Mrp3(Abcc3) is markedly induced following bile duct ligation (BDL) in the rat and in some human cholestatic liver diseases and is believed to ameliorate liver injury in this setting. Recently, the orphan nuclear receptor fetotoprotein transcription factor/cholesterol-7α-hydroxylase promoter factor (CPF/FTF/Lrh-1) has been shown to activate Mrp3 expression. However, whether inflammatory cytokines or elevated bile acid levels increased Lrh-1/Mrp3 expression in obstructive cholestasis was not known. We hypothesized that induction of Mrp3 would be associated with Lrh-1 up-regulation and would require intact cytokine signaling. Male tumor necrosis factor (Tnfr) receptor I (Tnfr−/−) mice and C57BLJ wild type (WT) controls were subjected to sham surgery or bile duct ligation. HepG2 cells were treated with bile acid or cytokines. Immunoblot assay and real time reverse transcriptase-PCR were used to determine expression of Mrp3/Mrp3, CPF/Lrh-1, Mrp2, and Bsep. CPF/Lrh-1 DNA binding to the MRP3/Mrp3 promoter was assessed using electrophoretic mobility shift assay, and promoter activity was determined by luciferase assay. Total bile acids and lactate dehydrogenase were measured using colorimetric assays, and cytokine abundance was determined by enzyme-linked immunosorbent assay. Lrh-1 and Mrp3 were significantly induced after BDL in WT but not Tnfr−/− mice. This was associated with more severe hepatocellular necrosis in Tnfr−/− mice. Lrh-1 binding to the Mrp3 promoter increased after BDL in WT but not in Tnfr−/− mice. Tnfr treatment of HepG2 cells also up-regulated CPF and Mrp3, increased CPF binding to the MRP3 promoter, and up-regulated MRP3 promoter activity. These results indicate that induction of Mrp3 after BDL is due to Tnfr-dependent up-regulation of Lrh-1. They provide strong evidence that induction of Mrp3 plays a significant role in hepatocyte protection during obstructive cholestasis.

Mrp3(Abcc3), the multidrug resistance associated protein 3, is one of the hepatocellular ATP-binding cassette transport proteins and is located on the basolateral surface of the hepatocyte, cholangiocyte, and enterocyte (1). Previous reports (2, 3) have demonstrated that Mrp3 transports bile salts, 17β-estradiol, and some anti-cancer drugs. Hepatic Mrp3 is markedly up-regulated in the liver following bile duct ligation (BDL) in the rat and in some forms of human cholestatic liver disease (4–7). It is thought to function primarily as an inducible basolateral efflux pump for bile salts, thereby limiting hepatocellular injury and playing a significant role in hepatocyte cytoprotection during cholestasis. However, the molecular basis for this response is not known.

The dramatic induction of Mrp3 seen in obstructive cholestasis indicates that substances that accumulate within the hepatocyte specifically regulate this gene. It has been reported previously that some nuclear hormone receptor (NHR) ligands including bile acids (8) and phenobarbital induce Mrp3, although the specific transcriptional pathways have not been elucidated. Phenobarbital induces Mrp3 without bile acid elevation, whereas the pregnane X receptor null mouse has normal Mrp3 expression (9), suggesting that more than one transcriptional pathway may exist. This implies that other regulators, which may include cytokines or other potential Mrp3 substrates, may lead to induction of Mrp3. Recently, adjacent response elements for the orphan NHR, foprotein transcription factor (FTF/CPF/Lrh-1), have been identified in the MRP3 promoter in the intestine (10, 11). In these studies bile acids were shown to up-regulate both Lrh-1 and Mrp3 DNA expression. The transcriptional function of Lrh-1 is negatively regulated by small heterodimer partner (Shp-1), which, in turn, up-regulated by the farnesoid X receptor (FXR) and its heterodimeric partner, the retinoid X receptor (RXR) (12, 13). We have observed previously (14) that BDL is associated with a rapid reduction in FXR:RXRa and Shp-1 and that this is associated with induction of Mrp3 expression.

Inflammatory cytokines including Tnfa, IL1β, and IL6 are also induced after BDL, and for IL1β this induction persists at significant levels for time points up to 14 days (15, 16). We have reported previously (17) that inflammatory cholestasis induced by LPS administration is associated with a reduction of RXRa protein and target genes including Mrp2 and the basolateral bile acid transporter Na+-taurocholate cotransporting polypeptide. Moreover, we have also reported that these NHR alter-
**Fig. 1. Hepatocellular necrosis is more severe in Tnfr−/− mice after BDL.** Liver H&E staining (magnified ×5000) WT Sham (n = 8) (A), Tnfr−/− Sham (n = 8) (B), WT BDL (n = 8) (C) and Tnfr−/− BDL (n = 6) (D), indicate unremarkable sham liver tissue and BDL-induced portal inflammation and bile duct reduplication with more severe necrosis (arrows) in Tnfr−/− mice (D).

