Intestinal bacterial translocation (BTL) is defined as the migration of viable micro-organisms from the gut lumen toward the mesenteric lymph nodes (MLNs) and extra-intestinal sites such as the peritoneal cavity.1 BTL has been documented in both animal and human cirrhosis, and its occurrence is considered detrimental in the natural history of a patient with chronic liver disease, because it either precludes hepatic decompensation or propagates further liver impairment, often culminating in multiorgan failure and death.2–9

Also, translocation of pathogen-associated molecular patterns such as endotoxins has been shown to drive progression of and complications in liver disease in cirrhotic patients.6 Ample evidence from observational studies in cirrhotic patients has shown that BTL increases with increasing degree of
hepatocellular failure, rather than the degree of portal hypertension, although animal studies have shown BTL to occur also in conditions of acute and chronic portal hypertension.\textsuperscript{1,5} Especially rapidly progressive jaundice appears to coincide with infection, because histological features of intrahepatic cholestasis such as ductular bilirubinosis predict the development of septicemia and worsen prognosis in patients with acute decompensating cirrhosis.\textsuperscript{10,11} Furthermore, obstructive jaundice has been found to promote BTL in humans, irrespective of the presence of advanced liver disease.\textsuperscript{12} Taken together, these findings suggest a strong association between BTL and cholestasis in liver disease.\textsuperscript{10–12}

Another pivotal element in BTL is increased intestinal permeability, which facilitates migration of bacteria and bacterial products such as endotoxins across the gut wall.\textsuperscript{13–16} In vivo, several studies have confirmed increased permeability in cirrhotic patients by means of functional assays such as \(^{51}\text{Cr}-\text{EDTA}\) or lactulose—mannitol tests.\textsuperscript{17,18} Recently, knowledge has been accumulating on the complexity, selectivity, and dynamic character of intestinal epithelial barrier. A distinction has been made between at least two key functional pathways: a high-capacity pore pathway allowing the passage of small ions and a low-capacity leak pathway determining large-molecule permeability, the first being regulated mainly by claudins and the latter by occludin and tight-junction proteins of the zonula occludens family.\textsuperscript{19} Furthermore, this tight junction—mediated permeability appears to be regulated by the local expression of pro- and anti-inflammatory interleukins, among which interferon gamma (IFN-\(\gamma\)) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) have received considerable interest.\textsuperscript{20–24}

The farnesoid X-activated receptor (FXR) has emerged as a promising target for numerous hepatobiliary and gastrointestinal disorders. In addition to its main function as a bile acid—responsive transcription regulator of hepatic bile metabolism, its expression and functionality has recently been documented in intestine (especially ileum), immune cells, and endothelial tissue.\textsuperscript{25–28} FXR knockout mice not only exhibit pronounced hepatic inflammation and fibrosis, but they also develop an inflammatory bowel disease—like phenotype, with increased intestinal inflammation and permeability and eventually BTL.\textsuperscript{25}

We hypothesized that, in a rat model of cholestatic liver injury, dysfunctional intestinal FXR signaling serves as the central molecular switch driving BTL, facilitated by increased intestinal permeability and orchestrated by intestinal inflammation, which can be restored by means of gavage of the highly selective and potent FXR agonist obeticholic acid [INT-747; provided by David Shapiro and Luciano Adorini (Intercept Pharmaceuticals, San Diego, CA)].

**Materials and Methods**

**Animal Models**

All animal experiments were performed according to guidelines of the local ethics committee. Male Wistar rats (Janvier Labs, Le Genest St Isle, France) weighing 200 to 250 g were divided into five experimental groups. The first group served as untreated healthy controls (\(n = 28\)). In the second group (\(n = 6\)), toxic compensated cirrhosis was induced through administration of thioacetamide in their drinking water for 18 weeks, as described previously.\textsuperscript{29} The other three groups underwent ligation of the common bile duct (BDL, \(n = 51\)) and were treated with vehicle, 5 mg/kg UDCA, or 5 mg/kg INT-747 (kindly provided by Intercept Pharmaceuticals) every 2 days during the period of BDL, based on dose-finding studies in which increasing mortality was observed with increasing doses of INT-747: 3/7 animals at 5 mg/kg per day and 7/7 animals at 30 mg/kg per day. Mortality was related to hepatic INT-747 accumulation after blockage of enterohepatic circulation. Animals were sacrificed at 10 days after BDL.

