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The Bile Acid Receptor FXR Is a Modulator of Intestinal Innate Immunity

Piero Vavassori,* Andrea Mencarelli,* Barbara Renga,* Eleonora Distrutti,† and Stefano Fiorucci2*

The farnesoid X receptor (FXR) is a bile acid-regulated nuclear receptor expressed in enterohepatic tissues. In this study we investigated whether FXR is expressed by cells of innate immunity and regulates inflammation in animal models of colitis. Acute (7 days) and chronic (8 wk) colitis were induced in wild-type and FXR−/− mice by intrarectal administration of trinitrobenzenesulfonic acid or by 7-day administration of 5% dextran sulfate in drinking water. The results of this experiment demonstrate that FXR is expressed by and exerts counterregulatory effects on cells of innate immunity. Exposure of LPS-activated macrophages to 6-ethyl chenodeoxycholic acid (6E-CDCA; INT-747) a synthetic FXR ligand, results in a reciprocal regulation of NF-κB dependent-genes (TNF-α, IL-1β, IL-6, COX-1, COX-2, and iNOS) and induction of SHP, a FXR-regulated gene. FXR activation stabilizes the nuclear corepressor NCoR on the NF-κB responsive element on the IL-1β promoter. Colon inflammation in Crohn’s disease patients and in rodent models of colitis is associated with a reduced expression of FXR mRNA. Using two rodent models of colon inflammation, we show that progression of these immune-mediated disorders is exacerbated in FXR−/− mice (p < 0.01).

In vivo treatment with INT-747 attenuates organ injury and immune cell activation. FXR activation increased the colon expression of I-BABP, FXR, and SHP while reducing IL-1β, IL-2, IL-6, TNF-α, and IFN-γ mRNA expression and attenuating disease severity. In aggregate, these findings provide evidence that FXR is an essential component of a network of nuclear receptors that regulate intestinal innate immunity and homeostasis. The Journal of Immunology, 2009, 183: 6251–6261.

The human intestine is the site of an extraordinarily complex and dynamic environmentally transmitted interaction of the local immune system with nutrients and microbial products (reviewed in Ref. 1). A network of regulatory genes links signals provided by luminal Ags to immune and inflammatory cells. In this context the regulation of resident intestinal macrophages by the recognition of conserved pathogen-associated molecular patterns by TLRs and intracellular sensors such as nucleotide-binding oligomerization domains is critical for understanding the homeostasis of gut-associated immunity. Deciphering the pathways involved in the regulation of intestinal macrophages promises to provide new host targets for treating diseases such as inflammatory bowel disease in which a dysregulation of innate immunity plays a role (2).

Nuclear receptors (NRs)3 are a growing family of regulatory factors that exert homeostatic functions at the interface between nutrient metabolism and innate immunity. Activation of members of the peroxisome proliferator-activated receptor (PPAR) family such as PPAR-α and -γ, the pregnane X receptor, and the liver X receptor by lipid mediators, bile acids, and oxysterols modulates lipid/cholesterol metabolism and provides counterregulatory signals for macrophages (3–7) and protection in rodent models of dysregulated innate immunity (8, 9).

NRs trans-repress the expression of inflammatory genes by several mechanisms, including the formation of direct complexes with AP-1 and NF-κB family members and/or the modulation of p38 MAPK activity (reviewed in Ref. 10). In addition, PPARγ and glucocorticoids inhibit the expression of inflammatory genes in a promoter-specific manner. This mechanism involves the ligand-dependent recruitment of corepressors such as the nuclear corepressor (NcoR) and SMRT (11) and the formation of a multiprotein complex in the promoter of target genes. Recruitment of the small ubiquitin-like modifier (SUMO), an 11-kDa protein structurally similar to ubiquitin, is required in this process (11).

Bile acids, the end products of cholesterol metabolism, exert an indispensable role in integrating nutrient absorption, lipid metabolism with liver, and intestinal homeostasis. This homeostatic function has been linked to the activation of the farnesoid X receptor (FXR), a NR expressed in enterohepatic tissues (12–14). Upon activation, FXR alters the transcription of target genes by interacting with FXR-responsive elements as a heterodimer with the 9-cis-retinoic acid receptor (14). In liver cells, FXR negatively regulates genes involved in bile acid synthesis and lipodigenesis but enhances the expression/activity of a number of the basolateral transporters required for bile formation and cholesterol and lipid secretion (15). In intestinal epithelial cells, FXR activates the transcription of the intestinal bile acid-binding protein (I-BABP) (16) and fibroblast growth factor factor 15 (17), with both genes regulating similar to ubiquitin, is required in this process (11).

Bile acids have long been known to exert direct regulatory function on cells of innate immunity with chenodeoxycholic acid (CDCA), a primary bile acid and FXR ligand (12–14) that...
negatively regulates IL-1β, IL-6, and TNF-α release from LPS-primed macrophages (18, 19).

In this study, we have investigated whether FXR regulates intestinal innate immunity and whether its activation protects against the development of colonic inflammation in rodent models of colitis.

Materials and Methods

Patients and colon samples

Colonic mucosal samples were obtained from macroscopically involved and uninvolved areas of seven patients with active Crohn’s disease undergoing colonoscopy for diagnostic purposes. Patients were at the first diagnosis and no treatment was taken for at least 1 wk before biopsies were taken. All patients (four men; mean age, 25.3 ± 5.3) had a ileocolonic or colonic localization of the disease. Macroscopically normal colon samples were also obtained from seven patients (three men; mean age, 26.7 ± 7.3) with irritable bowel syndrome having a colonoscopy for recurrent abdominal pain. Informed consent was obtained from all patients and control subjects.