The severity of liver injury following BDL would be dependent upon Lrh-1 expression and intact cytokine signaling. Our findings confirm that Mrp3 induction is hepato-protective in obstructive cholestasis. We hypothesized that up-regulation of hepatic Mrp3 and the severity of liver injury following BDL would be dependent upon Lrh-1 expression and intact cytokine signaling. In this study we have shown that Mrp3 may be transcriptionally regulated by Lrh-1, and the expression of both is dependent on Tnfα signaling. Our findings confirm that Mrp3 induction is hepato-protective in obstructive cholestasis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic oligonucleotides were obtained from Invitrogen. The primers and probes for human MRP3 and FGF and for murine Mrp2, Mrp3, Bsep, Lrh-1, FXR, RXRα, and Shp-1 were obtained from Applied Biosystems (Foster City, CA). The antibodies to Lrh-1, FXR, RXRα, actin, and Shptp1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to Mrp2 and Bsep were kindly provided by D. Keppler (Heidelberg, Germany) and B. Stieger (Zurich, Switzerland), respectively. Anti-human MRP3 antibody was purchased from Alexis Biochemicals (San Diego). ELISA was performed using the assay kit from Sigma. Gel shifts were performed using the digoxigenin Gel Shift Kit from Roche Applied Science. Reporter assay reagents and vectors were purchased from Invitrogen and Promega (Madison, WI). Immunoblot detection was performed using the Western blot chemiluminescence kit from PerkinElmer Life Sciences. Cell culture media and fetal bovine serum were obtained from Invitrogen. Serum and tissue bile acids were assayed using a colorimetric assay from Sigma. LDH assay was performed using a commercially available kit (Roche Applied Science). All other routine biochemicals and reagents were of the highest quality commercially available and were purchased from J. T. Baker Inc., Bio-Rad, Applied Biosystems (Foster City, CA), Stratagene (La Jolla, CA), Eastman Kodak Co., Invitrogen, Sigma, and Collaborative Biomedical (Bedford, MA).

**Bile Duct Ligation**—Male Tnf receptor I knockout (Tnfr1−/−) mice and age-matched C57BL/J wild type (WT) controls were obtained from The Jackson Laboratories. Animals were maintained in the Yale Animal Research Center and housed in a temperature- and humidity-controlled environment under a constant light cycle where they had free access to water. The protocol was approved by the Yale Animal Care and Use Committee, and all animals received humane care as outlined in Ref. 28. Common bile duct ligation was performed under sterile conditions as described previously (4). The common bile duct was exposed, ligated twice close to the hilum of the liver, immediately below the cystic duct, and then cut between the ligatures. Sham animals underwent sham surgery in which the bile duct was exposed but not ligated. The animals were exsanguinated; serum was saved, and the livers harvested 14 days after surgery.

HepG2 Cell Culture and Treatment—HepG2 cells were suspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, non-essential amino acids, and 1 × penicillin/streptomycin/glutamine (Invitrogen). The cells were plated (4–6 million cells/plate) on 10-cm plates (Collaborative Biomedical). Following adhesion the cells were treated with Tnfs (100 ng/ml), IL1β (100 ng/ml), IL6 (100 ng/ml), and chenodeoxycholic acid (100 μM). Control cells were treated with serum-free medium, and all cells were recovered after 24 h. Transient transfection was performed according to the LipofectAMINE 2000 method (Invitrogen). In general, HepG2 cells were plated in 12-well plates to 40–60% confluence and were washed three times with phosphate-buffered saline, and medium was replaced with 100 μl of Opti-MEM medium before a mixture of LipofectAMINE and DNA was added to the well. 1.25 μg of reporter DNA, 100 ng of pCMVGal (Promega, Madison, WI), and 200 ng of mouse FTF receptor expression plasmid DNA were used in each transfection experiment. Empty pcDNA3.1 expression vector was used as carrier DNA to maintain equal amounts of DNA for each transfection where required. After 12 h of transfection, the cells were cultured in serum-free medium Eagle’s medium or 1, 10, or 100 ng/ml human TNFα for 12–48 h. Then cells were harvested, washed twice with phosphate-buffered saline, and lysed with 200 μl of Luciferase Cell Culture Lysis Reagent (Promega). Luciferase activities were measured using Promega Luciferase assay system by luminometer and normalized by dividing the relative light units by β-galactosidase activity.