**RT-PCR of SHP, Tight-Junction Proteins, and Proinflammatory Cytokines in Ileum**

The relative expression of the FXR downstream signaling molecule small heterodimer partner (SHP), tight-junction proteins (claudin-1, claudin-2, occludin, and ZO-1), and the proinflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) was determined by RT-PCR. For this purpose, total RNA was extracted from jejunum and ileum samples (\(n = 5\) to 8 per group) in TRIzol reagent (Invitrogen; Life Technologies, Carlsbad, CA; Merelbeke, Belgium) and reverse transcribed. cDNA was amplified by PCR by use of rat-specific primers (Table 1). Primers were designed using sequence data and nucleotide BLAST software from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/nucleotide) and were manufactured by TIB

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers Used for Analysis of mRNA Expression Levels in Rat Ileum</th>
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<tbody>
<tr>
<td>Protein (Gene)</td>
<td>Forward</td>
</tr>
<tr>
<td>SHP (Nob2)</td>
<td>5’-CTTGGAGCTGGGCTCCCAAGGA-3’</td>
</tr>
<tr>
<td>Claudin-1 (Cldn1)</td>
<td>5’-ATGGGATGAGTGCATGAG-3’</td>
</tr>
<tr>
<td>Claudin-2 (Cldn2)</td>
<td>5’-GGCTATTAGGCACATCGAT-3’</td>
</tr>
<tr>
<td>ZO-1 (Tjp1)</td>
<td>5’-CCTGGTCATCATTTCCACACA-3’</td>
</tr>
<tr>
<td>Occludin (Ocln)</td>
<td>5’-GGTCTGTGAGCGCTTTTGG-3’</td>
</tr>
<tr>
<td>TNF-(\alpha) (Tnf)</td>
<td>5’-GATCGTCCCCACAAGAAGG-3’</td>
</tr>
<tr>
<td>IFN-(\gamma) (Ifng)</td>
<td>5’-TATCTGGAGGAACTGGCAAAAG-3’</td>
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MolBiol (Berlin, Germany). All samples were run in duplicate with a LightCycler 480 systems (Roche Applied Science, Mannheim, Germany; Indianapolis, IN). Samples were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (Hprt), and fold-change expression was compared with the healthy control group using the $2^{-\Delta\Delta CT}$ method, as described previously.30

Detection of BTL in Ascites Fluid and MLNs

To evaluate the presence of viable translocated bacteria, 1 mL of ascites fluid (peritoneal lavage in healthy controls) was aspirated from the abdomen, and MLNs were dissected under fully sterile conditions. The MLNs were then homogenized and cultured on multiple different growth plates: Columbia Agar—based blood agar with 5% horse blood (Thermo Fisher Scientific; Oxoid, Basingstoke, UK; Waltham, MA), Wilkens—Chalgren anaerobe broth, MacConkey agar, mannitol salt agar, Sabouraud agar with chloramphenicol, thioglycollate medium, and tryptone soy broth. MLNs lining the large bowel were divided into four anatomical regions, proximal to distal, and were scored according to the number of bacterial strains detected in culture.

Assessment of Intestinal Permeability in Ussing Chambers

Full-thickness intestinal tissue taken from mid-jejunum and terminal ileum (n = 5 rats per group) was mounted in triplicate in 3-mL modified Ussing chambers as described previously, with a 0.096-cm² area of tissue exposed.15 The mucosal side was exposed to 10 mmol/L mannitol and the serosal side to 10 mmol/L glucose in Krebs—Ringer bicarbonate buffer, and the samples were maintained at 37°C and carbogenated with 95%/5% O2/CO2 at all times, to preserve tissue viability throughout the experiment.31 Gut permeability was assessed by transepithelial electrical resistance (TEER) and dextran permeability. TEER was measured after induction of bipolar constant-current pulses of 50 μA every 1 minute, and average values were acquired from the first 60 minutes of measurement after a 30-minute stabilization period and expressed as Ω·cm². Macromolecular permeability was determined by the transepithelial paracellular passage of fluorescein isothiocyanate (FITC)—labeled 20-kDa dextran (Sigma-Aldrich, Diegem, Belgium; St. Louis, MO) from the mucosal to the serosal side. For this experiment, the average fluorescence levels in the serosal buffer samples, taken every 30 minutes after addition of 3 mg/mL labeled dextran to the mucosal buffer, were analyzed with a Fluoroskan Ascent microplate fluorometer (Thermo Fisher Scientific).

