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The Bile Acid Sensor Farnesoid X Receptor Is a Modulator of Liver Immunity in a Rodent Model of Acute Hepatitis

Andrea Mencarelli,* Barbara Renga,* Marco Migliorati,* Sabrina Cipriani,* Eleonora Distritti,† Luca Santucci,† and Stefano Fiorucci‡*

Immune-mediated liver diseases including autoimmune and viral hepatitis are a major health problem worldwide. In this study, we report that activation of the farnesoid X receptor (FXR), a member of the ligand-activated nuclear receptor superfamily and bile sensor highly expressed in the liver, attenuates liver injury in a model of autoimmune hepatitis induced by Con A. We found that FXR gene ablation results in a time-dependent increase of liver expression (up to 20-fold in a 9-mo-old mouse) of osteopontin, a NKT cell-derived extracellular matrix protein and immunoregulatory cytokine. In comparison to wild-type, FXR−/− mice are more susceptible to Con A-induced hepatitis and react to Con A administration by an unregulated production of osteopontin. Administering wild-type mice with a synthetic FXR agonist attenuated Con A-induced liver damage and liver expression of the osteopontin gene. By in vitro studies, we found that FXR is expressed by primarily isolated NKT cells and its ablation favors osteopontin production in response to Con A. Chromatin immunoprecipitation assay and coimmunoprecipitation experiments demonstrate that the short heterodimer partner (SHP), a nuclear receptor and FXR target, was expressed by NKT cell hybridomas and increased in response to FXR activation. FXR activates SHP that interacts with and inhibits c-Jun binding to the osteopontin promoter. These data indicate that in NKT cells, FXR activation causes a SHP-mediated inhibition of osteopontin production. These data support the notion that the bile acid sensor FXR regulates the activation of liver NKT cells. The Journal of Immunology, 2009, 183: 6657–6666.
array of hepatocellular abnormalities including adenomas and carcinomas. This pattern associates with an increased liver epithelial proliferation and expression of proinflammatory cytokines and oncogenes (40, 41).

In the present study, we demonstrate that FXR−/− mice spontaneously develop hepatic damage and liver immune dysregulation and show enhanced susceptibility to T cell-mediated hepatitis. In addition, we provide evidence that NKT cells express a functional FXR and that FXR ligands activate counterregulatory signals in NKT cells that prevent hepatitis by inhibiting Con A-induced OPN synthesis.

Materials and Methods

Mice

Specific pathogen-free C57BL/6 mice (5–7 wk old) were purchased from Charles River Laboratories. Homozygous FXR−/− mice (C57BL/6J genetic background) were originally provided by F. J. Gonzalez (National Institutes of Health, Bethesda, MD). Animals were maintained with a 12-h light/12-h dark cycle with free access to food and water. All procedures were approved by the Animal Care and Ethics Committees of the University of Perugia.

Cell lines and culture media

The HepG2 cell line was cultured in MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. DN32.D3 cells, a murine NKT cell hybridoma cell line (42), provided by Dr. A. Bendelac (Princeton University, Princeton, NJ), were cultured in DMEM supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Treatments

FXR WT and FXR−/− mice were injected i.v. with a freshly prepared Con A, type IV, in a pyrogen-free saline (Sigma-Aldrich). In a first set of experiments, we have investigated whether FXR gene ablation sensitizes WT mice to Con A-induced liver injury. To this end, FXR−/− mice were administered with a lethal dose of Con A (25 mg/kg), with or without pretreatment with 6E-CDCA (Bio-Rad), and then probed with primary anti-FXR Ab (Abcam). The anti-FXR Ab (Bio-Rad) was used as a secondary Ab, and specific protein bands were visualized by chemiluminescence using Super Signal West FEMTO reagent (Pierce).

Evaluation of FXR activity in ND32.D3 cells

Immunoprecipitation. DN32.D3 cells (42), a NKT hybridoma, were serum starved for 24 h and then incubated for 18 h with 10 μM 6E-CDCA and 1 μg/ml Con A at 37°C. 6E-CDCA was added 30 min before Con A/LPS treatment. Cellular lysates were first diluted with E1A wash buffer containing phosphate and protease inhibitors, sonicated, and then visualized by chemiluminescence using Ultra Signal West FEMTO reagent (Pierce, Rockford, IL).