**Murine Hepatic Histopathology**—Following organ harvest, 100-mg wedge of liver tissue was fixed in 10% formalin and embedded in paraffin. Hematoxylin and eosin (H & E) and trichrome staining were performed on each sample. The histology was assessed and scored blindly, and necrosis was quantified using an Olympus BX70 microscope and ImagePro software (ImagePro Inc., Boston).

**Serum and Tissue Bile Acid Levels**—At organ harvest, inferior vena cava serum was isolated from all animals and stored at −20 °C until analysis. Liver tissue (50 mg) was frozen in liquid nitrogen, and bile acids were extracted and measured as described previously (18). Briefly, 500 mg of frozen liver tissue was ground finely and incubated overnight at room temperature with 1:1 t-butyl alcohol/double distilled H2O. Following centrifugation, the supernatant was stored at −20 °C until analysis. All samples subsequently underwent analysis by standard enzymatic-colorimetric assay using a bile acid assay kit in accordance with the manufacturer’s protocols (Sigma). The absorption of each sample was assessed by spectrophotometry at a wavelength of 530 nm (Ultrospec 3000, Amersham Biosciences).

**Serum Lactate Dehydrogenase (LDH) Assay**—Serum stored at −20 °C was used for this assay (20, 21), which was performed according
Fig. 2. Hepatocellular necrosis is associated with elevated tissue bile acid levels. Software analysis of the following is shown: tissue necrosis (*, p < 0.005) (A); relative LDH concentration (*, p < 0.001 relative to sham; #, p < 0.0005 Tnf−/− BDL relative to WT BDL) (B); serum bile acid concentration (*, p < 0.001 relative to sham; #, p < 0.005 Tnf−/− BDL relative to WT BDL) (C); and liver bile acid concentration (*, p < 0.0001 relative to Tnf−/− sham; #, p < 0.02 relative to WT sham) (D).

Real Time Reverse Transcriptase (RT)-PCR Analysis—RT reactions were performed on 5 μg of the isolated mouse or HepG2 RNA using established protocols and the ProSTAR RT-PCR kit (Stratagene, La Jolla, CA). TaqMan real time quantitative PCR assay was performed on an ABI Prism 7700 Sequence Detection System, according to the manufacturer's protocol (Applied Biosystems). The following primers and probes were used for the TaqMan RT-PCR assay: murine Mrp2: forward, CGACCATCCGGAACGAGTT, reverse, GCAGTCGCTAGTGAGAAG, and probe, CAACGTGGCACACATTGGCCTCC; murine Mrp3: forward, TGAGATCGTCATTGATGGGC, and reverse, AGCTGGCATGGAGTG, and probe, AGGGCACGATCCTCTTCAACCCAGA; murine FXR: forward, AACTCCCGCTGATCGAACCT, reverse, TGATGGTTGAATGTCTGG, and probe, CTGGTGGTGAGGCTCCGTTCCCT; human FTF: forward, TGCACGGCAGCCTGC, reverse, GCACTCAGCTGTCTCA, and probe, CCAGTCACCTATGTGGTCCAG-AGGGTTTCCGT, and probe, CATCGCCCGCGCCCCTCA; human GAPDH was proprietary (Applied Biosystems, Foster City, CA); human FTF and GST were added to each well and incubated for 30 min at 15–25 °C. The absorbance of the samples was measured at 490 nm using an ELISA reader.

Western Blot Analysis—For Western immunoblot analysis, 50 μg of nuclear extract or membrane protein was loaded on 12 or 7.5% SDS-polyacrylamide gels. Following electrophoresis, gels were subjected to electroblot onto nitrocellulose membranes, and uniformity of loading and transfer was confirmed by Ponceau staining. The membranes were blocked overnight in TS complete (20 mM Tris-HCl, 150 mM NaCl, 5% non-fat dry milk, and 0.1% Tween 20) at 4 °C. Blots were incubated at 50 °C for 2 min and at 95 °C for 10 min and then cycled at 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The input amounts of cDNA of the unknown samples were then calculated for all target probes and normalized to GAPDH or β-actin.

Tissue ELISA Assay—Cytoplasmic extract isolated from 50 mg of liver tissue was prepared using the NE-PER Kit (Pierce) according to the manufacturer's protocol and as described previously (18). Concentrations of mouse Tnfa, IL-1β, and IL-6 were determined using a commercially available kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, 50 μl of sample and assay diluent were added to TNFα- or IL-1β-, or IL-6-coated 96-well plates and incubated for 2 h. After washing, 100 μl of conjugate was added to each well and incubated for 2 h, after which 100 μl of substrate solution was added to the manufacturer's protocol (Roche Applied Science). Briefly, 100 μl of serum was transferred into an optically clear 96-well flat-bottomed microtiter plate. To determine LDH activity, 100 μl of reaction mixture was added to each well and incubated for 30 min at 15–25 °C. The absorbance of the samples was measured at 490 nm using an ELISA reader.