Animals

Six- to 8-wk-old female C57BL/6J, BALB/c, and SCID mice were obtained from Charles River Laboratories. FXR−/− mice (C57BL/6J background) were originally provided by F. Gonzalez (National Institutes of Health, Bethesda, MD) (20). All mice were housed in a temperature-controlled room and had free access to food and water. The local Animal Care and Ethics Committee of the University of Perugia (Perugia, Italy) approved all experimental protocols.

Colitis induction, study design, and isolation and stimulation of lamina propria (LP) CD11b+ cells

Pharmacological activation of FXR was obtained by in vivo and in vitro treatment with 6-ethyl-CDCA (INT-747), a semisynthetic FXR ligand that activates the receptor with an EC50 of ~100 nM (21-23). The efficacy of INT-747 was tested in acute and chronic models of colitis induced by trinitrobenzenesulfonic acid (TNBS) or dextran sulfate (DSS) (24, 25). Lamina propria-derived CD11b+ cells were prepared as described previously using a MACS selection system and CD11b microbeads (Miltenyi Biotec) following the manufacturer’s instructions.

In a pilot study to test the dose-dependent prevention of colitis, acute TNBS colitis was induced in BALB/c mice by the intrarectal administration of TNBS (0.5 mg/mouse) in 50% ethanol. To assess whether activation of FXR would protect against the development of colitis, TNBS-treated mice were randomized to receive no treatment or INT-747 at a dose of 1, 3, or 5 mg/kg/day (n = 10 for each group) (23). INT-747 was administered i.p. for 3 days before TNBS injection and daily thereafter for 7 days. Control mice received 50% ethanol alone (n = 10). No differences in terms of mortality were observed in all TNBS groups (~40% of the animals). Seven days after TNBS administration, mice were sacrificed and the colons were removed and divided for histology and RNA extraction. The induction of chronic colitis was performed according the protocol proposed by Lawrance et al. (25). Briefly, 6- to 8-wk-old female mice were treated weekly for 8 wk with escalating doses (0.5 to 1.25 mg, with an 0.25 mg/kg increment) of TNBS in 30% ethanol via an intracolonic catheter. To test the influence of FXR in the inflammatory and fibrotic response to the chronic administration of TNBS, chronic colitis was induced in C57BL/6 FXR wild-type (FXR+/+; n = 20) and FXR−/− (n = 18). To test the efficacy of FXR activation to prevent the development of chronic colitis, BALB/c mice were administered TNBS (n = 15) or TNBS plus INT-747 (5 mg/kg/day via intragastric catheter; n = 15). In both experiments, control mice received 30% ethanol alone (n = 7). During the 8 wk of treatment, mice were examined weekly for weight loss, diarrhea, rectal bleeding and prolapse, ileorectal, left, and periorbital edemas. No differences in terms of mortality were observed among the TNBS-administered animals (~20% of the C57BL/6 mice, irrespective of the FXR genetic background, and ~40% of the BALB/c mice of the TNBS group as well as the TNBS plus INT-747 group). Two days after the last TNBS administration, mice were killed and the colons were removed, examined, and divided for histology and RNA extraction. The macroscopic appearance was analyzed considering the presence of induration, edema, thickening, and evidence of mucosal hemorrhage. For histologic examination, a section of the distal colon from each animal was fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E or Sirius red. Images from colon sections were acquired with a BX60 microscope (Olympus), digitalized using a SPOT-2 camera (Diagnostic Instruments) with a resolution of 1315 × 1033 pixels, and analyzed using a computerized image analysis system (Image Acquisition System, version 005; Delta Systems). Sections from the colon of each animal were thus examined in a blinded fashion. The degree of inflammation and fibrosis was evaluated on the basis of the density and extent of inflammatory infiltrates, the loss of goblet cells, bowel wall thickening, and the intensity of Sirius red-positive connective tissue staining.

To define the immune compartment that mediates the effect of the FXR ligand in vivo, the efficacy of INT-747 was also tested in T-cell- and B-cell-depleted SCID mice. TNBS colitis was induced by intrarectal administration of TNBS (0.5 mg/mouse) in 50% ethanol (21, 22). Mice receiving TNBS were randomized to receive no treatment or INT-747 at a dose of 5 mg/kg (n = 10 for each group). INT-747 was administered i.p. for 3 days before TNBS injection and daily thereafter for 7 days. Control mice received 50% ethanol alone (n = 10). No differences in terms of mortality were observed in all TNBS groups (~40% of the animals). Seven days after TNBS administration, mice were sacrificed and the colons were removed and divided for histology and RNA extraction. For histological examination, a specimen of the colon of each animal was used. Inflammation was graded semiquantitatively from 0 (no signs of inflammation) to 4 (transmural infiltrations, loss of goblet cells, high vascular density, and kening of the colon wall) (25).

Isolation and culture of human and mouse macrophages

Human PBMCs were obtained from normal individual donors to the Blood Bank Service of the University of Perugia Hospital. PBMCs were isolated by density gradient centrifugation through a Ficoll-Hypaque gradient (Pharmacia Biotech). Monocytes were isolated by positive selection using magnetic cell sorting according to the manufacturer’s instructions (Miltenyi Biotec). Human peripheral blood monocyte-derived macrophages (PBDMs) were obtained from PBMCs by adherence in plastic flasks. Mouse macrophages were obtained from the spleens of FXR wild-type and null mice (C57BL/6J6/J6 background) following a previous described protocol.