Chromatin immunoprecipitation (ChIP) assay. Cross-linking and ChIP assays were performed according to the manufacturer’s protocols (Abcam), with minor modifications. Briefly, DN32.D3 cells were cross-linked with 1% formaldehyde at room temperature and then the reaction was terminated by the addition of glycine at a final concentration of 0.125 M. Cells were washed in ice-cold PBS and lysed with SDS lysis buffer (50 mM Tris-HCl, pH 8). Cellular lysates were diluted with ChIP dilution buffer, sonicated, and immunoprecipitated with specific Abs: anti-SHP and anti-c-Jun from Santa Cruz Biotechnology. Immunoprecipitates were collected with protein A beads (Amersham Biosciences) and washed sequentially with a low-salt wash buffer and then with a high-salt wash buffer using the manufacturer’s recommended procedures. DNA was eluted at elution buffer (1% SDS, 0.1 M NaHCO3, and 1 M NaCl) and the cross-linking reactions were reversed by heating the mixture to 65°C overnight. The DNA was recovered from immunoprecipitated material by proteinase K treatment at 65°C for 1 h followed by phenol:chloroform (1:1) extraction, ethanol precipitation, and dissolved into 50 μl of water. For real-time PCR, 5 μl of ChIP products from DN32.D3 cells were used to perform amplification of the mouse Opn gene promoter using the following primers: sense: 5′-AGAGGAAATGTGAGGGCTAGTG-3′, antisense: 5′-GGCTCAGACCTCCCAGAATTTA-3′. Quantization of the expression of selected genes was performed by realtime qRT-PCR. Total RNA were obtained from livers and isolated with TRIzol reagent (Invitrogen), incubated with DNase I, and reverse-transcribed with Superscript II (Invitrogen) according to the manufacturer’s specifications. For real-time PCR, 100 ng of template was used in a 25-μl reaction containing a 0.3 μM concentration of each primer and 12.5 μl of 2× SYBR Green PCR Master Mix (Bio-Rad). All reactions were performed in triplicate using the following cycling conditions: 2 min at 95°C, followed by 50 cycles of 90°C for 10 s and 60°C for 30 s using an iCycler iQ instrument (Bio-Rad). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (Ct). The amount of gene expression was then calculated as the difference (ΔCt) between the Ct value of the target gene and the Ct value of an internal control (GAPDH). Relative expression was calculated as the difference (ΔΔCt) between the ΔCt values of the test and control samples for each target gene. The relative level of...
expression was measured as $2^{-\Delta\DeltaCT}$. All PCR primers were designed with the software PRIMER3-OUTPUT using published sequence data obtained from the National Center for Biotechnology Information database.

Mouse primers were as follows: FXR, TGTGAGGGCTGCAAAGGTT and ACATCCCCATCTTGGAC; IL-4, CCTCACAGCAACGAAGAACA and ATTCGGAAAGCCGGAAGAGT; IFN-$\gamma$, GCTGCTATTGAAATCA CACCTG and GACCTTGTTGGTTGACCT; OPN, CTGTGCGCCAG CTCTAGG and GACCTTGGAACCTGTTGAC; TNF-$\alpha$, AAGCC ATGGAATCTCAAGAC and GTGGGTGAGGAGCACGTAG; SHP, AAGGGCTTGTTGAGCAGTATA and TCTCTCTCTCCGCTTACATA; 18S, ACCGACGCTAGGAAATATGGA and GCCTCAGTCCGAAA AAC; and GAPDH, CTAATGTGCTGAGTCTAGT and GTTG GTGGGTGAGGATGATG. Human primers were as follows: FXR, TAMATGGAAGAAATGTCAGA and ACTGATTTCTCAAG GTTCTGA; GAPDH, GAAGGTGAAGGTCGGAGTCTAC and CATGAGTG GAATCATATTGGAA; and 18S, CGGTCTACACATCAAAGCAA and GCTTGAAATATCCGCGGT.

Cytokine measurements. Plasma cytokine concentrations were determined using Bio-Plex Cytokine Assays (Mouse 23-Plex Panel 171-FII241; Bio-Rad) according to the manufacturer’s directions. Plasma samples were incubated for 2 h at room temperature and cytokine concentrations were measured on a single plate with a Bio-Plex 200 system and then analyzed with Bio-Plex Manager 5.0 software.