Abi Prism 7700 SDS software. Briefly, 2.0, 1.0, 0.5, and 0.25 μl of synthesized mouse liver or HepG2 cDNA was amplified in triplicate for both GAPDH or β-actin and each target gene to create a standard curve. Likewise, 2.0 μl of cDNA was amplified in triplicate in all isolated mouse liver or HepG2 samples for each primer/probe combination and GADPH or β-actin. Each sample was supplemented with both respective forward and reverse primer and fluorescent probe and made up to 50 μl using TaqMan Master Mix (Applied Biosystems). Each target probe was amplified in a separate 96-well plate. All samples were incubated at 50 °C for 2 min and at 95 °C for 10 min and then cycled at 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The input amounts of cDNA of the unknown samples were then calculated for all target probes and normalized to GAPDH or β-actin.
added and then incubated, in the dark, for 30 min. Spectrophotometric absorbance was read at 450 nm.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear protein was prepared from sham and BDL mouse liver and treated HepG2 cells as described previously (18). A synthetic CPF/FTF oligonucleotide corresponding to the MRP3 promoter element (10) and a double-site mutated oligonucleotide were used in the experiments: CPF forward, GTGCTAACCCACCTCGGGCCAAATGCTCCCTCCAG, reverse, GTGGGTGAGGACTTTGGGGCAGAGGTGGGGGGGCCCTTGGCAC; CPF Mutant(1 + 2) forward, GTGCTACATGCCCCCCCACCTCTGCCCCAAAGTCCCTCCCAG, reverse, CTGGGAGGGAATGTAGGGCAGACTACATTCCCTCCCAGGGAGGC-3

The oligonucleotide was end-labeled with digoxigenin-11-ddUTP, and EMSA was performed using the digoxigenin Gel Shift Kit, according to the manufacturer’s protocol (Roche Applied Science). Briefly, 5 µg of nuclear extract per sample was preincubated in binding buffer. Labeled oligonucleotide was added, and the binding reaction was allowed to proceed for 30 min on ice. For competition assays, a 100-fold excess of unlabeled oligonucleotide was added to the binding reaction. The binding reaction 2 h before addition of the labeled oligonucleotide. The binding reaction 2 h before addition of the labeled oligonucleotide. OD was determined by densitometry.

Bsep (C) primer/probe sets. Representative blots from each time point are shown, and RNA levels were normalized to GAPDH. Signal intensity was determined by densitometry. OD, optical density; ng/mg, nanogram RNA/mg tissue. 1st *, p < 0.05 relative to sham RNA; 2nd *, p < 0.005 relative to sham RNA.

Western immunoblot and real time RT-PCR were performed using membrane protein and total RNA from 14-day sham and BDL liver and Mrp2 (A) and Bsep (C) antibodies and Mrp2 (B) and Bsep (D) primer/probe sets. Representative blots from each time point are shown, and RNA levels were normalized to GAPDH. Signal intensity was determined by densitometry.

For supershift assays, 0.2 ng/mg of polyclonal antibody was added to the binding reaction, in the dark, for 30 min. Spectrophotometric absorbance was read at 450 nm.

To characterize the 5′-flanking region of the human MRP3 gene transcriptional ability, a series of 5′/H11032 deletions ranging from −4134, −2000, −1000, −500, and −200 bp of human MRP3 upstream sequence from +19 nucleotides were amplified by the PCR from a BAC clone (hCIT.22.K.21 complete sequence, AC004590, Research Genetics Inc., Huntsville, AL), which contains the 5′-flanking region of human MRP3 gene. MRP3 upstream primers contained an internal SmaI restriction site, and one common downstream primer contained an internal Smal site. MRP3 reporter gene constructs are as follows: 4KBMRP3F, 5′-CGGGGTACCAACACCGATGGACACCTTC-3′; 1KBMRP3F, 5′-CGGGGTA-CGGGACACAGCGATGGGACACCTTC-3′; 2KBMRP3F, 5′-CGGGGTACCAACACCGATGGACACCTTC-3′; 2000 bp of human MRP3 upstream sequence

The PCR products were digested with KpnI/Smal and ligated into the luciferase promoterless vector pGL3-basic (Promega, Madison, WI) predigested with those indicated restriction enzymes. Primers were prepared, and sequence identity of promoter inserts of those constructs was verified by the W. M. Keck DNA Synthesis and Sequencing Facility at the Yale University School of Medicine.