Analysis of Immune Cells and Activation Markers in Spleen and MLNs by Flow Cytometry

Single-cell suspensions of white blood cells from spleen and MLNs (n = 4 rats per group) were obtained by use of 100 μmol/L cell strainers (BD Biosciences, Erembodegem, Belgium; San Jose, CA). Total cell count was performed using an ABX Micros 60 automated cell counter (Horiba Instruments, Irvine, CA). Per sample, 2.5 × 10⁶ cells were stained in phosphate-buffered saline containing 0.5% bovine serum albumin for 30 minutes at 4°C in the dark and were fixed in 1% formaldehyde solution. The following rat-specific antibodies were used for flow cytometry: CD45—FITC and CD45—phycoerythrin-Cy7 for white blood cells and CD45RA—allophyococyanin-Cy7 for B-cells (BD Biosciences); CD103—FITC for dendritic cells (BioLegend, Dedham, MA); NKR-PIA—phycoerythrin for natural killer cells (Invitrogen; Life Technologies); and CD11b/c2—peridinin chlorophyll protein complex (PerCP)-eFluor 710 for macrophages, CD4—allophyococyanin for helper T cells, and CD8a—phycoerythrin-Cy7 for cytotoxic T cells (eBioscience, Vienna, Austria; San Diego, CA). The following activation markers were assessed on CD11b/c2⁺ cells: CD80—allophyococyanin (Invitrogen; Life Technologies) and CD86—FITC (eBioscience). Flow cytometry was performed on a FACSCanto II cytometer (BD Biosciences).

Assessment of Direct Antibacterial Potential of INT-747 and UDCA

The bacteriostatic or bactericidal effects of both INT-747 and UDCA were assessed in vitro by Mueller—Hinton broth detecting culture growth inhibition at concentrations ranging from 1 to 256 μg/mL in cultured strains of Klebsiella oxytoca, K. pneumoniae, Staphylococcus aureus, S. warneri, Pseudomonas aeruginosa, Escherichia coli, Enterobacter cloacae, Stenotrophomonas maltophilia, Enterococcus faecalis, Proteus mirabilis, and Citrobacter freundii.

Detection and Quantification of Bile Acids in Blood and Urine

Samples of heparinized blood plasma and urine were collected from BDL rats and healthy controls at sacrifice (n = 5 per group). In each sample, deoxycholic acid (DCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCa), chenodeoxycholic acid (CDCA), taurochenodeoxycholic acid (TCDDA), and glycochenodeoxycholic acid (GCDCA) were detected and quantified by use of a liquid chromatography—mass spectrometry system (Thermo Fisher Scientific, Breda, The Netherlands) as described previously.32

In Vitro Assessment of Relationships between FXR, Intestinal Permeability, and BTL Under Inflammatory Conditions

Confluent monolayers of the human intestinal epithelial cell line Caco-2 were grown on Snapwell permeable support membranes (Corning Life Sciences, Tewksbury, MA) until steady-state TEER was achieved, as described previously.33 Cells were grown in modified Eagle’s medium (Life Technologies, Bleiswijk, The Netherlands) enriched with 10%
bovine serum albumin, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine and 1% antibiotic solution (Invitrogen; Life Technologies). Epithelial permeability was evaluated by TEER measurement with an EndOhm resistance meter (World Precision Instruments, Sarasota, FL); the trans-epithelial flux of 4-kDa FITC-labeled dextran (Sigma-Aldrich, Belgium) from the apical to the basolateral compartment was analyzed with a Fluoroskan Ascent microplate fluorometer (Thermo Fisher Scientific). Translocation of Texas Red-labeled E. coli bacteria (K-12 strain; Life Technologies) was assessed with a Fluoroskan Ascent microplate fluorometer (Thermo Fisher Scientiﬁc). At the start of experiments, the apical compartment was replaced with medium containing 2 × 10^8 E. coli particles per milliliter and 100 μmol/L dextran replenished with either vehicle (control conditions), 10 ng/mL IFN-γ (eBioscience), and/or 10 μmol/L of the FXR agonist INT-747. TEER and basolateral samples were analyzed every 24 hours during a 96-hour period. Average ΔTEER from the start to 48 hours and 72 hours, compared with control conditions, and average passage of dextran and bacteria between 48 and 96 hours, was compared between groups, based on experiments by Madara and Stafford.33 The experiments were repeated at least three times under identical conditions.