Myeloperoxidase (MPO) activity. Neutrophil infiltration in the liver was measured by assessing MPO activity. Liver lysates were prepared by homogenizing 100 mg of tissue in 1 ml of T-PER buffer. Liver homogenates were then centrifuged at 15,000 rpm for 15 min and supernatants were collected for MPO activity measurement by using a spectrophotometric assay with tetramethylbenzidine as a substrate. Standard curve was performed using purified MPO (Sigma-Aldrich).

Data analysis. All values are expressed as mean ± SE of mice per experiment. The variation between data sets were tested with the unpaired Student’s t test.

Results

FXR$^{-/-}$ mice spontaneously develop a progressive liver injury associated with liver immunity dysregulation

We first evaluated hepatic histopathology and expression of regulatory cytokines and chemokines at 2, 5, and 9 mo of age in FXR null and WT mice. Histopathological evaluation demonstrates that FXR deficiency results in a progressive liver injury characterized by hepatocytes vacuolization, lipid deposit, and focal necrosis (data not shown). As shown in Fig. 1, these changes associate with liver immunity dysregulation, as demonstrated by the robust induction of IFN-$\gamma$ (~9-fold increase vs WT mice) and OPN (~20-fold increase vs WT mice) in 9-mo-old mice ($p < 0.05$ vs WT; $n = 6$). These changes associated with a strong reduction in the expression of liver SHP, a FXR-regulated gene ($p < 0.05$ vs WT mice; $n = 6$).

**FIGURE 1.** Liver expression of regulatory cytokines and SHP, a FXR-regulated gene, in WT and FXR$^{-/-}$ mice at 2, 5, and 9 mo of age. *, $p < 0.05$ vs WT mice; n = 6.

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**FXR gene ablation exacerbates, while FXR activation protects, against Con A-induced liver injury**

To explore whether FXR gene ablation predisposes to hepatitis induced by Con A, we injected 8-wk-old FXR$^{-/-}$ and WT mice with a lethal dose of Con A (25 mg/kg), with or without pretreatment with 6E-CDCA (10 mg/kg). As shown in Fig. 2A, this treatment resulted in a 24-h mortality of 80% in WT mice. This figure was further exacerbated by FXR gene ablation, with an early higher mortality (50% at 4 h; $p < 0.05$ vs WT) and the 100% mortality at 24 h. Administering FXR WT mice with 6E-CDCA reduced lethality induced by Con A administration, highlighting the protective role of the receptor in this model of liver injury ($p < 0.05$ vs Con A alone).

In the next series of experiments, we measured AST plasma levels and assessed liver histology in mice exposed to a sublethal dose of Con A (10 mg/kg). As shown in Fig. 2B, administering WT mice with Con A resulted in a time-dependent increase in AST plasma levels. In comparison to WT mice, Con A administration to FXR$^{-/-}$ mice resulted in significantly higher AST levels at 24 h ($p < 0.05$, vs WT mice; $n = 8$). Interestingly, pretreatment with 6E-CDCA attenuated liver injury caused by Con A administration, as measured by AST plasma levels ($p < 0.05$; $n = 8$). Moreover, liver MPO activity (Fig. 2C), a measure of neutrophil accumulation into the liver, was significantly elevated in all groups within 4 h but remained markedly elevated at 24 h only in Con A-treated FXR$^{-/-}$ mice. Pretreatment with 6E-CDCA resulted in a significant reduction in Con A-induced liver MPO activity ($p < 0.05$ vs saline-treated mice, $n = 8$). In comparison to WT mice, the severity of liver injury caused by Con A was markedly exacerbated by FXR gene ablation, as demonstrated by the histopathological analysis (Fig. 2D).

In aggregates, these data establish that FXR
ablation exacerbates, while FXR activation protects against Con A-induced hepatitis.