Western immunoblot and real time RT-PCR were performed using membrane protein and total RNA from 14-day sham and BDL liver and Mrp2 (A) and Bsep (C) antibodies and Mrp2 (B) and Bsep (D) primer/probe sets. Representative blots from each time point are shown, and RNA levels were normalized to GAPDH. Signal intensity was determined by densitometry.

To characterize the 5′-flanking region of the human MRP3 gene transcriptional ability, a series of 5′ deletions ranging from −4134.

Fig. 3. Expression of hepatic Mrp2 and Bsep is similar in Tnfr−/− and WT mice after BDL. Western immunoblot and real time RT-PCR were performed using membrane protein and total RNA from 14-day sham and BDL liver and Mrp2 (A) and Bsep (C) antibodies and Mrp2 (B) and Bsep (D) primer/probe sets. Representative blots from each time point are shown, and RNA levels were normalized to GAPDH. Signal intensity was determined by densitometry. OD, optical density; ng/mg, nanogram RNA/mg tissue. 1st *, p < 0.05 relative to sham RNA; 2nd *, p < 0.005 relative to sham RNA.

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A pair of primers Mut1F and Mut1R or Mut2F and Mut2R was used to generate CPF site 1 mutant construct, Mut-CPF1, or CPF site 2 mutant, Mut-CPF2, that converted the sequence TGCCAAGGGCC to TGCtAcatGCC, or CCCCAAAGTCC to CCCtAcAtTCC, respectively. The CPF site 1 and site 2 double mutation construct was derived from Mut-CPF1 construct with Mut2F and Mut2R primer by the same method.

Statistical Analysis—All data were expressed as mean ± S.D. or mean ± S.E. Differences among groups were analyzed by analysis of variance, and statistical significance was accepted when \( p < 0.05 \). Differences between specific groups were determined using an unpaired Student's t test and analyzed by the Instat software package (GraphPad Software Inc., San Diego, CA).

RESULTS

Hepatic Necrosis After BDL Is More Severe in the Absence of Tnfr Signaling—Two weeks after BDL, Tnfr\(^{-/-} \) mice exhibited increased weight loss and liver injury relative to WT. We found that WT had lost 23.7 ± 3% of initial body weight versus 34.7 ± 4% in Tnfr\(^{-/-} \) mice (\( p < 0.05 \)). Liver weights were not significantly different. In addition, 4/18 Tnfr\(^{-/-} \) mice died following BDL in contrast to 2/18 WT. As shown in Fig. 1, histological examination revealed that sham liver (Fig. 1, A and B) did not differ between the two groups. Hepatocellular necrosis, portal inflammation, and bile duct proliferation were observed after BDL (Fig. 1, C and D). However, significantly more necrosis (Figs. 1D and 2A) was observed in Tnfr\(^{-/-} \) (8.44 ± 5.2%) liver relative to WT (1.7 ± 1.3%). To confirm differences in hepatocyte injury, we also measured serum LDH levels in all groups and, as shown in Fig. 2B, found a significantly higher serum LDH level in BDL Tnfr\(^{-/-} \) mice (2.5-fold) relative to WT. As accumulation of intrahepatic bile acids represented a likely source of the more severe liver damage seen in Tnfr\(^{-/-} \) mice following BDL, we examined bile acid levels in the serum and liver of all animals.

Bile Salts Accumulate in the Liver to a Greater Degree in the Absence of Tnfr Signaling—Previous reports from our laboratory and others have demonstrated the hepatotoxicity of bile acids (22) and therefore serum and liver tissue bile acids were measured in WT and Tnfr\(^{-/-} \) mice. As shown in Fig. 2C, serum bile acid levels rose in both groups after BDL, but to a significantly lower level in the serum of Tnfr\(^{-/-} \) mice (240.7 ± 71.6 \( \mu \)mol/liter) versus WT (458.3 ± 119 \( \mu \)mol/liter). We then measured the bile acid concentration in BDL liver tissue. As shown in Fig. 2D, we found a significantly higher concentration of bile acids in the liver of Tnfr\(^{-/-} \) mice (9.73 ± 2.38 mmol/kg) relative to WT (3.93 ± 2.12 mmol/kg). These findings suggested differences in the hepatocellular efflux of bile acids following...
We have demonstrated previously alterations in bile salt efflux pumps following BDL and thus proceeded to determine whether differences in apical Mrp2 or Bsep, or basolateral Mrp3 expression were associated with the differences in serum and hepatocellular bile acid levels in Tnfr/I/H11002/H11002 and WT bile duct ligated mice.

