice treated with DSS did not attenuate DSS-induced liver damage (fig.1C and supplementary table 1), suggesting that impaired bile secretion was not the main mechanism leading to biliary damage in our conditions.

As showed by others^{7, 22} after DSS-treatment, intestinal permeability increases allowing microbial products and endotoxins to reach the liver through the portal circulation. Our data show that the level of endotoxins is comparable in WT and *Cftr*-KO mice (supplementary fig.2) and that intestinal decontamination with neomycin and polymyxin-B significantly

decreased the portal level of endotoxins and inhibited the inflammatory response in Cftr-KO mice, consistent with a role for enteric derived endotoxins in determining a higher inflammatory response in Cftr-KO mice.

Our findings suggest that the increased susceptibility of Cftr-KO mice to endotoxin-mediated injury may be caused by an altered innate immunity response, rather than by impaired ion secretion. Because of its anatomical location, the liver is constantly exposed to gut-derived bacterial products, but it is able to rapidly clear most of the portal endotoxins^{10,11}. Furthermore, hepatocytes are able to uptake endotoxins and to secrete them into the bile³³. To protect itself against enteric pathogens the biliary epithelium expresses Toll-like receptors (TLRs), including TLR4 on the apical side of the membrane. TLR4 is activated by LPS, and signals to its downstream target NF- κ B, controlling the expression of several inflammatory cytokines. Excessive or prolonged activation of TLR can cause organ damage, and therefore, also the biliary epithelium, is highly tolerant towards TLR ligands. In normal conditions, little or no inflammation is generated due to a process of “endotoxin tolerance” that prevents an excessive inflammatory response after LPS stimulation^{10, 11}.

To investigate the response of Cftr-KO cholangiocytes to endotoxins, we isolated and cultured primary cholangiocytes from wild type and Cftr-KO mice and measured the apical and basolateral secretion of a panel of cytokines after exposure to apical or basolateral LPS (see figure 3 and supplementary table 2). The secretion of several inflammatory cytokines (G-CSF, KC, IL-1 α , IL-6, MCP-1, MIP-2 and LIX) was significantly higher in Cftr-KO cholangiocytes, with respect to wild type cholangiocytes. IL-1 α and IL-6 are proinflammatory cytokines that classically play a role in the acute primary response to pathogens, G-CSF and particularly KC (the murine homologue of human IL-8), MIP-2 and LIX are strongly involved in neutrophil proliferation, survival and chemotaxis^{34, 35}. Several of the above cytokines were already increased in Cftr-KO cells, before LPS challenge.

Expression of these cytokines is modulated by NF κ B, a downstream effector of the TLR4 pathway. Following ligand binding TLR4 dimerizes, resulting in the recruitment of TIR-domain containing adaptor molecules (MyD88, Mal, TRIF and TRAM), and the activation of intracellular signaling pathways that regulate proinflammatory gene expression, through the transcription factor NF- κ B²⁴. In our study we found that, after LPS stimulation, the nuclear expression of p65, a monomer of the p50/p65 NF- κ B complex, and NF- κ B gene activity were significantly higher in Cftr-KO cells than in wild type. Treatment with TAK-242, a potent and specific TLR4 inhibitor completely blunted the LPS stimulation, confirmed the role of TLR4. The exaggerated activation of TLR4/NF- κ B signaling, after exposure to LPS, in Cftr-KO cholangiocytes, suggests the loss of endotoxin tolerance.

Endotoxin tolerance can be modulated by several mechanisms, including the activation of specific negative regulators of TLR4^{36–39}. In our study, the expression of IRAK-M and A20, two regulators of TLR4 known to be involved in the tolerance mechanism in biliary⁹ and in intestinal²³ cells, was similar in Cftr-KO and WT cholangiocytes. Post-translational modifications are also important in the regulation of TLR signaling³⁸. In particular, phosphorylation of TLR4-TIR domains plays an essential role in creating docking platforms for the recruitment of down-stream signal-competent adapter proteins (e.g. MyD88) and kinases (e.g. IRAK-4 and IRAK-1) and thereby activates TLR4 signaling²⁸ and increases its sensibility to LPS. Src, a member of tyrosine kinase family, is able to phosphorylate the TIR domain of TLR4²⁸. In WT cholangiocytes, as expected from an endotoxins-tolerant epithelium, we found a low level of phosphorylation of TLR4, after 12 hours exposure to LPS. On the contrary, in CF-cholangiocytes TLR4 phosphorylation, higher already at baseline, further increased after exposure to LPS, but was completely inhibited by pre-treatment with PP2, a specific of Src kinase inhibitor²⁹. Phosphorylation of the Src

activation site, on its Tyrosine 418 residue, was also increased in Cftr-KO cholangiocytes. These observations and the finding that transfection with WT-CFTR decreases TLR4 response to LPS, in epithelial cells suggest a role for CFTR in maintaining the state of “endotoxin tolerance” of the biliary epithelium.