**FXR regulates liver NKT cell activation**

To gain information on the mechanisms involved in liver protection exerted by FXR activation, we then examined the immune phenotype of liver-infiltrating cells in WT mice administered Con A in the presence of a FXR ligand. In addition, we also investigated whether Con A administration to FXR-/- mice results in a different phenotype of liver-infiltrating leukocytes. We found that Con A administration resulted in an ~3-fold increase in the absolute number of leukocytes infiltrating the liver at 2 h (data not shown). The phenotypic characterization of these liver-infiltrating cells demonstrated that Con A administration associates with a robust increase in the percentage of CD14+ cells (macrophages) and Gr1+ cells (polymorphonuclear neutrophil (PMN)) while the percentage of CD3+ cells was slightly reduced (Fig. 3). This effect was attenuated by treating mice with 6E-CDCA at a dose of 10 mg/kg (Fig. 3). Moreover, flow cytometry analysis revealed that liver-infiltrating CD14+ and Gr1+ cells were significantly more activated than cells isolated from naive animals as assessed by measuring the expression of CD11b on both cell types. Again, treating the mice with a FXR agonist reduced this pattern. Administering mice with 6E-CDCA was also effective in reducing the expression of FasL on CD3+ and NKT cells (Fig. 3). Hepatic NKT cells, but not T cells, constitutively express FasL mRNA. Therefore, the rapid appearance of FasL on the surface of Con A-stimulated NKT cells may be predominantly mediated by the delivery of intracellularly stored FasL to the cell surface. A precedent work (6) indicated that NKT Fas/FasL cascade is involved in the early phase of Con A-induced hepatitis. Consistent with this finding, down-regulation of FasL on NKT cells in mice administered 6E-CDCA associated with a reduction of AST levels at 4–8 h after Con A administration. Although these data suggest that FXR activation limits the infiltration of activated leukocytes in this model, challenging FXR-/- mice with Con A did not generate a distinctive signature either in the number or the relative percentage of infiltrating cells in comparison to FXR WT mice (Fig. 3). This suggests that FXR agonism attenuates activation of NKT in the model of Con A-induced hepatitis.

**FXR regulates OPN liver expression**

We then investigated whether FXR gene ablation modulates the release of cytokines and chemokines known to play a pathogenic role in liver injury. Using a semi-synthetic FXR ligand, we found that treatment with 6E-CDCA reduced the expression of OPN, a chemokine involved in hepatic inflammation, in both FXR-/- and WT mice (Fig. 2). These results suggest that FXR activation limits the release of pro-inflammatory mediators in the liver, thereby contributing to the protective effect of FXR activation against Con A-induced liver damage.
role in the model of Con A-induced hepatitis. As shown in Fig. 4, Con A administration resulted in a robust induction of liver expression of several cytokines. This effect was insensitive to the presence of FXR, with the exception of OPN. Thus, FXR−/− mice react to Con A administration with a dramatic up-regulation of these cytokines in the liver (≈25-fold increase in comparison to WT mice; Fig. 4D). These findings were further corroborated by the measurement of OPN protein (ELISA and immunohistochemistry) in the liver (Fig. 5). Immunohistochemistry analysis (Fig. 5B) demonstrated a focal increase in the OPN expression. OPN expression was concentrated in areas of liver damage, suggesting a role for this glycoprotein in favoring emigration of inflammatory cells into necrotic tissues. FXR activation with 6E-CDCA in WT mice reduced mRNA liver expression of OPN and other proinflammatory cytokines measured 24 h after Con A injection (Fig. 4; p < 0.05 vs saline-treated mice, n = 8). The inhibitory effects of 6E-CDCA were lost in mice harboring a disrupted FXR gene. Confirming mRNA data, administering mice with 6E-CDCA caused a robust reduction in the liver expression of OPN protein content as measured by ELISA and immunohistochemistry (p < 0.05 vs saline-treated mice, n = 8; Fig. 5). Finally, 6E-CDCA administration to Con A-treated WT mice effectively reduced the plasma levels of several cytokines and chemokines (KC, MIP-1α, IFN-γ, and TNF-α; supplemental Fig. 1A).