Expression of Hepatic Mrp2 and Bsep Is Similar in Tnfr/I/H11002/H11002 and WT Mice after BDL—Previous studies from our laboratory and others have demonstrated down-regulation of Mrp2 and relative preservation of Bsep expression after 14 days BDL in the rat (23). To assess whether differences in the expression of these apical efflux pumps could account for the differences in serum and hepatocellular bile acid levels in Tnfr/I/H11002/H11002 and WT bile duct ligated mice.

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Lrh-1 Abundance and DNA Binding to the Mrp3 Promoter Is Increased after BDL—A recent study has identified adjacent CPF/FTF/Lrh-1-response elements on the MRP3 promoter in Caco2 cells. These response elements mediated bile acid up-regulation of MRP3 RNA. We therefore determined whether the differences in Mrp3 induction in Tnfr/I/H11002/H11002 and WT mice would be due to cytochrome-mediated alterations in the expression and/or function of regulating NHRs.

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operated animals (10- and 1.5-fold, respectively) but not in Tnfr−/− mice. We then examined Lrh-1 protein:DNA binding to the Mrp3 promoter after BDL (Fig. 5C). We found that Lrh-1 binding on EMSA was increased following BDL in WT but not in Tnfr−/− mice. Competition and supershift assays confirmed that the induced complex was specific and contained Lrh-1 (Fig. 5D). We have reported previously that expression of the negative regulator of Lrh-1, Shp-1, is reduced after BDL. This could also contribute to increased Lrh-1 activation of Mrp3. However, we found that Shp-1 was reduced to an equal degree in both WT and Tnfr−/− mice (data not shown). Therefore, up-regulation of Mrp3 was most likely due to increased Lrh-1 activation and binding activity. As disruption of Tnfr1 signaling impaired Lrh-1 and Mrp3 up-regulation, we then determined whether induction of other inflammatory cytokines, including IL1β and IL6, were also associated with the observed differences in the up-regulation of these proteins.

Tnfa and IL1β Up-regulation Is Reduced in Tnfr−/− BDL Mice—Previous studies have demonstrated up-regulation of inflammatory cytokines including Tnfa, IL1β, and IL6 after BDL (15, 16), and we have reported previously that IL1β suppresses RXRa expression in primary rat hepatocytes (18). We therefore determined the expression of these cytokines in liver after BDL. As shown in Fig. 6, Tnfa (Fig. 6A), IL1β (Fig. 6B), and IL6 (Fig. 6C) were induced in liver following BDL. This induction was less marked in Tnfr−/− mice, with significantly lower levels of induced Tnfa (8.3 ± 0.6 versus 24.8 ± 4.2 pg/mg) and IL1β (47.7 ± 3.7 versus 102 ± 11.6 pg/mg) relative to WT. We then proceeded to examine the direct effects of these cytokines and the bile acid CDCA upon CPF/FTF/Lrh-1 and Mrp3 expression in HepG2 cells.

MRP3 Is Up-regulated by Tnfa, IL1β, or CDCA Treatment of HepG2 Cells—HepG2 cells were treated with Tnfa, IL1β, IL6, or CDCA for 12, 24, or 48 h, and membrane protein, nuclear protein, and RNA were isolated. As shown in Fig. 7, treatment with Tnfa or CDCA significantly induced expression of MRP3 protein (4- and 3-fold, respectively, at 24 h) (Fig. 7A) and RNA (3- and 1.7-fold, respectively) (Fig. 7B). Treatment with IL1β significantly increased MRP3 protein but not RNA. Treatment with IL6 did not increase MRP3 protein abundance (data not shown). We then proceeded to determine whether the alterations in MRP3 expression were associated with CPF/FTF/ Lrh-1 expression and DNA binding activity.

Lrh-1 Abundance and DNA Binding to the MRP3 Promoter Is Increased by Tnfa or IL1β Treatment of HepG2 Cells—HepG2 cells were treated with Tnfa, IL1β, IL6, or CDCA for 12, 24, or 48 h. As shown in Fig. 8, we found a significant up-regulation of CPF/FTF/Lrh-1 nuclear protein (4-, 2-, and 8-fold, respectively, at 12 h) (Fig. 8A) and RNA (8-, 2-, and 2-fold, respectively) (Fig. 8B) with Tnfa, IL1β, or CDCA. These data confirmed that inflammatory cytokines could induce MRP3 and CPF/FTF expression. We then proceeded to examine DNA binding to the FTF-response elements in HepG2 cells treated with cytokines or CDCA. As shown in Fig. 8C, we found that DNA binding was significantly increased in cells treated with Tnfa (3-fold), IL1β (3-fold), or CDCA (4-fold). This increased FTF DNA binding could account for the up-regulation of MRP3 RNA expression observed in treated HepG2 cells and in BDL mice. These data confirmed that both inflammatory cytokines and bile acids could induce MRP3 and CPF/FTF/Lrh-1 RNA expression and that cytokines could increase MRP3 protein abundance. To determine whether Tnfa could directly activate the MRP3 promoter via CPF, we proceeded to determine luciferase activity of the promoter construct in transiently transfected HepG2 cells.