Quantitative RT-PCR (qRT-PCR), immunoblot analysis, immunoprecipitation, and immunohistochemistry

Quantization of the expression level of selected genes was performed by qRT-PCR, following a previously described protocol (supplemental Table 1). Immunoanalysis and immunoprecipitation were performed according to previously described protocols (26).

Three-dimensional structure of the ligand binding domain (LBD) of FXR and the mutagenesis of FXR

A putative consensus sequence for SUMO, KEE, was identified on the currently available three-dimensional structures of the LBD of FXR (11). At present, the three-dimensional structures of the LBD of the human FXR (in complex with exaramine, Protein Data Bank code 1OSH) and the mouse FXR (in complex with INT-747, Protein Data Bank code 1OSV, and in complex with 3-deoxyCDCA, Protein Data Bank code 1OT7) are available. The homologous sequence in mouse FXR (292KEE247) is localized in the short loop connecting H2 with H3 (see Fig. 9c).

The wild type (WT) 77R-mutated structure of the LBD was generated by classical PCR and cloning techniques. Briefly, the 5′-fragment corresponding to nt 91-1200 and the 3′-fragment corresponding to nt 1711-1772 of human FXR (GenBank accession no. NM_005123; www.ncbi.nlm.nih.gov/nucleotide/142360165) were amplified by using the Phusion polymerase enzyme according to the manufacturer’s guidelines (Finnzymes). The primers used were as follows: 5′-fragment, 5′-GATCGTAGAAGAAAGACTCA-3′ and 5′-CTCTGCACAGGACAGCTAGGC-3′; 5′-fragment, 5′-TTTTCGGAGAAAGTCCTTG-3′; 5′-TCAGTCCTCCATCAGATT-3′. The mutated nucleotides are underlined. The 3′-fragment was cloned in the pcRII.1 vector using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer’s guidelines to obtain pcRII.1–3′-FXR/27R7 vector. The 5′-fragment of FXR was subcloned upstream of the 3′-fragment of FXR into the SacI site of pcRII.1–3′-FXR/27R7 vector. The SacI site is in italics letters in the primers. The sequence of full
length mutated FXR K277R was sequenced and subcloned into mammalian vector expression pCMV-SPORT4 to obtain the construct pCMV-SPORT-FXRK277R.

Plasmids, cell culture, transfection, and luciferase assays

The human FXR-expressing plasmid was as described in Ref. 26. For luciferase assays, the minimal promoter region containing the NF-κB response element from inducible NO synthase (iNOS) or the IL-1β gene was amplified and cloned upstream of the luciferase reporter gene into the pGL3 vector (BglII site). The promoter primers used were as follows: IL-1β, 5'-CAGCTCTTCCACCTCCTCC-3' and 5'-AGCTCTAAACCCTTCCCT-3'; human iNOS, 5'-TCCTCCATTAGGACGCAATCT-3' and 5'-GCTACTGACCCAGCAGTCCC-3'. Their coordinates and positions relative to the IL-1β and iNOS NF-κB binding and transcription start sites were from Taylor et al. (27), and Chan et al. (28), respectively. HEK293 cells were transfected using the calcium phosphate coprecipitation method in the presence of 25 μM chloroquine. Transient transfections were performed using 500 ng of reporter vector, 200 ng of pCMV-βgal as internal control for transfection efficiency, and 100 ng of the FXR expression plasmid. The pGEM vector (Promega) was added to normalize the amounts of DNA transfected in each assay (2.5 μg). At 36–48 h posttransfection, cells were stimulated with INT-747 at 1 μM. Controls carried culture vehicle (0.1% DMSO) alone. HEK293 cells were lysed in 100 μl of diluted reporter lysis buffer (Promega), and 0.2 μl of cellular lysates was assayed for luciferase activity using the luciferase assay system from Promega. Luminescence was measured using an automated luminometer. Luciferase activities were normalized for transfection efficiencies by dividing the relative light units by β-galactosidase activity expressed from cotransfected pCMV-βgal. Plasmid mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s guidelines.

NCoR gene silencing by siRNA

THP-1 cells were transiently transfected with an Arrest-In transfection reagent (Open Biosystems) using guidelines provided by the manufacturer. After 72 h posttransfection, the cells were pretreated with INT-747 at 1 μM for 3 h and then induced with LPS at 1 μg/ml. RNA was extracted 18 h after LPS stimulation. Gene expression was evaluated by qRT-PCR. Experiments were repeated in at least three times.

Chromatin immunoprecipitation (ChIP) and NCoR silencing

ChIP assays were performed according to a previously published method (6). The following primers specific for the human IL-1β and iNOS promoters were used in ChIP assays: IL-1β promoter, 5'-CGTTGGAAAAATTCCAGTATTAAAATG-3' and 5'-CAATGATCAGGCAATAATGC-3'; iNOS promoter, 5'-TCCCTCATATTGGACGCAATCT-3' and 5'-GCTACTGACCCAGCAGTCCC-3'. Validated human NCoR small interfering RNA (siRNA) SMART pool and control siRNA were purchased from Dharmacon Research.

Immunoblot analysis, immunoprecipitation, and immunohistochemistry

Immunoblot analysis and immunoprecipitation were performed according to previously described protocols (6). Nitrocellulose membranes were probed with polyclonal Abs against anti-FXR (polyclonal H-130; Santa Cruz Biotechnology), anti-tubulin (Sigma-Aldrich), anti-SUMO (Cell Signaling Technology), anti-NCoR (Upstate Biotechnology), and anti-ubiquitin (Sigma-Aldrich). Immunoprecipitation was performed with 1 μg/ml anti-FXR or anti-CD3 (both from Santa Cruz Biotechnology) as a control Ab in the presence of 10 μl of protein A-Sepharose (GE Healthcare).