FXR/SHP directly regulates OPN gene expression on activated NKT cells

Because these data suggest that FXR regulates the liver expression of OPN, we have then designed a series of studies to investigate whether FXR regulates OPN expression in liver-derived NKT cells. The experiment shown in Fig. 6 demonstrates that liver-derived NKT cells express FXR (Fig. 6, A and B). CD3+CD1d−/− NKT cells (90% purity; n = 4) express FXR (Fig. 6, A–C). By using liver-derived NKT cells isolated from WT and FXR−/− mice, we found that FXR exerts a direct regulatory role on OPN generation. Thus, as shown in Fig. 6D, not only OPN expression was ≈3-fold higher in FXR−/− NKT cells in comparison to WT cells, but, while exposure of WT NKT cells to Con A resulted in a robust induction of OPN mRNA expression (p < 0.05 vs untreated), OPN expression was boosted up to 7-fold in FXR−/− NKT cells (p < 0.01 vs WT cells treated with Con A). To gain insight on the molecular mechanisms involved in OPN regulation by FXR in NKT cells, additional experiments were conducted using a NKT cell hybridoma (the murine Vα14/NKT TCRβ CD1d-specific T-T hybridoma DN32.D3 (39)). By qualitative RT-PCR, Western blot analysis and real-time qRT-PCR, we found that DN32.D3 cells, nonactivated by Con A, express FXR along with the FXR-regulated gene SHP (Fig. 7). This finding is in line with the observation that adult peripheral blood-derived CD56 NK cells express FXR, as reported by the gene atlas (http://symatlas.gnf.org). Treating DN32.D3 with Con A for 18 h resulted in a 10-fold increase in IFN-γ mRNA expression, while expression of the OPN gene was induced by 50-fold (p < 0.01 vs untreated, n = 6; Fig. 7D and E). Induction of cytokine expression by Con A was associated with a significant reduction in SHP mRNA expression (Fig. 7F). These changes were reversed by treating DN32.D3 cells with the FXR

The online version of this article contains supplemental material.
ligand 6E-CDCA (Fig. 7, D–F). Thus, exposure of DN32.D3 cells to 10 μM of the FXR ligand completely abrogated IFN-γ and OPN induction caused by Con A and restored SHP mRNA expression (p < 0.05; n = 6), highlighting the negative correlation existing between cytokines/chemokines and SHP gene expression in NKT cell hybridomas.

Previous studies have shown a regulatory function for the AP-1 consensus site located at +76 bp in the transcriptional regulation of the OPN gene (43–45). The importance of this AP-1 site for the transcriptional activity of the OPN promoter has been highlighted by a complete loss of basal and inducible OPN promoter activity after site-directed mutagenesis (43–45). We have previously shown that FXR activation induces a SHP-dependent inhibition of JunD and c-Jun binding to the AP-1 binding site (46, 47). We have therefore performed a ChIP assay and PCR amplification using primer pairs that cover the AP-1 consensus site at −76 in the OPN promoter. Results from these experiments demonstrated that this promoter region communoprecipitated with c-Jun in Con A-treated DN32.D3 cells (Fig. 8B) and that this effect was reduced in cells treated with 6E-CDCA. In addition, immunoprecipitation experiments demonstrated that exposure to 6E-CDCA induces SHP association with c-Jun in Con A-activated cells (Fig. 8A). Furthermore, by ChIP assay, we found that SHP associates with the OPN promoter in untreated cells (Fig. 8B). Although SHP binding to the OPN promoter was markedly reduced by exposure of DN32.D3 cells to Con A (Fig. 8B), this interaction was robustly reinduced by coexposing DN32.D3 cells treated with Con A to 6E-CDCA. In concert, these findings indicate that Con A-induced c-Jun binding to the AP-1 consensus site at +76 of the proximal OPN promoter is inhibited by FXR/SHP activation.

**FIGURE 5.** FXR gene ablation resulted in an unregulated generation of OPN. OPN liver expression was assessed in FXR−/− and WT mice 24 h after the mice had been treated with Con A (10 μg/kg). In addition, WT mice were administered 6E-CDCA (10 μg/kg). A, Quantification of OPN protein levels. The results were normalized with the protein sample concentrations (*, p < 0.05 vs WT Con A alone group. n = 8). B, OPN immunohistochemistry. B1, Naive WT mice; B2, WT mice administered Con A; B3, WT mice administered Con A and 6E-CDCA; B4, naive FXR−/− mice; and B5, FXR−/− mice administered Con A.