Statistical Analysis

Statistical data were tested for equal variance and normal distribution. Data were all normally distributed and therefore were expressed as means ± SEM and were subjected to parametrical statistics. When comparing two unpaired groups, the Student’s t-test was applied; for comparison of multiple unpaired groups, a one-way analysis of variance was applied. If positive, a subsequent pairwise comparison was performed by means of Bonferroni t-testing. P values below 0.05 were considered statistically significant. Statistical analysis was performed with GraphPad Prism software version 6.02 (GraphPad Software, La Jolla, CA).

Results

FXR Deactivation in Intestine of Cholestatic Rats Is Associated with Increased Gut Permeability and Intestinal BTL but Can Be Prevented by Selective FXR Reactivation in the Ileum

The median number of translocated bacterial strains dropped from 4 to 2 in MLNs of BDL rats after treatment with

Figure 1 Effects of FXR agonism on BTL, gut permeability, and FXR pathway in the several experimental groups. A: The median number of bacterial strains isolated from MLN was reduced from 4 to 2 in INT-747—treated, but not in UDCA-treated, BDL rats. No BTL was observed in healthy control or TAA toxic cirrhotic rats. B: TEER decreased in both jejunum and ileum of vehicle-treated BDL rats, compared with healthy controls, reflective of increased intestinal permeability. After treatment with INT-747, a selective restoration of TEER was observed in the ileum, but not in the jejunum, of BDL rats. C: INT-747 induced a selective reduction of dextran passage in the ileum of BDL rats, compared with both vehicle-treated BDL and healthy control rats, consistent with decreased intestinal permeability. D: Expression of the FXR downstream receptor SHP was reduced in the jejunum and ileum of vehicle-treated BDL rats, compared with healthy controls, and was significantly increased in the ileum, but not the jejunum, of INT-747—treated BDL rats. Data are expressed as means ± SEM. Rats per group: n = 5 [A (BDL+INT-747, BDL+UDCA), B, and C]; n = 6 [A (Control, TAA, BDL)]; n = 8 (D). *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001. BDL, bile-duct ligated; BTL, bacterial translocation; FXR, farnesoid X-activated receptor; INT-747, obeticholic acid; MLN, mesenteric lymph nodes; SHP, small heterodimer partner; TAA, thioacetamide; TEER, transepithelial electrical resistance; UDCA, ursodeoxycholic acid.
INT-747 \((P \leq 0.01)\) (Figure 1A). This was associated with sterile ascites in 5/6 INT-747-treated BDL rats, compared with only 1/6 vehicle-treated BDL rats. INT-747 significantly increased survival (19/19), compared with vehicle treatment (11/16) in BDL rats \((P = 0.01)\). UDCA treatment did not affect either BTL or survival. No BTL was observed in thioacetamide-treated rats (an anicteric model of toxic cirrhosis and portal hypertension) (Figure 1A). TEER decreased in the jejunum of vehicle-treated BDL rats, compared with healthy controls \((49 \pm 2 \text{ versus } 58 \pm 2 \text{ } \Omega \cdot \text{cm}^2; P \leq 0.01)\), and in the ileum \((48 \pm 1 \text{ versus } 60 \pm 4 \text{ } \Omega \cdot \text{cm}^2; P < 0.05)\), consistent with increased intestinal permeability (Figure 1B). With FXR agonist treatment, selective restoration of TEER (back to healthy control values) was observed in the ileum of BDL rats, but not in the jejunum (Figure 1B). The transepithelial passage of dextran remained unaffected in the jejunum of INT-747—treated BDL rats; in the ileum, however, macromolecular passage was clearly decreased \((14 \pm 2 \text{ pmol} \cdot \text{cm}^2)\), compared with both vehicle-treated BDL rats \((33 \pm 8 \text{ pmol} \cdot \text{cm}^2)\) and healthy control rats \((27 \pm 5 \text{ pmol} \cdot \text{cm}^2; P \leq 0.05)\) (Figure 1C). These findings corroborate the selective reduction of intestinal permeability in the ileum by FXR stimulation (Figure 1, B and C). In the gut of vehicle-treated BDL rats, the expression of the FXR downstream target receptor small heterodimer partner (SHP) was markedly reduced, compared with healthy controls, reflective of a deactivated FXR pathway, which appeared to be reactivated in the ileum by administration of INT-747, but not in the jejunum \((P \leq 0.05)\) (Figure 1D), which parallels the apparent selective functional restoration of the ileum mentioned earlier.