In conclusion, we propose that an exaggerated TLR4-mediated innate immune response to intercurring events plays an important role in the pathophysiology of liver disease. In fact, defective CFTR function is associated to an increased Src-mediated TLR4 phosphorylation. Further studies will clarify the mechanistic role of CFTR in this novel mechanism of regulation of TLR4 signaling in cholangiocytes.

Current treatment of CF liver disease is limited to the administration of choleric bile acids¹⁵. Our findings indicate that the correct pathogenetic approach should be to target the inflammatory process and TLR4 signaling. In addition to the classic anti-inflammatory agents, many new drugs able to target the signaling mechanisms studied in this work are in fact under development⁴⁰. Finally these concepts may apply also in other chronic cholangiopathies. For example, CF has several clinical features in common with Primary Sclerosing Cholangitis (PSC), a progressive cholestatic liver disease of unknown pathogenesis, associated with inflammatory bowel disease (IBD), where the translocation of bacterial products into the portal circulation, may injure the biliary epithelium⁷.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Support: This work was supported by Yale University Liver Center (National Institutes of Health), Grant Number: P30DK34989 and Cystic Fibrosis Foundation, Grant Number: CFF-FIOROT0810 to R.F.; Fondazione S. Martino (Bergamo, Italy) and PSC Partners Seeking a Cure Foundation grant to MS; R.S. was recipient of a fellowship from EXTRA Program supported by Fondazione Cariplo, Italy.

We thank Dr. Ruslan Medzhitov (Yale University Medical School, New Haven, CT, USA) for critical reading of this manuscript, Dr. Silvia Bompadre for the WT-hCFTR construct, Dr. Carol Soroka for assistance with LAL assay and Dr. Dhanpat Jain for histological examination of colonic tissues.

Abbreviations

CF	cystic fibrosis
PKA	protein kinase A
CLCa	Ca ²⁺ -dependent Cl ⁻ channel
TLR	toll-like receptor
LPS	lypopolysaccharide
DSS	dextran sodium sulfate
Nor-UDCA	24-Norursodeoxycholic Acid
Pan-CK	pan-cytokeratin
SDS	Sodium Dodecyl Sulphate
EGTA	ethylene glycol tetraacetic acid
NF-κB	nuclear factor-κB

IRAK-M	IL-1R-associated kinase M
BSA	Bovine serum albumin
SEAP	secreted embryonic alkaline phosphatase
G-CSF	Granulocyte colony stimulating factor
KC	Keratinocyte chemoattractant
IL	Interleukin
MCP-1	monocyte chemotactic protein-1
LIX	lipopolysaccharide-induced CXC chemokine
TIR	Toll/Interleukin-1 receptor
PP2	4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
MyD88	Myeloid differentiation primary response gene (88)
MAL	MyD88 adapter-like
TRIF	TIR-domain-containing adapter-inducing interferon- β
TRAM	TRIF-related adaptor molecule
PSC	Primary Sclerosing Cholangitis
IBD	Inflammatory Bowel Disease
EGF	Epidermal growth factor
PBS	phosphate-buffered saline
TBS	tris-buffered saline
DAPI	4',6-diamidino-2-phenylindole
VEGF	Vascular endothelial growth factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
IP-10	Interferon gamma-induced protein 10 kDa
LIF	leukemia inhibitory factor
MIG	Monokine induced by gamma interferon
MIP-2	macrophage inflammatory protein 2
RANTES	regulated upon activation; normal T-cell expressed and secreted
IFN-γ	Interferon-gamma
M-CSF	Macrophage Colony Stimulating Factor
TNF	Tumor necrosis factor

References

- Colombo C, Battezzati PM, Strazzabosco M, et al. Liver and biliary problems in cystic fibrosis. *Semin Liver Dis.* 1998; 18:227–35. [PubMed: 9773423]
- Cohn JA, Strong TV, Picciotto MR, et al. Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells. *Gastroenterology.* 1993; 105:1857–64. [PubMed: 7504645]