**FIGURE 6.** FXR expression and OPN regulation in liver-derived NKT cells. A and B, Isolation and characterization of liver NKT cells (CD3−NK1.1+ cells) from WT and FXR−/− mice. Purity of cell fractions was verified by two-color flow cytometry analysis using FITC-conjugated anti-mouse CD3 and PE-conjugated anti-NK1.1 mAbs. An example of liver mononuclear cells before extraction protocol is shown in A, 10% of cells are CD3− and NK1.1+. B, A cell population after the purification protocol, see Materials and Methods, with 90% of cells staining positively with anti-CD3 and anti-NK1.1 (n = 4). C and D, FXR expression in liver NKT cells as assessed by qualitative and quantitative RT-PCR. HepG2 cells, an hepatocellular human carcinoma cell line, was used as a positive control. E, OPN gene expression in liver NKT cells obtained from WT and FXR null mice, 5 mo old mice. Cells were incubated alone or in combination with 1 μg/ml Con A for 16 h. *, p < 0.05 vs WT cells not treated and **, p < 0.05 vs WT cells treated with Con A alone (n = 4).
**FIGURE 7.** FXR/SHP directly regulates OPN gene expression in activated DN32.D3 NKT hybridoma. A–C, Qualitative PCR and Western blotting analyses and real-time qRT-PCR demonstrating FXR expression by DN32.D3 cells, a murine NKT cell hybridoma, and HepG2 cells, an hepatocellular human carcinoma cell line used as a positive control. D–F, Modulation of IFN-γ, OPN, and SHP gene expression by Con A and 6E-CDCA in DN32.D3 cells. *, p < 0.05 vs cells treated with Con A alone. For these experiments, DN32.D3 cells were starved in medium alone. Treating DN32.D3 cells with Con A increased ~10-fold IFN-γ in the mRNA expression, while OPN gene expression was increased ~50-fold. *, p < 0.01 vs untreated cells; n = 6. In contrast, Con A decreased SHP mRNA expression. *, p < 0.01 vs untreated cells; n = 6. Cotreatment with a FXR ligand completely abrogated IFN-γ and OPN induction caused by Con A and restored SHP mRNA expression. *, p < 0.01 vs cells treated with Con A alone; n = 6. WB, Western blot.

**FIGURE 8.** FXR activation recruits SHP to the OPN promoter. In naive cells, c-Jun was coimmunoprecipitated with SHP. A, Coimmunoprecipitation of c-Jun with SHP was robustly inhibited by cell exposure to Con A and reinduced by FXR activation with 6E-CDCA. Data shown are representative of five experiments. Input represents a lane loaded with a total cell lysate (nuclear plus cytosol). B, ChIP analysis demonstrates that binding of c-Jun to its consensus site in the OPN promoter was robustly enhanced by exposing DN32.D3 cells to Con A and reversed by FXR activation with 6E-CDCA. The values of real-time PCR analysis are the mean ± SE of four experiments. *, p < 0.05 vs untreated cells and #, p < 0.05 vs Con A-alone treated cells. WB, Western blot; IP, immunoprecipitated.

**Discussion**

FXR is a ligand-regulated transcription factor expressed in the liver and gastrointestinal tract that functions as an endogenous sensor for bile acids regulating cholesterol and lipid homeostasis. In this study, we demonstrate a novel role for FXR in regulating key aspects of liver T cell biology. Thus, we demonstrated that FXR−/− old mice spontaneously develop hepatic damage associated with a marked increase in liver expression of proinflammatory cytokines such as IFN-γ and OPN. Moreover, using a rodent model of T cell-mediated hepatitis, we were able to provide evidence that although the progression of this immune-mediated disorder is severely exacerbated by FXR gene ablation, it could be prevented by the administration of a synthetic FXR agonist to WT mice.

Previous studies have linked metabolic nuclear receptors to the counterregulation of innate immunity (23–26). Thus, activation of peroxisome proliferator activated receptor γ and liver X receptor by lipid mediators generated by the arachidonic acid breakdown antagonizes the expression of inflammatory cytokines providing counterregulatory signals to cells of innate immunity (24–26). In the present study, we have further extended the list of lipid mediators and intermediates that regulate effector branches in the immune response by demonstrating that FXR gene ablation results in a proinflammatory phenotype characterized by a robust increase in the liver expression of OPN, a glycoprotein that is involved in immune-mediated liver diseases both in human and experimental models (13–16, 48–53). In addition, the fact that FXR-deficient mice express high levels of IFN-γ, a cytokine that plays a major role in regulating the immune response to virus, suggests that activation of FXR by bile acids might favor development of liver immune tolerance.