**Restoration of Intestinal Permeability in the Ileum of BDL Rats by INT-747 Relates to Altered Expression of the Tight-Junction Proteins Claudin-1 and Occludin**

In both the jejunum and ileum of vehicle-treated BDL rats, expression of the pore-forming tight-junction protein claudin-2 increased significantly, compared with healthy controls \((P \leq 0.01)\) (Figure 2B), whereas the expression of the counterbalancing pore-closing claudin-1 remained unaffected (Figure 2A). By contrast, INT-747—treated BDL rats exhibited a significant selective ileal increase in expression of pore-closing claudin-1, compared with both vehicle-treated BDL and healthy control rats \((P \leq 0.02)\) (Figure 2A). Furthermore, in the ileum of INT-747—treated BDL rats the expression of occludin, one of the chief inhibitory regulators of the leak pathway, was significantly up-regulated compared with vehicle-treated BDL rats \((P = 0.02)\) (Figure 2C). Ileal expression of ZO-1 was significantly up-regulated in INT-747—treated BDL rats, compared with healthy control rats \((\text{fold change } 2.19 \pm 0.34 \text{ versus } 1.01 \pm 0.05; P \leq 0.01)\); however, the difference failed to reach statistical significance in comparison with vehicle-treated counterparts \((P = 0.12)\).

The FXR Agonist INT-747 Induces a Strong Intestinal and Systemic Anti-Inflammatory Response in BDL Rats, Associated with Decreased Local Expression of IFN-γ in the Ileum

The average spleen/body weight ratio was significantly reduced in BDL rats treated with INT-747, compared with vehicle-treated BDL rats and even with healthy control rats \((0.24 \pm 0.04 \text{ versus } 0.39 \pm 0.03 \text{ and } 0.36 \pm 0.04, \text{ respectively}; n = 4 \text{ rats per group}; P \leq 0.05)\). This was associated with an overall decrease in total white blood cell count in spleen of BDL rats receiving INT-747, compared with vehicle \((1.24 \pm 0.54 \times 10^7 \text{ versus } 2.93 \pm 0.45 \times 10^7; P \leq 0.05)\) (Figure 3A), and similarly in MLNs \((0.38 \pm 0.08 \times 10^7 \text{ versus } 1.23 \pm 0.26 \times 10^7; P \leq 0.05)\).
versus $1.04 \pm 0.15 \times 10^7; P \leq 0.01$) (Figure 3B). In terms of white blood cell subtypes in spleen, vehicle-treated BDL rats exhibited significantly increased numbers of both natural killer cells (NKCs) [$2.3 \pm 0.4 \times 10^6 (8.2\% \text{ of viable cells})$] and macrophages [$8.6 \pm 1.6 \times 10^6 (27.3\%)$], compared with healthy controls [$1.4 \pm 0.2 \times 10^6 \text{ NKCs (7.4\%)}$ and $2.2 \pm 0.9 \times 10^6 \text{ macrophages (11\%)}; P \leq 0.05$] (Figure 3A). Similarly, in MLNs, vehicle-treated BDL rats exhibited significantly increased numbers of both NKCs [$6.3 \pm 0.1 \times 10^6 (0.7\%) \text{ of viable cells}$] and macrophages...
INT-747 Reduces Bacterial Translocation

[12.8 ± 2.0 × 10^4 (1.2%)], compared with healthy controls [2.6 ± 0.1 × 10^4 NKCs (0.3%) and 4.2 ± 0.8 × 10^4 macrophages (0.6%); P ≤ 0.05] (Figure 3B). Treatment with INT-747 induced a reduction in dendritic cells, cytotoxic and helper T cells, and B cells in both spleen and MLN of BDL rats (data not shown), but the decreased number of NKCs was particularly notable, in both spleen [0.8 ± 0.4 × 10^6 (6.1% of viable cells)] and MLNs [2.0 ± 0.7 × 10^4 (0.7%)], compared with vehicle-treated BDL rats (P ≤ 0.05) (Figure 3, A–C).