3. Feranchak AP, Sokol RJ. Cholangiocyte biology and cystic fibrosis liver disease. *Semin Liver Dis.* 2001; 21:471–88. [PubMed: 11745036]
4. Strazzabosco M, Fabris L, Spirli C. Pathophysiology of cholangiopathies. *J Clin Gastroenterol.* 2005; 39:S90–S102. [PubMed: 15758666]
5. Strazzabosco M, Mennone A, Boyer JL. Intracellular pH regulation in isolated rat bile duct epithelial cells. *J Clin Invest.* 1991; 87:1503–12. [PubMed: 2022723]
6. Castaldo G, Fuccio A, Salvatore D, et al. Liver expression in cystic fibrosis could be modulated by genetic factors different from the cystic fibrosis transmembrane regulator genotype. *Am J Med Genet.* 2001; 98:294–7. [PubMed: 11170070]
7. Blanco PG, Zaman MM, Junaidei O, et al. Induction of colitis in *cftr*^{-/-} mice results in bile duct injury. *Am J Physiol Gastrointest Liver Physiol.* 2004; 287:G491–6. [PubMed: 15064232]
8. Chuang YH, Lan RY, Gershwin ME. The immunopathology of human biliary cell epithelium. *Semin Immunopathol.* 2009; 31:323–31. [PubMed: 19533127]
9. Harada K, Isse K, Sato Y, et al. Endotoxin tolerance in human intrahepatic biliary epithelial cells is induced by upregulation of IRAK-M. *Liver Int.* 2006; 26:935–42. [PubMed: 16953833]
10. Seki E, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology.* 2008; 48:322–35. [PubMed: 18506843]
11. Szabo G, Dolganiuc A, Mandrekar P. Pattern recognition receptors: a contemporary view on liver diseases. *Hepatology.* 2006; 44:287–98. [PubMed: 16871558]
12. Li Y, Chang M, Abar O, et al. Multiple variants in toll-like receptor 4 gene modulate risk of liver fibrosis in Caucasians with chronic hepatitis C infection. *J Hepatol.* 2009; 51:750–7. [PubMed: 19586676]
13. Scholte BJ, Davidson DJ, Wilke M, et al. Animal models of cystic fibrosis. *J Cyst Fibros.* 2004; 3 (Suppl 2):183–90. [PubMed: 15463956]
14. Spirli C, Fiorotto R, Song L, et al. Glibenclamide stimulates fluid secretion in rodent cholangiocytes through a cystic fibrosis transmembrane conductance regulator-independent mechanism. *Gastroenterology.* 2005; 129:220–33. [PubMed: 16012949]
15. Fiorotto R, Spirli C, Fabris L, et al. Ursodeoxycholic acid stimulates cholangiocyte fluid secretion in mice via CFTR-dependent ATP secretion. *Gastroenterology.* 2007; 133:1603–13. [PubMed: 17983806]
16. Halilbasic E, Fiorotto R, Fickert P, et al. Side chain structure determines unique physiologic and therapeutic properties of norursodeoxycholic acid in *Mdr2*^{-/-} mice. *Hepatology.* 2009; 49:1972–81. [PubMed: 19475687]
17. Emmelot CH, van der Waaij D. The dose at which neomycin and polymyxin B can be applied for selective decontamination of the digestive tract in mice. *J Hyg (Lond).* 1980; 84:331–40. [PubMed: 6300222]
18. Spirli C, Okolicsanyi S, Fiorotto R, et al. ERK1/2-dependent vascular endothelial growth factor signaling sustains cyst growth in polycystin-2 defective mice. *Gastroenterology.* 2010; 138:360–371. e7. [PubMed: 19766642]
19. Spirli C, Okolicsanyi S, Fiorotto R, et al. Mammalian target of rapamycin regulates vascular endothelial growth factor-dependent liver cyst growth in polycystin-2-defective mice. *Hepatology.* 2010; 51:1778–88. [PubMed: 20131403]
20. Spirli C, Fabris L, Duner E, et al. Cytokine-stimulated nitric oxide production inhibits adenylyl cyclase and cAMP-dependent secretion in cholangiocytes. *Gastroenterology.* 2003; 124:737–53. [PubMed: 12612912]
21. Miki H, Zhou Z, Li M, et al. Potentiation of disease-associated cystic fibrosis transmembrane conductance regulator mutants by hydrolyzable ATP analogs. *J Biol Chem.* 285:19967–75. [PubMed: 20406820]
22. Neilly PJ, Gardiner KR, Kirk SJ, et al. Endotoxaemia and cytokine production in experimental colitis. *Br J Surg.* 1995; 82:1479–82. [PubMed: 8535797]
23. Wang J, Ouyang Y, Guner Y, et al. Ubiquitin-editing enzyme A20 promotes tolerance to lipopolysaccharide in enterocytes. *J Immunol.* 2009; 183:1384–92. [PubMed: 19570823]
24. Barton GM, Medzhitov R. Toll-like receptor signaling pathways. *Science.* 2003; 300:1524–5. [PubMed: 12791976]

25. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine*. 2008; 42:145–51. [PubMed: 18304834]
26. Ii M, Matsunaga N, Hazeki K, et al. A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. *Mol Pharmacol*. 2006; 69:1288–95. [PubMed: 16373689]
27. Kawamoto T, Ii M, Kitazaki T, et al. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *Eur J Pharmacol*. 2008; 584:40–8. [PubMed: 18299127]
28. Medvedev AE, Piao W, Shoenfelt J, et al. Role of TLR4 tyrosine phosphorylation in signal transduction and endotoxin tolerance. *J Biol Chem*. 2007; 282:16042–53. [PubMed: 17392283]
29. Smolinska MJ, Horwood NJ, Page TH, et al. Chemical inhibition of Src family kinases affects major LPS-activated pathways in primary human macrophages. *Mol Immunol*. 2008; 45:990–1000. [PubMed: 17875324]
30. Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol*. 1997; 13:513–609. [PubMed: 9442882]
31. Leu TH, Charoenfuprasert S, Yen CK, et al. Lipopolysaccharide-induced c-Src expression plays a role in nitric oxide and TNFalpha secretion in macrophages. *Mol Immunol*. 2006; 43:308–16. [PubMed: 15869794]
32. Sheth P, Delos Santos N, Seth A, et al. Lipopolysaccharide disrupts tight junctions in cholangiocyte monolayers by a c-Src-, TLR4-, and LBP-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol*. 2007; 293:G308–18. [PubMed: 17446308]
33. Mimura Y, Sakisaka S, Harada M, et al. Role of hepatocytes in direct clearance of lipopolysaccharide in rats. *Gastroenterology*. 1995; 109:1969–76. [PubMed: 7498663]
34. Clarke CN, Kuboki S, Tevar A, et al. CXC chemokines play a critical role in liver injury, recovery, and regeneration. *Am J Surg*. 2009; 198:415–9. [PubMed: 19716886]
35. Ohtsuka Y, Lee J, Stamm DS, et al. MIP-2 secreted by epithelial cells increases neutrophil and lymphocyte recruitment in the mouse intestine. *Gut*. 2001; 49:526–33. [PubMed: 11559650]
36. Kobayashi K, Hernandez LD, Galan JE, et al. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell*. 2002; 110:191–202. [PubMed: 12150927]
37. Wang J, Hu Y, Deng WW, et al. Negative regulation of Toll-like receptor signaling pathway. *Microbes Infect*. 2009; 11:321–7. [PubMed: 19146978]
38. Carpenter S, O'Neill LA. Recent insights into the structure of Toll-like receptors and post-translational modifications of their associated signalling proteins. *Biochem J*. 2009; 422:1–10. [PubMed: 19627256]
39. Muir A, Soong G, Sokol S, et al. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol*. 2004; 30:777–83. [PubMed: 14656745]
40. Hennessy EJ, Parker AE, O'Neill LA. Targeting Toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov*. 2010; 9:293–307. [PubMed: 20380038]

Figure 1.

A) DSS-colitis causes biliary damage in Cftr-KO, but not WT mice WT and Cftr-KO mice received DSS by oral administration, as described⁷. Liver tissue was stained with the cholangiocyte-specific marker K19. Cftr-KO but not WT mice developed biliary damage, characterized by bile duct proliferation and inflammation. Original magnification: 10X; inset: 40X. **B) Computer-assisted morphometric analysis of K19 positive areas**, expressed as percentage with respect to the whole area analyzed. Ductular reaction was significantly increased in Cftr-KO mice treated with DSS (Cftr-KO + DSS) (p<0.01). **C) Effects of norUDCA vs polymyxin-B/neomycin treatment on DSS-induced biliary damage.** Cftr-KO mice and WT littermates were exposed to DSS in the presence or absence of norUDCA (15 mg/mouse/day), a bile acid able to stimulate biliary secretion in Cftr-KO mice. Ductular reaction in Cftr-KO mice treated with DSS was not decreased by treatment

with nor-UDCA ($p=n.s$). On the contrary, treatment with polymyxin B (100 mg/Kg·body-weight/day) plus neomycin (220 mg/Kg·body-weight/day) prevented the increase in ductular reaction ($p<0.01$).