Statistical analysis

All values in the text, graphs, and tables are expressed as mean ± SEM. Variations in the data set were tested with Student’s t test or ANOVA, and significance was tested with unpaired t tests with a Bonferroni modification for comparison of more than two groups of data.

Results

FXR-null mice show proinflammatory and profibrotic phenotype of the colon under naive conditions

FXR is expressed in the small and large intestines (17, 19, 20); however, the role of FXR in the colon is poorly investigated. FXR-null mice on a C57BL/6Jb6 genetic background were used in these studies (20–23). Although no macroscopic and histopathologic abnormalities were detected in 8-wk old mice (not shown), a mild to moderate cellular infiltration of the colonic mucosa LP with enhanced collagen deposition in the superficial layers of the intestinal wall was observed in 14- to 16-wk-old FXR−/− mice in comparison with wild-type mice (FXR+/+) (Fig. 1a). Analysis of LPS-derived cells from wild-type and FXR−/− mice revealed that, in comparison with wild-type mice, FXR gene ablation manifests itself by an enhanced infiltration of CD11b+ cells (18.1 ± 0.1 vs 8.4 ± 0.1%, respectively; p < 0.05, Student’s t test, n = 5; Table I), whereas the relative percentage of LP-infiltrating CD4+, CD8+, CD19+, and Gr1+ cells was unchanged. Accompanying these features, an enhanced expression of inflammatory genes along with proteins of the extracellular matrix was detected in the colons of mice harboring a disrupted FXR. By qRT-PCR analysis, a 5- to 60-fold increase in the expression of TNF-α, IL-1β, IFN-γ, TGFβ1, α1(I) collagen, TIMP-1, and α-smooth muscle actin mRNA was detected, indicating that FXR gene ablation leads to dysregulation of intestinal immunity and tissue remodeling (Fig. 1b). To investigate the role of macrophage influx on this proinflammatory phenotype, LP-derived CD11b+ cells obtained from wild-type and FXR-null mice were tested for their ability to release IL-1β, IFN-γ, and TNF-α (Fig. 1c). FXR−/− macrophages released, either under basal conditions and in response to stimulation with LPS, increased amounts of IL-1β, IFN-γ, and TNF-α compared with wild-type cells. In addition, whereas levels of IL-10 secretion by FXR−/− and wild-type CD11b+ cells were unchanged (data not shown), an increased release of IL-6 was measured either in the basal state and after treatment with LPS (27.5 ± 32.7 vs 64.1 ± 34.9 pg/ml; n = 5, p < 0.05) and in response to LPS (718 ± 276.4 vs 298.0 ± 54.0 pg/ml; n = 4, p < 0.05). This setting of immune dysfunction and enhanced colon collagen deposition was maintained during the entire life span of FXR−/− mice as shown by the enhanced expression of TNF-α, IL-1β, TGFβ1, α1(I) collagen, fibronectin, and MMP-2 mRNA in the colons of 14-mo-old FXR−/− mice (Fig. 1d). Consistent with these finding, CD11b+ cells isolated from the spleens of FXR−/− mice generated higher amounts of IL-1β, IFN-γ, and TNF-α when challenged with LPS than wild-type macrophages (Fig. 2e), indicating that FXR gene ablation results in a systemic dysregulation of macrophage function.

FXR gene ablation predisposes to uncontrolled immune reaction in the colon

Administering TNBS to 6- to 8-wk-old FXR+/+ C57BL/6 mice resulted in mild chronic inflammatory infiltration in the superficial layers of the colon, with minor collagen deposition in the mucosal and submucosal layers in < 30% of mice (Fig. 2, b and c). This attenuated manifestation is consistent with the relative resistance of the C57BL/6 background to colon inflammation induced by TNBS (24). The disease, however, was dramatically exacerbated in FXR−/− mice (Fig. 2a). By histopathology analysis a moderate to severe colitis with acute and chronic inflammatory infiltrates located in the mucosal and submucosal layers was observed in 70–90% of these mice (Fig. 2, b and c), and by qRT-PCR analysis a robust induction of proinflammatory and profibrogenic genes was detected in the colons of FXR−/− mice exposed to TNBS (Fig. 2d). The dysregulated immune response of FXR-null mice to the haptenizing agent reflects itself also in the ability of intestinal macrophages to release IL-1β, IFN-γ, and TNF-α. Thus, in comparison with wild-type mice, LP CD11b+ cells isolated from FXR−/−, TNBS-treated mice released significantly higher amounts of these cytokines either in basal state and after treatment with LPS (Fig. 2e).
We then investigated whether colon inflammation results in altered expression of FXR. The colonic expression of FXR was assessed in colon samples of TNBS-treated mice and human samples obtained at colonoscopy from Crohn’s disease patients (n = 7) and control subjects (n = 7). In comparison with control mice, colon expression of FXR mRNA was reduced by TNBS administration (Fig. 3a). In comparison with control subjects, FXR mRNA expression was similarly reduced in colonic biopsies obtained from areas of macroscopically inflamed mucosa of Crohn’s patients (Fig. 3b). In contrast, FXR expression was maintained in those areas of the colonic mucosa of Crohn’s patients showing normal appearance at colonoscopy. The change in FXR gene expression was specific and not mirrored by similar changes in the expression of I-BABP and SHP (Src homology region 2 domain-containing phosphatase) (24), two FXR-regulated genes.