In the ileum of vehicle-treated BDL rats, expression of IFN-γ, a cytokine produced mainly by NKCs, increased significantly; with INT-747 treatment, however, IFN-γ expression decreased to below healthy control values (P ≤ 0.01) (Figure 3D). The functionality of macrophages in the ileum, as reflected by expression of activation markers such as CD80 (Figure 3E), was unaffected by INT-747 treatment; CD86 expression and local production of TNF-α in the ileum were also unaffected (data not shown).

The FXR Agonist INT-747 Does Not Significantly Alter Composition of the Bile Acid Pool during 10 Days of BDL

In BDL rats, we observed an important increase in circulating bile acids in blood, especially GDCA and CDCA, along with increased urinary excretion of DCA, TDC, CDCA, TDCA, and GCDCa, all consistent with cholestasis. After FXR administration, however, neither blood and urinary bile acid concentrations nor conjugation of bile acids was significantly affected (Supplemental Table S1).

IFN-γ Induces E. coli BTL in Vitro, Which Remains Unaffected by Local FXR Stimulation

The functional relevance of IFN-γ was substantiated in vitro by incubating a human intestinal epithelial monolayer with 10 ng/mL IFN-γ. This induced increased E. coli BTL, compared with healthy control (26 ± 8 versus 8 ± 4 colony-forming units (CFU)/mL) (P ≤ 0.03) (Figure 4). This translocation remained unaffected by simultaneous incubation with 10 μmol/L INT-747 (26 ± 8 versus 41 ± 20 CFU/mL; P = 0.43) (Figure 4). Intestinal permeability, as assessed by TEER and dextran passage, also remained unaffected after INT-747–addition to an IFN-γ–exposed intestinal epithelium of Caco-2 cells (data not shown).

UDCA and INT-747 Have No Clear Direct Bacteriostatic Potential in Vitro

In Mueller–Hinton broth, no inhibition of in vitro growth of the bacterial strains was obtained for concentrations of ≤128 μg/mL of either UDCA or INT-747. At 256 μg/mL, both agents showed similar complete inhibition of bacterial growth in all strains tested; however, these concentrations are not obtained in vivo and classically are considered to reflect dose-related toxicity.

Discussion

BTL is considered a harmful event in the natural history of chronic liver disease, because it either preludes hepatic decompensation or acts as a pacemaker to further liver insufficiency and multiorgan failure. Both intestinal bacterial overgrowth and a leaky gut are considered to be key events, but to date the available clinical approaches toward prevention and treatment of BTL have been limited to gastrointestinal decontamination by means of antibiotics. The increasing rate of treatment failures, due to numerous infections with Gram-positive and multiresistant microorganisms, points to the need for novel therapies in treatment and prevention of BTL and of spontaneous bacterial peritonitis, the most common infection in patients with cirrhosis.

From this perspective, we focused on FXR, a bile acid–responsive nuclear transcription factor that is crucial as a chief regulator of hepatic bile acid, lipid, and carbohydrate metabolism. Interestingly, FXR is also highly expressed in the intestine, so the effects extend beyond the liver. More specifically, the pivotal role of FXR in the preservation of intestinal homeostasis is increasingly recognized, along with its involvement in the gut–liver axis. An example is the recent finding that FXR-dependent signaling from the gut through fibroblast growth factor-15 (FGF-15) protects mice against cholestasis. FXR deficiency has therefore been linked to increased intestinal permeability and BTL in non–liver-related gastrointestinal disorders, such as inflammatory bowel disease. However, whether FXR dysfunction also relates to BTL in chronic liver disease (and also what are the underlying pathophysiological mechanisms) remains unclear.
In the present study, we confirmed intestinal FXR pathway deficiency in a rat model of chronic cholestatic liver injury. This deficiency was associated with high rates of BTL and increased intestinal permeability, which in turn was related to a disproportional up-regulation of the pore-forming tight-junction protein claudin-2 throughout the small bowel.