Figure 2. Periportal inflammatory infiltrate in DSS-treated Cftr-KO mice

A) Immunohistochemical analysis of liver specimens from DSS-treated WT and Cftr-KO mice shows a portal inflammatory cell infiltrate (CD45-positive cells) in Cftr-KO mice. The CD45-positive cell infiltrate is prevalently positive for the neutrophil marker NIMP-R14 and for the macrophage marker F4/80. (Original magnification: 40X). **B)** FACS analysis of the non-parenchymal CD45⁺ population isolated from DSS-treated Cftr-KO mice confirmed the presence of macrophages and showed very low percentages of NK, T and B cells.

Figure 3. Cftr-KO cholangiocytes are more responsive to LPS-stimulated cytokine secretion
 Biliary cells isolated from WT and Cftr-KO mice were cultured as polarized monolayer in transwell inserts with a 0.4 μ m pore semi permeable membrane, as previously described¹⁵. Once established the confluence of the monolayer, by measuring transepithelial resistance and membrane potential, cells were exposed to LPS (100 ng/ml) for 6–12–24h from the apical (A) or basolateral (B) side. At the end of the treatment apical and basolateral media were collected and analyzed by Luminex and data were normalized for the cell protein content. Kinetics of cytokine secretion by WT (blue line) and Cftr-KO (red line) cholangiocytes in response to LPS exposure (0h, 6h, 12h, 24h) are showed. The plots represent the means of 4 experiments for the six cytokines showing the most robust differences between WT and Cftr-KO cells (* p <0.05, ** p <0.01 vs WT at the respective time point). Statistical analysis and raw data are also reported in supplementary table 2.

Figure 4. Cfr-KO cholangiocytes respond to LPS with increased TLR4-dependent NF-κB activation

Cells were untreated (Controls) or exposed to LPS (100 ng/ml) for 6 and 12h. At the end of the treatment nuclear fraction were extracted. **A**) NFκB activity was assessed by western blot in nuclear fractions using an antibody against NFκB component p65. Histone-3 was used to normalize for nuclear protein content. **B**) Bar graphs represent optical density quantification of p65 in the nucleus normalized for histone-3. (* $p < 0.05$ vs WT; $p < 0.05$ vs Cfr-KO 6h). **C**) WT and Cfr-KO cells were exposed to LPS (100 ng/ml) for 6h in presence/absence of the TLR4 inhibitor TAK-242; NF-κB activity was measured by Luciferin/luciferase reporter gene assay. After LPS stimulation, NF-κB activity significantly increased in Cfr-KO, as compared to WT cells (*, $p < 0.05$ vs WT+LPS); NF-κB activity induced by LPS was significantly inhibited by treatment with TAK 242 (#, < 0.01 vs LPS). Bar graphs represent mean $\pm$ SD of the relative luciferase activity normalized to control in $n=3$ different experiments repeated in triplicate for each condition.

Figure 5. Increased TLR4 phosphorylation is caused by increased Src activity in Cftr-KO cholangiocytes

A) WT cells were exposed to LPS (100 ng/ml) for 12h; Cftr-KO cells were exposed to LPS (100 ng/ml) in the presence or absence of PP2 (10 μ M) (Src inhibitor) for 12h; untreated cells served as control (Ctrl). TLR4 phosphorylation was determined by western blot using an antibody against the TLR4 tyrosine phosphorylation site (Y674). **B)** Bar graph represents the ratio between p-TLR4 (pY674) and total TLR4. WT cholangiocytes show a significant tyrosine phosphorylation of TLR4 after 12 hours of exposure to LPS while in CF-cholangiocytes the level of TLR4 phosphorylation is significantly higher at basal condition and increased upon LPS stimulation. Pretreatment with PP2 (10 μ M) completely blunted TLR4 phosphorylation in Cftr-KO cells. **C)** Src activity was determined by western blot using an antibody against the active form of Src (pY418) in cells at basal conditions. **D)** Bar graphs represent the optical density ratio of p-Src (pY418) vs total Src. Cftr-KO cells show an increased amount of p-Src ($p < 0.01$) as compared to WT cells, suggesting an increased in the activity of the kinase. Data represent mean $\pm$ SD of $n=3$ different experiments.

Figure 6. CFTR expression in HEK-Blue cells decreases NF- κ B activation after LPS stimulation
 HEK-Blue cells were transfected with WT-CFTR or with an empty vector plasmid cDNA. **A)** As shown in the western blot, on the contrary to cells transfected with WT-CFTR DNA, there is no endogenous expression of CFTR protein in cells transfected with the empty vector. **B)** Bar graph represents % changes in NF- κ B activation in response to LPS in HEK-Blue cells transfected with WT-CFTR as compared to the increase in NF- κ B activation measured in control cells transfected with empty vector (average $\pm$ SD, n=3 independent experiments, *p<0.05).