**FXR activation protects against colitis development**

Because these data predict a protective role for FXR in the context of colonic inflammation, we then investigated whether in vivo FXR activation with 6-ethyl-CDCA (INT-747) (24, 28), a synthetic ligand, would protect against the development of colitis in mice administered TNBS. BALB/c mice were used for these studies because they develop a more aggressive disease than C57BL/6 mice administered TNBS. BALB/c mice were used for these studies because they develop a more aggressive disease than C57BL/6 mice administered TNBS. BALB/c mice were used for these studies because they develop a more aggressive disease then C57BL/6 mice in response to TNBS (24). In a preliminary dose-finding study we found that administering mice with INT-747 at doses of 3 and 5 mg/kg/day (23) effectively protects against colitis development in mice administered TNBS for 1 wk (Fig. 4). Because this is a rather short model, confirmation of these results was obtained by treating TNBS-administered BALB/c mice with INT-747 at 5 mg/kg/day for 8 wk. As shown in Fig. 5, whereas mild to moderate inflammation and fibrosis were seen in virtually all mice administered TNBS alone, up to 60% of mice coadministered INT-747 and INT-747 treatment also attenuated the immune dysfunction caused by TNBS as demonstrated by the qRT-PCR analysis of proinflammatory genes and Th1-type cytokines on whole colon homogenates (Fig. 5c) and the measurement of IL-1β, IFN-γ, and TNF-α release from LP-derived CD11b+ cells (Fig. 5d).

Table I. Flow cytometry analysis of LP mononuclear cells isolated from colons of naive FXR wild-type (FXR+/+ ) and null (FXR−/− ) mice

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>FXR+/+ (%)</th>
<th>FXR−/− (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b+</td>
<td>8.4 ± 0.2</td>
<td>18.1 ± 0.1*</td>
</tr>
<tr>
<td>CD11c+</td>
<td>3.1 ± 0.1</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>CD4+</td>
<td>23.6 ± 0.7</td>
<td>22.6 ± 0.4</td>
</tr>
<tr>
<td>CD4+/CD25+</td>
<td>16.5 ± 1.4</td>
<td>13.5 ± 0.5</td>
</tr>
<tr>
<td>CD4+/FAS+</td>
<td>30.6 ± 1.0</td>
<td>29.0 ± 2.0</td>
</tr>
<tr>
<td>CD8A+</td>
<td>8.0 ± 1.5</td>
<td>7.0 ± 0.5</td>
</tr>
<tr>
<td>CD19+</td>
<td>55.3 ± 1.1</td>
<td>44.7 ± 4.1</td>
</tr>
<tr>
<td>GR1+</td>
<td>6.5 ± 0.2</td>
<td>7.5 ± 0.3</td>
</tr>
</tbody>
</table>

* Analysis of LP cells was carried out by flow cytometry as described in Ref. 24. Mice were 14–15 wk old.

* p < 0.05 (Student’s t-test) vs FXR+/+ (n = 5 mice per group).
A similar beneficial effect of the FXR ligand was observed in a second model of colitis induced by administering mice with 5% DSS in drinking water (supplemental Fig. 1) (24). To obtain confirmation of the specificity of the effect of INT-747 in modulating intestinal inflammation, we administered INT-747 to TNBS-treated FXR\(^{-/-}\) mice and to their wild-type counterparts at 5 mg/kg (7 days). As shown in supplemental Fig. 2, INT-747 failed to protect against colitis development in TNBS-treated FXR\(^{-/-}\) mice.

Of interest, FXR activation with INT-747 (5 mg/kg/day) protected against colitis development in T cell- and B cell-depleted SCID mice to a similar extent as that observed in wild-type mice, indicating that FXR exerts its counterregulatory function by acting on the innate immunity of cells (Fig. 6).

**FIGURE 2.** Exacerbation of TNBS-induced colitis in FXR-null mice (C57BL/6J background). a, Exacerbation of wasting diseases induced by 8 wk of administration of TNBS in FXR\(^{-/-}\) mice. Group sizes were as follows: FXR\(^{+/+}\) TNBS, n = 16; FXR\(^{-/-}\) TNBS, n = 14; and control FXR\(^{+/+}\) mice n = 7. *, p < 0.05 vs control and FXR\(^{+/+}\) TNBS mice. b, Histologic scoring of inflammation (I) and fibrosis (F) of distal colons. c, H&E (left panels) and Sirius red (central and right panels) staining of representative paraffin-embedded sections from distal colons after 8 wk of administration of TNBS or vehicle (control mice). d, qRT-PCR analysis of inflammatory and profibrogenetic genes in colon homogenates from control mice (white bars) and FXR\(^{+/+}\) (blue bars) and FXR\(^{-/-}\) (red bars) administered TNBS; n = 7–9 mice per group. *, and ○, p < 0.05 vs control and FXR\(^{+/+}\) TNBS mice. e, IL-1\(\beta\), IFN-\(\gamma\), and TNF-\(\alpha\) production by LP-derived CD11b\(^{+}\) cells cultured with (black bars) or without (white bars) LPS (1 \(\mu\)g/ml); n = 3, p < 0.05 vs unstimulated cells (*), control cells (○), or FXR\(^{+/+}\) TNBS (∧) (by ANOVA followed by Bonferroni’s Multiple Comparison).