Based on these findings, we then aimed to reactivate the FXR pathway by oral administration of the first-in-class highly potent and selective FXR agonist 6-a-ethyl-chenodeoxycholic acid (obeticholic acid; INT-747), a semisynthetic bile acid derivative whose value has recently been authenticated in phase II and III clinical trials for nonalcoholic fatty liver disease and primary biliary cirrhosis. To discriminate between FXR-specific and general bile acid–related effects, INT-747–gavaged rats were compared with rats gavaged with equal amounts of ursodeoxycholic acid (UDCA), a bile acid similar in molecular structure to INT-747 but without FXR agonist properties. Interestingly, after treatment with INT-747, a reactivation of the FXR pathway was observed only in the ileum and not the jejunum of BDL rats, which paralleled selective ileal restoration of intestinal integrity and coincided with a marked reduction in BTL, thus suggesting the ileum as a primary source for BTL in liver disease. The finding that FXR is expressed mainly in the ileum is in accord with previous reports by others and is not surprising, given the pivotal role of FXR in regulation of enterohepatic reabsorption of bile acids and ileal clustering of gut immunity. Although increased gut permeability in the jejunum was equally associated with reduced SHP expression, it appeared unresponsive to FXR agonists, which thus failed to restore jejunal permeability. The reason remains unclear, but it might relate to the effects of FXR agonists on the immune system, which is more abundant in the ileum than in the jejunum; alternatively, it might relate to a non–FXR-dependent mechanism.

Functionally, restored ileal integrity was related to a marked increase in ileal expression of pore-closing claudin-1, but the leak pathway also was targeted by INT-747, via up-regulation of occludin and ZO-1. Thus, it seems that the relative overexpression of the culprit pore-forming tight-junction protein claudin-2 is overcome by these compensatory protective mechanisms. Given that the tight junction–mediated regulation of paracellular intestinal permeability is highly regulated by local expression of pro- and anti-inflammatory cytokines, we investigated both the recruitment and functionality of all relevant components of the innate and adaptive immunity in this context. Vehicle-treated BDL rats exhibited a significant increase in NKCs and macrophages in both spleen and MLNs. Of additional interest in this context is the observation that the increase in NKCs in vehicle-treated BDL rats was concurrent with a marked and specific increase of ileal expression of IFN-γ. IFN-γ is a pro-inflammatory cytokine that is predominantly expressed by NKCs as a part of the innate immune response and is considered particularly important in intestinal permeability, for several reasons. First, IFN-γ induces increased permeability in cultured intestinal epithelial monolayers, allowing the passage of E. coli–derived lipopolysaccharide in vitro. Second, recent findings suggest that IFN-γ also enhances the increased permeability induced by other interleukins such as TNF-α. Third, IFN-γ promotes the internalization of epithelial tight-junction proteins and, fourth, IFN-γ also appears to promote passage of bacteria such as Escherichia coli via a transcellullar route. After treatment with INT-747 restored FXR activity, we observed a general
anti-inflammatory response with, in particular, a remarkable
decrease in the number of NKCs, both locally in the MLNs
and systemically in the spleen, consistent with findings by
others on the hepatic level.50 As a logical consequence, IFN-γ
levels (in parallel with the decreased number of NKCs in the
INT-747—treated BDL group) were equally suppressed in the
gain-of-function group, strengthening the arguments in favor
of a fundamental role for IFN-γ in intestinal permeability.

Given that we were observed absent BTL in an anicteric
model of toxic compensated cirrhosis with portal hyperten-
sion, but clearly present BTL in a model of short-term
cholestatic injury, the next question is whether the observed
effects are mediated either directly by obeticholic acid on the
intestine or indirectly through an altered bile acid meta-
bolism.53 Interestingly, FXR stimulation did not significantly
c change the increased bile acid pool or its composition, did
not increase the conjugation of primary or secondary bile
acids, and did not affect urinary excretion. Thus, taken
together with the fact that the observed effects on BT and
permeability were obtained in the absence of bile acids and
that INT-747 and UDCA lack inhibition on bacterial growth
in vitro on Mueller—Hinton broth, the findings support a
primary and bile acid—inddependent effect of INT-747 on the
intestinal barrier.