Tnfa Increases MRP3 Promoter Activity via the CPF in HepG2 Cells—The 5′-flanking region from -4134 to +19 of the human MRP3 gene was isolated by PCR from a BAC clone, which was confirmed by sequencing. Luciferase assay (Fig. 9B) showed that the MRP3 gene promoter activity was induced by mFTF in a dose-dependent manner. MRP3 promoter activity was maximally up-regulated by mFTF cotransfection at doses ≥200 ng of plasmid. To explore further the CPF functional role in MRP3 gene regulation in human hepatocytes, the two consensus CPF sites were, individually or in combination, mutated by site-directed mutagenesis. These single and double CPF mutants of the p-500Luc construct were cotransfected with mouse FTF expression plasmid in HepG2 cells (Fig. 9C). Murine FTF significantly increased the promoter activity of p-500Luc. The basal luciferase activity of the doubly mutated CPF construct was reduced relative to wild type, and the response to CPF/FTF/Lrh-1 was abolished by the double mutation. Treatment with human TNFα at concentration of 1, 10, and 100 ng/ml for 12 h and 10 ng/ml for 24 h significantly increased the luciferase activity of p-500Luc in HepG2 cells.
(data not shown), an effect that was abolished by the double mutant (Fig. 9D). EMSA was subsequently performed using oligonucleotides for CPF WT and double mutation of the CPF elements (Fig. 9E), and we confirmed that DNA binding to the CPF oligonucleotide was abolished by the double mutant. Protein:DNA binding was completely competed away with excess of unlabeled WT CPF probe, and no competition was observed with the double mutant. These data indicated that TNFα may transcriptionally activate MRP3 via the NHR Lrh-1/FTF/CPF.

**DISCUSSION**

The multidrug resistance-associated protein 3 (Mrp3) is an inducible organic anion transport protein located on the basolateral membrane of the hepatocyte, cholangiocyte, and enterocyte, which has been shown to transport bile salts, 17β-estradiol, and some anti-cancer drugs (1–3). Our group and others (4–7) have characterized previously the induction of hepatic Mrp3 expression in experimental models of cholestatic liver disease, including bile duct ligation. Induction of Mrp3 is thought to increase elimination of bile salts from the hepatocyte and play a role in hepatic cytoprotection during cholestasis. However, the molecular mechanisms that provide the basis for the observed regulation of Mrp3 in obstructive cholestasis were not known, and its cytoprotective effect had not been directly demonstrated previously. In this study we have identified a cytokine-dependent mechanism to account for the hepatic up-regulation of Mrp3 expression, via alterations in abundance and DNA binding of the Lrh-1 nuclear receptor and subsequent up-regulation of MRP3 promoter activity. We have confirmed that induction of this transporter is hepatoprotective in obstructive cholestasis.

The relatively recent identification and characterization of nuclear hormone receptors has contributed significantly to our understanding of the regulation of hepatobiliary metabolic and transport systems in normal hepatic physiology. The realization that substrates for these transport proteins, such as bile acids, are also ligands for regulatory NHRs that may control expression of critical target genes has resulted in the understanding that intracellular accumulation of a transporter substrate may induce expression of that transporter, thereby limiting the intracellular accumulation of toxic substances (12). The FXR ligand, bile acids,
and the CAR ligand, phenobarbital, have been shown to up-regulate Mrp3 RNA expression (8, 9). More recently, Shwu-Jen et al. (10) and Inokuchi et al. (11) have described up-regulation of the rat Mrp3 promoter by Sp3 and the human MRP3 promoter by CPF/FTF. Liver receptor homologue (Lrh-1) is negatively regulated by Shp-1. Shp-1 expression is induced by bile acid-activated FXR and the heterodimeric partner for the class II NHRs, RXRα (12). We had determined previously that expression of FXR:RXRα and Shp-1 is reduced following BDL, and that these alterations were associated with hepatic Mrp3 induction (14). These studies indicated that up-regulation of Mrp3 after BDL could be due to either increases in Lrh-1 or decreases in Shp-1 activity. Data in this study support the former mechanism.