Other than in naive conditions, dysregulation of liver immunity in mice lacking FXR manifests itself by an increased susceptibility to develop immune-mediated liver injury. The hepatitis induced in susceptible mice by Con A administration represents a useful model to explore key aspects of T cell-mediated immunity in the liver. An important observation made in this report was that challenge of FXR−/− mice with Con A results in a form of hepatitis manifested by an unusually high rate of early mortality (4–8 h). In vivo, FXR activation with the semisynthetic ligand 6E-CDCA rescued FXR WT mice from the lethality induced by Con A and protected against hepatitis development induced by a sublethal dose of this lectin. The fact that the same agent failed to protect against injury caused by Con A in FXR−/− was a striking demonstration of the role FXR exerts in regulating liver immune homeostasis (Fig. 2).

Despite that NKT cells trigger hepatitis after Con A injection, it is uncertain how the mitogen locally activates NKT cells. Liver NKT cells can be locally activated either specifically through the...
TCR (e.g., by glycolipid-presenting cells) or nonspecifically through cytokines such as IL-12 or IL-18 (54). In vivo, activation of T cells is an APC-dependent process, but the APC type that presents the lectin in the liver in this model is not defined. Using herpes virus entry mediator (HVEM)-deficient mice, Wahl et al. (54) have shown that nonparenchymal APC are essential for activating NKT cells in the Con A model. Because HVEM is expressed by most cells of the immune system (T cells, B cells, NK cells, dendritic cells, and monocytes) and by some nonimmune cells such as hepatocytes, the specific cell subtypes that function as APC in this model were not strictly defined. However, because intrahepatic dendritic cells express HVEM on the surface and NKT maturation in the Con A model requires IL-12, these professional APC are an attractive candidate (54). In addition to professional APC, it is suggested that liver sinusoidal cells could play a role in NKT cell activation. NKT cells in the liver do not transmigrate outside the vascular system and remain within the sinusoids. Gessimann et al. (55) have elegantly shown that NKT cells patrol within hepatic sinusoids at 10–20 μm/min and stop upon TCR-mediated activation, thus providing a local, intravascular immune surveillance (56). Liver NKT cells are CXCR6 positive (55). CXCR6 is a chemokine receptor that can serve in conjunction with CD4 as a coreceptor for infection with some human and most simian immunodeficiency viruses (HIV-1, HIV-2, and SIV) and similarly to CCR5 and CXCR3 has an expression pattern restricted to memory/effector T cells such as NKT cells. CXCR6 has one known ligand, CXCL16, a transmembrane chemokine highly expressed by liver sinusoidal cells (55). CXCR-deficient mice have a decreased susceptibility to hepatitis induced by Con A. Because sinusoidal cells are the main site of lymphocyte adhesion/transmigration and adhesion molecule expression in the hepatic venous system during Con A-induced hepatitis (57), it is has been suggested that these cells can function as nonprofessional APC for NKT cells. Interestingly, endothelial cells (58) and liver sinusoidal cells express a functionally active FXR that regulates multiple effector functions in these cells (59).

Activation of NKT cells in response to Con A can be also partially modulated in an autocrine fashion. Previous studies have demonstrated that OPN derived from activated NKT cells is essential in the development of hepatitis induced by Con A (12–14). Thus, Con A administration results in activation of liver NKT cells that secrete OPN. The interaction of NKT cells with the thrombin-cleaved form of OPN through αβ and αβ integrins further activates NKT cells in an autocrine fashion (12). However, it is intriguingly that in contrast to the cleaved form (that is mostly acting through integrin receptors), the intact isoform of OPN binds to CD44 (60). Because CD44 directly activates NKT cells with a different mechanism in comparison to conventional T cells (61, 62), this interaction could further strengthen the pathogenic role of the OPN-NKT cell pathway in the model of Con A-induced liver injury.