This then leads to the question of whether the observed
restoration of ileal epithelial integrity is a direct protective
effect on the enterocyte with secondary decrease in
inflammation or an indirect effect primarily through its
NF-kB—mediated anti-inflammatory potential in intesti-
nal immune cells, similar to effects observed by others in
animal models of inflammatory bowel disease.26,51,52 For
this purpose, we performed auxiliary in vitro experiments
on a human monolayer of intestinal epithelial Caco-2 cells
endowed with enterocyte-like properties, including the
formation of tight junction—mediated cell-to-cell adhesions,
and we assessed changes in permeability and the trans-
location of E. coli, a species frequently translocated in BDL
rats in the present study. We confirmed the IFN-γ—mediated
increase in BTL in vitro, but BTL and permeability remained
unaffected by costimulation with INT-747. This finding
favors indirect anti-inflammatory properties of INT-747,
rather than a primary direct effect on the intestinal barrier
(Figure 5). In addition to decreased NKCs and IFN-γ levels,
the observed anti-inflammatory effect was further substanti-
ated by the fact that in INT-747—treated BDL rats, the
average spleen weight (as well as the total number of white
blood cells in spleen and MLNs) decreased even to below the
level of healthy controls.

Although we cannot exclude an additional direct effect of
INT-747 on gut microbiota, we do not expect this to alter the
observed beneficial effects of treatment with the FXR agonist
INT-747 in BDL rats, because we observed absence of direct
inhibition by INT-747 of in vitro bacterial growth of strains
frequently translocated in the present study. This argues
against a functionally relevant effect of FXR agonists on
intestinal bacterial overgrowth and gut microbiota in general.

A final question might be whether the observed effects on
BTL in this model of cholestatic injury relate to the broader
context of human cirrhosis, which is primarily toxic or viral
in origin. First, here we have demonstrated absent BTL in a
model of compensated toxic cirrhosis, comparable to the
human condition.5 Second, others have recently shown that
INT-747 reduces BTL in carbon tetrachloride—intoxicated
rats, a representative model of decompensated toxic
cirrhosis.53 Interestingly, a similar reactivation of FXR in
the ileum of INT-747—treated cirrhotic rats was found to be
associated with marked reduction of ileal inflammatory
mediators such as IL-17 and IFN-γ,53 similar to what was
found in the present study. This suggests not only that the
positive effects of FXR agonism on BT extend beyond
cholestatic liver injury, but also that they can be extrapo-
lated to the broader context of decompensated cirrhosis,
with high comparability for the mechanism of action.

In conclusion, we have demonstrated that the FXR agonist
INT-747 exerts a protective effect on gut and liver in a rat
model of cholestasis. This relates to an anti-inflammatory
effect, mainly through decreased NKC-mediated IFN-γ
expression in the ileum, resulting in restored epithelial
integrity and a subsequent decrease in intestinal BTL. This
approach should be further explored in patients with chronic
liver disease.

Acknowledgments

We thank David Shapiro and Luciano Adorini ( Intercept
Pharmaceuticals) for kindly providing the obeticholic acid
compound.

L.V. was responsible for the study concept and design,
acquisition of data analysis and interpretation of data,
 obtaining funding, drafting of the manuscript, and statistical
analysis; R.F., B.V., K.C., T.V., J.V., M.K., T.R., J.T.,
S.C., and P.A. provided technical support, acquisition of data,
and design, acquisition of data analysis and interpretation
of data, and obtaining funding; and W.L. provided study con-
cept and design, acquisition of data, analysis and interpreta-
tion of data, drafting of the manuscript, and statistical
analysis, obtaining funding, and study supervision.

Supplemental Data

Supplemental material for this article can be found at
http://dx.doi.org/10.1016/j.ajpath.2014.10.009.

References

1. Berg RD, Garlington AW: Translocation of certain indigenous bacteria
from the gastrointestinal tract to the mesenteric lymph nodes and other


INT-747 Reduces Bacterial Translocation