FXR is expressed by macrophages and exerts counterregulatory functions

We then assessed whether, in addition to epithelial cells, intestinal macrophages express FXR. As illustrated in Fig. 7, a–e, expression of FXR in LP-derived CD11b\(^{+}\) cells was demonstrated by qualitative RT-PCR and qRT-PCR, Western blot analysis, and immunohistochemistry. In addition, by qualitative RT-PCR, FXR mRNA expression was detected on myeloid cells of mouse (spleen-derived macrophages and RAW264.7 cells) and human origin (PBDM, and THP-1 cells) (Fig. 7f). The sequencing of human FXR fragments demonstrates that these PCR products show 100% homology with the human FXR gene (sequence between exons 5 and 6) (supplemental Fig. 3). By
qRT-PCR (Fig. 7g) we found that although mouse livers and human HepG2 cells express higher content of FXR, FXR mRNA was also expressed in macrophage cell lines (THP-1 and RAW264.7), PBDM, and spleen-derived macrophages. Skeletal muscle cells were used as negative control. FXR protein expression and nuclear localization were further confirmed in mouse and human macrophage cell lines by Western blotting (Fig. 7h) and immunohistochemistry analyses (Fig. 7, i–m).

To investigate whether FXR activation exerts counterregulatory activities on macrophages, LP-derived Cd11b<sup>+</sup> cells and THP-1 cells were treated with LPS alone or in combination with INT-747 (1 μM). As shown in Fig. 8a, induction of IL-1β and TNF-α caused by LPS was attenuated by INT-747. In this setting, INT-747 caused a 3-fold increase in SHP expression. Furthermore, FXR activation reduced IL-1β production (Fig. 8b). These effects were insensitive to the activity of GW9662, a PPARγ antagonist.

FIGURE 4. INT-747 protects against the development of TNBS-induced colitis in BALB/c mice in a dose-dependent manner. Percentage of body weight variation (a), histologic score (b), and qRT-PCR analysis of inflammatory genes in whole colon homogenates (c) from control-treated (white bars), TNBS-treated (blue bars), and TNBS plus INT-747-treated (5 mg/kg/day) mice (red bars) are shown. In all panels, data represent mean ± SEM (n = 6–10) and, in c, indicate the relative mRNA expression in comparison to control mice (value = 1). * and ○, p < 0.05 (ANOVA followed by Bonferroni’s multiple comparison test) vs control and TNBS groups.

FIGURE 5. FXR activation by INT-747 (5 mg/kg/day) protects against the development of chronic intestinal inflammation and fibrosis. a, Histologic scoring of inflammation (I) and fibrosis (F) of distal colons from BALB/c mice in naive conditions (control, n = 7) and after 8 wk of administration of TNBS (n = 9) or TNBS plus INT-747 (n = 10). b, H&E (upper panels) and Sirius red (lower panels) staining of representative paraffin-embedded sections from distal colons. c, qRT-PCR of inflammatory and profibrogenic genes in whole colon homogenates. Data indicate the relative mRNA expression in comparison with control mice (value = 1) and are expressed as mean ± SEM (n = 7–9), * and ○, p < 0.05 vs control and TNBS group, respectively. d, Cytokine production by LP-derived CD11b<sup>+</sup> cells cultured with (black bars) or without (white bars) LPS (1 μg/ml). Results represent the mean ± SEM; n = 3. p < 0.05 vs unstimulated (+) and control and TNBS plus INT-747 (○) (by ANOVA followed by Bonferroni’s test).
sumoylation of transcription factors correlates with transcriptional activation and/or transcriptional repression (11). We have investigated whether INT-747 causes FXR sumoylation. Results from immunoprecipitation experiments demonstrate that sumoylated FXR decreases rapidly in cells exposed to LPS, an event that was robustly inhibited by INT-747 (Fig. 9b).

The primary amino acid sequence of human FXR shows at least two sumoylation consensus sequences (wKX/E/D); where w represents an hydrophobic amino acid and X refers to any amino acid) (26). To further investigate the role of sumoylation in regulating the expression of target genes by FXR, we generated a human FXR protein mutant lacking the sumoylation consensus sequence located in the LBD of the receptor, position K277 (Fig. 9c; the homologous sequence of mouse FXR is K272). Transfection of HEK293-TLR4 cells with a protein in which the K277 of human FXR was mutated to arginine (K277R) greatly impaired the inhibition of the TNF-α gene expression caused by the FXR ligand INT-747, whereas the wild-type FXR retained full trans-repression activity (Fig. 9d). Thus, sumoylation of FXR is required for FXR-mediated trans-repression of cytokine expression induced by INT-747.

We then investigated whether NCoR is required for FXR-mediated trans-repression of TLR-4-induced cytokines expression. THP-1 cells transfected with an anti-human NCoR siRNA validated for efficacy (supplemental Fig. 5) were used for these experiments. In comparison with wild-type cells, the NCoR knockout mice responded to LPS by enhanced production of IL-1β and iNOS mRNA (Fig. 9e). Similar results were observed for TNF-α (data not shown). These observations predict that NCoR-containing complexes associate with the IL-1β and iNOS promoters. Results of the ChIP analysis confirmed that NCoR was present on the promoter of these genes under basal conditions and cleared within 30 min following LPS stimulation (Fig. 9e). We next used ChIP assays to evaluate whether an ordered sequence of events was required for NCoR clearance in response to LPS stimulation in the presence or absence of an FXR ligand and found that pretreatment of cells with INT-747 prevented the clearance of NCoR from the promoters of IL-1β and iNOS (Fig. 9, f and g).

Discussion
The NR FXR is a ligand-regulated transcription factor expressed in the liver and gastrointestinal tract that regulates bile acid homeostasis (12–14). Previous studies have linked NRs to the counterregulation of macrophage biology (3–7). Activation of PPARγ and the liver X receptor by lipid mediators generated by the arachidonic acid breakdown in the COX pathways and oxysterols antagonizes the expression of inflammatory cytokines, providing counterregulatory signals that limit macrophage activation by pathogen-associated molecular patterns (3–7). In addition to these receptors, we now provide evidence that the bile acid sensor FXR counterregulates myeloid cell activation by TLR-4. Support for this view comes from a number of different experimental approaches, including quantitative and qualitative PCR, sequencing of the transcript products, Western blot analysis, and immunohistochemistry, as well as functional studies on FXR-null macrophages. The results of these studies support a regulatory role for FXR on macrophages. Indeed, FXR activation by natural and synthetic and steroidal and nonsteroidal, ligands represses the expression of a set of TLR-4-regulated genes, including proinflammatory genes.