There is circumstantial evidence to support the concept that activated NKT cells expressing FasL contribute to Con A-induced liver cell injury (12). On the other hand, OPN, along with MIP-2, recruits neutrophils into the liver (12). Upon interaction of OPN with its receptors on neutrophils, the latter cells became activated, secreted MPO, and contributed to additional liver cell damage (12). Confirming this scenario, we demonstrated that FXR activation exerts protective activity in Con A-induced hepatitis by counteracting OPN expression. This view is confirmed by the following observations: 1) FXR−/− old mice spontaneously develop liver damage associated with a strong (20-fold) increase of liver OPN mRNA expression, 2) induction of OPN liver expression by Con A administration is amplified in FXR−/− mice, and 3) FXR activation in WT mice reduces liver OPN gene expression. In aggregate, these data support the notion that attenuation of liver expression of OPN could be the pivotal mechanism through which FXR exerts its counterregulatory role in this model. Thus, attenuation of OPN synthesis observed in mice treated with the FXR agonist could manifest its effects by a reduction of liver-activated NKT cells harboring FasL on their surface. Because these cells are instrumental to the liver damage caused by Con A, this effect is likely to play an important role in the protective effect we observed in response to FXR activation (6). In addition, because of the essential role OPN plays in recruiting PMN into the liver in this model, inhibition of OPN synthesis by the FXR agonist is also likely to contribute to the protective effect we measured on the number of liver-infiltrating PMN, another cell type that contributes to liver injury in this model (12, 62).

One important observation we made was that treating WT mice with a FXR agonist attenuates changes in blood plasma levels of KC (63) and MIP-1α (62) (supplemental Fig. 1). Because MIP-1α levels are elevated in the serum of patients with T cell-mediated liver diseases and this chemokine appears to be involved in recruitment of CD4+ cells in the Con A-induced hepatitis (64), it is tempting to speculate that MIP-1α represents an additional target for FXR.

Previous studies have shown that SHP mediates some of the regulatory activities exerted by FXR (33, 36, 46, 47). SHP is an atypical nuclear receptor, lacking a ligand-binding domain. SHP activation by FXR ligands in hepatocytes leads to the repression of cholesterol 7α-hydroxylase expression, the rate-limiting enzyme in the neutral pathway that leads to bile acid production from cholesterol (33). SHP is also a known target for other nuclear receptors, including the estrogen receptors and PPARγ (65, 66), and is essential in the regulation of the expression/activity of hepatocyte NF4 and retinoid X receptor (66). A body of evidence suggest that SHP represents an important mediator of FXR activity and acts as a corepressor of FXR target genes in different physiological contexts (33, 36, 46, 47). To investigate the molecular mechanism(s) by which FXR regulates OPN production, we conducted in vitro experiments using a NKT cell hybridoma. The results of these experiments demonstrate that DN32.D3 cells express FXR and SHP and that FXR activation leads to induction of SHP expression. Because the analysis of the OPN promoter failed to demonstrated a FXR-responsive element and OPN is positively regulated by interaction of AP-1 elements to its promoter (43–45), we have investigated whether FXR activation inhibits the interaction of AP-1 with the OPN promoter. The results of these studies demonstrate that activation of FXR leads to a displacement of c-Jun from its consensus site in the OPN promoter. The mechanism through which FXR mediates this effect requires SHP, because FXR activation leads to a direct interaction of SHP with c-Jun, an AP-1 constituent, and the formation of a SHP-c-Jun protein-protein complex. Coimmunoprecipitation experiments and ChIP analysis demonstrated that SHP binds to the OPN promoter in untreated cells, but is released in cells exposed to Con A. This interaction is restored by treating the cells with the FXR ligand. In concert, these findings indicate that Con A-induced c-Jun binding to the AP-1 consensus site at −76 of the proximal OPN promoter is inhibited by FXR/SHP activation.

In summary, ablation of the bile acid sensor FXR results in a dysregulated generation of OPN in the liver and exacerbation of the liver injury caused by Con A. Activation of FXR by a synthetic ligand rescues from liver damage caused by Con A by a mechanism that involves inhibition of NKT cell activation and OPN production. Regulation of OPN production by NKT cells represents a
new mechanism for regulation of liver-immune homeostasis by the bile acid sensor FXR.

Disclosures

The authors have no financial conflict of interest.

References