Inflammatory cytokines TNFα, IL1β, and IL6 are induced in the liver following bile duct obstruction (15, 16), and we have demonstrated previously (18) that these cytokines, particularly IL1β, then reduce expression of the NHRs RARα:RXRα and the apical transporter, Mrp2. We hypothesized that cytokines might also regulate hepatic induction of Mrp3 via up-regulation of Lrh-1. In this study we found that induction of hepatic Mrp3 following BDL was associated with Lrh-1 up-regulation, at both the protein and RNA levels. Targeted disruption of TnfrI prevented up-regulation of Lrh-1 and Mrp3. This in vivo induction of Mrp3 and Lrh-1 was reproduced by TNFα or IL1β treatment of HepG2 cells, thus confirming that these changes could be directly mediated by these inflammatory cytokines. While recognizing the fact that HepG2 cells, like primary mouse hepatocytes, may synthesize cytokines (26, 27), we would suggest that cytokine protein levels were likely many-fold lower than those used in our experiments. It is important to note that there also appears to be a TNFα-dependent post-translational effect on Lrh-1 protein abundance in vivo. In addition, IL1β levels were significantly lower in TnfrI−/− mice relative to WT following BDL, and MRP3 was up-regulated by IL1β in vitro. These data indicate that IL1β may contribute to up-regulation of MRP3 in vivo. We have further demonstrated that MRP3 promoter activity

**Fig. 8.** Lrh-1 abundance and DNA binding to the MRP3 promoter are increased in HepG2 cells treated with cytokines. Western immunoblot, real time RT-PCR, and EMSA were performed using nuclear protein and total RNA from HepG2 cells treated with Tnfa, IL1β, or CDCACPF/Lrh-1 protein (A), CPF/Lrh-1 RNA (B), and DNA binding (C) to the MRP3 promoter CPF-response element. Representative blots at each time point are shown, and RNA levels were normalized to GAPDH. Signal intensity was determined by densitometry. OD, optical density. ng/mg, nanogram RNA/mg tissue. (*, p < 0.05 relative to control.)
may be increased by both CPF and TNFα and that a double mutation of both CPF-response elements on the promoter abolishes these effects and binding to the MRP3 promoter element. These data reveal that the two CPF-like elements may play a key role in MRP3 basal expression in human liver and that TNFα may increase MRP3 expression via the Lrh-1/FTF/CPF pathway. Both CPF-like elements seem to be required for up-regulation of MRP3 in response to TNFα in HepG2 cells.

Importantly, we confirmed that induction of Mrp3 conveyed significant hepatoprotection following bile duct obstruction in mice. The absence of Mrp3 induction in TnfrI−/− mice was associated with increased hepatic bile acid concentration, hepatocellular necrosis, weight loss, and mortality. Although previous work in Tnfa−/− mice has demonstrated improved survival and decreased fibrosis (we could detect no significant difference in the histologic degree of fibrosis between the 2 groups) after 3 weeks BDL compared with WT (25), TNF signaling via the type II receptor was intact in our model and may, in part, account for the observed differences.

The cytokine-dependent and hepatoprotective effects of Lrh-
1-associated induction of Mrp3 has profound implications for hepatobiliary transport in both normal physiologic conditions and in certain liver diseases, including obstructive cholestasis. As we have already suggested, the alteration in hepatobiliary transport systems by inflammatory cytokines, either directly or indirectly by up-regulation of NHR abundance, may serve as a protective and regulatory impetus in certain liver disease states, just as specific ligand-activated NHRs control the expression of genes involved in hepatic biosynthesis and transport under normal physiologic conditions. First, it would be predicted that induction of Mrp3 expression should be beneficial to patients with cholestasis. For example, induction of genes such as Mrp3 by compounds such as phenobarbital or other xenobiotics, or by pharmacological manipulation of the expression of specific NHRs, including Lrh-1, may significantly reduce hepatocellular accumulation of bile salts and potentiate the liver damage.

In conclusion, our combined results indicate that induction of Mrp3 is hepatoprotective in cholestasis and suggest that this transporter may be transcriptionally regulated by Lrh-1. Up-regulation of both Mrp3 and Lrh-1 is dependent on intact TNFα signaling pathways. We speculate that this great flexibility of the hepatocyte to respond to multiple signals in order to maintain hepatic bile acid homeostasis is extremely relevant to putative pharmacological manipulation of these pathways. Further study, understanding, and manipulation of NHR and cytokine mediated regulation of hepatobiliary transporters should lead to more specific treatments for cholestatic liver diseases.

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