**Sumoylation of FXR mediates the counterregulatory activities of FXR ligands**

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**Discussion**

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cytokines, chemokines, and their receptors. Because these counterregulatory effects are lost in FXR−/− macrophages, they selectively depend on FXR expression and are not mediated by other receptors, such as the recently discovered G protein-coupled receptor TGR5, a seven-transmembrane domain receptor for bile acids that is also expressed in macrophages (29). Furthermore, the preventive effect of INT-747 was lost in FXR−/− mice (supplemental Fig. 2).

The present studies also define sequential steps of the pathway mediating a ligand-dependent trans-repression of inflammatory response genes by FXR ligands. Several of the macrophage genes inhibited by FXR ligands are established targets for NF-κB and AP-1 (27), which are central transcriptional regulators in cells of innate immunity and adaptive immunity. The iNOS, TNF-α, and IL-1β were used for these studies for their relevance in inflammation and their ability to respond to TLR-4 ligation by a NF-κB-mediated pathway (27). The promoters of these genes are marked in the basal state by the presence of NCoR-containing complexes linked to the NF-κB-responsive element (11). Previous studies have provided evidence that LPS signaling results in NCoR clearance from the promoters of these genes, allowing a switch from active repression to transcriptional activation (30). By ChIP and gene overexpression we demonstrated that INT-747 targets FXR to the iNOS and IL-1β promoters and stabilizes the NCoR complexes on the promoters of these two genes (11, 30). NCoR stabilization is essential for FXR-mediated iNOS and IL-1β trans-repression caused by INT-747 as demonstrated by knockdown experiments with anti-NCoR siRNA. This pathway is initiated by a ligand-induced sumoylation of FXR. In this study we demonstrate that the human FXR protein has two sumoylation consensus sequences. Mutation of the K277, located in the FXR LBD, to arginine (K277R) resulted in a protein that was unable to trans-repress TNF-α expression in cells induced with LPS (28, 31).
Inhibition of inflammatory signaling by FXR is not limited to isolated macrophages but manifests itself in vivo. Thus, FXR gene ablation results in a proinflammatory phenotype characterized by spontaneous development of mild to moderate intestinal inflammation and fibrosis. In comparison to FXR-/-, LP CD11b+ cells isolated from FXR-null mice show an increased activation in the resting state and enhanced release of TNF-α in response to TLR-4 ligation. Complementary to our data, Inagaki et al. (19) have shown that mice lacking FXR have increased ileal levels of bacteria and a compromised epithelial barrier. Thus, by modulating the integrity of the intestinal epithelium and the functions of resident macrophages, bile acids appear to participate in a FXR-regulated manner in protection of the host against foreign pathogens and maintenance of the intestinal homeostasis. Because other NRs, such as PPARγ, are also involved in regulating intestinal macrophage functions, a network of NRs exists to modulate TLR-activated signals to limit inflammatory/immune response to luminal pathogens. The restriction of FXR expression to the enterohepatic system with intestinal macrophages acting as high gain sensors of luminal Ags might provide the requested tissue specificity to this regulatory network.

Other than in naïve conditions, dysregulated intestinal innate immunity in mice lacking FXR manifests itself also in an increased susceptibility to the development of inflammation in acute and chronic models of colitis. These murine models show similarities with inflammatory bowel disease, a family of chronic and destructive inflammatory disorders for which dysregulation of innate immunity plays a mechanistic role (24). The model of chronic colitis induced by 8 wk of administration of TNBS, in which inflammation caused by the haptenizing agent associates with enhanced generation of TNF-α, IL-1β, IL-2, IL-6, and intestinal fibrosis, presents similarity with Crohn’s disease (24). The finding that mice lacking FXR were more susceptible to this chronic model than other mice of their genetic background and develop moderate to severe fibrosis along with proinflammatory phenotype provides robust evidence that FXR functions to limit innate immunity responses and immune-mediated activation of intestinal fibroblasts. A further confirmation of the inverse correlation existing between FXR expression/function and host susceptibility to inflammation in the intestinal compartment comes from the observation that acute exposure to TNBS results in a robust down-regulation of colonic expression of FXR. At a translational level, we found that
FXR mRNA expression was almost undetectable in colon biopsies obtained from macroscopically inflamed mucosa of patients with Crohn’s disease. In contrast, in the same patients the expression of two FXR related genes, IBAPB and SHP, was maintained online. Because the expression of PPARγ has also been reported to be reduced in the inflamed mucosa (9), these data suggest that a down-regulation of the NR network is required to mount an inflammatory response in the human gut (32).

Further evidence of the regulatory role of FXR on innate immunity comes from the results of pharmacological studies with a synthetic FXR ligand. Treating BALB/c mice with INT-747 protects against the development of intestinal inflammation induced by TNBS and DSS. In both models, exposure to FXR ligands attenuates the release of the signature cytokines IL-1β and IL-6 in the TNBS model and IL-4 and IL-5 in the DSS model (24), demonstrating that FXR exerts a counterregulatory function on both branches of the intestinal immune system. However, because INT-747 also protected against colitis development in lymphocyte-deficient SCID mice, it appears that myeloid cells in the innate immunity compartment are the predominant target.

In summary, we have provided evidence that FXR-deficient mice respond to intestinal inflammation with an exaggerated production of proinflammatory cytokines and inflammation-driven fibrosis in the colon. By demonstrating that FXR activation with potent synthetic ligand protects against the development of inflammation in murine models of colitis, our results establish that FXR might represent a novel therapeutic target in inflammatory bowel diseases.

FIGURE 9. Recruitment of FXR to iNOS promoter requires sumoylation of the receptor. a, ChIP experiments demonstrate that FXR is recruited to the iNOS promoter within 30 min (30’) of INT-747 cotreatment in LPS-stimulated THP-1 cells. Experimental conditions were the same described in Fig. 2. * and **, p < 0.05 vs unstimulated and LPS-treated cells, respectively. Results represent mean ± SEM (n = 4) and indicate the relative mRNA expression in comparison to the unstimulated condition (Unst; value = 1). b, INT-747 induces sumoylation of FXR in THP-1 cells treated as in a and cellular lysates that were Western blotted using an anti-SUMO (top panel) and an anti-FXR Ab (third panel from top). This figure is representative of three independent experiments. IP, Immunoprecipitation. c, Potential site of sumoylation on the LBD of mouse FXR. Three-dimensional structure of the LBD of rFXR (Protein Data Bank code: 1OSV). Secondary element structures are colored according to the experimental B factors. The putative sumoylation sequence is located in the H2-H3 loop, which is region of medium to high mobility. The side chain of K272 is exposed to the solvent. d, Mutated human FXR in the K277 sumoylation site does not inhibit LPS-induced TNF-α gene expression. TLR-4-expressing HEK293 cells were transfected with the wild-type or mutated FXR (K227R). e, qRT-PCR analysis demonstrating that siRNA directed against NCoR abolishes INT-747-dependent trans-repression of IL-1β and iNOS expression induced by LPS. THP-1 cells transfected with human NCoR-1-specific siRNA or control siRNA were pretreated with INT-747 (1 μM) for 3 h and then stimulated with LPS (1 μg/ml) for 18 h. *, p < 0.05. f and g, ChIP analysis demonstrates that INT-747 prevents dissociation of NCoR-1 complex from the IL-1β and iNOS promoters. Cells were pretreated with INT-747 (1 μM) for 3 h and then exposed to LPS (1 μg/ml) for the indicated times, *, and **, p < 0.05 vs unstimulated and LPS-treated cells, respectively. In a and b, results represent mean ± SEM (n = 3–5) and indicate the relative mRNA expression in comparison with the unstimulated condition (value = 1).


Legends to Supplementary Figures

**Supplementary Figure 1.**

FXR activation by INT-747 (5 mg/kg/die) prevents the development of colitis induced by DSS in balb/c mice. Percentage of body weight variation (a), histologic score (b) and qRT-PCR analysis of inflammatory genes in whole colon homogenates (c). In all panes, data represent mean ± s.e.m. (n=6-8) and, in panel c, indicate the relative mRNA expression in comparison to control mice (value=1); (*) and (°) P <0.05 (ANOVA followed by Bonferroni’s Multiple Comparison test) versus control and DSS group, respectively.

**Supplementary Figure 2.**

INT-747 fails to protect against development of TNBS-induced colitis in C57BL6 FXR-/- mice (a), weight changes (b) diarrhea score (c) macroscopic score and (d) mucosal cytokines expression (qRT-PCR) were not affected by INT-747 (5 mg/kg/die) treatment. In all panels, data represent mean ± s.e.m. (n=6-7 animals). Panel d, is the relative mRNA expression in comparison to control mice (value=1); (*) and (°) P <0.05 (ANOVA followed by Bonferroni’s Multiple Comparison test).

**Supplementary Figure 3**

PCR products were sequenced by CEQ8000 DNA sequencer. BLAST alignment reveals 100% homology between these sequences and the human FXR mRNA present in Gene Bank (Accession Number NM_005123).
The PCR products show 100% homology with the human FXR gene (sequence between exon 5 and exon 6).

**Supplementary Figure 4.** The PPAR\(\gamma\) antagonist GW9662 has no effect on the counter-regulatory effects of FXR on LPS-primed lamina propria derived CD11b+ cells. Data are mean ± SE of 4 experiments. Cells were prepared by naive wild type mice and stimulated with LPS alone or in combination with the FXR agonist INT-747 (10 \(\mu\)M) and the PPAR\(\gamma\) antagonist GW9662 (1 \(\mu\)M) for 18 hours. Data are mean ± SE of 4 experiments.

**Supplementary Figure 5.** Validation of anti-NCoR siRNA. Efficiency of knockdown was evaluated in THP-1 cells transfected with specific anti-human NCoR and control siRNAs. The levels of NCoR mRNA were assessed by qRT-PCR. Results indicate the relative mRNA expression of NCoR in comparison to the cells transfected with control siRNA (value=1) and represent mean ± s.e.m (n=4); (*) \(P < 0.05\) (Student's t-test) versus control siRNA.
**Supplementary Tables**

**Supplementary Table 1. Primers used for qRT-PCR**

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DSS colitis in balb/c mice

Vavassori P. et al. – Supplementary Figure 1
FXR-/- mice

Vavassori P. et al. – Supplementary Figure 2
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sample 121  AGT  123
NR1H4  680  AGT  682

Vavassori P. et al. – Supplementary Figure 3
The PPARγ antagonist GW9662 fails to revert attenuation of IL-1β and SHP caused by FXR on LP-derived CD11b+ cells

Vavassori P. et al. – Supplementary Figure 4
NCoR siRNA validation

Vavassori P. et al. – Supplementary Figure 5